UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

November 6, 2014

MEMORANDUM FOR DR (b)(6)
(b)(6)

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on November 6, 2014:

Animal Protocol Title: (b)(4)
(b)(4)

USUHS Protocol Number: (b)(6)

Expiration Date: November 5, 2017

Supporting Grant(s) Number: TBD

Name of Principal Investigator: Dr (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

Revised

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE:
(b)(4)

GRANT TITLE (if different from above):
(b)(4)

USUHS PROJECT NUMBER:

FUNDING AGENCY:
(b)(4)

EARLIEST ANTICIPATED FUNDING START DATE:
(b)(4) 2015

PRINCIPAL INVESTIGATOR:
(b)(6) — 9/9/2014

Principal Investigator Signature | Department | Office/Lab Telephone | Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) — Chair — (b)(6) — 9/9/2014

Research Unit Chief / Dept Head Signature | Title | Telephone | Date

Typed Name. (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the stu

(b)(6) — (b)(6) — 10/16/2014

St

Typed Name. (b)(6) PhD

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) — LAM — (b)(6) — 10/16/2014

Attending/Consulting Veterinarian Signature | Department | Date

Typed Name (b)(6) DVM, MPH

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR:
(b)(6)

ANIMAL PROTOCOL TITLE:
(b)(4)

GRANT TITLE (if different from above):
(b)(4)

USUHS PROJECT NUMBER:

CO-INVESTIGATOR(S):

TECHNICIANS(S):
(b)(6)

I. NON-TECHNICAL SYNOPSIS:

The proper regulation of the hypothalamic-pituitary-adrenal (HPA) axis is important for optimum physiological function and survivability of an individual (b)(4) (b)(4) Not only does the HPA axis control hormonal responses to stress, but its activity also synchronizes physiological systems with environmental cues and rhythms. When the HPA axis does not function appropriately and circulating glucocorticoid levels are inappropriate during the day, there is a correlation with the development of numerous physiological and psychological disorders. These include metabolic disruption, cardiovascular disease, immunological variations, sleep/wake inefficiencies (including reduced performance in operational environments), neuropsychiatric disease, neurodegenerative diseases and putative increased cancer risks. *These are all areas of significant concern within the military community.* The goal of this study is to examine how circadian disruption alters the HPA axis. Specifically, we are interested in determining the fundamental changes that occur in the stress axis regulation. Despite known correlations, work environments both in the civilian world as well as in the military does not always allow for the optimal diurnal schedules. This study will tell us how changes to the daily circadian schedule may potentially have impact on the stress physiological systems.

II. BACKGROUND:

II.1. Background:

(b)(4)

(b)(4)

(b)(4)

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:

- PubMed
- Biomedical Research Database (BRD)
- RePORTER – The replacement for CRISP is the RePORTER (http://projectreporter.nih.gov/reporter.cfm)

II.2.2. Date of Search:

October 6, 2014

II.2.3. Period of Search:
No Period Restriction

II.2.4. Key Words and Search Strategy:
(b)(4)

II.2.5. Results of Search:

There are over 4000 papers that study the interaction of the stress system and the circadian system (context of rhythm or disruption) going back to 1966. Many of these studies focus on the autonomic system and the response of peripheral organs to stress during circadian shifts or changes. With the identification of CLOCK genes, many of these studies switch their focus on how stress may alter CLOCK genes in brain and periphery. (b)(4)

(b)(4)

The conclusion of our literature search are: a. this is a unique approach and b. these approaches will significantly advance our understanding of the (b)(4) neuronal biology and thus, by default our understanding of the stress system.

A similar conclusion is made from a review of Biomedical Research Database (BRD) and the RePORTER.

(b)(4)

In the NIH RePORTER, a search was conducted in all dates (from 1990 onward) and search terms included title, abstract and terms. There were 163 "hits" representing approximately 50 funded grants. All these studies examine stress in context of hormonal response (b)(4)

III. OBJECTIVE\HYPOTHESIS:

(b)(4)

IV. MILITARY RELEVANCE:

When the (b)(4) does not function appropriately and circulating (b)(4) are inappropriate in amplitude or timing, the development of numerous physiological and psychological disorders is facilitated. These include metabolic disruption, cardiovascular disease, immunological variations, sleep/wake inefficiencies (including reduced performance in operational environments), neuropsychiatric disease, neurodegenerative diseases and putative increased cancer risks. *These are all areas of significant concern within the military community.*

V. MATERIALS AND METHODS:

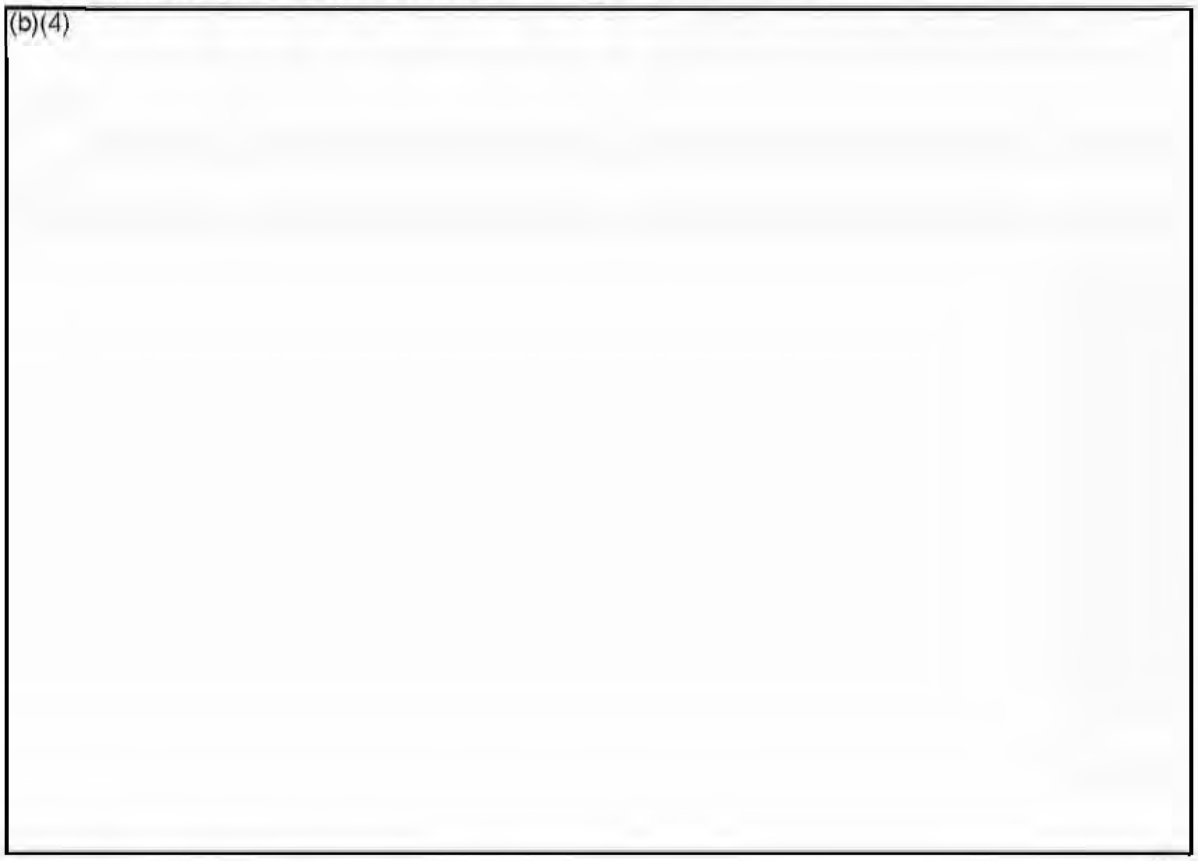
V.1. Experimental Design and General Procedures:

(b)(4)

(b)(4)

To reduce the number of mice used, Experiment 1 will have 2 parts, Exp 1a and Exp 1b; Exp 1a will only examine the 4 week time point. Exp 1b will follow up on the initial examination of global gene expression in Exp 1a.

(b)(4)



(b)(4)

(b)(4)

V.2. Data Analysis:

In Exp 1a, we base our numbers estimated from (b)(4) and from Dr. (b)(6) (b)(6) experiment running RNASeq experiments to examine global gene expression.

For (b)(4) the number of animals per group is based on a culmination of ours and others' previous studies. Group size is determined using a statistical package, SPSS with an alpha = 0.05. A two tailed test with an (b)(4) will give a power of 0.8 to detect differences between group means of 1.33 standard deviations or greater. The differences of this magnitude has been shown in previous studies in response to dexamethasone suppression test and to restraint stress. The data collected for both experiments will be analyzed with the appropriate ANOVA followed by Fishers' LSD post-hoc test.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

This aspect of the study involves whole animals since there are no known current cell models or computer models to (b)(4) (b)(4)

V.3.2. Animal Model and Species Justification:

(b)(4)

(b)(4)

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	Mice	Mice
V.3.3.2. Strain/Stock:	(b)(4)	
V.3.3.3. Source/Vendor:		
V.3.3.4. Age:	2 – 8 months	2 – 8 months
V.3.3.5. Weight:	20-45 g	20-45 g
V.3.3.6. Sex:	**Male**	**Male**
V.3.3.7. Special Considerations:		

V.3.4. Number of Animals Required (by Species): (b)(4)

	Species #3	Species #4
V.3.3.1. Genus & Species:	Mice	Mice
V.3.3.2. Strain/Stock:	(b)(4)	
V.3.3.3. Source/Vendor:		
V.3.3.4. Age:	2 – 8 months	2 – 8 months
V.3.3.5. Weight:	20-45 g	20-45 g
V.3.3.6. Sex:	**Female**	**Female**

V.3.3.7. Special Considerations:

V.3.4. Number of Animals Required (by Species): (b)(4)

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: Mice will be handled at least 3-4 times a week to reduce stress during experimentation. In addition, mice will be handled at approximately the same time each day. For terminal experiments, the mice will be anesthetized prior to euthanizing. Refinements considered but not used: adjusted early end-point, unless the animals become ill. Under that circumstance, the animals will be euthanized. The decision to euthanize the sick mouse will be in consultation with the PI and the Veterinarian.

(b)(4)

V.3.5.2. Reduction: In experiment 1, we designed the experiment into 2 parts to significantly reduce the number of mice used. If we utilize the original 2X2X2X2X3 (48 total treatment groups), we will utilize a total of (b)(4) mice. By designing the experiment into 2 parts, we reduced the numbers to (b)(4) In addition, we will conduct the studies using a latin square design where once statistical difference is achieved, we can stop the experiment. This allows us to use the minimum number of mice possible to still be statistically significant. In addition, every effort has also been made to use every tissue part for the current and other studies without compromising the integrity of the experimental design and for not compromising data analysis. We will not use any more animals than needed.

V.3.5.3. Replacement: At this time, it is not possible yet to study the impact of the the environment on the animal's stress axis. It is possible that in the future, when more information has been garnered, that we would be able to test behavioral responses, endocrine responses or other physiological responses using computer modeling.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. **APHIS Form 7023 Information:**

V.4.1.1.1. **Number of Animals:**

	Species #1	Species #2	Species #3	Species #3
V.4.1.1.1.1. **Column C:**	(b)(4)			
V.4.1.1.1.2. **Column D:**				
V.4.1.1.1.3. **Column E:**				

V.4.1.2. **Pain Relief / Prevention:**

V.4.1.2.1. **Anesthesia/Analgesia/Tranquilization:**
Carbon dioxide (for deep anesthesia).
The mice will be deeply anesthetized by CO_2 prior to decapitation. These will utilize the CO_2 chambers available at DLAM. According to the SOP attached to the DLAM CO_2 chambers, the mice will be in standard mouse cages (5L volume) where the flow rate will displace 20% of the chamber volume per minute by introducing 100% CO_2. This translates to 1 L per minute. Deep anesthesia is tested with toe pinch – a lack of response indicated deep anesthesia. (b)(4)
(b)(4)

V.4.1.2.2. **Pre- and Post-procedural (not surgery) Provisions:**
Not applicable.

V.4.1.2.3. **Paralytics:** NA

V.4.1.3. **Literature Search for Alternatives to Painful or Distressful Procedures:**

V.4.1.3.1. **Sources Searched:**
MEDLINE, AGRICOLA

V.4.1.3.2. **Date of Search:**
October 16, 2014

V.4.1.3.3. **Period of Search:**
1968 to present

V.4.1.3.4. **Key Words of Search:**
Animal, pain, surgery, analgesia, mice, (b)(4)

V.4.1.3.5. **Results of Search:**
Animal studies are needed to further test the physiological and behavioral significance of any result procured from cell culture studies. There are no

alternatives (cell or computer modeling) to study the effect of (b)(4) (b)(4) a complex stress system that is regulated by feedback and through cellular connectivity. The methods described in this proposal are standard procedures that have been used for many decades and have been translated eventually to human studies and drug development.
Based on a literature search:

1. the proposed research is not duplicative of research which has already been done;
2. Alternatives to the use of procedures that may cause more than momentary or slight pain or distress are not appropriate or available. This is to establish an animal model for studying how changes in the circadian rhythm may alter the stress system. The 20 min restraint stress is a commonly used model to test the responsiveness of the (b)(4) (b)(4)
3. We have proposed to utilize the appropriate pain management methods in the protocols.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: NA

V.4.2. Prolonged Restraint: Not applicable

V.4.3. Surgery: Not applicable

V.4.3.1. Pre-surgical Provisions: NA

V.4.3.2. Procedure: NA

V.4.3.3. Post-surgical Provisions: NA

V.4.3.4. Location: NA

V.4.3.5. Surgeon: NA

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: NA

V.4.3.6.2. Scientific Justification:

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

V.4.4.2. Biosamples: Entire organs will be harvested during routine necropsy – brain, pituitary, liver, uterus. Maximum obtainable trunk blood will be obtained after euthanasia.

V.4.4.3. Adjuvants: Not applicable

V.4.4.4. Monoclonal Antibody (MAbs) Production: NA

V.4.4.5. Animal Identification: The animals will be identified on the cage card and when group housed, they will be identified by a non-toxic marker on the tail.

V.4.4.6. Behavioral Studies: Not applicable

V.4.4.7. Other Procedures: Daily vaginal lavage will be conducted using a well established method described in (b)(4) (b)(4) This is a non-invasive method to stage the estrous cycle. An exception is to use sterile physiological saline as opposed to ddH_2O. Daily lavages will be conducted for at least 2 consecutive estrous cycles. No anesthesia is required.

Briefly, the mouse is firmly grasp by the tail to expose the rear end. The vagina is gently perfused by gently placing about 50 ul physiological saline at the opening of the vaginal canal. Draw the saline back into the tip. Repeat 4-5 times using the same tip. Place the fluid on a glass slide for analysis.

V.4.4.8. Tissue Sharing: Yes. Excess tissues will be made available to other investigators upon request.

V.4.5. Study Endpoint: Based on the respective time-points as described in Exp 1 and 2, animals will be euthanized by deep carbon dioxide anesthesia followed by decapitation (Exp 1) or deep carbon dioxide anesthesia followed transcardiac perfusion by physiological saline and buffered 4% paraformaldehyde (Exp2).

Any mouse that becomes ill, shows a greater than 20% weight loss, becomes lethargic, dehydrated, unkempt, or shows any other clinical signs of a medical problem will be evaluated by the veterinarian and PI (when available). In the event of a debilitating illness or adverse reaction, the PI and the veterinarian will consult on a course of treatment. Mice may be euthanized for humane reasons at the discretion of the veterinarian, but attempts to contact the PI will be made before the mouse is euthanized.

V.4.6. Euthanasia:

All animals will be euthanized by CO2 anesthesia followed by decapitation or cardiac perfused with physiological saline and buffered 4% paraformaldehyde. The mice will be deeply anesthetized by CO_2 prior to decapitation. These will utilize the CO_2 chambers available at DLAM. According to the SOP attached to the DLAM CO_2 chambers, the mice will be in standard mouse cages (5L volume) where the flow rate will displace 20% of the chamber volume per minute by introducing 100% CO_2. This translates to 1 L per minute. Deep anesthesia is tested with toe pinch – a lack of response indicated deep anesthesia. In Exp 1, the mice will be decapitated and in Exp 2, the mice will be pericardially perfused.

Briefly, Perfusion will be conducted using standard methods (eg, described in http://www.ebiomethods.com/methods/mouseperfusion) utilizing a butterly needle (26G) and the sequential perfusion with physiological saline (approximately 20 ml) and buffered paraformaldehyde (approximately 100 ml). CO2 anesthesia will be conducted according to USU Policy 013, "Rodent Anesthesia with CO2 prior to Euthanasia".

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. Routine cage changes will be conducted by the PI's laboratory staff.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Mice will be house in separate rooms with special light cycles according to the experimental design. It is requested that the mice be placed in:

i. (b)(4) L:D cycle, or

ii. (b)(4) L:D cycle.

(b)(4)

Food Restriction: Yes _____ No ___X___

Fluid Restriction: Yes No X

V.5.1.3. Exceptions:

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
Mice will be observed at least once daily by LAM personnel and by investigative staff. Mice will be weighed regularly by investigative personnel. Any mice that becomes ill, shows a greater than 20% weight loss by visual appraisal and by additional weighing if necessary, becomes lethargic, dehydrated, unkempt, or shows any other clinical signs of a medical problem will be evaluated by the veterinarian and PI (when available). In the event of a debilitating the PI and the veterinarian will consult on a course of treatment. Mice may be euthanized for humane reasons at the discretion of the veterinarian, but attempts to contact the PI will be made before the mouse is euthanized.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies.

Mice will be group housed (up to 4 mice per cage) for both male and female mice and all Enrichment Strategies will be conducted in accordance to USU Policy 008 (Laboratory Animal Exercise and Environmental Enrichment Program). The mice will be give nestlets as enrichment (Section F under Environmental Enrichment).

V.5.3.2. Enrichment Restrictions: NA

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Basic Training	(b)(6)	Greater than 20 years	(b)(6)

Course			Rodent Handling Course (Taught by Dr. (b)(6) USUHS Investigator Training (between January and June, 2001)
Euthanasia	(b)(6)	Greater than 20 years	(b)(6) Euthanasia Course (Taught by Dr. (b)(6) (b)(6) DVM, PhD) (b)(6) USUHS Euthanasia Training Course (I don't remember the exact date)
CITI Training		Completed	9/10/2014
Basic Training Course		4 years	DLAM Investigator Training Course (9/2013) (b)(6)
Injections		4 years	DLAM Rodent Handling Course (9/2011)
CITI Training		Completed	5/9/2014
Basic Training Course		10 years	DLAM Investigator Training Course and Rodent Handling Course (6/2004)
Euthanasia		10 years	DLAM Euthanasia Course (we do not remember the exact date)
Injections		10 years	DLAM Rodent Handling Course (6/2004)/Dr (b)(6)
CITI Training		Completed	9/11/2014
Basic Training Course		2 years	DLAM Investigator Training Course and Rodent Handling

			Course (March 2012)
Euthanasia	(b)(6)	2 years	DLAM Rodent Handling Course (March 2012)/Dr. (b)(6) Supervision
Injections		2 years	DLAM Rodent Handling Course (March 2012)/Dr. (b)(6)
CITI Training		Completed	9/16/2014
Basic Training Course		Greater than 10 years	DLAM Investigator Training Course (2002 – unfortunately we do not remember the month)
Euthanasia		Greater than 10 years	DLAM Rodent Handling Course (2002 – unfortunately we do not remember the month)
Injections		Greater than 10 years	DLAM Rodent Handling Course (2002 – unfortunately we do not remember the month)
CITI Training		In progress	

VII. BIOHAZARDS/SAFETY:

When handling animals all personnel will wear personal protective equipment including lab coats, face masks and gloves to minimize exposure to rodent allergens. The personnel will also annually visit the nurse to discuss their needs.

All perfusions with a paraformaldehyde-based buffer will be conducted to minimize exposure to vapors. Precautions include personnel wearing gloves, lab coats and face masks to protect from noxious fumes and perfusions be conducted in a fume hood or a ventilated table (DLAM necropsy room).

VIII. ENCLOSURES:

(b)(4)

(b)(4)

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User

(b)(6)

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting hum

(b)(6)

Principal Investigator Signature Date

I.

Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL** or **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title:

(b)(4)

C. Principal Investigator:

(b)(6)

D. Performing Organization:

Uniformed Services University

E. Funding:

The funding agency requires IACUC approval with grant submission.

F. Objective and Approach:

(b)(4)

G. Indexing Terms (Descriptors):

(b)(4)

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

June 3, 2016

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF OBSTETRICS AND GYNECOLOGY

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on June 3, 2016:

Title of Application: (b)(4)
(b)(4)

USUHS Protocol Number: (b)(6)

Expiration Date: June 2, 2019

Supporting Grant(s) Number (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is A3448-01. The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet yoar needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

final version

USUHS FORM 3206 ANIMAL STUDY PROPOSAL

PROTOCOL COVER SHEET

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: (b)(4)
(b)(4)

GRANT TITLE (if different from above): (b)(4)
(b)(4)

USUHS PROJECT NUMBER/ DAI GRANT NUMBER: (b)(6)

FUNDING AGENCY: (b)(4)

EARLIEST ANTICIPATED FUNDING START DATE: (b)(4)

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6)			2/15/2016
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	Chair	(b)(6)	2/15/2016
Research Unit Chief / Dept. Head Signature	Title	Telephone	Date

Typed Name: (b)(6)
(b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)			2/15/2016
Statistician Signature	Department	Telephone	Date

Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics. All signatures are required prior to submission to the IACUC Office.

	LAM		
Attending/Consulting Veterinarian Signature	Department	Telephone	Date

Printed Name:

USUHS Form 3206 – Revised January 2016
Previous versions are obsolete

USUHS FORM 3206 ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

PROTOCOL NUMBER:

PROTOCOL TITLE: (b)(4)
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GRANT TITLE (if different from above): (b)(4)
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PRINCIPAL INVESTIGATOR: (b)(6)
(b)(6)
Principal Investigator Signature | Department | Office/Lab Telephone | 2/15/2016 Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice

(b)(6)
Research Unit Chief / Dept. Head Signature | Chair Title | (b)(6) Telephone | 2/15/2016 Date
Typed Name: Col (b)(6)

(b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)
Statistician Signature | Department | Telephone | 2/15/2016 Date
Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics. All signatures are required prior to submission to the IACUC Office.

(b)(6)
Attending/Consulting Veterinarian Signature | LAM Department | (b)(6) Telephone | 2/11/2016 Date
Printed Name: (b)(6)

USUHS Form 3206 – Revised January 2016
Previous versions are obsolete

USUHS Form 3206 – Revised January 2016
Previous versions are obsolete

USUHS FORM 3206 ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

PROTOCOL NUMBER:

PROTOCOL TITLE: (b)(4) (b)(4)

GRANT TITLE (if different from above): (b)(4) (b)(4)

USUHS PROJECT NUMBER/ DAI GRANT NUMBER: (b)(6)

FUNDING AGENCY: (b)(4)

EARLIEST ANTICIPATED FUNDING START DATE: (b)(4)

PRINCIPAL INVESTIGATOR: (b)(6)

Principal Investigator Signature	(b)(6) Department Office/Lab Telephone	2/15/2016 Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

Research Unit Chief / Dept. Head Signature Typed Name: Col (b)(6)	Chair Title	(b)(6) Telephone	2/15/2016 Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

Statistician Signature Typed Name: (b)(6)	OBG Department	(b)(6) Telephone	2/15/2016 Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics. All signatures are required prior to submission to the IACUC Office.

Attending/Consulting Veterinarian Signature Printed Name:	LAM Department	Telephone	Date

The aims of the supporting grant must be submitted along with the 3206. If funding is from NIH please also submit the Vertebrate Animal Section (VAS).

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6)

PRINCIPAL INVESTIGATOR EMAIL: (b)(6)

ANIMAL PROTOCOL TITLE: (b)(4) (b)(4)

GRANT TITLE (if different from above): (b)(4) (b)(4)

USUHS PROJECT NUMBER:

DAI GRANT NUMBER:

CO-INVESTIGATOR(S):

TECHNICIANS(S): (b)(6) (b)(6)

I. NON-TECHNICAL SYNOPSIS:

The consequences of sleep loss and sleep disorders are dire. Sleep loss has been attributed to some of the sensational result in significant financial loss as well as long term impact on the environment and human health (b)(4) (b)(4) The economic cost is in the millions (b)(4) According to the CDC, "more than one-quarter of the US population report occasionally not getting enough sleep, while nearly 10% experience chronic insomnia (b)(4) " It is well documented that there is a strong correlation between sleep disorders and neuropsychiatric disorders, such as post-traumatic stress disorder (PTSD) and depression (b)(4) the relationship between sleep and neuropsychiatric disorders remain to be determined. To date, the underlying mechanism mediating the transition to neuropsychiatric disorders is limited. (b)(4) (b)(4)

II. BACKGROUND:

II.1. Background:

The consequences of sleep loss and sleep disorders are dire. Sleep loss has been attributed to some of the sensational result in significant financial loss as well as long term impact on the environment and human health (b)(4) (b)(4) The economic cost is in the millions (b)(4) According to the CDC, "more than one-quarter of the US population report occasionally not getting enough sleep, while nearly 10% experience chronic incomnia (b)(4) " It is well documented that there is a strong correlation

between sleep disorders and neuropsychiatric disorders, such as post-traumatic stress disorder (PTSD) and depression (b)(4) the relationship between sleep and neuropsychiatric disorders remain to be determined. It is interesting to note that sleep disruption (or partial disruption) may be a predictor of developing PTSD and depression

(b)(4)

(b)(4)

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: DTIC, Pubmed, RePORTER

II.2.2. Date of Search: February 19, 2016

II.2.3. Period of Search: No Period Restriction

II.2.4. Key Words and Search Strategy:

(b)(4)

II.2.5. Results of Search:

Pubmed: Of 2,451 studies published to date on sleep disruption, there are 17 that evaluate (b)(4) after sleep disruption in clinical studies or reviews. (b)(4)

(b)(4)

(b)(4) in a single point representing the altered homeostasis after sleep disruption or apply a stress to examine a behavioral or biochemical outcome. (b)(4)

(b)(4)

The conclusions' of our literature search are: a. this is a unique approach and b. these approaches will significantly advance our understanding of the neuro-circuitry involving the (b)(4) neuronal biology and thus, by default our understanding of (b)(4) (b)(4) and finally, c. there is limited information on how sleep disruption may result in dysregulated biological systems in a sex-dependent manner.

A similar conclusion is made from a review of DTIC and the RePORTER.

In the DTIC, there were 12 citations that study sleep and the HPA axis. There are no studies that examine the impact of sleep on the molecular components of the HPA axis and (b)(4) Most of the studies are in aquatic mammals or clinical studies.

In the NIH RePORTER, a search was conducted in all dates and search terms included title, abstract and terms. There were 17 "hits" in which in which sleep disorders and (b)(4) were mentioned in the same grant representing 7 grants. (b)(4) (b)(4)

III. OBJECTIVE\HYPOTHESIS:

(b)(4)

IV. MILITARY RELEVANCE:

Do changes in the (b)(4) contribute to pathological conditions or do they merely provide evidence of it? Given that many physiological systems are either under circadian input or control, this proposal represents a novel shift in the way one could look at treatment of disease and clinical diagnoses. Particularly, this proposal describes the mechanism(s) whereby circadian and (b)(4) (b)(4) interact can lead to novel approaches to the promotion of warfighter resilience or the prevention or treatment of stress-related problems.

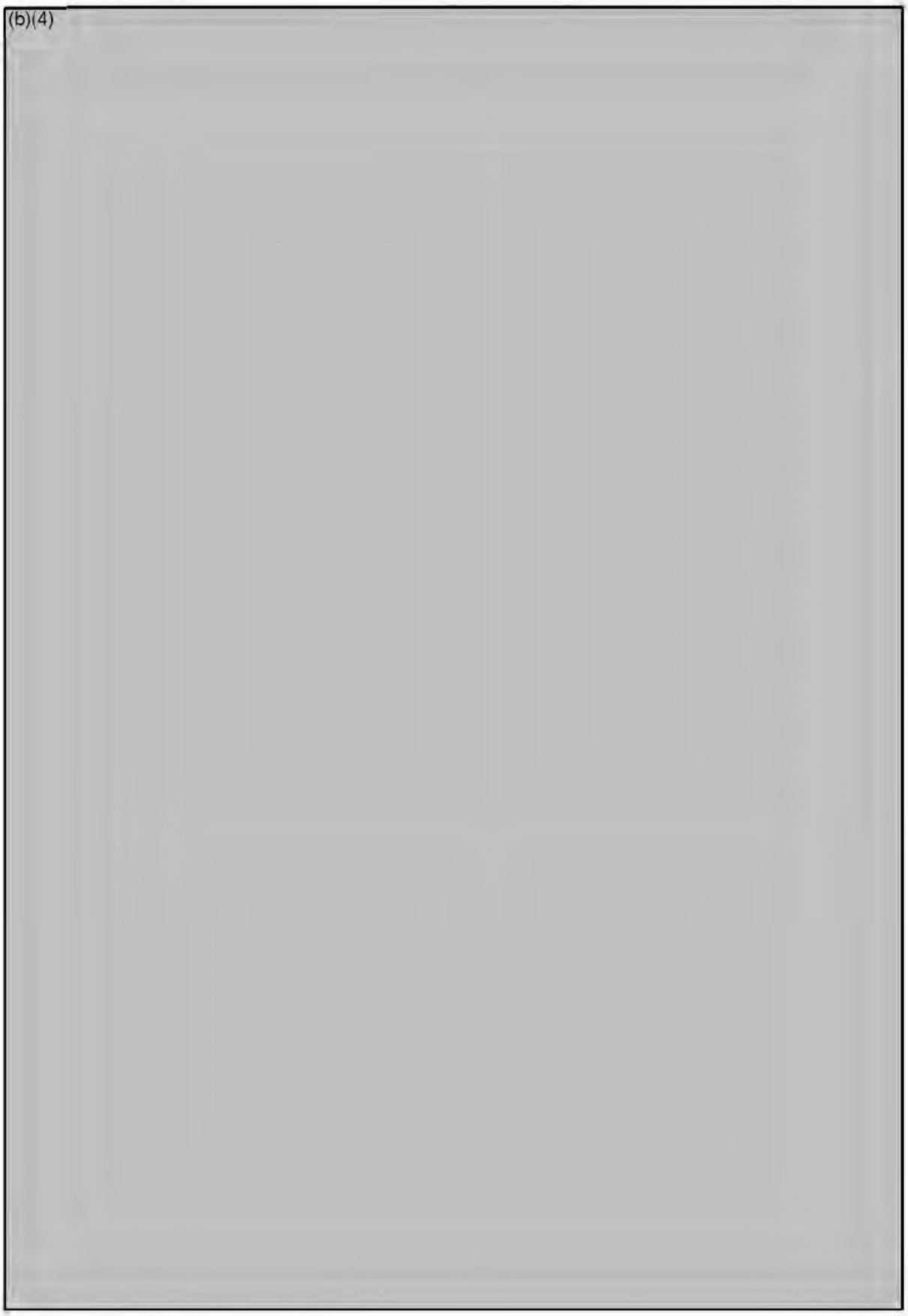
This project will explore the neurobiological consequences of sleep quality that result in long-term alterations (b)(4) The relationship between sleep disorders and the development of neuro-psychological disorders is unknown and there is also little understanding of how sex may be impactful. (b)(4) (b)(4)

V. MATERIALS AND METHODS:

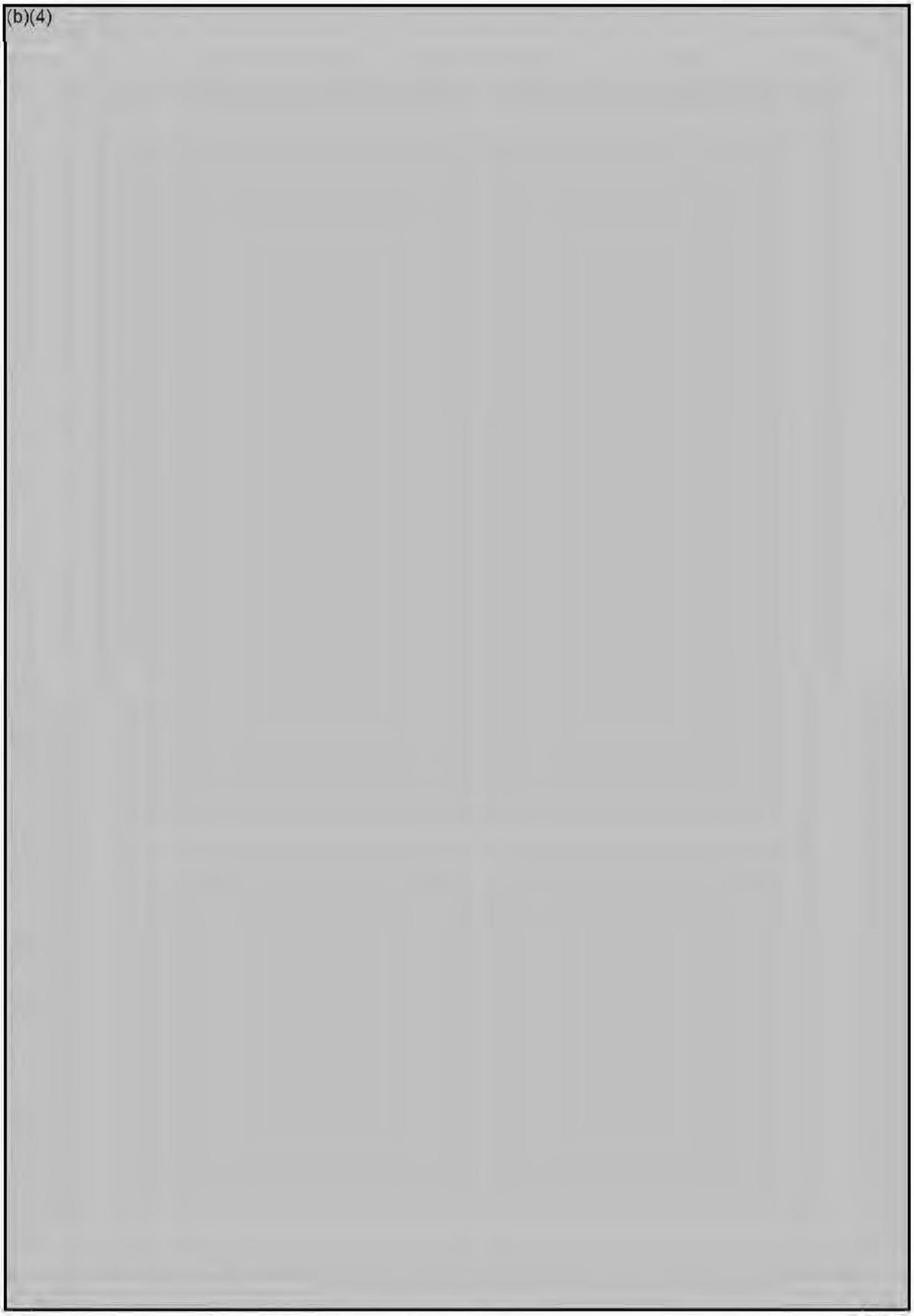
V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1:

(b)(4)



(b)(4)



(b)(4)

(b)(4)

V.2. Data Analysis:

All experiments will be conducted in a block design in which animals from each treatment group will be tested. Standard statistical analyses will be conducted. Briefly, an appropriate analysis of variance (ANOVA) in a block design will be conducted that is specified in each aim. For each run, we will run 2 mice per treatment group.

(b)(4)

(b)(4) the number of animals per group is not only based on a culmination of the literature, our experience and others' previous studies but also by confirming using a power analysis. Group size is determined using a statistical package, SPSS with an alpha = 0.05. A two tailed test with an (b)(4) will give a power of 0.8 to detect differences between group means of 1.33 standard deviations or greater (for the molecular studies). A two tailed test with an (b)(4) will give a power of 0.8 to detect differences between group means of 1.33 standard deviations or greater (for the anatomical studies). The differences of this magnitude has been shown in previous studies in response to dexamethasone suppression test and to restraint. The data collected for both experiments will be analyzed with the appropriate ANOVA followed by Fishers' LSD post-hoc test.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

This aspect of the study involves whole animals since there are no known current cell models or computer models to simulate sleep disruption to study the hypothalamic-pituitary-adrenal axis regulation.

V.3.2. Animal Model and Species Justification:
We have chosen the mouse as a model for several reasons. The most important reason is that the mouse stress axis is well characterized. Moreover, there are numerous mouse genetic models available allowing us to test our hypothesis. (b)(4)

(b)(4)

(b)(4) To this end and also in response to future NIH policy on inclusion of males and females in pre-clinical studies (Clayton and Collins, 2014), all studies in the present proposal will be conducted using both adult male and female mice (1.5 - 6 months old).

V.3.3. Laboratory Animals

ALTERNATIVES CONSIDERATIONS: Does the protocol have any provisions that would qualify it to be identified as one that Refines, Reduces, or Replaces (3R's) the use of animals in relation to other protocols or procedures performed in the past?

YES

SECTION V.3.5.

Exceptions to the *Guide for the Care and Use of Laboratory Animals* (Please check all applicable):

[ ] Use of Paralytics (V.4.1.2.3.)
[ ] Prolonged Restraint (V.4.2.)
[ ] Multiple Major Survival Surgery (V.4.3.6.)
[ ] Use of Non-pharmaceutical grade chemicals (V.4.4.1.)
[ ] Use of Complete Freund's Adjuvant (V.4.4.3.)
[ ] Death as an endpoint (V.4.5.)
[ ] Food/Water Restriction (V.5.1.2.
[ ] Single Housing of Social Species (V.5.1.3)
[ ] Restriction of Environmental Enrichment (V.5.3.2.)
[ ] Drug Use/Controlled Substances (Appendix A)

IDENTIFICATION OF SPECIES AND STRAIN: In accounting for animal numbers, please ensure that the strain of animal as well as the species is identified. If more than one strain of any species will be used, please list each proposed strain in a separate column. If more than two species/strains are to be used, duplicate Sections V.3.3.1 – V.3.4 , and Section V.4.1.1.1, on subsequent pages to cover all requested strains.

Species #1 **Species #2**

V.3.3.1. **Genus & Species:**	*Mus musculus*	*Mus musculus*
V.3.3.2. **Strain/Stock:** V.3.3.3. **Source/Vendor:**	(b)(4)	
V.3.3.4. **Age:**	2 – 8 months	2 – 8 months
V.3.3.5. **Weight:**	20-45 g	20-45 g
V.3.3.6. **Sex:**	**Male**	**Female**
V.3.3.7. **Special Considerations:**	N/A	N/A
V.3.4. **Number of Animals Required (by Species):**	(b)(4)	

	Species #3	**Species #4**
V.3.3.1. **Genus & Species:**	*Mus musculus*	*Mus musculus*
V.3.3.2. **Strain/Stock:** V.3.3.3. **Source/Vendor:**	(b)(4)	
V.3.3.4. **Age:**	2 – 8 months	2 – 8 months
V.3.3.5. **Weight:**	20-45 g	20-45 g
V.3.3.6. **Sex:**	**Male**	**Female**
V.3.3.7. **Special Considerations:**	N/A	N/A
V.3.4. **Number of Animals Required (by Species):**	(b)(4)	

	Species #5
V.3.3.1. **Genus & Species:**	*Mus musculus*
V.3.3.2. **Strain/Stock:**	(b)(4)
V.3.3.3. **Source/Vendor:**	

V.3.3.4. **Age**: 2 – 8 months

V.3.3.5. **Weight**: 20-45 g

V.3.3.6. **Sex**: **Male**

V.3.3.7. **Special Considerations**: N/A

V.3.4. **Number of Animals Required (by Species)**: (b)(4)

V.3.5. **Refinement, Reduction, Replacement (3 Rs)**:

V.3.5.1. **Refinement**: Mice will be handled at least 3-4 times a week to reduce stress during experimentation. In addition, mice will be handled at approximately the same time each day. For terminal experiments, the mice will be anesthetized prior to euthanizing. Refinements considered but not used: adjusted early end-point, unless the animals become ill. Under that circumstance, the animals will be euthanized. The decision to euthanize the sick mouse will be in consultation with the PI and the Veterinarian.

(b)(4)

V.3.5.2. **Reduction**: We will conduct the studies using a factorial design where once statistical difference is achieved, we can stop the experiment. This allows us to use the minimum number of mice possible to still be statistically significant. In addition, every effort has also been made to use every tissue part for the current and other studies without compromising the integrity of the experimental design and for not compromising data analysis. We will not use any more animals than needed.

V.3.5.3. **Replacement**: At this time, it is not possible yet to study the impact of the environment on the animal's (b)(4) It is possible that in the future, when more information has been garnered, that we would be able to test behavioral responses, endocrine responses or other physiological responses using computer modeling.

V.4. **Technical Methods**:

V.4.1. **Pain / Distress Assessment**:

V.4.1.1. **APHIS Form 7023 Information:**

V.4.1.1.1. **Number of Animals:**

Species:	(b)(4)
V.4.1.1.1.1. Column C:	
V.4.1.1.1.2. Column D:	
V.4.1.1.1.3. Column E:	

V.4.1.2. **Pain Relief / Prevention:**

V.4.1.2.1. **Anesthesia/Analgesia/Tranquilization:**
Carbon dioxide (for deep anesthesia).
The mice will be deeply anesthetized by CO_2 prior to decapitation or perfusion. These will utilize the CO_2 chambers available at LAM. According to the SOP attached to the LAM CO_2 chambers, the mice will be in standard mouse cages (5L volume) where the flow rate will displace 20% of the chamber volume per minute by introducing 100% CO_2. This translates to 1 L per minute. Deep anesthesia is tested with toe pinch – a lack of response indicated deep anesthesia. In Exp 1, the mice will be decapitated and in Exp 2, the mice will be pericardially perfused.

V.4.1.2.2. **Pre- and Post-procedural (not surgery) Provisions:**
Not applicable.

V.4.1.2.3. **Paralytics:**
Not applicable.

V.4.1.3. **Literature Search for Alternatives to Painful or Distressful Procedures:**

V.4.1.3.1. **Sources Searched:**
MEDLINE, AGRICOLA

V.4.1.3.2. **Date of Search:**
February 25, 2016

V.4.1.3.3. **Period of Search:**
1968 to present

V.4.1.3.4. **Key Words of Search:**
Animal, pain, surgery, analgesia, mice, (b)(4)

V.4.1.3.5. **Results of Search:**

Animal studies are needed to further test the physiological and behavioral significance of any result procured from cell culture studies. There are no alternatives (cell or computer modeling) to study the effect of changes in circadian rhythms on a complex (b)(4) system that is regulated by feedback and through cellular connectivity. The methods described in this proposal are standard procedures that have been used for many decades and have been translated eventually to human studies and drug development.
Based on a literature search:

1. the proposed research is not duplicative of research which has already been done;
2. Alternatives to the use of procedures that may cause more than momentary or slight pain or distress are not appropriate or available. This is to establish an animal model for studying how (b)(4) may alter the (b)(4) system. The 20 min (b)(4) is a commonly used model to test the responsiveness of (b)(4) (b)(4)
3. We have proposed to utilize the appropriate pain management methods in the protocols.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
Not applicable

V.4.2. Prolonged Restraint:
Not applicable

V.4.3. Surgery:
Not applicable

V.4.3.1. Pre-surgical Provisions:

V.4.3.2. Procedure:

V.4.3.3. Post-surgical Provisions:

V.4.3.4. Location:

V.4.3.5. Surgeon:

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures:

V.4.3.6.2. Scientific Justification:

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

(b)(4) DEX [redacted] ug/kg, s.c.; Water soluble DEX is dissolved in saline) or no DEX (vehicle = saline, s.c.) with a needle size range between 22-25 gauge. Up to 2 ml will be administered.

V.4.4.2. Biosamples: Entire organs will be harvested during routine necropsy – brain, pituitary, liver, uterus. Maximum obtainable trunk blood will be obtained after euthanasia.

V.4.4.3. Adjuvants: Not applicable

V.4.4.4. Monoclonal Antibody (MAbs) Production: NA

V.4.4.5. Animal Identification: The animals will be identified on the cage card and when group housed, they will be identified by a non-toxic marker on the tail.

V.4.4.6. Behavioral Studies:

(b)(4) will be used to evaluate anxiety-like behaviors

(b)(4) [redacted]

(b)(4) Animals will be placed in an open field environment (40 cm x 40 cm) and allowed to move freely for up to one hour. (b)(4)

(b)(4) [redacted]

(b)(4) These measures allow conclusions regarding exploration, locomotion and anxiety-like states of the rodents.

(b)(4) to assess depressive behavior. (b)(4)

(b)(4) [redacted]

(b)(4)

V.4.4.7. Other Procedures:

1. Daily vaginal lavage will be conducted using a well-established method described (b)(4) This is a non-invasive method to stage the estrous cycle. Daily vaginal lavage (at least 1-2 weeks is needed to determine the stage of the estrous cycle) is conducted to understand which stage of the estrous cycle the mice were tested on and when samples are collected. Because of the significant hormonal fluctuations throughout the estrous cycle and the impact these ovarian steroids have the HPA axis, this method will be used to monitor estrous cycle stage.
   An exception is to use sterile physiological saline as opposed to ddH_2O. Daily lavages will be conducted for at least 2 consecutive estrous cycles. No anesthesia is required. Briefly, the mouse is firmly grasped by the tail to expose the rear end. The vagina is gently perfused by gently placing about 50 ul physiological saline at the opening of the vaginal canal. Draw the saline back into the tip. Repeat 4-5 times using the same tip. Place the fluid on a glass slide for analysis.

Multiple platform method to induce sleep disruption. The image above is an image for rats. The modification is described in the text.

2. Restraint. The mice will be restrained for 20 min and euthanized 10 or 60 minutes after the end of the restraint. Mice will be restrained using a standard plexiglass restrainer (b)(4)
   (b)(4)
   (b)(4) The mice in these restrainers will be awake, unanesthetized and will have no other procedures conducted on them during this 20-min transient restraint. Restraint will be conducted in a LAM facility. (b)(4)
   (b)(4)

3. Transient sleep disruption. We will utilize the well-established multiple platform method used to induce sleep disruption (b)(4)
   (b)(4)
   (b)(4) Experiment groups will comprise of 3-5 mice per run. All mice will be from the same cage. Mice will be able to move around the container by leaping between platforms.

Briefly, mice will be placed on platforms that are submerged in approximately 1 cm of water during the light hours. Each platform is 3 cm in diameter with each mice having access to the platforms (up to 12 platforms per container, 3 cm in diameter each). Mice allocated to receive the sleep disruption treatment will be acclimated in the chambers for 1 h/day for up to 3 days prior to testing during the active circadian phase. On day of testing, the mice will be placed onto the 3-cm platforms that are immersed in water with 1 cm below the surface. Actual testing will occur during the light hours (12 h) for the mice (inactive period) for up to 3 days. To maintain the water temperature (70-76 F), a water-submersible heater(s) will be placed in the tank. Water temperature will be checked during the testing to ensure the stability of temperature. The water tank will be located in a procedure room in DLAM. Each day of treatment is equivalent to a bad night of sleep. Mice will be able to move around the container by leaping between platforms thus allowing the mice to interact with each other. *This model is unique as it prevents stress by controlling social isolation and preserving social hierarchy.*

4. Handling and weighing. The mice will be handled 3-4 times a week. During handling, they will be weighed.

V.4.4.8. Tissue Sharing: Yes. Excess tissues will be made available to other investigators upon request.

V.4.5. Study Endpoint:

Based on the respective time-points as described, animals will be euthanized by deep carbon dioxide anesthesia followed by decapitation (Exp 1 and 2) or deep carbon dioxide anesthesia followed transcardiac perfusion by physiological saline and buffered 4% paraformaldehyde (Exp 3).

Any mouse that becomes ill, shows a greater than 20% weight loss, becomes lethargic, dehydrated, unkempt, or shows any other clinical signs of a medical problem will be evaluated by the veterinarian and PI (when available). In the event of a debilitating illness or adverse reaction, the PI and the veterinarian will consult on a course of treatment. Mice may be euthanized for humane reasons at the discretion of the veterinarian, but attempts to contact the PI will be made before the mouse is euthanized.

V.4.6. Euthanasia:

All animals will be euthanized by CO_2 anesthesia followed by decapitation or cardiac perfusion with physiological saline and buffered 4% paraformaldehyde. The mice will be deeply anesthetized by CO_2 prior to decapitation. These will utilize the CO_2 chambers available at LAM. According to the SOP attached to the LAM CO_2 chambers, the mice will be in standard mouse cages (5L volume) where the flow rate will displace 20% of the chamber volume per minute by introducing 100% CO_2. This translates to 1 L per minute. Deep anesthesia is tested with toe pinch – a lack of response indicates deep anesthesia.

In Exp 1 and 2, the mice will be decapitated and in Exp 3, the mice will be pericardially perfused.

Briefly, Perfusion will be conducted using standard methods (eg, described in http://www.ebiomethods.com/methods/mouseperfusion) utilizing a butterfly needle (26G) and the sequential perfusion with physiological saline (approximately 20 ml) and buffered paraformaldehyde (approximately 100 ml). CO_2 anesthesia will be conducted according to USU IACUC Policy 013, "Rodent Anesthesia with CO_2 prior to Euthanasia".

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. Routine cage changes will be conducted by the PI's laboratory staff.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Mice will be housed (b)(4) (b)(4) standard light cycle of 12:12 L:D. It is requested that lights go off at 1300 h.

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes ___X___ No _____

Fluid Restriction: Yes _____ No ___X___

V.5.1.3. Exceptions:

Food is restricted during the sleep disruption period (light hours) when mice do not normally eat.

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:

Mice will be observed at least once daily by LAM personnel and by investigative staff. Mice will be weighed at least 2 times a week by investigative personnel. Any mice that becomes ill, shows a greater than 20% weight loss by visual appraisal and by additional weighing if necessary, becomes lethargic, dehydrated, unkempt, or shows any other clinical signs of a medical problem will be evaluated by the veterinarian and PI (when available). In the event of a debilitating the PI and the veterinarian will consult on a course of treatment. Mice may be euthanized for humane reasons at the discretion of the veterinarian, but attempts to contact the PI will be made before the mouse is euthanized.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies.

Mice will be group housed (up to 5 mice per cage) for both male and female mice and all Enrichment Strategies will be conducted in accordance to USU IACUC Policy 008 (Laboratory Animal Exercise and Environmental Enrichment Program). The mice will be given nestlets only as enrichment (Section F under Environmental Enrichment).

V.5.3.2. Enrichment Restrictions: n/a

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Basic Training Course	(b)(6)	Greater than 20 years	(b)(6) University Rodent Handling Course (Taught by Dr. (b)(6) USUHS Investigator Training (between January and June, 2001)
Decapitation Cardiac perfusion Injections Vaginal lavage Anesthesia monitoring	(b)(6)	Greater than 20 years	(b)(6) Euthanasia Course (Taught by Dr. (b)(6) DVM, PhD) (b)(6) USUHS Euthanasia

			Training Course (I don't remember the exact date)
CITI Training	(b)(6)	Completed	9/10/2014
Basic Training Course		4 years	LAM Investigator Training Course (9/2013) (b)(6) Investigator Training Course (9/2011)
Decapitation Injections Anesthesia Monitoring		4 years	LAM Rodent Handling Course (9/2011) and (b)(6) (b)(6) Investigator Training Course (9/2011)
CITI Training		Completed	5/9/2014
Basic Training Course		New	LAM Investigator Training Course and Rodent Handling Course (5/2016)
CITI Training		In progress	
Basic Training Course		1 year	DLAM Investigator Training Course and Rodent Handling Course
Decapitation Cardiac Perfusion		1 year	LAM Rodent Handling Course (March 2012)/Dr. Supervision (b)(6)
Injections		1 year	LAM Rodent Handling Course (March 2012)/Dr. (b)(6)
CITI Training		Completed	9/5/14
Basic Training Course		1 year	DLAM Investigator Training Course and Rodent Handling Course (11/5/15)
Decapitation Cardiac Perfusion		1 year	LAM Rodent Handling Course (11/5/15)/Dr (b)(6) Supervision
Injections		1 year	LAM Rodent Handling Course

			(11/5/15)/Dr. (b)(6)
CITI Training	(b)(6)	Completed	10/21/15
Basic Training Course		Greater than 10 years	LAM Investigator Training Course (2002? – unfortunately we do not remember the month)
Decapitation Cardiac perfusion Injections Vaginal lavage Anesthesia monitoring		Greater than 10 years	LAM Rodent Handling Course (2002 – unfortunately we do not remember the month)
CITI Training		In progress	To be completed

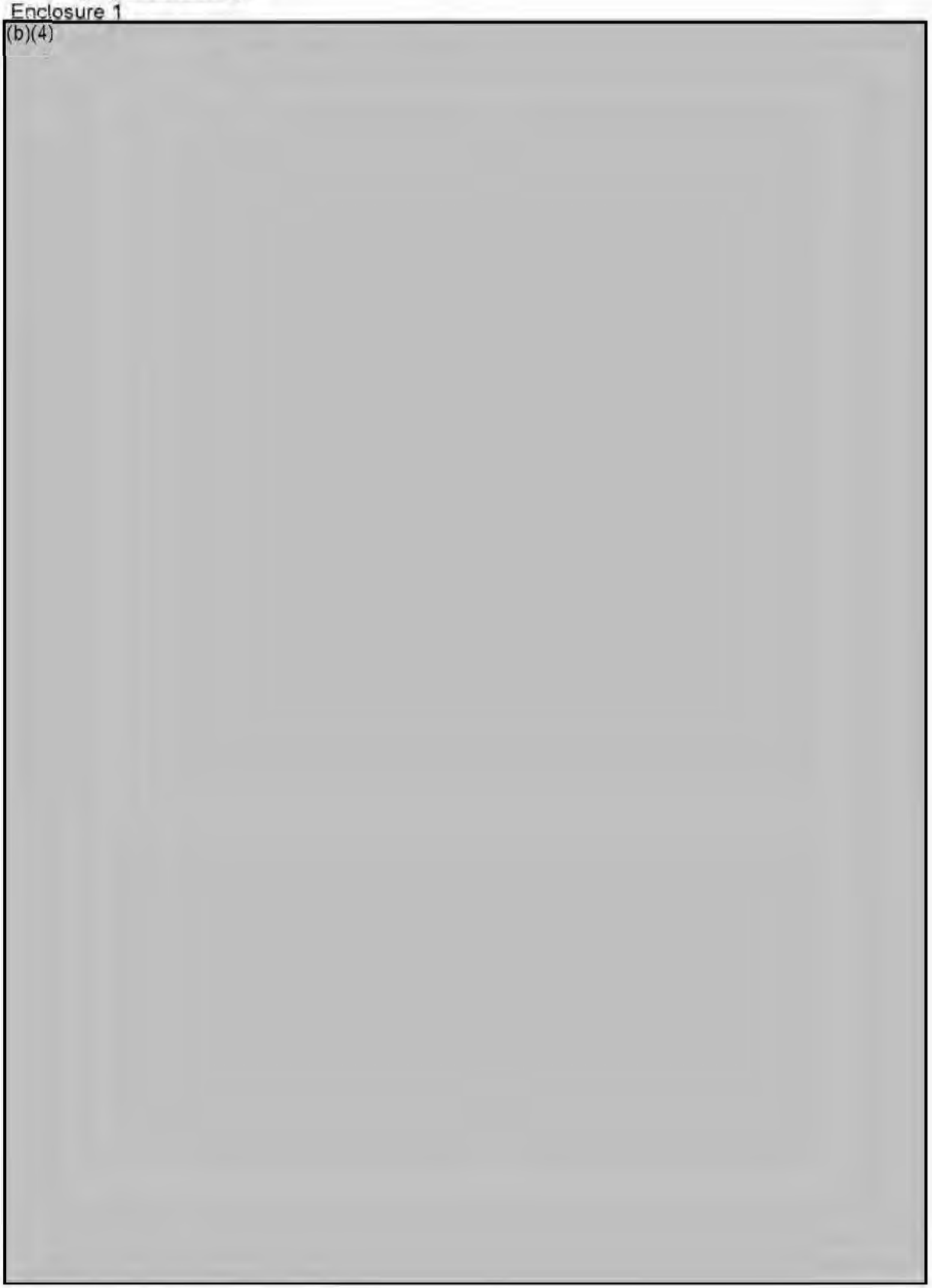
VII. BIOHAZARDS/SAFETY:

A. Zoonotic Disease: No
B. Safety Hazards: No
C. Isoflurane: No
D. Isoflurane Exposure: No
E. Sharp Instruments: No
F. Infectious Agents that do not cause Zoonoses: No

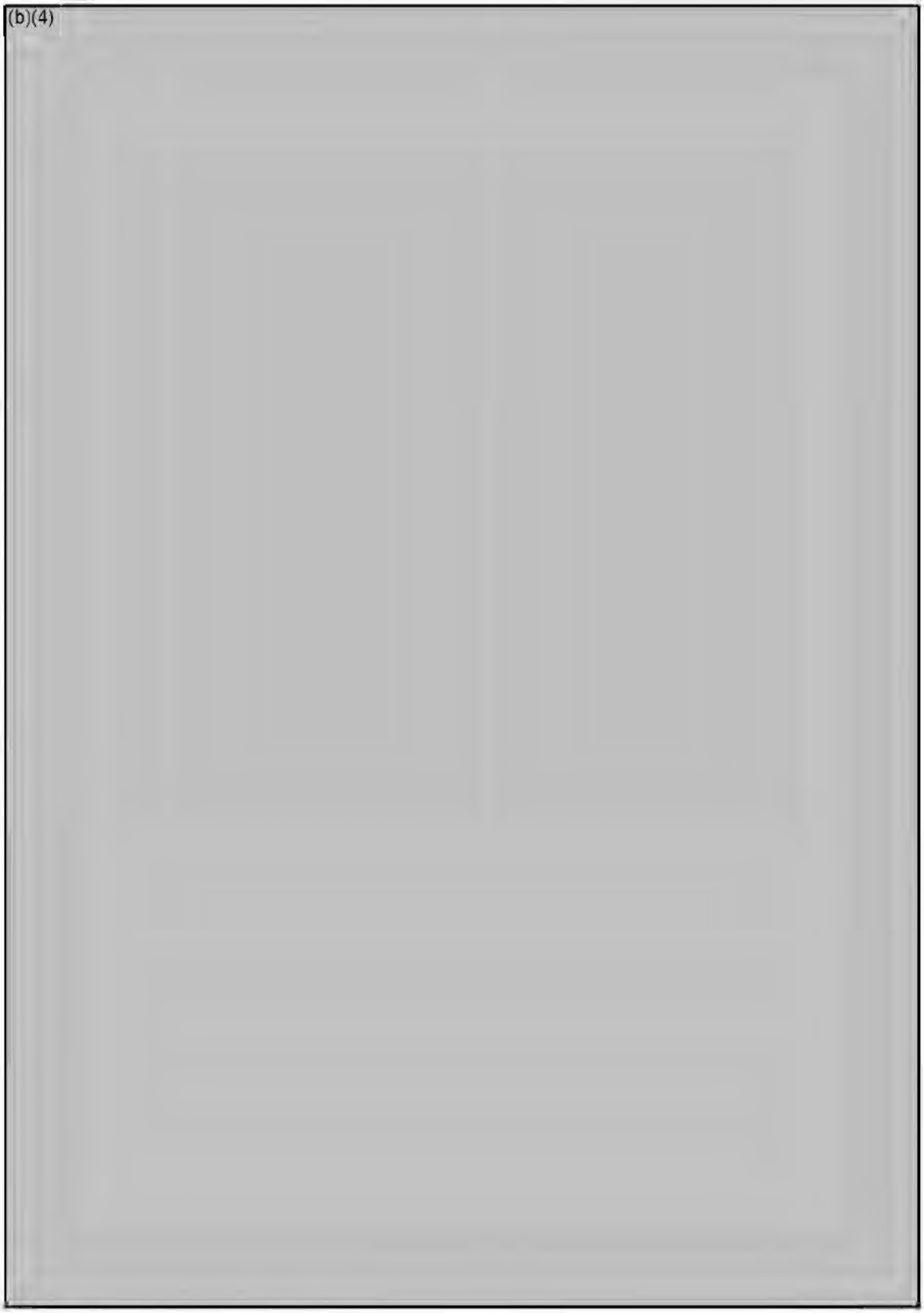
VIII. ENCLOSURES:

Enclosure 1

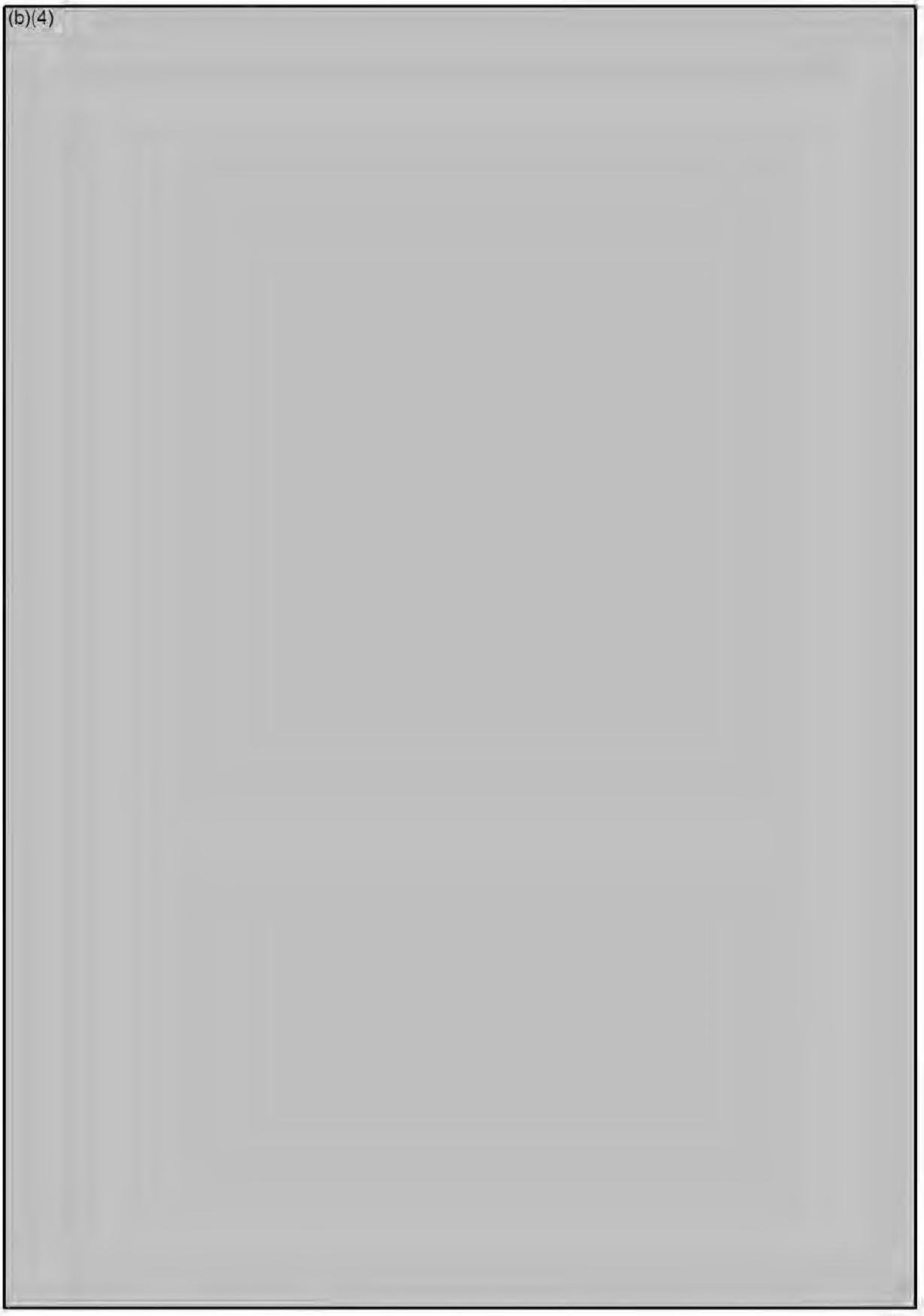
(b)(4)



(b)(4)



(b)(4)



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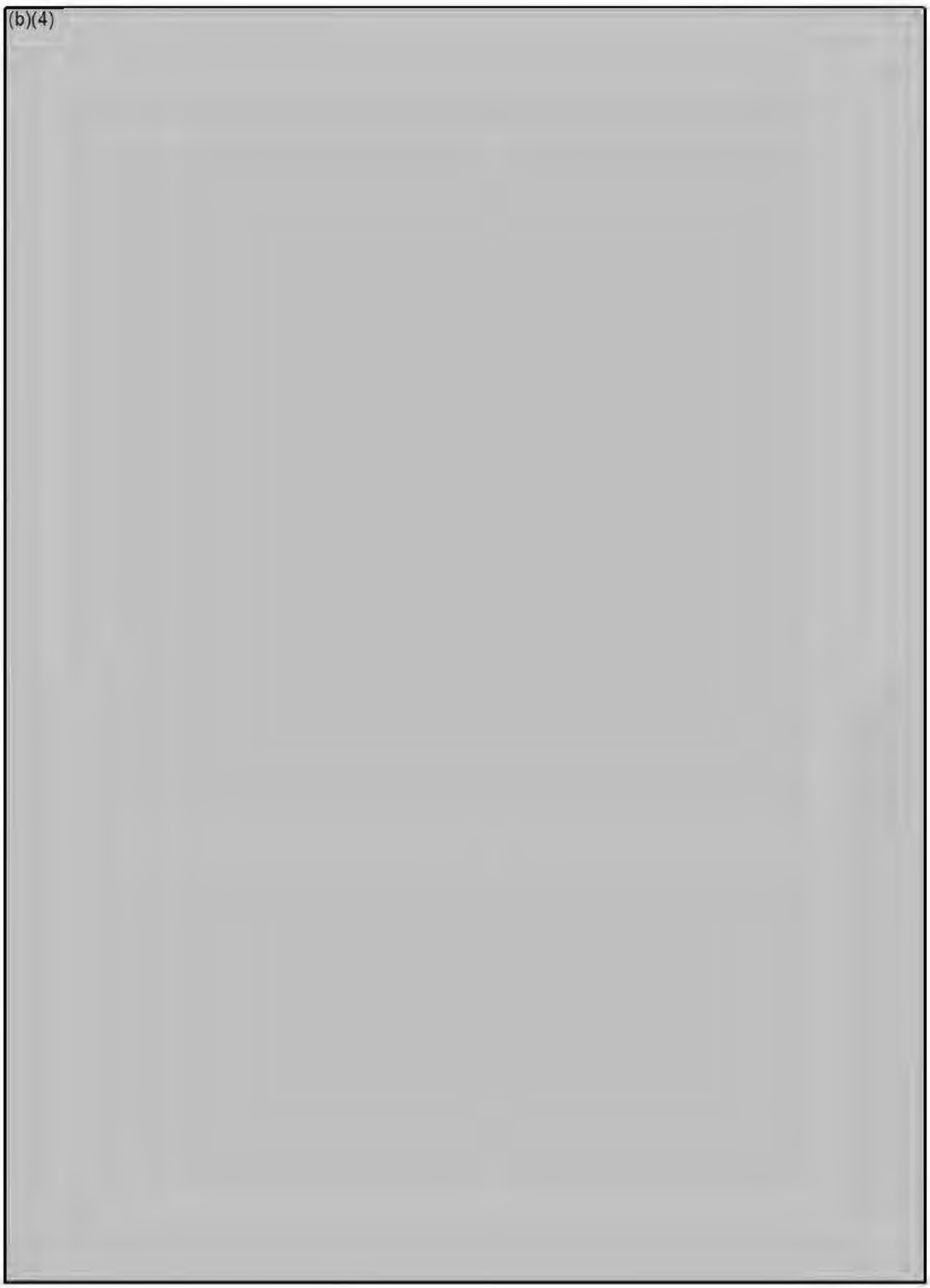

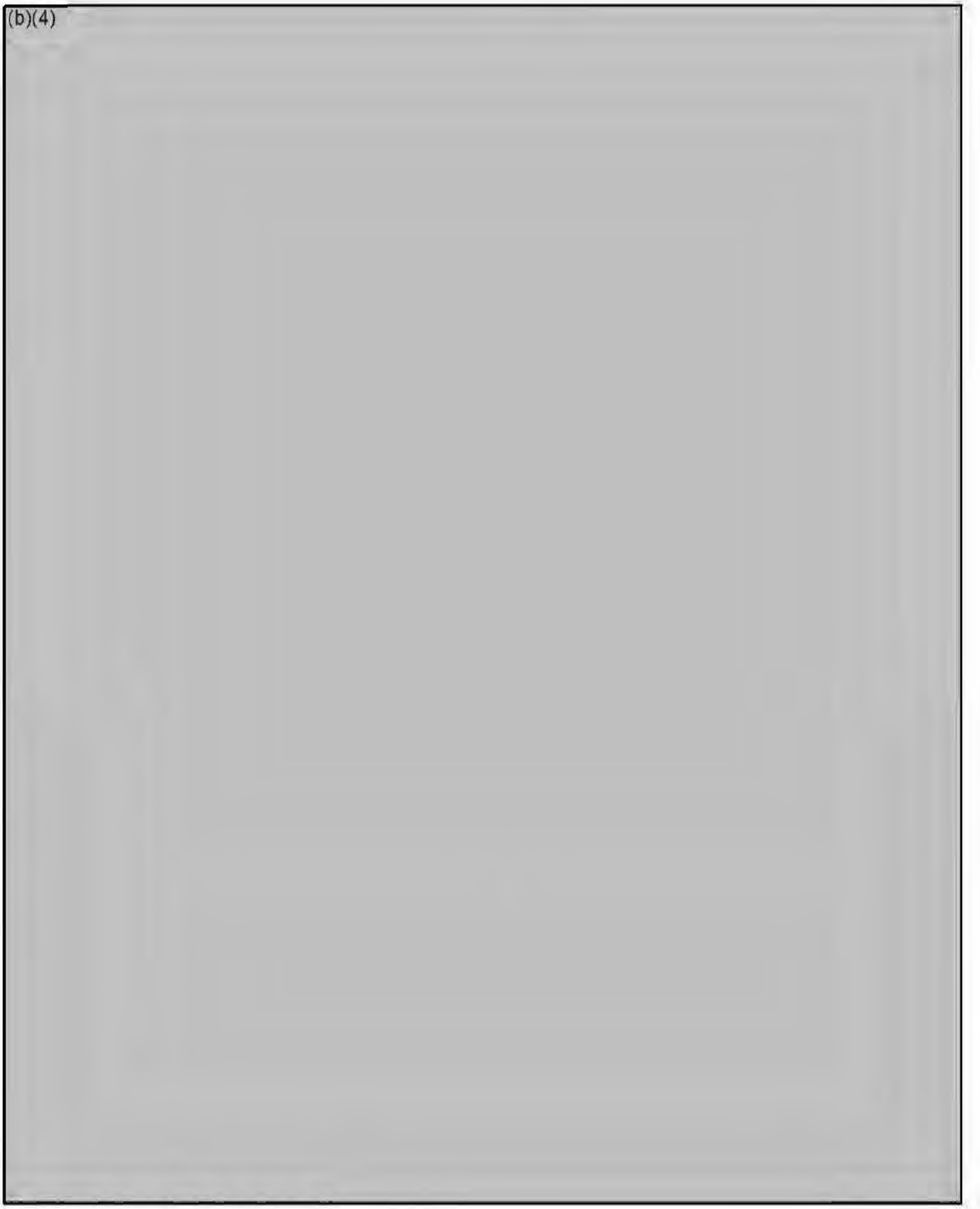
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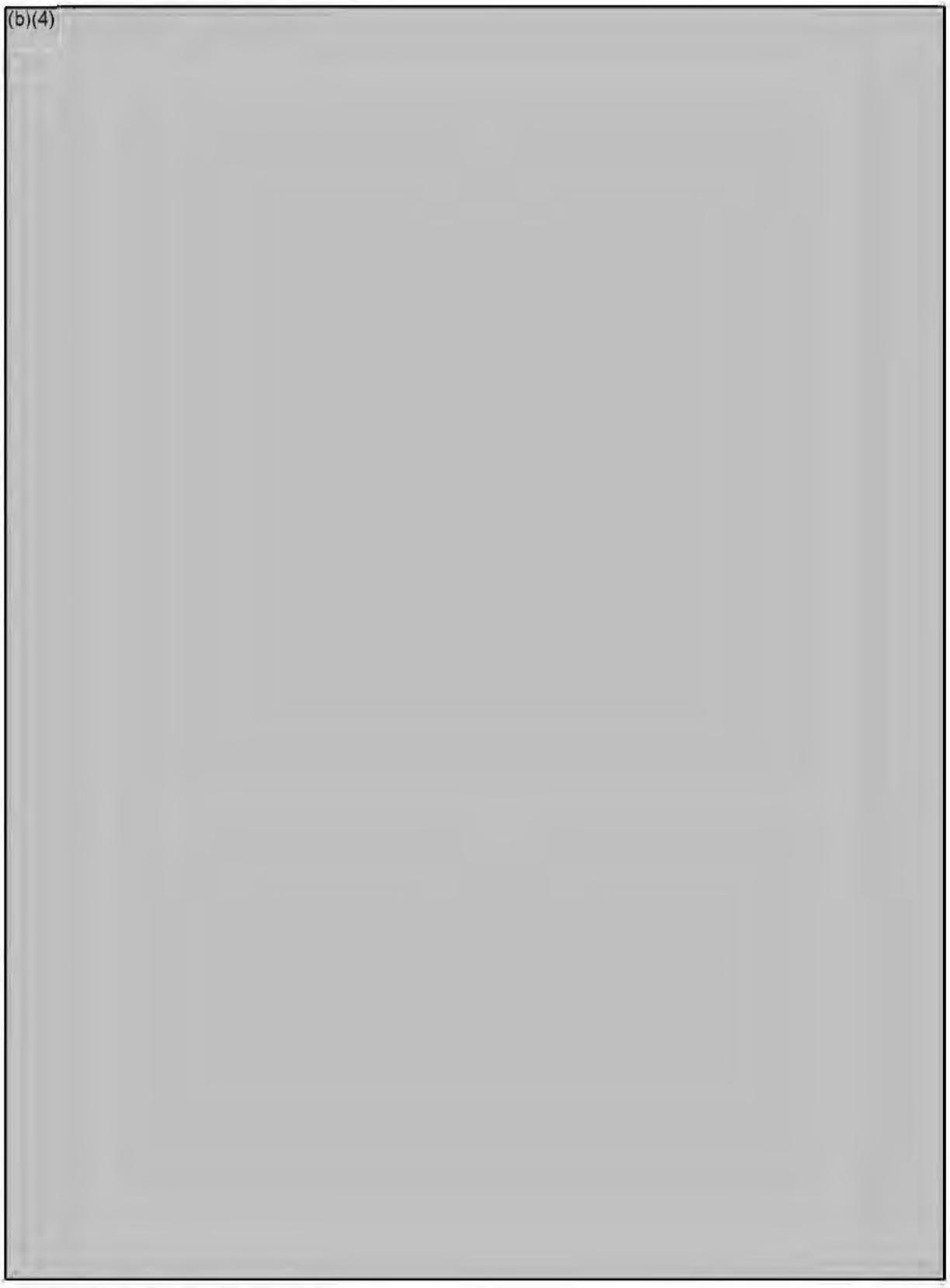
Enclosure 2: Specific Aims Page

(b)(4)



IX. EXTRAMUAL COLLABORATION:

Not applicable

X. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6)

2/26/2016

Principal Investigator Signature | Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure

that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6)

2/26/2016

Principal Investigator Signature | Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

2/26/2016

Principal Investigator Signature | Date

XI. PROTOCOL ABSTRACT:

A. **Animal Protocol Number:** (b)(6)

B. **Animal Protocol Title:**
(b)(4)

C. **Principal Investigator:**
(b)(6)

D. **Performing Organization:**
Uniformed Services University

E. **Funding:**
(b)(4)

F. **Objective and Approach:**
(b)(4)

G. **Indexing Terms (Descriptors):**
(b)(4)

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (JANUARY 2012)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	5/3/2012	2nd Resubmission Date:	6/21/2012
Full Committee Review:	5/31/2012	**Approved**/Approval Withheld by IACUC:	6/26/2012
1st Resubmission Date:	6/5/2012	**EXPIRATION DATE:**	6/25/2015
Approved/**Returned for Revision**:	6/20/2012	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and *subsection*, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Evaluation of Mechanisms and Involvement of Non-targeted Radiation Effects in Radiation and Depleted Uranium Induced Leukemias in mice (*Mus musculus*) (Intramural)

IV. PRINCIPAL INVESTIGATOR: (b)(6) PhD
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **MD, CPT, MC, USN** Date
Head, Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date

Head, Veterinary Sciences Department
Telephone (b)(6) Fax (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department
Telephone (b)(6) Fax (b)(6)
(b)(6)

IX. SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD** Date

Scientific Director
Telephone (b)(6) Fax (b)(6)
(b)(6)

X. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **PhD, CAPT, MSC, USN**

IACUC Chair
Telephone (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: Evaluation of Mechanisms and Involvement of Non-targeted Radiation Effects in Radiation and Depleted Uranium Induced Leukemias in mice (*Mus musculus*) (b)(4)

PRINCIPAL INVESTIGATOR: (b)(6) **PhD**

CO-INVESTIGATOR(S): None

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS

Military personnel are potentially exposed to radiation in a variety of scenarios as well as the radioactive heavy metal depleted uranium (DU). It is known that radiation or heavy metal exposure can induce cancer under certain circumstances. To study these carcinogenic phenomena we need to use a murine model. It is therefore important to characterize the carcinogenic effects of these exposures in this murine model to better understand carcinogenic mechanisms and to enable targeted development of safe and efficacious cancer chemoprevention for military personnel using these models. The overall research objective of this proposal is to characterize the ^{60}Co gamma radiation- and depleted uranium (DU) - induced leukemia mouse models used in our laboratory by examining two possible mechanisms i.e., epigenetic (methylation), and genetic (genomic instability). These results, in conjunction with *in vitro* results evaluating non-targeted radiation effects (NTE) will assist in the characterization of these two induced leukemias. This mechanistic information will assist us in developing more effective and safer late radiation effects countermeasures.

II. BACKGROUND

II.1. Background

Radiation health risks of concern to the Department of Defense (DOD) include carcinogenesis and particularly, leukemia. Military personnel can potentially be exposed to a variety of radiation qualities including gamma, neutron, and protons (astronauts) depending on the type of military operation. Internal contamination from military relevant heavy metals like depleted uranium (DU), an alpha particle emitter, during military operations is a potential threat to military personnel as well (1). The health risks of internally deposited alpha-emitters like uranium remain a question for military personnel since it is both a chemical and radiation hazard (2, 3). Our laboratory has demonstrated using cellular models, that the alpha radiation from DU does play a role in the induction of biological damage and neoplastic transformation (4-16). Development and characterization of an effective "late effects" *in vivo* model is critical to a better understanding of radiation- or DU- induced leukemogenic effects and to the development of countermeasures.

The overall goal of this project is to characterize the ^{60}Co- and DU- induced leukemia mouse models used in our laboratory by examining three possible mechanisms. These

include 1) epigenetic, i.e. methylation, 2) genetic, i.e. genomic instability, and 3) non-targeted radiation effects (NTE). The leukemia model used in our studies is unique in comparison to other radiation leukemia mouse models because immature (non-tumorigenic) hematopoietic cells (FDC-P1) are injected into a ^{60}Co-irradiated (17-19) or DU-exposed mice (4, 6, and 7) and the development of acute myeloid leukemia (AML) occurs within 4 to 8 months (4, 6, 7, 17-20). This is in contrast to the 14-18 months in standard murine leukemia models that also require significantly more mice for statistical significance.

A complex picture of radiation leukemogenesis has emerged however in which, additional to any damage induced directly in target stem cells, the hemopoietic microenvironment can be a source of damaging signals and cellular interactions which are involved in the cancer development process (21, 22). To study the process of radiation leukemogenesis, specifically from the standpoint of the hematopoietic microenvironment, we have selected a novel murine model for the development AML which allows an analysis of the indirect effects of irradiation or DU exposure separate from the mutagenic effects on hematopoietic cells (17-19). In this model, murine hematopoietic stem cells of the IL-3-dependent line, FDC-P1, consistently transform to AML when injected into DBA/2 mice irradiated with 1 to 3.5 Gy gamma radiation (17-19) or exposed to internalized DU (4, 6-7). This leukemia model, in which non-tumorigenic immature hematopoietic cells are introduced into an irradiated or DU exposed bone marrow environment, and followed by the development of leukemia, suggests that it could be characterized as a "non-targeted effects" (NTE) leukemia model. In radiation biology, the "Linear No Threshold" hypothesis has been challenged by a large number of observations of the so-called NTE, i.e., genomic instability, bystander effects, which consists of the induction of damage in cells not directly traversed by radiation, most likely as a response to molecular messengers released by directly irradiated cells (23, 24). This DBA/2/FDC-P1model presents the unique opportunity to characterize a leukemia model in which NTEs like genomic instability can be evaluated.

(b)(4)

AML is a clonal disorder resulting from uncontrolled proliferation of hematopoietic progenitor or stem cells (25) and transformation into a leukemic cell is considered a multistep process implicating both genetic and epigenetic aberrations (25-28). The most important genetic findings in AML are chromosomal translocations involving different transcription factors and activating point mutations in multiple signal transduction pathways (28). Epigenetic disturbances, such as aberrant promoter hypermethylation and covalent histone modifications, have been implicated in the pathogenesis of human leukemia as well (29-32). Signaling pathways such as the Wnt and CDK pathways are involved in hematopoietic malignancies. The Wnt signaling pathway has a key function in stem cell maintenance and differentiation of hematopoietic progenitors. Secreted Frizzled-related protein genes (SFRPs), functioning as Wnt signaling antagonists, have been found to be down-regulated by promoter hypermethylation in AML and have been linked to genetic instability as well (29). This methylation controlled silencing of the Wnt gene is critical in AML development. Another pathway disrupted by aberrant methylation in human AML involves the cyclin dependent kinase inhibitor pathway with the cycle inhibitors p16 (CDKN2A or p16INK4a) and p15 (CDKN2B or p15INK4b) (30-31). These pathways and their methylation status have not been examined in the DBA/2/FDC-P1leukemia models (^{60}Co or DU) and the data from human studies suggest that these pathways would be a good target to evaluate in leukemogenesis in the radiation- or DU-murine models

As stated above, the overall objective of this study is to fill the gap in our understanding of ^{60}Co- or DU- leukemia risks, by focusing on the mechanisms of these induced leukemias. In this project we will concomitantly use *in vitro* studies to address whether NTEs could be involved in ^{60}Co- or alpha particle- induced hematopoietic cells transformation and whether cell-cell communication or reactive oxygen species (ROS) are involved in the NTE. The *in vivo* studies described in this animal use protocol with the murine model will assess whether methylation and genomic instability are involved in the development of ^{60}Co- or DU- induced leukemia. The data collected in this proposal would also assist in determining whether this model can be used to develop biological countermeasures to radiation or DU late effects.

(b)(4)

General Study Design:

Section A. Objective/Hypothesis/Specific Aims

The overall goal of this project is to initiate a characterization of the ^{60}Co- and DU-induced leukemia mouse models used in our laboratory by examining possible

mechanisms, i.e. DNA methylation changes and genomic/chromosomal instability and evaluating whether non-targeted radiation effects (NTEs) may be involved.

Hypotheses and Specific Aims:
Hypothesis# 1: A mechanism of gamma or alpha particle leukemogenesis involves a NTE on hematopoietic stem cells through an initial radiation-induced alteration of supportive bone marrow stromal cells. (*In vitro* studies)
Hypothesis #2: The induction of leukemia by gamma radiation or DU exposure is associated with altered DNA methylation in spleen and bone marrow that occurs within 1 month after exposure. (*In vivo* studies).
Hypothesis #3: The induction of leukemia by gamma radiation or DU exposure is associated with hematopoietic stem cell genetic instability that occurs within one month after exposure. (*In vivo* studies).

The following specific aims are designed to address these hypotheses.

Specific Aim #1: NO ANIMALS INVOLVED.
To determine whether ^{60}Co or alpha particle radiation exposure of murine bone marrow stromal cells (cell line) induces malignant transformation of un-irradiated co-cultivated hematopoietic stem cells.

Specific Aim #2: NO ANIMALS INVOLVED.
To determine whether the induction of malignant transformation of un-irradiated hematopoietic stem cells co-cultivated with murine bone marrow stromal cells (cell line) irradiated with ^{60}Co or alpha particle radiation involves cell-cell communications.

Specific Aim #3: NO ANIMALS INVOLVED
To determine whether the induction of malignant transformation of un-irradiated hematopoietic stem cells co-cultivated with murine bone marrow stromal cells (cell line) irradiated with ^{60}Co or alpha particle radiation involves reactive oxygen species (ROS).

Radiation Safety Issues: This protocol will involve the use of depleted uranium (DU). DU is a radioactive heavy metal. (b)(4),(b)(6)

(b)(4),(b)(6)

(b)(4)

Specific Aim #4/Experiment 1: ***To examine DNA methylation in mouse hematopoiesis before and after in vivo exposure to a leukemogenic exposure of dose of gamma radiation or internalized DU exposure and evaluate whether DNA methylation status has a role in gamma radiation or internalized DU exposure leukemogenesis.***

Experiment #1 will assess methylation in mouse hematopoiesis following gamma radiation or DU exposure. Animals will be either exposed to ^{60}Co radiation (total body irradiation) or DU implants (low and high dose implanted in the hind limb). The methylation studies are divided into two parts, a) to evaluate the global genomic content of 5-methylcytosine in spleen and bone marrow and b) to examine the methylation status of specific genes (Wnt family antagonists, SFRP1, -2, -4, -5, p15, p16), in spleen and bone marrow of ^{60}Co or DU- exposed mice. Methylation status following exposure to a leukemogenic dose of ^{60}Co (0.05, 2.0, 3.5 Gy, 0.6 Gy/min) or implanted DU (2 DU + 4 tantalum (Ta) pellets or 6 DU pellets, 1 month exposure) will be examined in bone marrow and spleen. Immediately following radiation exposure or at 1 month post-DU implant, mice will be tail-vein injected with hematopoietic stems cells (FDC-P1 cells). FDC-P1 cells are obtained from ATCC and they are certified to be pathogen free. Cells (2 to 5 E6 cells) will be tail-vein injected (27 gauge needle) in a volume of 100 to 200 ul of sterile saline. FDC-P1 cells are non-tumorigenic hematopoietic stem cells that were derived from DBA/2 mice (same mice used in this study). They are maintained in cultured using IL-3 as a growth supplement. Sterile IL3 is obtained from BD labs and added to the Dulbecco's media used to culture these cells.

Seven days after cell injection animals will be euthanized. Spleen and bone marrow will be obtained for analysis. Spleen and bone marrow tissues previously obtained from ^{60}Co- and DU- induced leukemic mice will be used for comparison. For Aim 4a, global DNA methylation in bone marrow and spleen will be measured by direct analysis of the 5-methylcytosine content of DNA and by Southern blot analysis of genomic repeat sequences. In Aim 4b, dysregulation of the Wnt signaling antagonists also known as secreted frizzled-related proteins (SFRPs) and cyclin-dependent kinase pathway proteins, p15Ink4b and p16Ink4b will be examined using real-time reverse transcription PCR (RT-PCR) in the same samples analyzed for methylation.

Specific Aim #5/Experiment 2: ***To assess genomic/chromosomal instability by examining chromosomal aberrations in mouse ^{60}Co radiation- and DU- induced leukemia's and the clonal descendants of ^{60}Co- irradiated and DU-exposed bone marrow hematopoietic stem cell and compare to controls.***

Experiment#2 will assess chromosomal/genomic instability following radiation or DU exposure. The induced genetic instability should be detectable in the irradiated target untransformed hematopoietic stem cell population and the clonal ^{60}Co- or DU- induced

leukemia by measuring chromosomal aberrations in leukemic and sub-clonal variants. Bone marrow from animals exposed to ^{60}Co radiation at 3 doses (0.5, 2.0, and 3.5 Gy at 0.6 Gy/min) or implanted DU (2 DU + 4 tantalum (Ta) pellets or 6 DU pellets, 1 month exposure) will be evaluated. Immediately following radiation exposure or at 1 month post-DU implant, mice will be injected with hematopoietic stems cells (FDC-P1 cells). At 7 days post-cell injection, animals will be euthanized. Spleen and bone marrow will be obtained for analysis. Spleen and bone marrow tissues previously obtained from ^{60}Co- and DU- induced leukemic mice will be used for comparison. A comparison to un-irradiated/non-implanted controls will also be done. Fluorescence in situ hybridization (FISH) will be used to screen for chromosomal aberrations in ^{60}Co- or DU- induced leukemias (obtained from previous studies/frozen spleen samples) and in the clonal descendants of control, irradiated, or DU-exposed bone marrow hematopoietic stem cells at 7 days post-radiation/cell injection or 7 days post-DU (30 days)/cell injection, using the *in vitro* clonogenic CFU-A colony assay.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

1) Biomedical Research Database (BRD); 2) Pubmed; 3) RePORTer replaced Retrieval of Information of Scientific Projects (CRISP); 4) Federal Research in Progress (FEDRIP).

II.2.2. Date of Search

BRD **Jan 20, 2012**
Pubmed **Jan 3, 4, 20, 2012**
RePORTer **Jan 5, 20, 2012**
FEDRIP **Jan 5, 20, 2012**

II.2.3. Period of Search

BRD **1970-2012**
Pubmed **1980-2012**
RePORTer **1980-2012**

II.2.4. Key Words of Search

DBA/2, mice, male, female, radiation, depleted uranium, tantalum. Epigenetics, genetic, methylation, DNA damage, cytosine extension assay, LD_{50}, radiation carcinogenesis, leukemia, leukemogenesis, toxicity, animal models, survival, lifespan, pain, suffering.

II.2.5. Results of Search

The searches demonstrated that there have been no previous or current studies that have investigated or are in the process of studying the type of mechanism involved in DU-induced carcinogenic effects in a mouse model. There are studies evaluating the

effects of radiation on leukemia development but they are focusing on a different radiation quality, primarily protons and heavy ions, found in space radiations. Secondly, these mouse models are different than the model I will be using which allows us to examine the role of non-targeted radiation effects. The other studies on radiation leukemia do not evaluate non-targeted radiation effects. The searches demonstrated that there have been no previous or current studies that have investigated or are in the process of studying the extent of DNA damage, methylation status, histone status, or gene expression (p15 or p16) in a DU animal model. Therefore the proposed exposure study evaluating epigenetic processes *in vivo* is not a duplication of current or previous experiments. In terms of specific science issues related to DU-carcinogenesis, the searches revealed the following: 1) that the DBA/2 mouse has been used in carcinogenesis studies; pathology, histology, behavior, morbidity, and mortality are well known [19-20]. 2) the background section above provides a summary of the DU publications regarding their transforming, mutagenic, genotoxic, and carcinogenic characteristics; 3) there is one publication on DU and methylation/epigenetics [7] (PI of this protocol is the first author of that publication).

III. OBJECTIVE / HYPOTHESIS

The overall goal of this project is to initiate a characterization of the ^{60}Co- and DU-induced leukemia mouse models used in our laboratory by examining two possible mechanisms, i.e. methylation and genomic instability. The third mechanism, evaluating whether non-targeted radiation effects (NTEs) *in vitro* may be involved, is conducted with *in vitro* models only. In this animal use protocol, we will compare the methylation status and genomic instability in animals exposed to DU for 1 month to those animals exposed to DU (7 months) that developed leukemia. We will also be able to compare the methylation status/genomic instability in animals exposed to ^{60}Co to those animals exposed to ^{60}Co that developed leukemia.

IV. MILITARY RELEVANCE

Military personnel could potentially be exposed to ionizing radiation or DU during the course of their duties. (b)(4)

(b)(4)

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

General Approach: For the DU experiments, animals will be internally exposed to DU, (high and low doses) for 30 days, injected with non-tumorigenic hematopoietic stem cells (FDC-P1) and at 7 days post-cell injection, euthanized. The spleen and bone marrow will be obtained for analysis of epigenetic (methylation changes, gene silencing) and genetic alterations (genetic instability). These samples will be contemporaneously compared to samples previously collected (and stored/frozen) from DU-exposed mice diagnosed with leukemia. For the ^{60}Co experiments, animals will be irradiated and at 7 days post-radiation/cell injection, will be euthanized. The spleen and bone marrow will be obtained for analysis of epigenetic (methylation changes, gene silencing) and genetic alterations (genetic instability). These samples will be compared to samples previously collected from ^{60}Co exposed mice diagnosed with leukemia.

V.1.1. Experiment 1 – Aim 4 of Intramural Proposal

Experiment 1: ***To examine DNA methylation in mouse hematopoiesis before and after in vivo exposure to a leukemogenic exposure of dose of gamma radiation or internalized DU exposure and evaluate whether DNA methylation status has a role in gamma radiation- or internalized DU exposure- leukemogenesis.***

Hypothesis Experiment 1: The induction of leukemia by ^{60}Co radiation or DU (embedded exposure) in this murine model is associated with altered DNA methylation status in spleen and bone marrow that occurs within 1 month post exposure.

Rational Experiment 1: Previous studies have demonstrated that hypomethylation is associated with DU-induced leukemia and human leukemia (7, 21-23).

Methodology Experiment 1: Experiment #1 will assess methylation in mouse hematopoiesis following gamma radiation or DU exposure. The methylation studies are divided into two parts, a) to evaluate the global genomic content of 5-methylcytosine in spleen and bone marrow and b) to examine the methylation status of specific genes (Wnt family antagonists, SFRP1, -2, -4, -5, p15, p16), in spleen and bone marrow of ^{60}Co or DU- exposed mice. Methylation status following exposure to a leukemogenic dose of ^{60}Co (0.5, 2.0, 3.5 Gy, 0.6 Gy/min) or DU (2, 6 pellets, 1 month exposure) will be examined in bone marrow and spleen. Immediately following radiation exposure or at 1 month post-DU implant, mice will be injected with hematopoietic stems cells (FDC-P1 cells). FDC-P1 cells are obtained from ATCC and they are certified to be pathogen free. Cells (2 to 5 E6 cells) will be tail-vein injected (27 gauge needle) in a volume of 100 to 200 ul of sterile saline. Seven days after cell injection animals will be euthanized. Spleen and bone marrow will be obtained for analysis. Bone marrow will be obtained from both femurs. Spleen and bone marrow tissues previously obtained from ^{60}Co- and DU- induced leukemic mice will be used for comparison. These mice were diagnosed approximately 4 to 12 months post exposure. For Aim 4a, global DNA methylation in bone marrow and spleen will be measured by direct analysis of the 5-methylcytosine content of DNA and by Southern blot analysis of genomic repeat sequences. In Aim 4b, dysregulation of the Wnt signaling antagonists also known as secreted frizzled-related proteins (SFRPs) and cyclin-dependent kinase pathway proteins, p15Ink4b and p16Ink4b will be examined using real-time reverse transcription PCR (RT-PCR) in the same samples analyzed for methylation. Bone marrow and spleen of control, ^{60}Co-irradiated mice (0.5, 2.0, and 3.5 Gy doses; 0.6 Gy/min), and internalized DU-exposed

(2 DU + 4 Tantalum (Ta) pellets, 6 pellets + No Ta; 1 month) mice will be assessed. Table 1 details the control and exposure groups. Groups A, B, C, and D are control groups (± FDC-P1 cell injection ± ^{60}Co or DU exposure) and will be immediately euthanized following exposure. Group E will be ^{60}Co exposed, injected with FDC-P1 cells and euthanized at 7 days post-cell injection. Group F (un-irradiated mice) will be injected with ^{60}Co-irradiated FDC-P1 cells and euthanized at 7 days post-cell injection. Group G will be DU exposed (30 days), injected with FDC-P1 cells and euthanized a 7 days post-cell injection. For group H, FDC-P1 cells will be exposed to DU-uranyl nitrate (1, and 100 uM, 4 hrs) prior to injection into non-DU exposed mice and euthanized at 7 days. Following euthanasia, tissues will be prepared for methylation analyses. To accomplish Exp 1a global DNA methylation will be measured in bone marrow and spleen by direct analysis of the 5-methylcytosine content of DNA and by Southern blot analysis of genomic repeat sequences. In Exp 1b, dysregulation of the Wnt signaling antagonists also known as secreted frizzled-related proteins (SFRPs) and cyclin-dependent kinase pathway proteins, p15Ink4b and p16Ink4b will be examined using real-time reverse transcription PCR (RT-PCR) in the same samples analyzed for 5-methylcytosine analysis. Frozen spleen tissues and bone marrow cells obtained in a previous study of ^{60}Co and DU leukemia will be concomitantly used to evaluate bone marrow and spleen samples from animals diagnosed with leukemia. These animals were generally 5-8 months post radiation or DU exposure. These tissues are from our previous studies here at AFRRI and will not be brought into VSD; they are currently stored in my laboratory. No tissue sharing agreement is necessary since they are tissues from my previous studies in which I was also the principal investigator.

Table 1 is on the next page for ease of reading.

Table 1 – Experiment 1. Exposure and Control Groups

	Status of FDC-P1 Cells (± Inj or ± Rad or ± DU)	Status of Mouse ± Rad or DU	Euthanasia	Number of animals
A	No Cell Injection	No Rad/No DU	Day 0	5
B	With Cells Injected	No Rad/No DU	Day 7	5
C	No Cell Injection	With Mouse ^{60}Co Rad	Day 7	15 (5 ea @ 3 doses 0.5 Gy, 2 Gy, 3.5 Gy)
D	No Cell Injection	With Mouse DU Implant	Immediately post-37 Days DU	10 (5 ea @ 2 doses; 2 DU, 6 DU pellets)
E	With Cells Injected	With Mouse ^{60}Co Rad	7 days post-rad	15 (5 ea @ 3 doses 0.5 Gy, 2 Gy, 3.5 Gy)
F	With ^{60}Co Irradiated Cells Injected	No Mouse Rad	7 days post- cell rad	15 (5 ea @ 3 doses 0.5 Gy, 2 Gy, 3.5 Gy)
G	With Cells Injected	With DU Implant	37 days post DU implant (7 days post cell injection)	10 (5 ea @ 2 doses; 2 DU, 6 DU pellets)
H	With DU Exposed Cells Injected	No DU Implant	7 days post cell injection exposure to cells	10 (5 ea @ 2 doses, 1, 100 uM)
I	With cells Injected	No Rad No DU + Tantalum	37 days post Ta implant (7 days post cell inject)	5 (5 ea @ 6 Ta)

This aim will allow us to determine if methylation alterations are involved in ^{60}Co-or DU-exposure and with ^{60}Co-, or DU- induced leukemia in this unique leukemia model. There will be 5 animal tissues per dose point. Bone marrow cellularity and spleen weight will be analyzed as well.

V.1.2. Experiment 2 - Aim 5 of Intramural proposal: *To assess genomic instability by examining chromosomal aberrations in mouse ^{60}Co radiation- and DU-induced leukemia's and the clonal descendants of ^{60}Co- irradiated and DU-exposed bone marrow hematopoietic stem cell and compare to controls.*

Hypothesis Exp 2: Following ^{60}Co- or DU- exposure, genetic instability occurs within 1 month post exposure.

Rationale Experiment 2: Published studies have shown that DU induces genomic instability *in vitro* (2, 5, and 13) and that gamma-induced leukemia is associated with chromosomal instability (22-24). Both indicate genetic damage.

Methodology Experiment 2 To address this hypothesis, we intend to employ fluorescence *in situ* hybridization (FISH) to screen for chromosomal aberrations in mouse ^{60}Co- and DU- induced leukemias (obtained from frozen samples) and in the clonal descendants of control, ^{60}Co-irradiated bone marrow hematopoietic stem cells

(0.5, 2.0, and 3.5 Gy doses; 0.6 Gy/min), and internalized DU-exposed (either 2 DU + 4 Ta pellets or 6 DU + No Ta pellets; 1 mo.) bone marrow hematopoietic stem cells using the *in vitro* clonogenic CFU-A colony assay. The induced genetic instability should be detectable in the gamma irradiated or DU-exposed untransformed hematopoietic stem cell population and the clonal radiation-, or DU- induced leukemia by measuring chromosomal aberrations in leukemic and sub-clonal variants. Groups A, B, C, and D are control groups (± FDC-P1; ± ^{60}Co or DU) and will be euthanized at 7 days post exposure. Group E will be ^{60}Co exposed, injected with FDC-P1 cells and euthanized at 7 days post-cell injection. Group F (un-irradiated mice) will be injected with ^{60}Co-irradiated FDC-P1 cells and euthanized at 7 days post-cell injection. Group G will be DU exposed (30 days, either 2 DU + 4 Ta pellets or 6 DU + No Ta pellets) and will be injected with FDC-P1 cells; euthanasia will occur at 7 days post-cell injection. For group H, FDC-P1 cells will be exposed to DU-uranyl nitrate (1, and 100 uM, 4 hrs) prior to injection into non-DU exposed mice and euthanized at 7 days. Following euthanasia, bone marrow will be collected from both femurs. Bone marrow cells will be obtained by flushing the femurs. FISH will be used to screen for chromosomal aberrations in the clonal descendants of control-, irradiated, or DU exposed- bone marrow hematopoietic stem cells at 7 days post-radiation or 7 days post-pellet removal and in ^{60}Co- or DU-induced leukemias (obtained from previously frozen bone marrow cell samples), using the *in vitro* clonogenic CFU-A colony assay to assess genetic instability (30-34). Seven days post-radiation was selected since radiation or DU exposure may induce cell cycle arrest. Bone marrow cells from control, irradiated, or DU-exposed mice will be cultured *in vitro* for 6 to 8 days post-euthanasia to generate CFU-A colonies as described (36-40) and will then be selected and fixed for FISH analysis (36-40).

Table 2 is on the next page for ease of reading.

Table 2 – Experiment 2. Exposure and Control Groups

	Status of FDC-P1 Cells (± Inj or Rad)	Status of Mouse ± Rad or DU	Euthanasia	No animals
A	No Cell Injection	No Rad/No DU	Day 0	5
B	With Cells Injected	No Rad/No DU	Day 7	5
C	No Cell Injection	With Mouse ^{60}Co Rad	7 Days post-rad	15 (5 ea @ 3 doses 0.5 Gy, 2 Gy, 3.5 Gy)
D	No Cell Injection	With Mouse DU Implant	Immediately post-37 Days DU	10 (5 ea @ 2 doses; 2 DU, 6 DU pellets)
E	With Cells Injected	With Mouse ^{60}Co Rad	7 Days post-rad	15 (5 ea @ 3 doses 0.5 Gy, 2 Gy, 3.5 Gy)
F	With ^{60}Co Irradiated Cells Injected	No Mouse Rad	7 Days post-cell injection	15 (5 ea @ 3 doses 0.5 Gy, 2 Gy, 3.5 Gy)
G	With Cells Injected	With Mouse DU Implant	37 Days post-DU implant	10 (5 ea @ 2 doses; 2 DU, 6 DU pellets)
H	With DU Exposed Cells Injected	No Mouse DU	7 Days post-DU cell exposure	10 (5 ea @ 2 doses; 1 , 100 uM)
I	With Cells Injected	No DU No Rad + Tantalum	37 days post Ta implant (7 days post cell inject)	5 (5 ea @6 pellets)

Table 3.
Research Design Tables
(Table shows number of mice needed per Experiment)

Exp 1	Exp 2
90	90

General Methods:

Mice

Six- to 10- week old male DBA/2 mice will be purchased from the appropriate vendor and be housed (5 per cage) in an air-conditioned facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. The mouse facility room is maintained at 64-79°F with 10-15 hourly cycles of fresh air and a relative humidity of 30 to 70%. The mice will be held in quarantine for two weeks upon arrival. Microbiology, serology, and histopathology examination of representative samples will ensure the absence of *Pseudomonas aeruginosa* and common murine diseases. Mice will receive certified rodent rations (Harlan Teklad Rodent Diet, Harlan

Teklad, WI) and acidified water (with HCL, pH 2.5-2.8) *ad libitum*. All mice will be kept in rooms with a 12h light/dark cycle with lights on from 0600 to 1800. All animal procedures will be performed based on a protocol approved by the AFRRI Institutional Animal Care and Use Committee. Research will be conducted according to the most recent *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources, National Research Council, and US National Academy of Sciences.

Surgical Procedures.

Metal pellets. Each pellet (DU or Tantalum (TA)) will be a cylinder 1 mm in diameter and 2 mm long. *Surgery.* Pellets will be cleaned and chemically sterilized prior to implantation. Pellets will be immersed in absolute alcohol (15 mins), soaked in 50% nitric acid solution for 3 min, and then thoroughly rinsed 2X with sterile saline (0.9%). The procedure has been shown to remove the oxide formation on the surface of the DU pellet. A small heating pad will be used during surgery to maintained body temperature. Thirty to sixty minutes prior to surgery, each animal will receive 1 SC injection (27 gauge sterile needle) of buprenorphine (0.05 mg/kg) in the back of the neck. If mice appear to be in pain following implantation surgery they will receive another injection of the buprenorphine (same, dosage, same location). In our recent experiences with mouse DU surgery a second injection was not necessary but the project personnel will be prepared to provide additional analgesia as required. The criteria for analgesia following surgery will be 1) limping; 2) not eating/drinking; 3) ruffled coat; or 4) huddling. One criterion is sufficient to require analgesia. Anesthesia will be induced with isoflurane (~2-4%) and then maintained with isoflurane at the lowest possible rate (~1-2%). The animal will remain under isoflurane anesthesia for 5-8 minutes. Pellets will be implanted in the gastrocnemius muscle of each back leg, spaced approximately 2-4 mm apart. A single surgical site per rear limb will be shaved prior to surgery and then cleaned with betadine (i.e. standard sterile surgical preparation). A single scalpel incision (using a sterile surgical blade, #15 for example) will be made through the skin and pellets inserted into the muscle with a 16-gauge needle with plunger. The 16-gauge needle resembles a trochar and the pellets are loaded in order either (3 DU pellets) of 1 Ta + 1 DU + 1 Ta pellet. Each animal will receive a total of 6 pellets - three per hind limb. For high DU dose there are 3 DU pellets per limb. For the low DU dose there are 2 Ta and 1 DU pellet per hind limb. There is little chance that an air bolus could be introduced and it has never been seen in our previous pellet surgeries. The incision is generally 5-7 mm in length. One incision will enable the implantation of 3 pellets per limb. The syringe is preloaded with the pellets to be implanted. The incision will be closed with absorbable sutures (for example, 5-0 vicryl or, as recommended by the veterinarian). Mice will be closely monitored following surgery until ambulatory (usually within 10 mins). Animals will be monitored every 1 hr for the first 4 hrs post-surgery to determine if another dose of analgesia is necessary. However animals will be monitored closely for up to 6-8 hrs after surgery. Analgesia (buprenorphine, 0.05 mg/kg SC; injection back of neck) will be used if an animal demonstrates 1 criterion of pain. The criteria for analgesia will be 1) limping; 2) not eating/drinking; 3) ruffled coat; or 4) huddling. Animals will then be monitored by project personnel a minimum of 2X daily for the duration of the experiment but, during the first two weeks, four or more daily

observations by project personnel will be made if a single animal behaves as if he is in distress at any time point. An attending veterinarian will be consulted if any abnormalities are found. A technician experienced in this surgical technique, and previously observed by VSD veterinarians, will perform the surgeries. *Animal Observations/assessment of health status.* Mice will be monitored after pellet implantation and the PI or PI's technician will observe the mice a minimum of 2X daily for morbidity and mortality. Body weights will be recorded weekly. Moribund animals (NOT EXPECTED) will be euthanized, and necropsied. Previous studies in our laboratory have demonstrated that adverse effects following pellet surgery are rare [1-8].

Euthanasia:
At the appropriate time, the animal will be euthanized by CO_2 inhalation followed/confirmed by cervical dislocation.

Tissues Collection for Data Analysis:
Tissues will be collected following animal euthanasia.

V.2. Data Analysis

Experiment 1:
The endpoint for 1A will be southern blot/gel analysis of DNA methyl content. The endpoint of 1B will be southern gel analysis of methylation of the promoter region of two gene. The gels will be analyzed using laser densitometry. Levels of global genome DNA methylation in the spleen and bone marrow of exposed and control mice will be measured by the cytosine extension assay on treatment of DNA with a methylation-sensitive restriction enzyme HpaII that cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands. The enzyme leaves a 5V-guanine overhang after DNA cleavage that is used for subsequent single nucleotide extension with labeled [3H]dCTP. The extent of [3H]dCTP incorporation opposite the exposed guanine is directly proportional to the number of cleaved and thus unmethylated CpG sites and inversely proportional to the levels of methylation (i.e., the higher the methylation, the lower is the incorporation of [3H]dCTP). A significant difference from control will be obtained by ANOVA (Analysis of variance of groups).

Experiment 2:
The endpoint of Experiment 2 will be the measurement of chromosomal aberrations in the CFU-A colonies derived from either the ^{60}Co-exposed bone marrow or the DU-exposed bone marrow. These chromosomal aberrations will be compared to the chromosomal aberrations measured in the bone marrow samples of mice previously diagnosed with leukemia following either radiation or DU exposure.

For gene expression studies signals will be quantified using NIH Image J 1.63 software and normalized relative to glyceraldehyde-3-phosphate dehydrogenase or the Mr 50,000 protein band. A comparison of the bands will be made using ANOVA analysis.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

Approaches using cellular models have already been conducted to study both radiation and DU-induced leukemic cell transformation. (b)(4),(b)(6)

(b)(4),(b)(6)

(b)(4) Radiation studies have demonstrated that methylation and histone modification (epigenetic changes) are observed following radiation exposure (23, 24). These *in vitro* studies implicate epigenetic mechanisms in the development of radiation late effects. However these findings must be confirmed in an *in vivo* model before they will be accepted by the risk assessment community (US ARMY Public Health Command (Prov.) and NCRP). Risk assessment requires that animal models be used to verify *in vitro* findings. Secondly, *in vitro* models do not allow us to evaluate whether the mechanism involved in the neoplastic transformation of cells *in vitro* would be affected by physiological, genetic, and environmental factors that are important to cancer induction *in vivo*. Finally, computer modeling could not be used to answer the research question I am addressing because modeling is dependent upon established *in vivo* results and there are currently no results regarding the mechanism of heavy metal induced late effects *in vivo*.

V.3.2. Animal Model and Species Justification

This murine model for toxicity studies is a well-characterized animal model system used in a variety of toxicological studies [41]. It has also been commonly used to study the mechanisms involved in the induction of biological effects following exposure to radiation and heavy metals [4-5, 7-8]. (b)(4)

(b)(4)

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species

Mus musculus

V.3.3.2. Strain / Stock

DBA/2

V.3.3.3. Source / Vendor

Jackson Labs, NCI Frederick, Taconic, or Charles River

V.3.3.4. Age
6-10 weeks

V.3.3.5. Weight
18 – 30 grams

V.3.3.6. Sex
Male

V.3.3.7. Special Considerations
None

V.3.4. Number of Animals Required (by species)
180 mice

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement
Based upon our current *in vitro* human cell methylation studies we are ready to evaluate mechanisms in an *in vivo* model. (b)(4)

(b)(4)

V.3.5.2. Reduction
By using tissue samples from previous studies we are reducing the number of animals needed in this study. We are able to compare the results from this study in which the tissues are collected at a short time after exposure (1 month) to results from animals that had a longer exposure (7 months) and had been diagnosed with AML.

V.3.5.3. Replacement
None

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals 180

V.4.1.1.1.1. Column C ___70__ (# of animals)

V.4.1.1.1.2. Column D ___50__ (# of animals)

V.4.1.1.1.3. Column E ___60__ *(# of animals)*

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
Exp 1 Controls Groups A and B	No surgery, No radiation Groups A and B	10			10
Exp 1 – Mice receiving any dose of radiation; Groups C and E	Mice receive 60Co gamma radiation +/- FDC-P1 cells			30	30
Exp 1 – Mice receive DU implant Groups D and G	Mice receive DU implant +/- FDCp1 cells		20		20
Exp 1 – Mice receive cell injection Group F	Mice receive an injection of FDC-P1 cells that were irradiated; No mouse radiation, No DU implant	15			15
Exp 1 – Mice receive cell injection Group H	Mice receive an injection of FDC-P1 cells that were exposed to DU *in vitro*. No mouse radiation and No DU implant	10			10
Exp 1 – Mice receive Ta implant	Mice receive Ta implant + FDC-P1 cells		5		5
Exp 2 - Controls Groups A and B	No surgery, No radiation Groups A and B	10			10
Exp 2 – Mice receiving any dose of radiation; Groups C and E	Mice receive 60Co gamma radiation +/- FDC-P1 cells			30	30
Exp 2 – Mice receive DU implant Groups D and G	Mice receive DU implant +/- FDCP1 cells		20		20
Exp 2 – Mice receive cell injection Group F	Mice receive an injection of FDC-P1 cells that were irradiated; No mouse radiation, No DU implant	15			15
Exp 2 – Mice receive cell injection Group H	Mice receive an injection of FDC-P1 cells that were exposed to DU *in vitro*. No mouse radiation and No DU implant	10			10
Exp 2 – Mice receive Ta implant	Mice receive Ta implant + FDC-P1 cells		5		5
Totals:		70	50	60	180

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

Anesthesia (isofluorane) will be used to anesthetize the mice during the pellet implantation procedure. We have been doing this in our recently completed project. One hr prior to surgery, each animal will receive 1 SC injection (27 gauge sterile needle) of buprenorphine (0.05 mg/kg) in the back of the neck. If mice appear to be in pain following implantation surgery they will receive another injection of the buprenorphine (same, dosage, same location). The criteria for analgesia following surgery will be 1) limping; 2) not eating/drinking; 3) ruffled coat; or 4) huddling. One criterion is sufficient to require analgesia. During surgery and anesthesia administration, the animals will be placed on a small heating pad.

V.4.1.2.2. Pre- and Post-procedural Provisions

Pre-procedural Provisions: None needed.

Post-procedural Provisions: For the all aims/Experiment 1: Animals will be monitored a minimum of twice daily for 5 days post-pellet implantation procedure. Animals will be weighed weekly by trained personnel on the PI's staff. The pellet implantation procedure is not expected to cause pain, suffering or morbidity. At 30 days post-pellet implantation, animals will be euthanized and tissues collected. While it is unexpected, any animal in pain will be immediately euthanized. We will monitor the animals as described and any animal that appears to be in pain based upon the following specific criteria will be immediately euthanized. Mice will be closely monitored following surgery until ambulatory (usually within 10 mins). Animals will be monitored every 1 hr for the first 4 hrs post-surgery to determine if another dose of analgesia is necessary. However animals will be monitored closely for up to 6-8 hrs after surgery. Analgesia (buprenorphine, 0.05 mg/kg SC; injection back of neck) will be used if an animal demonstrates 1 criterion of pain. A VSD veterinarian will also be consulted if any abnormalities are encountered. The abnormal criteria include: dehydration, changes in body weight (>10% increase or decrease from baseline), abnormal posture, hypothermia (shivering), swelling, tissue masses, ruffled fur, eye discharge, head tilted, excessive salivation, dyspnea, diarrhea, hypo activity, and prostration. The endpoint of this study is euthanasia (to collect tissues for mechanism studies).

V.4.1.2.3. Paralytics

None used.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched

AltWeb
PubMed
Agricola

V.4.1.3.2. Date of Search

Jan 20, 23, 2012
Jan 20, 2012
Jan 23, 2012

V.4.1.3.3. Period of Search

1967-2011

V.4.1.3.4. Key Words of Search

Radiation, Depleted uranium, leukemia, alpha radiation, DBA/2, euthanasia, lethality, survival, pellets, implantation, epigenetics, leukemogenesis, model, pain, suffering, alternatives, distress, carcinogenesis, injection.

V.4.1.3.5. Results of Search
The implantation of heavy metals into the gastrocnemius muscle does not appear to cause pain in rodents based on the published results with similarly implanted heavy metals [1-7]. Studies have been published indicating that the surgical addition of prosthetic devices involving the heavy metal tantalum, a normal component to prosthetic limb attachments, do not cause medical problems or pain due to the surgical implantation of the heavy metal [1-8]. Furthermore, medical surveillance studies from soldiers wounded with DU shrapnel do not discuss whether the symptomology of the soldiers includes pain at the sites of the metal shrapnel; these papers do not indicate that the soldiers are in pain [6].

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification
The animals in this protocol will receive a dose of 0.5, 2.0 or 3.5 Gy of radiation and will not receive any analgesia. In my experience with this mouse model (11 years) the animals have not exhibited any signs of being in pain from these low doses of radiation. Furthermore, the effects of analgesia administration on the study endpoints are unknown and could compromise the experiment so no analgesia will be used unless the animals unexpectedly exhibit pain criteria. The criteria include but are not limited to: dehydration, changes in body weight (>10% increase or decrease from baseline), abnormal posture, hypothermia (shivering), swelling, tissue masses, ruffled fur, eye discharge, head tilted, excessive salivation, dyspnea, diarrhea, hypo activity, and prostration.

V.4.2. Prolonged Restraint
None Used.

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions
None Used.

V.4.3.2. Procedure(s) Surgical Procedures.
Each pellet (DU) will be a cylinder 1 mm in diameter and 2 mm long. *Surgery*. Pellets will be cleaned and chemically sterilized prior to implantation. Pellets will be immersed in absolute alcohol (15 mins), soaked in 50% nitric acid solution for 3 min, and then thoroughly rinsed 2X with sterile saline (0.9%). The procedure has been shown to remove the oxide formation on the surface of the DU pellet. Thirty to sixty minutes prior to surgery, each animal will receive 1 SC injection (27 gauge sterile needle) of buprenorphine (0.05 mg/kg) in the back of the neck. If mice appear to be in pain

following implantation surgery they will receive another injection of the buprenorphine (same, dosage, same location). The criteria for analgesia following surgery will be 1) limping; 2) not eating/drinking; 3) ruffled coat; or 4) huddling. One criterion is sufficient to require analgesia. Anesthesia will be induced with isoflurane (~2-4%) and then maintained with isoflurane (~1-2%). The animal will be under isoflurane anesthesia for 5-8 minutes. Fragments will be implanted in the gastrocnemius muscle of each back leg, spaced approximately 2-4 mm apart. A single surgical site per rear limb will be cleaned with betadine and shaved prior to surgery (i.e. standard sterile surgical preparation). A single scalpel incision (using a sterile surgical blade, #15 for example) will be made through the skin and pellets inserted into the muscle with a 16-gauge needle with plunger. One incision will enable the implantation of 3 pellets. The syringe is preloaded with the pellets to be implanted. The incision will be closed with absorbable sutures (for example, 5-0 vicryl or, as recommended by the veterinarian). Mice will be closely monitored following surgery until ambulatory (usually within 10 mins). However animals will be monitored closely for up to 4 hrs after surgery. Analgesia (buprenorphine, 0.05 mg/kg SC; injection back of neck) will be used if an animal demonstrates 1 criterion of pain. The criteria for analgesia will be 1) limping; 2) not eating/drinking; 3) ruffled coat; or 4) huddling. Animals will be monitored a minimum of 2X daily for the duration of the experiment but, during the first two weeks, three or more daily observations will be made if a single animal behaves as if he/she is in distress. An attending veterinarian will be consulted if any abnormalities are present. *Animal Observations/assessment of health status.* Mice will be monitored after pellet implantation and observed a minimum of 2X daily for morbidity and mortality. Body weights will be recorded weekly. Moribund animals (NOT EXPECTED) will be euthanized immediately and necropsied. Previous studies in our laboratory have demonstrated that adverse effects following pellet surgery are rare [1-7, 15-19].

V.4.3.3. Post-surgical Provisions
Mice will be closely monitored following surgery until ambulatory. Analgesic (buprenorphine 0.05/mg/kg, SC back of neck) will be used if necessary. Animals will be monitored a minimum of 2x daily for duration of experiment.

V.4.3.4. Location
Surgery location is (b)(6) or other suitable room as designated by VSD staff.

V.4.3.5. Surgeon
Trained technicians on the PI's staff

V.4.3.6. Multiple Major Survival Operative Procedures

V.4.3.6.1. Procedures
None Used.

V.4.3.6.2 Scientific Justification

None Used.

V.4.4. Animal Manipulations
1) Permanent individual identification using the ear notching method: This method requires a special tool to punch or notch holes at various positions in the ears; this technique is commonly used in rodents. Procedure will take place under sterile conditions in VSD after released from quarantine. Manual restraining is required to perform this technique.

V.4.4.1. Injections
Experiment 1 and Experiment 2

1) Animals will receive an injection of sterile cells (sterile FDC-P1 cells; 2-5 E6 cells/0.1-0.2 ml) immediately (within 30 mins) post-radiation into the tail vein. A 25-28 gauge needle will be used. Choice of needle gauge size can vary based on the animal size. This is necessary since this cell injection was required in the previous studies in which leukemia was induced.

V.4.4.1.1. Pharmaceutical Grade Drugs
None

V.4.4.1.2. Non-Pharmaceutical Grade Drugs
None

V.4.4.2. Biosamples
None, animals will be euthanized and samples will be taken post-mortem. My understanding is that biosamples are obtained from a live animal. However, after euthanasia, blood samples, liver, spleen, and bone marrow from both femurs will be taken.

V.4.4.3. Adjuvants
None used.

V.4.4.4. Monoclonal Antibody (MAb) Production
None used.

V.4.4.5. Animal Identification
Ear notches and cage cards will be used.

V.4.4.6. Behavioral Studies
None used.

V.4.4.7. Other Procedures
None used.

V.4.4.8. Tissue Sharing
Not at this time.

V.4.4.9. Animal By-Products
None.

V.4.5. Study Endpoint
The study endpoint for the animals is euthanasia at 1 month + 7 days post-metal implantation or 7 days post-gamma radiation.

V.4.6. Euthanasia
All mice will be euthanized by CO_2 inhalation. Euthanasia will be conducted using a euthanasia chamber. Compressed CO_2 gas in a cylinder with a regulator will be used for euthanasia in accordance with the 2007 AVMA Panel on Euthanasia. Cervical dislocation will then be used to ensure that the animal has been euthanized.

V.5. Veterinary Care

V.5.1. Husbandry Considerations
Animals will be housed no more than 5 per cage in a micro-isolator cage. Animals will receive food and water ad libitum. Cages are changed three times weekly in accordance with the current VSD Rodent Husbandry SOP. Animals will have a 12 hour/12 hour light and dark cycle. Radiation safety procedures as detailed in the general experiment section pages 7 and 8 will be followed. While it is unexpected based on previous experience with this model, if an animal dies prior to the study endpoint VSD staff has been trained to put the carcass in freezer that is posted for radioactive materials. Before we begin this entire study (involving DU) VSD staff and project personnel will be re-trained by the RSO with a radiation safety refresher lecture. All these procedures will be reviewed.

V.5.1.1. Study Room
Assigned by VSD.

V.5.1.2. Special Husbandry Provisions
None

V.5.1.3. Exceptions
None

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care
Study personnel will monitor all animals 2X daily for 5 days post metal implantation. The animals will be individually observed at each observation period and weighed weekly. At euthanasia, the appropriate tissues for the *in vitro* analyses will collected and a necropsy will be performed by project personnel and hematological and molecular

assessment will be made by project personnel. This information could assist with the assessment of animals in future studies.

If an animal becomes ill or debilitated during the course of the study (prior to a planned euthanasia), the principal investigator, study personnel and the clinical veterinarian or VSD technical staff will be notified immediately to determine severity of condition.

VSD animal care technicians will observe all colony animals during morning and afternoon rounds in addition to the husbandry caretakers' twice daily observations (b)(4) (b)(4)

Criteria used for health evaluation: dehydration, changes in body weight (>10% increase or decrease), abnormal posture, hypothermia (shivering), swelling, tissue masses, ruffled fur, eye discharge, head tilted, excessive salivation, dyspnea, diarrhea, hypo-activity, and prostration.

V.5.2.2. Emergency Veterinary Medical Care

Emergency veterinary medical care will include euthanasia (and necropsy) if the animal meets the criteria described in section V.5.2.1.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

Animals can be given items in accordance with VSD/IACUC policy.

V.5.3.2. Enrichment Restrictions

None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				Surgical pellet implantation, Radiation, Injection, Euthanasia, Ear notch, Tail vein injection; All procedures are currently used in other projects. Codes 1-6	(b)(6)
				General animal handling, Radiation, drug Injection; Codes 1; 5	
				General animal handling; Codes 1; 3; 5	

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (DU pellet implantation; provide detail here)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY

(b)(4),(b)(6)

This project involves radiation hazards. The hazards were initially detailed in a paragraph in the general procedures section. It is repeated here for additional clarity.

(b)(4),(b)(6)

(b)(4) The VSD room where the mice will be housed will be posted as a "radiation materials work area". As in our currently approved DU protocol we (and all personnel handling mice and the cages) will have special safety wear (gloves, gowns); radiation safety practices will be used in Rad posted rooms in VSD in regard to waste/trash collection, and the carcasses will be stored in a locked frig/freezer. The VSD room will be posted with a sign indicating that the use of radioactive materials is occurring in that room. The animal bedding will be collected after each cleaning and will be inspected for any unexpected loss of DU pellets. The

bedding will be stored as radioactive waste. After the animals are euthanized, the DU pellets will be removed and accounted for.

In all non-radioactive procedures, standard laboratory safety precautions and programs will be observed in this project.

VIII. ENCLOSURES

None

X. ASSURANCES

Protocol Title: Evaluation of Mechanisms and Involvement of Non-targeted Radiation Effects in Radiation and Depleted Uranium Induced Leukemia's in mice (*Mus musculus*) (Intramural)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) PhD		
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

References

(b)(4),(b)(6)

(b)(4),(b)(6)

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(b)(4),(b)(6)

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DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	8/14/2012	2nd Resubmission Date:	11/21/2012
Approved/Returned for Revision:	9/5/2012	Approved/Approval Withheld by IACUC:	12/3/2012
1st Resubmission Date:	9/13/2012	EXPIRATION DATE:	12/2/2015
Approved/Returned for Revision:	11/5/2012	Previous Protocol Number *(if related)*	(b)(6)
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6) (renewal of (b)(6)

III. PROTOCOL TITLE: (b)(4)

(b)(4)

IV. PRINCIPAL INVESTIGATOR:

(b)(6) Ph.D. Date

(b)(6)

(b)(6) office (b)(6) fax: (b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) Ph.D. Date

(b)(6)

(b)(6) office (b)(6) fax: (b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician
Telephone (b)(6) Fax (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI
Telephone (b)(6) Fax (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department, USUHS
Telephone (b)(6) Fax (b)(6)
(b)(6)

IX. SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD** Date

Scientific Director, AFRRI
Telephone (b)(6) Fax (b)(6)
(b)(6)

X. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **PhD, CAPT, MSC, USN**

IACUC Chair (b)(6) AFRRI
Telephone (b)(6) Fax (b)(6)
(b)(6)

PROTOCOL TITLE: (b)(4)
(b)(4),(b)(6)

PRINCIPAL INVESTIGATOR:

(b)(6)

Date

CO-INVESTIGATOR(S):

(b)(6)

Date

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS

The terrorist attack of September 11, 2001 against the United States has increased the concern that terrorists may acquire and use radioactive sources (e.g. dirty radioactive bombs) to expose military and civilian populations to ionizing radiation. However, despite this concern, there are currently no FDA approved drugs that can be used for the general population to prevent death that is likely to result from exposure to ionizing radiation (b)(4) irradiation in humans can result in both (b)(4) (b)(4) injuries. However, because rodents are more resistant to (b)(4) injuries than humans, there is currently no murine model that has been developed to study both (b)(4) radiation injuries in the same animal.

(b)(4)

protects (b)(4) from radiation-induced genotoxicity, and that (b)(4) treatment increases the mean survival time from (b)(4) irradiation by 50%. (b)(4) (b)(4) Several laboratories have shown that a naturally occurring, non-toxic drug (b)(4) an inhibitor of (b)(4) is a protective agent against (b)(4) induced by chemotherapy agents (b)(4) (b)(4) enhances the activity of (b)(4) for the prevention of (b)(4). Our proposed studies here will examine the effects of (b)(4) in combination with (b)(4) for protection of (b)(4) radiation injuries in an animal model for both injuries. Our aim is to develop an effective, non-toxic radioprotectant/radiotherapeutic treatment strategy that can be used for protection against both (b)(4) effects of radiation exposure in a murine model for both types of radiation injury.

(b)(4),(b)(6)

II. BACKGROUND

II.1. Background

The acute toxic effects of exposure to high doses of ionizing radiation in humans have been shown to occur due to two sequential effects: first the ablation of immune cells, resulting in loss of immune function and increasing likelihood of pathogenic infection (b)(4) and subsequently the loss of epithelial progenitor cells of the gastrointestinal (GI) crypt (b)(4) Both of these events have been shown to be due to the loss of fast-growing pluripotent and/or progenitor cell types. The time course of the acute injuries correlates with the life span of the mature cell types of the tissue, which are not replaced because of radiation damage to the adult pluripotent cells and adult stem cells. There is currently no FDA approved prophylactic or treatment for prevention of acute radiation injuries following high-dose (b)(4) radiation exposure.

In some cases supportive measures can be used to treat acute radiation syndrome, and survival from (b)(4) radiation exposure is now possible (b)(4) This results in current efforts to mitigate or treat later stage effects of ionizing radiation exposure. (b)(4)

(b)(4)

In humans, death from radiation (b)(4) can occur following survival from (b)(4) Cumulative evidence from a number of laboratories, including our own, shows that there is a threshold dose of radiation of (b)(4) to induce sufficient (b)(4) for the (b)(4) to occur in mice; below these doses, no permanent injury manifests, although some short-term effects are observed (b)(4) (b)(4) However, in humans, there is currently no accepted protocol for preventative treatment of radiation-induced (b)(4) and morbidity as high as 50% is associated with each phase (b)(4) (b)(4) injuries from radiation is considered to be a serious problem in the field for the development of agents to prevent these radiation injuries.

(b)(4) **as a radioprotectant:** The biochemical properties of the isoflavones and the investigation of (b)(4) as a protectant against gamma radiation-induced lethality have been investigated by (b)(4),(b)(6) (b)(4),(b)(6) (b)(4) A single i.p. dose of (b)(4) administered (b)(4) hr prior to a lethal dose of radiation resulted in (b)(4) day survival rates of 65%, 91%, and 85% respectively. Mice receiving saline or PEG vehicle alone survived at 8% and 15% respectively. All doses of (b)(4) protected mice significantly ($p < 0.001$) better than vehicle or saline control groups.

The behavioral toxicity was investigated for male mice receiving a single i.p. injection (0.1 ml) of saline, PEG vehicle, or (b)(4) Locomotor activity (total distance traveled), grip strength and motor coordination were evaluated on days (b)(4) post-injection and body weight was measured over (b)(4) days. On day (b)(4) tissues from the testes, liver, adrenal gland, mesenteric lymph node, spleen, and bone marrow of the femur and sternum were collected, fixed in buffered formalin, paraffin embedded, sectioned, and stained by hematoxylin and eosin. The results indicated that there were no significant effects of (b)(4) or vehicle on locomotor activity, grip strength, motor coordination, or body weight, compared to the saline-treated control group. In addition, no gross morphological changes or histopathological alterations were observed (b)(4)

(b)(4) **as a radioprotectant and an antifibrotic agent:** (b)(4) and its proteolytic product (b)(4) are believed to have their primary biological functions in blood pressure homeostasis. However, (b)(4) have been demonstrated to be upregulated in fibrotic tissues. Inhibition of (b)(4) or blockade of the (b)(4) receptors on cells has been shown to ameliorate drug-induced (b)(4) in murine and rat models for (b)(4) (b)(4) receptor antagonists also prevent cardiac remodeling following ischaemia/reperfusion in rat models of cardiac remodeling diseases (b)(4) Several studies have also shown that inhibition of (b)(4) signaling mitigates radiation-induced injuries, including to the heart and (b)(4) One study shows that the (b)(4) reduces acute damage to the jejunal mucosa in mice following exposure to (b)(4) This work examined only initial tissue injury and did not extend findings past days, and did not examine mortality. A second (b)(4) study found a reduction in (b)(4) in rats exposed to (b)(4) radiation (b)(4)

Preliminary Studies:

(b)(4)

(b)(4),(b)(6)

[redacted]

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

1) Biomedical Research Database (BRD) (http://www.dtic.mil/biosys/org/brd/)
2) Computer Retrieval of Information of Scientific Projects (RePORTER) database (http://projectreporter.nih.gov/reporter.cfm)
3) PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed)

II.2.2. Date of Search

Data base searches conducted on 8/06/12.

II.2.3. Period of Search

Searches conducted for the entire time periods available in each data base. For BRD, 1998-2012; for RePorter, 1972-2012. For PubMed, 1970-2012.

II.2.4. Key Words of Search

Searches were performed using the following key word combinations:

(b)(4)

[redacted]

II.2.5. Results of Search

1) BRD: (b)(4) [redacted] returned 0 results.
One grant was funded to examine the activity of (b)(4) [redacted]

(b)(4)

2) A RePORTER search revealed 8 current grants studying the effects of radiation on (b)(4)

(b)(4)

None of the funded studies duplicate our research of (b)(4)

(b)(4)

3) PUBMED Search *8/3/09*

None of the publications available described a murine model for both (b)(4)

(b)(4 injuries from radiation.

(b)(4)

(b)(4)

Summary of other publications:

(b)(4)

III. OBJECTIVE / HYPOTHESIS

Our objective is to: 1) continue development of a murine model for both the (b)(4) (b)(4) injuries; and 2) examine protective effects of (b)(4) against these two

injuries in the developed model. (b)(4)
(b)(4)

IV. MILITARY RELEVANCE

The Department of Defense is prepared to execute military missions within radiation environments, to manage radiation crises associated with terrorist activities, and to manage consequences in the event of nuclear weapons detonation. Recent studies indicate that there is an increasing risk of exposure to ionizing radiation (IR) to military personnel due to increasing terrorist activities, including potential use of stolen nuclear weapons or dispersal of radioactive material in a "dirty bomb" (b)(4) Because of potential exposure of military personnel to radiation, the DoD is committed to protect members of the Armed Services from the adverse health effects of ionizing radiation. High dose IR causes short term, acute injuries including hematopoetic and GI syndromes. However, both high and low dose IR (as may result from a radiation dispersal device) can cause (b)(4) as demonstrated by Japanese atomic bomb survivor data as well as accidental exposures to Russian populations (b)(4) (b)(4) While early effects of radiation injury now have treatment/ prophylactic strategies, the delayed effects currently have no treatment. Lack of treatment for late effects of radiation, including (b)(4) will likely lead to delayed morbidity and mortality for personnel exposed to IR.

The Department of Defense (DoD) Chemical and Biological Defense Program 2007 Annual Report to Congress, Chapter 2.7.6 Medical Radiological Defense, addresses the requirements for the development of radioprotectants. This priority is outlined for the development of protectants for both acute and delayed radiation injuries, with an emphasis on (b)(4) According to this report, the mitigation of (b)(4) is a major research thrust area (b)(4)
(b)(4)

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

The goal of this project is to: 1) finish development of a murine model for both (b)(4) (b)(4) effects of radiation; and 2) investigate the protective effects and mechanisms of (b)(4) treatment for radiation-induced (b)(4) injuries.

(b)(4)

(b)(4)

(b)(4) Mice will be exposed to both (b)(4) (b)(4) irradiation to induce the total radiation doses required to induce both acute and delayed injuries, to simulate the injuries that occur in humans as a result of (b)(4) irradiation.

(b)(4) irradiation will be performed (b)(6) using the standard Lucite holders for irradiation. Sham irradiation will be performed by placing the mice in Lucite holders for equal time periods without irradiation. These experiments will involve short end points (less than days) for examination of (b)(4)
(b)(4) five criteria. Survival at days will be used to evaluate the (b)(4)

(b)(4) radiation used to induced (b)(4) For (b)(4) irradiation, we will utilize (b)(6) and modified Lucite jigs designed based on those used in the Radiation Research Branch, NIH/NCI. The use of these jigs was reviewed and approved by AFRRI veterinary staff. Sham irradiation will be performed by placing the mice in jigs for equal lengths of time with no irradiation. As measurements of (b)(injury and cellular injury in the (b) from radiation, five criteria will be examined:

(b)(4)

V.1.1. Experiment 1

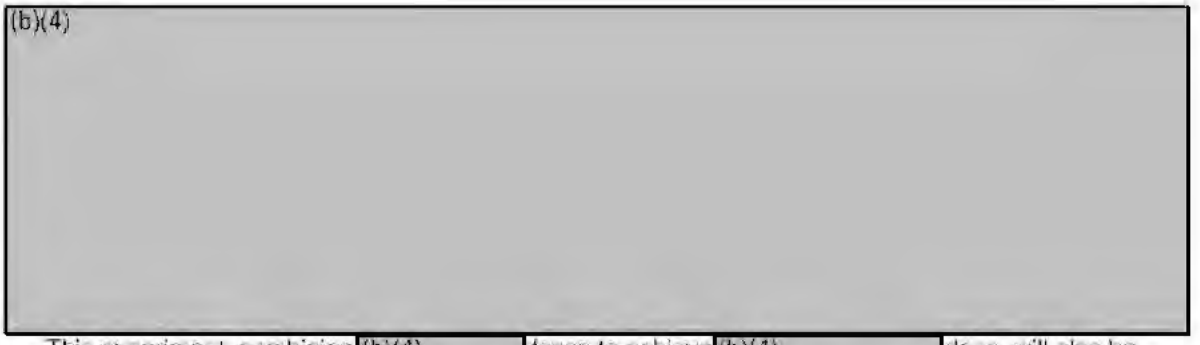
Experiment 1 Final development of a murine model that incorporates both (b)(4) radiation injuries.

(b)(4) mice: n pain category E)

Rationale: (b)(4)

(b)(4)

(b)(4)



This experiment, combining (b)(4) doses to achieve (b)(4) dose, will also be used to determine that we do not encounter a significant effect from (b)(4) the dose. To minimize a possible (b)(4) effect, we will keep the interval between radiation exposures as low as possible, (b)(4) min.

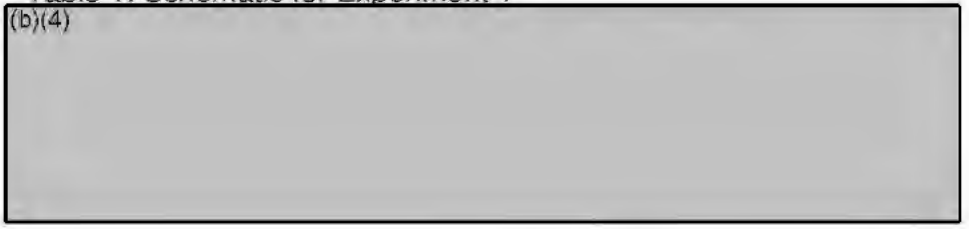
To complete our development of the model, we will use five doses of (b)(4) between (b)(4) (b)(4) Groups will be: 1) (b)(4) irradiation only; and 2) (b)(4) irradiation + (b)(4) irradiation. Time points will be: survival at (b)(4) days postirradiation. (b)(4) irradiation will be given to equal a total exposure of (b)(4) irradiated mice. Mice will first be exposed to (b)(4) irradiation in the (b)(6) facility, (b)(4) then anesthetized and exposed to X-ray irradiation of the (b)(6) facility, dose rate (b)(4) Control mice will be anesthetized and placed in holders to provide a sham for (b)(4) irradiation. This will control experiment will be performed to determine whether anesthesia given after (b)(4) irradiation affects survival. (b)(4) showed that anesthetics decrease bone marrow sensitivity to radiation; (b)(4) showed that pentabarbitol and steroids provide protection from radiation (b)(4) but not from (b)(4) In our experiments, we have observed significant radiation-induced (b)(4) injury although all of our experiments utilized anesthesia (telazol/xylazine) for the duration of the (b)(4) irradiation. Telazol is a combination of equal parts by weight of base of tiletamine hydrochloride (2-[ethylamino]-2-[2-thienyl]-cyclohexanone hydrochloride), an arylaminocycloalkanone dissociative anesthetic, and zolazepam hydrochloride (4-[o-fluorophenyl]-6, 8-dihydro-1,3,8-trimethylpyrazolo [3, 4-e] [1,4] diazepin-7 [1H]-1-hydrochloride), a non-phenothiazine diazepinone having minor tranquilizing properties. Tiletamine is a dissociative anesthetic and pharmacologically classified as an NMDA receptor antagonist. In comparison, ketamine is not a drug combination as is telazol, and is also classified as an NMDA receptor antagonist. Since tiletamine and ketamine are both NMDA receptor antagonists, we do not anticipate large alterations in the (b)(4) response to irradiation. The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) Mice needed for experiment: (b)(4) group to reach statistical significance for survival.
(b)(4) (b)(4) mice/group) x (6 conditions) x (1 time point) = (b)(4) animals.
The experiment will be repeated 1 time to confirm findings (total (b)(4) animals).

(b)(4) Endpoint: The endpoint will be (b)(4) days. We will measure survival at both (b)(4) days for the study. Early time points will be taken if any animal exhibits signs or symptoms of morbidity or moribundity in accordance with AFRRI IACUC Policy #10 (score of >11 as described in the AFRRI IACUC pain score sheet). Such animals will be euthanized at the time of observation.

Table 1: Schematic for Experiment 1

(b)(4)



(b)(4)

V.1.2. Experiment 2

Experiment 2. Determine the effect and mechanism of (b)(4) on (b)(4) following (b)(4) (b)(4) irradiation. (mice: in category E)

Rationale: Experiment 1 will be used to determine overlapping effects of (b)(4) irradiation on (b)(4) injury by (b)(4) irradiation. This section objective will be to determine the effects and the mechanisms of (b)(4) for the prevention of (b)(4) induced by exposure to (b)(4) irradiation. The approach will be to use murine model of both (b)(4) (b)(radiation injuries developed in Experiment 1. We will assess the mechanisms of protection using (b)(4) cellular, histological, molecular, and biochemical markers. The rationale for undertaking this research is that once the mechanisms of (b)(4) prevention of (b)(4) are known, these may help designing therapeutic strategies against radiation-induced (b)(injury.

PEG-400 (0.1 ml/mouse) vehicle only or (b)(4) will be administered by a single (b)(4) i.p. injection (b)(4) before radiation exposure. (b)(4) will be provided in the drinking water at (b)(4) to give a dosage of (b)(4) per day for the duration of the experiment starting on day 0. Mice will be exposed to (b)(4) using (b)(4) dose rate. Subsequently, mice will be anesthetized and exposed to (b)(4) x-ray irradiation to give exposures equal to (b)(4) irradiation, (b)(4) dose rate. Treatment groups will be: 1) PEG; 2) PEG + (b)(4) 3) (b)(4) We will not use (b)(4) alone as we have already determined that it is not effective alone for injury. Acidified water will be provided until after irradiation, and at which time it will be replaced with (b)(4) in acidified water (b)(4) water bottles will be prepared by the laboratory, and will be replaced 2X per week in accordance with VSD protocols. Extra water bottles will be prepared in advance in case they are needed to replace a leaking water bottle.

Mice will be monitored monthly using non-invasive, unrestrained (b)(4) to determine (b)(4) (b)(4) For these studies mice are required per radiation dose. The time point for (b)(4) is days, based on data from previous studies by others (b)(4) The time point for (b)(4) is (b)(4) days, based on previous studies by others (b)(4) The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) (mice per group) x (3 treatment groups) x (1 time point) x (3 radiation levels) = (b)(4)

(b)(4) mice/experiment) x (2 experiments) = mice

Endpoints: The final endpoint for the experiment is days. Mice displaying obvious dyspnea, extreme lethargy, or other markers of morbundity as described in the AFRRI IACUC pain score sheet (score of >11), and which appear to be in distress will be euthanized in accordance with AFRRI IACUC Policy #10 (b)(4) will be histologically examined for (b)(4) remodeling.

Table 2: Schematic for Experiments 2 and 3.

(b)(4)

V.1.3. Experiment 3

Experiment 3: Determination of the mechanism of reduction of (b)(4) injury by (b)(4)

(b)(4) (b)(4) mice/ in category C; in category E)

(b)(4) We have determined that between (b)(4) days, cells exhibit markers o (b)(4) (b)(4) following (b)(4) rradiation. We will investigate the mechanism of action of (b)(4) and (b)(4) examining these markers of cell injury.

We will use 4 time points (b)(4) days postirradiation, using the same doses of radiation listed in Table 2. Treatment groups will be: 1) PEG; 2) PEG + (b)(4) 3) (b)(4) (b)(4) The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) mice per group) x (3 treatment groups) x (4 time points) x (3 adiation levels) = (b)(4) (b)(4)
(b)(4) (b)(4 mice/experiment) x (2 experiments) = mice
(b)(4) **sham-irradiated mice will be required as controls.**

Endpoint: Endpoints for the experiments will be (b)(4) days. Early time points will be taken if any animal exhibits signs or symptoms of morbidity or moribundity (scoring over 11 on the AFRRI IACUC-recommended pain score sheet) in accordance with AFRRI IACUC Policy #10. Such animals will be euthanized at the time of observation.

V.2. Data Analysis Mean with standard error, or percentage will be reported if applicable. A significance level is set at 5% for each test. All statistical tests are two-sided. A statistical software, SAS will be used for statistical analyses.

Experiment 1: Log-Rank test will be used for comparison among groups. If significant, Fisher's exact test will be used to detect significant differences across the groups in survival rates at day Kaplan (b)(4)
(b)(4) Meier curves will be displayed. N = mice per group will have 80% power in detecting a significant difference between two groups if any, given type I error of 5% where the treatment group survival rate is at least 68%, compared to a control group survival rate of 20%. Similar statements would apply, if the treatment group survival rates are at least 83%, 78%, or 73%, compared to vehicle group survival rates of 35%, 30%, and 25%, respectively (b)(4)

Experiments 2 and 3: Analysis of variance (ANOVA) will be used to detect significant difference among the groups. If significant, then the pair-wise comparison by Tukey-Kramer will be used to identify which group is different from the other. For experiment 2, N = mice per group could have 80% power (b)(4) to detect any significant difference between groups if any, given type I error of 5% and delta/sigma has at least 1.4, where the delta is the estimated mean differences between the two groups and sigma is the pooled standard deviation of the two groups (b)(4) For experiment 3, we only need N = (b)(4) based on our current data from radiation experiments and induction of (b)(4) markers.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

For the last 8 years, we have utilized primary cell culture systems to examine anti-apoptotic mechanisms; however, we must determine whether our culture findings are relevant in whole animals. We will continue to use primary cell culture whenever possible to avoid unnecessary use of animals. We have also considered computer models as alternatives to animals. However, this alternative also lacks the knowledge base and complexity needed to evaluate radioprotective and radiotherapeutic agents.**V.3.2. Animal Model and Species Justification**Tissue culture conditions cannot fully substitute for the

complexities of whole organisms in response to radiation injury. Such injury involves the interactions between the (b)(4) system and other organ systems. In order to develop viable treatments for humans, we find that our research must be conducted in whole animals.

Work by (b)(6) also advocates the mouse as the best species to use for radiation protection and radiotherapy protocols: 1) mice provide the least sentient species available whose response to radiation has been clearly identified, with physiological responses to radiation and radioprotectants that are similar to human physiology, 2) extensive literature is available on responses of mice to radiation and countermeasures, 3) more reagents are available to study the relevant cell types and molecules in mice than in any other species except humans, and the availability of transgenic and knockout mice make this model extremely attractive for further research into molecular mechanisms, and 4) (b)(6) have extensive experience with this model in radiation injury and radioprotection research (b)(6)

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species *Mus musculus*

V.3.3.2. Strain / Stock CBA, female. CBA mice have (b)(4) (b)(4) in a shorter time course than C57BL/6 mice, and in a pattern that more closely resembles (b)(4) in humans (b)(4)

V.3.3.3. Source / Vendor
(b)(4) for CBA (fully accredited by AAALAC Int)

V.3.3.4. Age 10-15 weeks

V.3.3.5. Weight Normal adult weight range for this strain: 17-21 g.

V.3.3.6. Sex
Female. Only females will be used in this study for the following reasons: 1) we have so far utilized female mice to lay the foundation of our studies for radiation-induced (b)(4) injuries; 2) because of long housing periods, it is advantageous to use female mice that are less aggressive toward each other.

V.3.3.7. Special Considerations Commercially procured animals must be adventitious disease free. Mice- Pathogen-free, including *Pseudomonas aeruginosa*, and Pasteurella: All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus; *Helicobacter* spp. *Mycoplasma pulmonis*, and *Clostridium piliforme*. Endoparasite- and ectoparasite-free.

V.3.4. Number of Animals Required (by species) *Mice* (b)(4)

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement Animals demonstrating moribundity due to radiation exposure (b)(4) (b)(4) will be considered to have arrived at the study endpoint and will be immediately euthanized (See sections V.4.5 Study Endpoint and V.4.6 Euthanasia). (b)(4) will likely minimize pain and

(b)(4) distress, while [redacted] has been shown in studies of chemotherapy agents to function as a repair and [redacted] factor.

Ketamine/xylazine anesthesia will be used during X-ray irradiation to prevent stress from the time the mice are placed in restraint. Under the training protocol at AFRRI, under the supervision of AFRRI veterinary staff, we found that in female C57BL/6J mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia with no morbidity or mortality [redacted] We propose to use this range of concentration of anesthetics, together with a heating pad to keep mice warm during the period of recovery from anesthesia. Standard intraperitoneal injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which all laboratory personnel have received training at AFRRI.

V.3.5.2. Reduction Whenever possible, control groups will be shared between experimental groups for our experiments. Tissues will be collected for multiple assays from the same animals further reducing the total number of animals needed. At every stage of the research, planned experiments will be reevaluated, in an attempt to reduce the numbers of experiments and eliminate repeats of experiments where statistical significance has already been obtained. We also plan to take as many tissues as we can reasonably analyze for experiments examining protein oxidation.

V.3.5.3. Replacement At this time, it is not feasible to use non-animal systems in place of animal models to address the research questions in this protocol.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C [redacted] (# of animals)

V.4.1.1.1.2. Column D [redacted] (# of animals)

V.4.1.1.1.3. Column E [redacted] *(# of animals)*

V.4.1.1.1.4. Pain Category Assignments

[redacted]

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization We will be unable to use analgesics since they have effects on [redacted] function that would interfere with the interpretation of our results [redacted] Some sedatives have been reported to contribute to postoperative [redacted] which likely reduce some functions of radiation. Additionally, it has been shown that the biometabolism of analgesics can induce protein oxidation, which would interfere with sample analysis [redacted]

Animals in experiments involving blood collection will be deeply anaesthetized immediately prior to sample collection. For blood draws we will use 150 mg/kg ketamine plus 18 mg/kg xylazine given i.p. with a 25 G needle. For euthanasia, animals will be given 0.1-0.2 ml/mouse pentobarbital (50 mg/ml) given i.p. with a 25 G needle.

An anesthesia protocol has been developed for use during the X-ray irradiation. Ketamine will be used with xylazine for longer anesthesia in mice to be place in restraints for (b)(4) irradiation. Under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff, we found that in female CBA mice, ~12 weeks old,i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia (25 G needle) with no morbidity or mortality (b)(4) We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. Standard i.p. injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which all laboratory personnel have received training at AFRRI.

V.4.1.2.2. Pre- and Post-procedural Provisions Animals will be housed and cared for as described in Section V.5. Veterinary Care both before and after dose administrations and irradiation procedures.

V.4.1.2.3. Paralytics N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched Sources searched: DoD Bioedical Research Data Base (BRD), CRISP, PubMED

V.4.1.3.2. Date of Search 8/7/12

V.4.1.3.3. Period of Search
Search covered published manuscripts from ~1965-present. (BRD search included all available years 1998 -2012)

V.4.1.3.4. Key Words of Search
Pain, analgesia, anesthesia, mice, radiation, ionizing radiation, humane, (b)(4)

V.4.1.3.5. Results of Search
The literature search revealed that, at present, there are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. In some cases radiation has been demonstrated to reduce pain in laboratory mice (b)(4) and has been in humans reported to alleviate cancer related bone pain (b)(4) A recent report showed that administration of botulinum toxin A was effective for reduction of pain and muscle spasms after clinical radiation for treatment of tumors (b)(4) Application of natural honey 20 min prior to radiation exposure was also shown to reduce mucositis with associated pain in humans following head/neck radiotherapy (b)(4) it has not been determined whether honey acts as a local radioprotectant. Spinal cord stimulation has also been reported in humans to alleviate pain resulting from radiotherapy (b)(4) however, this method would not be practical for use in mice because of the extensive spinal cord manipulation required. Human administration of morphine also relieves pain (b)(4) but again this would not be practical for mice in our experiments because of side effects of the drug. In humans, exposure to (b)(4) irradiation may induce vomiting and diarrhea, causing pain and distress. However, mice are not susceptible to vomiting. The animals that will be administered (b)(4) will likely receive pain relief as phytoestrogens have been

reported to reduce neuropathic pain in rodents (b)(4) preliminary results also indicate that (b)(4) ameliorates acute radiation-induced injuries (b)(4)

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification

Anesthesia will be used before (b)(4) irradiation to avoid stress from restraint (b)(4) (b)(4) (b)(4) we will be unable to use analgesics after irradiation since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4) (b)(4) (b)(4) In experiments where tissue collection is needed, animals will be euthanized with pentobarbitol prior to sample collection as described elsewhere.

V.4.2. Prolonged Restraint

No prolonged restraint *is* required for (b)(4) irradiation. Whole body (b)(4) is non-invasive and unrestrained. (b)(4) irradiation will require restraint in designed jigs for 20-40 min to receive X-ray exposure (b)(4) ; animals will be anesthetized during this period (see Anesthesia above) and tails will be taped to one side with surgical tape. The jigs for use during (b)(4) irradiation were examined and approved by the AFRRI Veterinary staff. Under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff, we found that in female CBA mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia (25 G needle) with no morbidity or mortality (b)(4) We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. Standard i.p. injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which all laboratory personnel have received training at AFRRI. AFRRI Veterinary staff have provided training and supervision for the placement of anesthetized animals in the approved jigs for irradiation.

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions N/A

V.4.3.2. Procedure(s) N/A

V.4.3.3. Post-surgical Provisions N/A

V.4.3.4. Location N/A

V.4.3.5. Surgeon N/A

V.4.3.6. Multiple Major Survival Operative Procedures

V.4.3.6.1. Procedures N/A

V.4.3.6.2 Scientific Justification N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections

Intra-peritoneal injections of pentobarbital for euthanasia 0.1-0.2 ml/mouse Fatal Plus (39-78 mg pentobarbital/mouse), will be performed with a 25 G needle. I.p. injection of 150 mg/kg ketamine plus 18

mg/kg xylazine will be done using a 25 G needle. This has been extensively examined by the AFRRI Veterinary staff and shown to effectively produce non-lethal anesthesia (b)(4)

V.4.4.1.1. Pharmaceutical Grade Drugs

(b)(4)

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

(b)(4),(b)(6)

Endotoxin in the drugs will be measured by the PI, or information from the company will be provided prior to administration of drugs to the animals.

V.4.4.2. Biosamples

Blood will be taken under deep anesthesia. All other biosamples will be taken after euthanasia (b)(4) (b)(4) or analysis will removed *en bloc*. Intestinal tissue will be taken in smaller amounts (the first segment of the small intestine). All other tissues will be available to other researchers for tissue sharing.

For some experiments, blood will be obtained from the animals. Blood will be taken by percutaneous intra-cardiac collection using a 23 – 22 g needle. All other tissues will be available for tissue sharing with other investigators. Intra-cardiac puncture must be performed under deep anesthesia and is considered a non-survival procedure. The mouse is deeply anesthetized and toe pinch response will be used to determine the depth of anesthesia. The ventral chest will be wiped with 70% ethanol. A 23 – 22 g needle will then be inserted at the base of the sternum, bevel up (b)(4) at a 15-20° angle directed to the left of the midline. Once the needle is inserted, the blood is slowly aspirated. If no blood is observed, the needle is repositioned with slow aspiration. Once the blood volume is collected, the mouse is euthanized by cervical dislocation while still under deep anesthesia. Typically 0.5-0.75 ml of blood are collected; sometimes less blood is available after radiation exposure

V.4.4.3. Adjuvants N/A

V.4.4.4. Monoclonal Antibody (MAb) Production N/A

V.4.4.5. Animal Identification

All cages will have cage cards identifying the treatment groups for each experiment. For experiments 2 and 3, each animal within a cage will receive ear punches for individual identification (1 = no cut; 2 = right ear upper corner; 3 = left ear upper corner; 4 = both ears).

V.4.4.6. Behavioral Studies N/A

V.4.4.7. Other Procedures

(b)(4) **irradiation**

(b)(4) radiation exposures will be carried out within (b)(4),(b)(6) facility (b)(4) Mice will be irradiated at (b)(4) minutes in order to deliver the radiation doses required (b)(4) experiments 1 and 2). Mice will be placed in well-ventilated Plexiglas boxes (standardized for (b)(6) facility) for up to 45 min, but usually less time, during acute irradiation procedures. Following radiation exposures mice will be immediately moved to standard sized cages that house either 4-8 animals per cage. Holders will be sanitized after each use.

(b)(4) irradiation

The methodology for (b)(4) irradiation of mice in (b)(6) facility, including approval of holding jigs and development of anesthesia, was developed in conjunction with the AFRRI Veterinary Staff.

(b)(4) irradiation will be performed using (b)(6)
Three mice can be irradiated in one group (b)(6) Groups of three mice will be brought to the (b)(6) facility. The mice will be anesthetized by injection and placed in ventilated jigs, which restrain the animals to prevent lateral or vertical movement. Prior to irradiation, scans will be performed to identify the (b)(4) region to be targeted for radiation; this will take approximately 2 minutes per animal. Mice will be irradiated at (b)(4) doses, including dosage from (b)(4) For studies with (b)(4) irradiation prior to (b)(4) irradiation, for which only (b)(4) min of radiation will be required per animal, three animals will be prepared at one time. For animals that require the full (b)(4) irradiation, one animal will be irradiated at a time. Following radiation exposure, mice will be immediately returned to the VSD and allowed to recover from anesthesia under supervision, with heating pads. Immediately upon recovery, animals will be returned to standard cages that house 4-8 animals per cage. Lucite jigs will be sanitized after each use.

Whole body, non-invasive (b)(4)
Non-anesthetized, unrestrained (b)(4) will require individual animals to be placed in a clean plexiglass chamber (~ 12 cm in diameter, ~15 cm high) for 45-60 min. Animals will not have food or water during this time, but they will have a continuous flow of fresh room air. Their (b)(4) is measured based on warming and humidifying of the air in the chamber. The animals will experience no restraint, no anesthesia, no drugs, or other manipulation during this time. Following the measurement, to be performed in the room in which they are housed, the animals will be returned to their original cages. Chambers will be sanitized after each use.

Ear punch

The ear punch will be soaked in alcohol to disinfect it before use and between animals. The device will be placed on the pinna of the ear (external ear) in a location where the mouse will be marked for identification. The punch will be pressed firmly to punch a circular hole through the ear, being careful not to rip the delicate membrane of the pinna. The ear will then be gently separated from the device. The ear punch will be cleaned and disinfected in alcohol before use in another mouse.

V.4.4.8. Tissue Sharing For most studies (b)(4) (b)(4) will be obtained. All other tissues will be available for tissue sharing with other investigators. Our laboratory has made extensive use of shared tissues for training and technique development (b)(4) tissue.

V.4.4.9. Animal By-Products None.

V.4.5. Study Endpoint

The endpoint for (b)(4) survival studies will be [redacted] days and pattern of survival over the [redacted] day (b)(4) post-irradiation period. The endpoint for radiation-induced (b)(4) will be [redacted] days postirradiation (b)(4) and the endpoint for the (b)(4) studies will be (b)(4) days postirradiation. Early endpoints will be used in the case of morbidity and morbundity. Specific health assessment criteria including rapid weight loss, ruffled fur, dried lacrimal fluid accumulated in the cul-de-sac of their eyes, are cool to the touch, decreased appetite, diarrhea, difficulty breathing or panting (b)(4) will used to determine early study endpoints for euthanasia. Mice exhibiting these signs will be considered morbid and will be monitored by the PI or other members of the investigative team at least twice daily, early morning and late afternoon. Mice will be considered moribund when, in addition to showing some or all of the signs of morbidity, they exhibit labored breathing plus either an inability to remain upright or are unmotivated to move. Morbid or moribund mice will be automatically euthanized as described in

accordance with IACUC Policy 10. Moribund mice scoring above 11 on the AFRRI IACUC-recommended pain score sheet will be euthanized.

V.4.6. Euthanasia

Animals will be euthanized at the stated endpoints of the experiments by injection of 0.1-0.2 ml/mouse Fatal Plus (39-78 mg pentobarbital/mouse), IP using a 25 G needle. Euthanasia will be confirmed by cervical dislocation. Animals which appear to be experiencing pain or distress as a result of radiation exposure or are found moribund will also be euthanized by pentobarbitol and necropsied. At the end of experiments, unused animals will be transferred to other protocols or euthanized by exposure to 100% CO_2 from a compressed gas cylinder in accordance with the current American Veterinary Medical Association (AVMA) Guidelines for Euthanasia. Exsanguination, cervical dislocation, and/or (b)(4) will be used to ensure euthanasia.

V.5. Veterinary Care

V.5.1. Husbandry Considerations All of the animals used in this study will be housed in the VSD facility in cages of 4-8 mice. Food and acidified (2.5 – 3.0 pH) water (to minimize *Pseudomonas* infection) and Harlan Teklad Rodent Diet #8604 will be available *ad libitum*. The 12:12 hr (lights off at 6 pm) VSD room light cycle is fine for all the studies.

V.5.1.1. Study Room

(b)(6)

V.5.1.2. Special Husbandry Provisions

V.5.1.3. Exceptions

Mice will be group housed except during the irradiation procedures as described above or in the event that their cage mates have had to be removed from the study. Under these circumstances they may be housed individually

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care

Experimental animals will be observed at least twice a day by one of the study investigators or technicians. VSD personnel will also monitor the animals twice a day during their rounds. As described in another section (study endpoint) morbid animals will be monitored at least twice daily, morning and afternoon. The experimental design does not contemplate supportive therapy. The justification for not providing supportive care has been described in the study end point section as well as in the pain category section. Criteria to be used for health evaluation while the animals are on study include: weight loss (mice will be weighed every other day), ruffled fur, dehydration, decreased activity, and hunched body position, dyspnea, or panting. Animals exhibiting these signs may be euthanized, and considered to be at the study endpoint.

V.5.2.2. Emergency Veterinary Medical Care

Emergency veterinary medical care is available 24 hours a day, 7 days a week, including weekends and holidays.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy Environment enrichment for the mice will be provided with the addition of nestlets and/or hiding tubes to each cage. Group housing of the mice also provides social enrichment.

V.5.3.2. Enrichment Restrictions None.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)	Ph.D.	(b)(6)		1, 3a, 5, 7b	7 yr
	Ph.D.			1	8+ yr
	Ph.D.			1,3a, 5	5 yr
	Ph.D.			1,5	>10 yr
	B.S.			1,3a,5	4 yr
	B.S.			1, 3a, 3b, 4a, 5, 7a, 7b, 7c	8+yrs
	B.S.			1, 3, 5	6 months

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)
7a: tail vein injection for drug delivery
7b: plethysmography
7c: ear punch

VII. BIOHAZARDS/SAFETY

All the personnel will be trained as radiation users on a regular basis. All investigators and technicians while in the vivarium will use appropriate procedures as described by VSD, including the wearing of appropriate PPE, during observation of mice. No additional biohazards will be used for these experiments.

VIII. ENCLOSURES

Drug safety sheet for (b)(4) is attached.

References:

(b)(4)


(b)(4)


(b)(4)


(b)(4)

X. ASSURANCES

Protocol Title: (b)(4)

(b)(4)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM** / ~~**AM NOT**~~ conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / **WILL NOT** **(both)** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator (Printed Name) _____ Principal Investigator (Signature) _____ (Date)

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (AUGUST 2012)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

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Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD Instructions.

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This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

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Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	12/11/2012	2nd Resubmission Date:	
Full Committee Review :	1/31/2013	Final Approval by IACUC:	4/5/2013
1st Resubmission Date:	2/6/2013	**EXPIRATION DATE:**	4/4/2016
Approved/Returned for Revision:	2/26/2013	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:	2/28/2013	Approved/Returned for Revision:	3/25/2013
Approved/Returned for Revision:	3/7/2013	2nd Resubmission Date:	4/2/2013
1st Resubmission Date:	3/20/2013	ACURO Approved/Approval Withheld:	4/4/2013

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- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and *subsection*, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Evaluation of novel biodosimetry technologies using non-human primates (NHP; *Macaca mulatta*) radiation model

IV. CO- PRINCIPAL INVESTIGATOR:

[Signature on File] 11 Dec 2012

(b)(6) te

(b)(6)

PRINCIPAL INVESTIGATOR:

[Signature on File] 11 Dec 2012

(b)(6) Date

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

[Signature on File]

(b)(6) Date

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

[Signature on File] 11 Dec 2012

(b)(6) Date

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

[Signature on File] 10 Dec 2012

(b)(6) Date

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

[Signature on file] 11 Dec 2012

(b)(6) Date

IX. SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

[Signature on file] 11 Dec 2012

(b)(6) Date

X. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6)

PROTOCOL TITLE: Evaluation of novel biodosimetry technologies using non-human primates (NHP; *Macaca mulatta*) radiation model

CO-PRINCIPAL INVESTIGATOR:

[Signature on file] 11 Dec 2012

(b)(6) Date

PRINCIPAL INVESTIGATOR:

[Signature on file] 11 Dec 2012

(b)(6) Date

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS

In all potential radiation exposure scenarios, it is unlikely that radiation exposure detectors will be available for dose assessment to aid clinical management of mass casualties. Strategies for early-phase rapid diagnostic triage of the injured, contaminated and non-contaminated casualties are needed. The Armed Forces Radiobiology Research Institute (AFRRI) monkey biodosimetry study is part of a Biomedical Advanced Research and Development Authority (BARDA) Interagency Agreement (IAA) - Phase 3 plans to generate samples that will be used in the development of a device to determine injury from potential radiation exposures by measuring various biomarkers in a blood sample. Baseline or pre-irradiated samples will be taken from all animals to establish normal baseline levels of candidate biomarkers. Most studies will involve total-body irradiation (TBI) exposures to graded doses of gamma rays and the use of "Minimum Supportive Care Therapy" model reflecting the anticipated limited medical-care situation in the early-phase of a mass-casualty radiological incident. A pilot high-dose discovery study will be performed to identify and develop new biomarkers. A treatment experiment will be completed to characterize the influence of cytokine and conventional treatment on candidate biomarkers. In this study, we plan to use a "Conventional Treatment Supportive Care Therapy" approach that will include more aggressive therapy (i.e., cytokine treatment (granulocyte colony stimulating factor or G-CSF), administration of intravenous (IV) fluids, antibiotics, blood transfusion, etc.) similar to current treatment practice to characterize any effects of these treatments on the radiation biomarkers profile. See Table 1 below for a comparison of the two therapies. A dose-

and time-course experiment will then be performed to validate the selected biomarkers for the dose and injury assessment. Tissue sharing to test other candidate biomarkers will be considered via amendments to maximize the benefits from the study. Early and rapid dose estimates will be required in radiation disasters that involve a large number of victims and a finite amount of medical resources available to responders and healthcare providers. Our expectations are that this project will contribute to bridge a gap that exists in the current capabilities to identify and then rapidly as well as effectively to assess radiation exposure early after a radiation exposure and especially after a mass-casualty radiological incident. In particular, these efforts contribute to validating an early test to distinguish individuals exposed and injured by radiation in order to assist the physicians to choose the appropriate medical treatments and hence reducing the adverse acute effects or long-term risks associated with radiation exposure.

Table 1. Definition of Minimum and Conventional Treatment Supportive Care Therapy

Specific supportive care regime	**Minimum supportive care therapy**	**Conventional treatment supportive care therapy**
Supplementary oral fluids, citrus-free and soft food, analgesic for distress and mouth ulcers or bloody stools, therapeutics for anti-gas, anti-gastric reflux, anti-emesis, anti-diarrhea, elevated temperature, and topical antiseptic for local infection	Yes	Yes
Analgesic for prolonged restraint due to conventional treatment supportive care, cytokine (G-CSF) countermeasure, systematic antibiotics for bacterial infection, IV fluids for dehydration, anti-ulcerative therapeutic for bloody stool, and whole blood transfusion for platelet depression.	No	Yes
Note. See Appendices D and E for additional details		

II. BACKGROUND

II.1. Background

Since the terrorist attack of 9/11/2001, risks of potential radiation exposure scenarios include the of detonation of nuclear weapons, terrorist attacks on nuclear reactors, covert placement of large sources in public places, and dispersal of radioactive substances with the use of conventional explosives. Lack of availability or inaccurate initial absorbed dose estimates can result in suboptimal medical intervention. In the early-phase of a radiation incident, we currently rely on clinical signs (i.e., hematology changes), symptoms (i.e., onset of vomiting), and chromosome biodosimetry. Gaps have been identified in triage tools to aid first-responders, especially in the case of a mass-casualty radiological incident to rapidly identify individuals at risk of life-threatening exposures, exposed but not life-threatening, and non-exposed or worried well.

Early treatment of populations exposed to ionizing radiation requires accurate and rapid biodosimetry with a precision as high as possible to determine an individual's exposure dose, organ-specific radiation injury level, and severity levels for potential life-threatening acute radiation syndromes (ARS). The early medical-management situation requires quantitative

indications for early initiation (within one day after radiation exposure) of cytokine therapy in individuals exposed to life-threatening radiation doses and/or bone marrow ARS as well as effective triage tools for first-responders in mass-casualty radiological incidents (MacVittie et al. 2005; Waselenko et al. 2004).

There are two radiation scenarios for which radiological countermeasures are being developed. The first scenario is a small scale incident where victims will have access to medical facility and clinical support. This proposal, however, focuses primarily on the second scenario, a catastrophic event involving large number of casualties. In this scenario, there will be limited medical and physical resources. Our interests are to develop biodosimetric assays to permit early and rapid radiation exposure assessment applicable for a forward-field application. The focus of the main study design here is modeled to permit assessment of radio-responses prior to use of supportive-care medical treatment.

AFRRI scientists have advocated use of multiple parameter biodosimetry with emphasis on use of hematology and plasma biomarkers to assist in early-phase biodosimetry triage (b)(6),(b)(4) (b)(6),(b)(4) Studies have been performed in mouse and non-human primate models following total-body irradiation (TBI) exposures to gamma radiation; see publications at AFRRI's (b)(6),(b)(4) (b)(6),(b)(4) and in Appendix J. The radioresponse for hematology and body-weight changes from our earlier dose-response study is shown in Appendix A. Proteomic biodosimetry provides diagnostic information both to estimate absorbed dose and for the assessment of the severity of injury for relevant organ systems associated with the ARS (b)(6),(b)(4) Sigal et al. 2012). Plasma (cell free) blood-cell surface markers were recently used as surrogates for blood-cell counts and demonstrated to be diagnostically useful for dose assessment in the early-phase after irradiation. Recent mouse studies involving combined injury (i.e., radiation and wounding) identified a subset of radiation biomarkers that are not influenced by wounding, which were successfully used to accurately estimate radiation absorbed dose. A similar biodosimetry panel was used to accurately estimate radiation absorbed dose using archived samples from a prior NHP dose-response study using Meso Scale Diagnostics' (MSD) technology that permits multiplex measurements over a wide dynamic range.

BARDA has asked us to sustain our interactions with their contractor (MSD), who has adopted AFRRI's biodosimetry panel and collaborated with AFRRI to expand the panel to include additional organ-specific biomarkers additional to the panel described in three patent applications (see Appendix J). Follow-on studies using animal model systems are needed to further develop this diagnostic system.

Planned experiments include: 1) baseline measurements of candidate biomarkers in all animals prior to irradiation, 2) high-dose discovery experiment to provide key samples for testing of candidate antibody pairs of novel biomarkers of organ injury, 3) treatment experiment to evaluate the influence of conventional treatment supportive care therapy (i.e., G-CSF, IV fluids, antibiotics, etc.) (See Appendix E) on candidate biomarkers, and 4) dose-response study to permit testing of selected biomarkers for dose assessment accuracy.

In addition, a second goal of the study is to characterize the time course for biomarkers of acute phase and ARS organ injuries expressed during the evolution of the clinical case (0- 60 d). Note that full supportive care treatments involve IV injections likely to affect the plasma levels of some biomarkers and hematological parameters. We will request histopathology on selected animals and measure ARS severity levels in this model using a NHP ARS severity-scoring system developed in our laboratory. This will allow us to use biomarkers levels as prognostic indicators of radiation injury effects. We also plan to include tissue sharing when possible to maximize the benefits from the study.

The planned studies are needed to further discovery and validation of radiation-responsive biomarkers and develop a validated biodosimetry device, which represents the major goal of the biodosimetry program at AFRRI.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

Biomedical Research database (BRD): http://brd.dtic.mil/

NIH Research Portfolio Online Reporting Tools electronic resource (RePORTER): http://projectreporter.nih.gov/reporter.cfm

PubMed: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed

II.2.2. Date of Search

September 11, 2012; November 1, 2012

II.2.3. Period of Search

BRD: 1998-2009

RePORTER: 1987-2012

PubMed: 1950 to 9/2012

II.2.4. Key Words of Search

September 11, 2012:

Gamma-rays and biomarkers and non-human primates and whole-body: BRD (0), RePORTER (0), PubMed (0).

Gamma-rays or x-rays or radiation and biomarkers or bioindicators or bioassays or biological dosimetry or biodosimetry and non-human primates and whole body: BRD (0), RePORTER (0), PubMed (1028875).

Gamma-rays and biomarkers: BRD (0), RePORTER (937), PubMed (674).

Gamma-rays and biomarkers and non-human primates and whole-body: BRD (0), RePORTER (0), PubMed (0).

X-rays and biomarkers: BRD (0), RePORTER (1214), PubMed (11545).

X-rays and biomarkers and non-human primates and whole-body: BRD (0), RePORTER (0), PubMed (0).

Radiation and biomarkers: BRD (0), RePORTER (942), PubMed (14003).

Radiation and biomarkers and non-human primates and whole-body: BRD (0), RePORTER (0), PubMed (2).

Radiation and non-human primate and whole-body and dose response: BRD (0), RePORTER (0), PubMed (8).

Radiation and non-human primate and whole-body and G-CSF: BRD (0), RePORTER (0), PubMed (3).

Radiation and non-human primate and whole-body and G-CSF pilot: BRD (0), RePORTER (0), PubMed (0).

High dose radiation and non-human primate and whole-body: BRD (0), RePORTER (0), PubMed (7).

High dose radiation and non-human primate and whole-body and dose response: BRD (0), RePORTER (0), PubMed (4).

High dose radiation and non-human primate and whole-body and G-CSF: BRD (0), RePORTER (0), PubMed (0).

High dose radiation and non-human primate and whole-body and G-CSF pilot: BRD (0), RePORTER (0), PubMed (0).

Radiation and biomarkers and non-human primates and whole-body and blood: BRD (0), RePORTER (0), PubMed (1).

Radiation and non-human primate and whole-body and dose response and blood: BRD (0), RePORTER (0), PubMed (5).

Radiation and non-human primate and whole-body and G-CSF and blood: BRD (0), RePORTER (0), PubMed (3).

Radiation and biomarkers and non-human primates and whole-body and urine: BRD (0), RePORTER (0), PubMed (0).

Radiation and non-human primate and whole-body and dose response and urine: BRD (0), RePORTER (0), PubMed (0).

Radiation and non-human primate and whole-body and G-CSF and urine: BRD (0), RePORTER (0), PubMed (0).

Radiation and biomarkers and non-human primates and whole-body and hair follicle: BRD (0), RePORTER (0), PubMed (0).

Radiation and non-human primate and whole-body and dose response and hair follicle: BRD (0), RePORTER (0), PubMed (0).

Radiation and non-human primate and whole-body and G-CSF and hair follicle: BRD (0), RePORTER (0), PubMed (0).

Ionizing radiation and non-human primates: BRD (8), RePORTER (3), PubMed (19).

Ionizing radiation and non-human primates and whole-body: BRD (0), RePORTER (0), PubMed (6).

Ionizing radiation and NHP and G-CSF: BRD (0), RePORTER (0), PubMed (0).

Gamma-rays and biomarkers and non-human primates: BRD (0), RePORTER (1), PubMed (1).

Non-human primates and radiation: BRD (0), RePORTER (59), PubMed (134).

Non-human primates and radiation and whole-body: BRD (0), RePORTER (8), PubMed (25).

Radiation and biomarkers and non-human primates and whole-body and nail clipping: BRD (0), RePORTER (0), PubMed (0).

November 1, 2012:

Gamma-rays and biomarkers and non-human primates: BRD (0), RePORTER (1), PubMed (1).

Gamma-rays or x-rays or radiation and biomarkers or bioindicators or bioassays or biological dosimetry or biodosimetry and non-human primates: BRD (0), RePORTER (0), PubMed (1031838).

Gamma-rays and biomarkers: BRD (0), RePORTER (1189), PubMed (680).

Gamma-rays and biomarkers and non-human primates: BRD (0), RePORTER (1), PubMed (1).
X-rays and biomarkers: BRD (0), RePORTER (1542), PubMed (11732).
X-rays and biomarkers and non-human primates: BRD (0), RePORTER (0), PubMed (3).
Radiation and biomarkers: BRD (28), RePORTER (1216), PubMed (14179).
Radiation and biomarkers and non-human primates: BRD (2), RePORTER (1), PubMed (10).
Radiation and non-human primates and dose response: BRD (2), RePORTER (15), PubMed (24).
Radiation and non-human primates and G-CSF: BRD (0), RePORTER (3), PubMed (5).
Radiation and non-human primates and G-CSF pilot: BRD (0), RePORTER (0), PubMed (0).
High dose radiation and non-human primates: BRD (5), RePORTER (4), PubMed (16).
High dose radiation and non-human primates and dose response: BRD (1), RePORTER (1), PubMed (9).
High dose radiation and non-human primates and G-CSF: BRD (0), RePORTER (0), PubMed (0).
High dose radiation and non-human primates and G-CSF pilot: BRD (0), RePORTER (0), PubMed (0).
Radiation and biomarkers and non-human primates and blood: BRD (2), RePORTER (0), PubMed (6).
Radiation and non-human primates and dose response and blood: BRD (1), RePORTER (8), PubMed (11).
Radiation and non-human primates and G-CSF and blood: BRD (0), RePORTER (10), PubMed (3).
Radiation and biomarkers and non-human primates and urine: BRD (0), RePORTER (0), PubMed (0).
Radiation and non-human primates and dose response and urine: BRD (0), RePORTER (0), PubMed (1).
Radiation and non-human primates and G-CSF and urine: BRD (0), RePORTER (0), PubMed (0).
Radiation and biomarkers and non-human primates and hair follicle: BRD (0), RePORTER (0), PubMed (0).
Radiation and non-human primates and dose response and hair follicle: BRD (0), RePORTER (0), PubMed (0).
Radiation and non-human primates and G-CSF and hair follicle: BRD (0), RePORTER (0), PubMed (0).
Ionizing radiation and non-human primates: BRD (11), RePORTER (3), PubMed (19).
Ionizing radiation and NHP and G-CSF: BRD (0), RePORTER (0), PubMed (1).
Gamma-rays and biomarkers and non-human primates: BRD (0), RePORTER (1), PubMed (1).
Non-human primates and radiation: BRD (0), RePORTER (59), PubMed (134).
Non-human primates and radiation: BRD (30), RePORTER (59), PubMed (135).
Radiation and biomarkers and non-human primates and nail clipping: BRD (0), RePORTER (0), PubMed (0).

II.2.5. Results of Search

Searches were performed using BRD, RePORTER, and PubMed on September 11, 2012.

Using the above keywords, the literature search of DoD Biomedical Research (BRD) showed no past or ongoing project that was duplicative to our proposed study to evaluate the combined blood-based proteomic and hematological approach on biodosimetry endpoints dose-response relationships for radiation dose assessment, dose-dependent discrimination index of radiation exposure, and the response category severity score of ARS in non-human primate TBI model. In the literature search using the PubMed data base, one of the identified publications focused to compare the efficacy of autologous cell therapy after irradiation combined with G-CSF injections with G-CSF treatment alone in a heterogeneous model of irradiation representative of an accidental situation (Int. J. Radiation Oncology Biol. Phys., Vol 63, No. 3, pp 911-920, 2005). In another PubMed publication two cytokines G-CSF and interleukin-6 (IL-6) were identified as the candidate biomarkers of CBLB502's radioprotective/mitigative efficacy (J Pharmacol Exp Ther. 2012 Jul 26). In our study the focus is to investigate the modifying effects of cytokine (G-CSF) treatment on panel of biomarkers in non-human primate TBI model to identify and validate panels of protein biomarkers that can be used in the radiation dose and injury assessment, ARS diagnosis, and the influence of cytokine and conventional treatments (i.e., blood transfusion, antibiotic therapy, etc.).

The search in Research Portfolio Online Reporting Tools electronic resource (Reporter) does not show any duplicative projects to our proposed study.

Searches were performed using BRD, RePORTER, and PubMed on November 1, 2012.
The 1 Nov 2012 keyword literature search for duplication, omitting the word "whole body", revealed few more hits. Notably, in PubMed search with keyword "radiation and non-human primates and G-CSF", one study (J. Rad. Res. 2012, 53, 640-853) with mice showed that the survival-enhancing effects of 5-AED (5-androstendiol) are dependent on endogenous G-CSF and are associated with functional activation of innate immune cells during the acute radiation syndrome (ARS). Another report popped up under BRD database with the keyword "ionizing radiation and NHP and G-CSF" regarding a study initiated at AFRRI in 2008. The study was aimed to determine the ability of G-CSF and CBLB612 to mobilize stem cells into the peripheral blood of NHP after transfusion against gamma-irradiation. Later the study was abandoned due to technical difficulties and expenses. Other 10 hits with RePORTER-database with the keyword, "radiation and non-human primates and G-CSF and blood" were found to be irrelevant to the mode of our study.

III. OBJECTIVE / HYPOTHESIS

General Hypothesis:
Multiple blood protein biomarkers along with hematological surrogates will provide enhanced diagnostically useful indices to discriminate between injured and irradiated individuals. A panel of protein biomarkers, each with different radiation responses, coupled with peripheral blood cell counts or hematology surrogates will provide accurate assessment as well as an enhanced discrimination index of radiation exposure.

Using a NHP total-body radiation model the proposal's **objectives** are to:

1) Establish baseline levels of candidate biomarkers (Experiment #1).

2) Perform a pilot high dose experiment to permit testing of the measurement of novel organ specific biomarkers in isolated peripheral blood using commercially available antibodies (Experiment #2).
3) Evaluate the effects of "Conventional Treatment Supportive Care Therapy (G-CSF, IV fluids, antibiotics, blood transfusion, etc.) for radiation injury on the candidate biomarkers (Experiment #3).
4) Measure biomarker responses in a time- and dose-response study to evaluate the suitability of the selected biodosimetry biomarker panel to accurately assess radiation dose (Experiment #4).
5) Characterize radioresponse of animals following radiation exposure to permit correlation of biomarker levels to effect as measured by severity score of histopathology or ARS (Experiments #1-4).
6) Consider tissue sharing (i.e., urine, fingernail clippings, etc.) in these studies to maximize benefit from the study (Experiments #1-4) via subsequent amendments/tissue sharing if required.

IV. MILITARY RELEVANCE

Radiological terrorist attack or accidental mass-casualty exposures is highly possible. Military personnel responding to such emergencies can be exposed to radiation. Recognizing unpredictable mass casualties, U.S. Department of Defense has given top priority, as stated in the "Defense Technology Objectives (DTO)" to the development of medical countermeasures to radiation exposure" against both early- and late-arising health effects. In the case of mass casualties, it is difficult to screen out the severely exposed patient from the less exposed or not exposed individuals due to lack of immediate and convenient dose measurement technology. This study will determine if the combined proteomic and hematological approach is feasible for biodosimetry whole-body radiation dose assessment and also provide an enhanced discrimination index of radiation exposure to effectively manage radiation-casualty incidents or nuclear events. In the case of such an event, rapid and accurate assessment of radiation dose would be necessary to guide treatment decisions. This strategy addresses the need for developing a high-throughput multiparametric "***Field Radiological Biodosimetry***" system and promotes effective command decisions and force structure planning to ensure mission success. This system should be compatible with military field laboratories, homeland security applications, as well as with radiation therapy centers to assess radiation exposure based on blood protein biomarkers capability. In addition, assessment of a population's exposure in other radiation threats, such as nuclear accidents and terrorism mass-casualty scenarios addresses the need for a "***Clinical Radiological Biodosimetry***" system to provide physicians with the ability to triage radiation victims, make appropriate treatment decisions, and reduce uncertainties associated with the variability of individual response to radiation exposure. This proposed study is designed to support a BARDA funding contractor (MSD) to develop and validate a biodosimetry device. In a recent survey of requirements from the warfighters conducted by the Joint Requirements Office, medical diagnostics including radiation biodosimetry was rated with the highest priority (#14) among the radiological countermeasure requirements (i.e., medical countermeasures – protectant, treatment).

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

Baseline levels of candidate biomarkers and an ARS severity score will be measured in all animals pre-irradiation (**Experiment #1**). Non-human primates (NHPs), rhesus monkeys will be exposed to total-body gamma irradiation in AFRRI's Cobalt Facility. Pilot single high-dose discovery (**Experiment #2**) and conventional treatment supportive care therapy (**Experiment #3**) experiments will be performed for discovery and evaluation of promising new biomarkers as well as for evaluation of G-CSF cytokine treatment and additional supportive care therapy on the candidate radiation biomarker profile. In support of Experiment #3, six NHPs will be used as whole blood donors. These donor animals will be permitted a 30-60 d recovery period and if they demonstrate a normal hematology profile, they will be used as study animals in other experiments. In addition a range of radiation doses (0, 1, 3.5, 5, 6.5, and 8.5 Gy at ~0.56 Gy/min) will be used to explore the dose dependency of the various technologies (**Experiment #4**). See section "Study Endpoint (V.4.5.) for details regarding the expected outcome for NHPs in this study. Samples will be acquired at a range of time points before and after radiation (as below) to determine the time course of the biomarkers in this system.

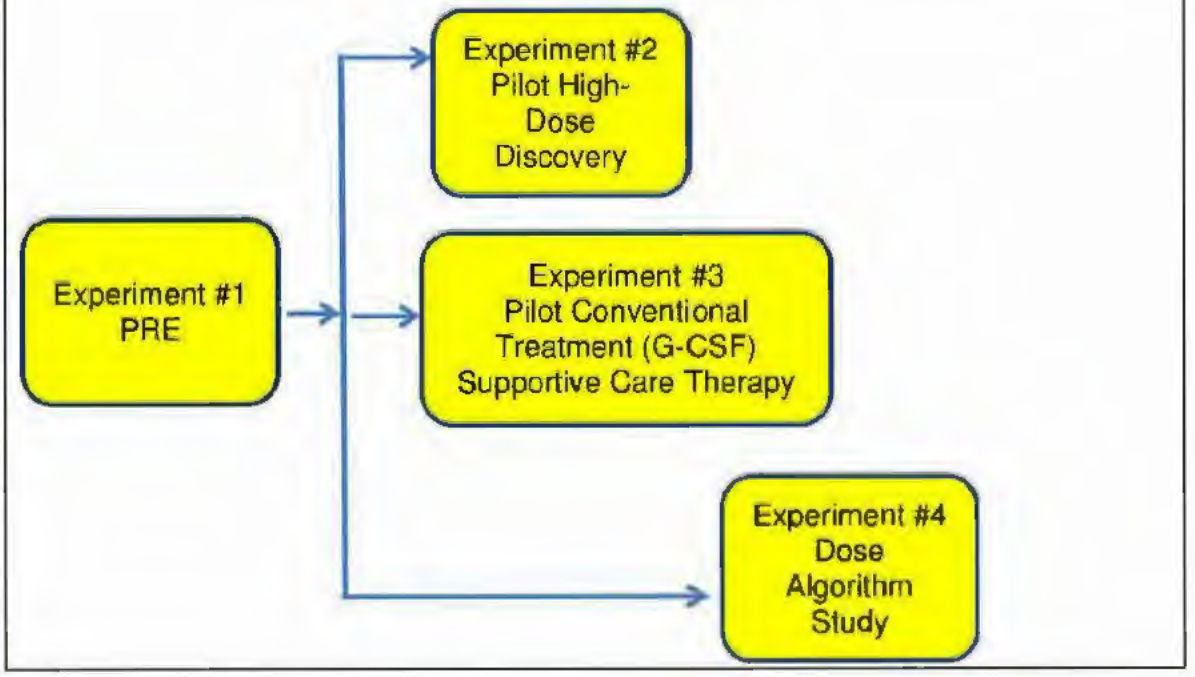
An ARS response severity score will be assessed prior to radiation (baseline; **Experiment #1**), 3 - 4 times per week after irradiation, including complete blood counts with differentials (CBC/diff), body weights, body temperatures, etc. (see attached ARS Severity Scoring System sheet, Appendix B). Body widths will be measured prior to irradiation to support accurate dosimetry. CBC/diff will be determined using the VSD clinical hematology analyzer or in another approved laboratory (e.g., Bio Reliance). Other biomarkers will be assessed at multiple time points as described below.

Rhesus macaques (*Macaca mulatta*) will be housed singly in stainless steel cages. On the morning of irradiation, animals will not be fed.

The times of emesis for each animal will be recorded during the first 12 h after irradiation as described in Appendix B. Each animal will be observed at least twice daily for evidence of pain or distress by VSD techs or caretakers during rounds. Research staff will also monitor the status of NHPs daily. As the animals start to become ill, the research staff will increase the monitoring frequency accordingly. A rhesus macaque ARS response severity scoring system has been developed and will be used in these studies (See Appendix B). Clinical assessment of the NHPs will be conducted throughout the study. This would include such parameters as hydration status, presence or absence of fever, lethergy or diarrhea, assessment of the respiratory and cutaneous systems and changes in the CBC. Blood for CBC will be collected and analyzed at 1 and 2 weeks before irradiation and at least 3 times per week after irradiation. Body temperature will be determined 3 times per week using implanted microchips (IPTT-200; BMDS, Seaford, DE) in chaired monkeys in daily rounds beginning approximately one week prior to the first treatment. (Balcombe et al. 2004). The time of each temperature collection will be recorded for each monkey. Body weights will be determined at least once prior to radiation exposure and at least 3 times per week after irradiation for 60 days. The primary endpoint for the studies is the radioresponse of plasma biomarkers. AFRRI's additional primary study endpoints will be mortality (or moribundity) along with ARS- subsyndromes biomarkers (i.e., kinetics of neutrophil, platelet, and lymphocyte counts, other blood count and differential leukocyte count variables, GI severity score), acute injury- and ARS organ injury plasma biomarkers, and serum amylase activity.

Figure 1 illustrates the overall schedule for the four experiments. The Summary Tables – Schedule A, B, and C shown below provide a more detailed sequence for individual animals.

Figure 1. Novel Biodosimetry Technology: Schedule



Summary Table – Schedule A

Number	Animal Code	1st reuse	Shipment	Exp Code	Dose, Gy		[-2 Month]	[-1 Month]	1st Month	2nd Month	3rd Month	4th Month	5th Month	6th Month
							APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV
Number of NHPs in 45 d quarantine							22	22			14	14		
Running number of NHPs in protocol									22	20	34	34	29	28
Expected number of NHPs losses due to radiation									2	0	0	3	1	0
Expected numbe of NHP for necropsy									[1] cytokine			[1] cytokine		2 (5 Gy)
Expected number of surviving NHPs									20	20	34	31	28	26
Number of NHPs returned to issue pool									0	0	0	2 (6.5 Gy)	0	0
Running total of NHPs under study									20	20	34	29	28	26
Number of NHPs under active study						[new + old]			[4]	[4+4]	[0+4]	[6+0]	[6+4]	[0+6]
1	NIO-1		1st			[Bld Donors]			S	S			S	
2	NIO-2		1st			[Bld Donors]			S	S			S	
3	NIO-3		1st			[Bld Donors]			S			S	S	
4	NIO-4		1st			[Bld Donors]			S			S	S	
5	NIO-5		1st			[Bld Donors]				S		S		
6	NIO-6		1st			[Bld Donors]				S		S		
7	NIO-7		1st	Exp 2-1	6.5	HD Study			X					
8	NIO-8		1st	Exp 2-2	0	HD Study			S					
9	NIO-9		1st	Exp 2-3	6.5	HD Study				S				
10	NIO-10		1st	Exp 2-4	0	HD Study				S				
11	NIO-11		1st	Exp 3-1	6.5	G-CSF +			X/N					
12	NIO-12		1st	Exp 3-2	0	G-CSF +			S					
13	NIO-13		1st	Exp 3-3	0	G-CSF +				S				
14	NIO-14		1st	Exp 3-4	6.5	G-CSF +				S				
15	NIO-15		1st	Exp 3-5	0	G-CSF +						S		
16	NIO-16		1st	Exp 3-6	6.5	G-CSF +						X/N		
17	NIO-17		1st	Exp 3-7	0	G-CSF +							S	
18	NIO-18		1st	Exp 3-8	6.5	G-CSF +							S	
19	NIO-19		1st	Exp 4-1	0	Dose Response						S		
20	NIO-20		1st	Exp 4-2	0	Dose Response						S		
21	NIO-21		1st	Exp 4-3	8.5	Dose Response						X		
22	NIO-22		1st	Exp 4-4	8.5	Dose Response						X		
23	NIO-23		2nd	Exp 4-5	6.5	Dose Response							X	
24	NIO-24		2nd	Exp 4-6	6.5	Dose Response							S	S
Code: S = Survival	X = Expectant		N: Necropsy											

Summary Table – Schedule B

Number	Animal Code	1st reuse	Shipment	Exp Code	Dose, Gy		7th Month	8th Month	9th Month	10th month
							DEC	JAN	FEB	MAR
Number of NHPs in 45 d quarantine										
Running number of NHPs in protocol							26	24	20	17
Expected number of NHPs losses due to radiation							0	2	1	0
Expected numbe of NHP for necrospy								2 (3.5 Gy); 2 (1Gy)		
Expected number of surviving NHPs							26	20	19	17
Number of NHPs returned to issue pool							2 (6.5 Gy)		2 (1Gy)	2 (1 Gy); 2 (3.5 Gy)
Running total of NHPs under study							24	20	17	13
Number of NHPs under active study						(new + old)	[6+0]	[6+6]	[6+4]	[0+6]
		NIO-8	1st	Exp 4-7	3.5	Dose Response	S	S/N		
		NIO-10	1st	Exp 4-8	3.5	Dose Response	S	S/N		
25	NIO-25		2nd	Exp 4-9	5	Dose Response				
26	NIO-26		2nd	Exp 4-10	5	Dose Response				
27	NIO-27		2nd	Exp 4-11	1	Dose Response	S	S/N		
28	NIO-28		2nd	Exp 4-12	1	Dose Response	S	S/N		
29	NIO-29		2nd	Exp 4-13	0	Dose Response	S			
30	NIO-30		2nd	Exp 4-14	0	Dose Response	S			
		NIO-1	1st	Exp 4-15	8.5	Dose Response		X		
		NIO-2	1st	Exp 4-16	8.5	Dose Response		X		
		NIO-3	1st	Exp 4-17	1	Dose Response		S		
		NIO-4	1st	Exp 4-18	1	Dose Response		S		
		NIO-5	1st	Exp 4-19	3.5	Dose Response		S		
		NIO-6	1st	Exp 4-20	3.5	Dose Response		S		
31	NIO-31		2nd	Exp 4-21	5	Dose Response			S	
32	NIO-32		2nd	Exp 4-22	5	Dose Response			S	
33	NIO-33		2nd	Exp 4-23	6.5	Dose Response			S	
34	NIO-34		2nd	Exp 4-24	6.5	Dose Response			S	
35	NIO-35		2nd	Exp 4-25	0	Dose Response			S	
36	NIO-36		2nd	Exp 4-26	0	Dose Response			S	
Code: S = Survival	X = Expectant		N: Necrospy							

Summary Table – Schedule C

Number	Animal Code	1st reuse	Shipment	Exp Code	Dose, Gy		11th Month	12th Month	13th Month	14th Month	15th Month	
							APR	MAY	JUNE	JULY	AUG	
Number of NHPs in 45 d quarantine												
Running number of NHPs in							13	10	7	7	4	
Expected number of NHPs losses due to radiation							0	3	0	0	0	12
Expected numbe of NHP for necrospy												6
Expected number of surviving NHPs							13	7	7	7	4	
Number of NHPs returned to issue pool							2 (5Gy); 1 (6.5 Gy)	0		1 (6.5 Gy); 2(3.5 Gy)	2 (1 Gy); 2 (5Gy)	18
Running total of NHPs under study							10	7	7	4	0	
Number of NHPs under active study						[new + old]	[0]	[6+0]	[6+4]	[0+4]		
		NIO-12	1st	Exp 4-27	8.5	Dose Response		X				
		NIO-13	1st	Exp 4-28	8.5	Dose Response		X				
		NIO-15	1st	Exp 4-29	6.5	Dose Response		X				
		NIO-17	1st	Exp 4-30	6.5	Dose Response		S				
		NIO-19	1st	Exp 4-31	3.5	Dose Response		S				
		NIO-20	1st	Exp 4-32	3.5	Dose Response		S				
		NIO-29	2nd	Exp 4-33	1	Dose Response			S			
		NIO-30	2nd	Exp 4-34	1	Dose Response			S			
		NIO-35	2nd	Exp 4-35	5	Dose Response			S			
		NIO-36	2nd	Exp 4-35	5	Dose Response			S			
Code: S = Survival	X = Expectant		N: Necrospy									

V.1.1. Experiment 1

PRE:

Animals: all animals in the protocol study (36)
Injection: microchips will be injected into animals under ketamine (10 mg/kg; via intramuscular (IM) injection using 12 gauge needle and syringe; see Section V.4.4.1.
Radiation doses: NA
Radiation exposure: NA
Treatment: NA
Assays: baseline levels of body weights, body widths, body temperatures, hematology (see Appendix C), blood chemistry (see Appendix C), proteomic biomarker parameters (see Appendix C), and ARS severity scores (see Appendix B) will be measured prior to irradiation
Time course: 1-2 times per week, 2-3 weeks (see Appendix C for specific details)

V.1.2. Experiment 2

Pilot high-dose discovery study:

Animals: 2 per dose (n = 4)
Radiation doses: 0 and 6.5 Gy [2 doses].
Radiation exposure: TBI will be given as a single exposure
Treatment: "Minimum supportive care therapy"; See Appendix D for additional details.
Assays: body weights, body temperatures, hematology (see Appendix C), blood chemistry (see Appendix C) proteomic biomarker parameters, and ARS severity scores (see Appendix B) will be measured after irradiation.
Time course: (Pre), 5 h, 1, 2, 3, 4, 5, 6, 7, 10 days and later time-points (2-3x weeks up to 60 days) in survived animals; see Appendix C for specific details.

V.1.2. Experiment 3

Pilot "Conventional Treatment Supportive Care Therapy" study:

Animals: 4 per dose (n = 8)
Radiation doses: 0 and 6.5 [2 doses].
Exposure: TBI will be given as a single exposure.
Treatment: "Conventional Treatment Supportive Care Therapy" including G-CSF treatment (10 µg/kg, once a day; 22-25 G needle; SC, 1-ml syringe) starting on day 1 after exposure and continuing until the recovery of absolute neutrophil count (ANC) >= 2,000 μL^{-1} for 2 consecutive days along with "conventional supportive care therapy" approach that will include more aggressive therapy (i.e., administration of IV fluids, whole blood transfusions, etc.); see Appendices E and H for additional details. In the case of 6.5-Gy irradiated NHPs where we expect to perform whole blood transfusions, we expect the duration of G-CSF treatment to continue for ~20 days.

Assays: body weights, body temperatures, hematology (see Appendix C), blood chemistry (see Appendix C) proteomic biomarker parameters, and ARS severity scores (see Appendix B) will be measured after irradiation.
Time course: (Pre), 5 h, 1, 2, 3, 4, 5, 6, 7, 10 days and later time points (2-3x weeks up to 60 days) in survived animals; see Appendix C for specific details.

Whole blood donor animals: n=6 [Study animals are planned to be used for blood donation. If there is a need where study blood donor animals are not available, then Protocol PIs will request for use of VSD residence pool animals, which have been approved for use for this purpose].

Radiation dose: NA
Exposure: NA
Treatment: NA
Assays: baseline levels of body weights, body widths, body temperatures, hematology (See Appendix C), blood chemistry (see Appendix C) and ARS severity scores (see Appendix B) will be measured to assure health suitability for blood donation.
Note. Guidelines for blood donations volumes for whole blood transfusions to support the G-CSF Treatmont Study guidelines (See Appendix E) are shown in Appendix H. Six animals will be used to this purpose in the early-phase of the study design. Animals selected for donation will be rotated to assure sufficient recovery period (1 month) between repeated whole blood donations. The recovery period for repeated blood donation is expected to be 1 month, which will be confirmed by a normal hematology profile.

V.1.2. Experiment 4

Dosimetry algorithm study:

Animals: 6 per dose (36) (We plan to re-use surviving sham animals for later use in radiation cohorts).
Radiation doses: 0, 1, 3.5, 5, 6.5, and 8.5 Gy [6 doses].
Exposure: TBI will be given as a single exposure.
Treatment: "Minimum Supportive Care Therapy"; See Appendix D for additional details.
Assays: body weights, body temperatures, hematology (see Appendix C), blood chemistry (see Appendix C) proteomic biomarker parameters (see Appendix C), and ARS severity scores will be measured after irradiation
Time course: (Pre), 5 h, 1, 2, 3, 4, 5, 6, 7, 10 days and later time points (2-3x weeks up to 60 days) in survived animals; see Appendix C for specific details.

V.2. Data Analysis

All hematology, vital signs (including weights), serum chemistry, plasma protein biomarkers levels data will be summarized using descriptive statistics on each assessment time for each dose cohort. Continuous variables will be summarized using sample size, mean, standard deviation, minimum and maximum values.

Statistical software, PC SAS, will be used for statistical analyses. If applicable, analysis of variance or multivariate analysis of variance (MANOVA) will be used for comparison among

the groups. If significant, appropriate pairwise comparisons will be used. The significance level will be set at 5% for each test. Linear regression, Fisher's exact test or McNemar test will be used if applicable. All statistical tests will be two-sided. Adjustment of multiple tests will not be made.

Justification of sample sizes: for any primary outcome variable in each experiment, the sample size of 6 monkeys will give 80% power to detect any difference between any two groups, if any, by assuming that type I error = 5%, type II error = 20%, and delta/sigma at least 1.8, where delta is equal to the absolute difference between the means of two groups, and sigma = the pooled standard deviation in the applicable statistical model.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

The purpose of this study is to evaluate radiation biodosimetry diagnostics involving use of organ specific targets that have been initially evaluated in rodent models, tested in NHP models, and in selected cases used in human radiation accidents. This technology is expected to be used with human samples in a forward deployment scenario, considering portability, accuracy, and ease-of-use. It is not possible to experimentally irradiate human subjects to test this technology, nor can presently available computer modeling or cell cultures studies adequately meet the research objectives.

V.3.2. Animal Model and Species Justification

The FDA has accepted rhesus macaque as an appropriate animal model for testing radiation countermeasures under the "Animal Efficacy Rule", where it cannot be performed in humans. Rhesus macaques are the model of choice for measurements of experimental radiation dose by using the current radiation biomarker panel. The biomarker technology has already been demonstrated in rodent animal radiation models and in selected cases in NHP radiation models. NHPs are more relevant to humans than other animals. Antibodies for NHP biomarkers are more readily available for measurement of candidate radiation biomarkers than in the minipig model. Therefore, this study in rhesus macaques is being undertaken to guide future development of the radiation blood plasma biomarker panel device.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species *Macaca mulatta*

V.3.3.2. Strain / Stock N/A

V.3.3.3. Source / Vendor

Animals will be obtained from Veterinary Sciences Department (VSD) approved vender.

V.3.3.4. Age 3 to 7.5 yrs

V.3.3.5. Weight 3 to 8 kg

V.3.3.6. Sex Males and Females (~50% split requested from vendor)

V.3.3.7. Special Considerations

Healthy NHPs are ordered in accordance with VSD requirements outlined in the NHP procurement contract. NHPs will be pole and collar trained by the vendor.

V.3.4. Number of Animals Required (by species)

Rhesus monkey (Macaca mulatta): 36

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement

Implantable microchips (IPTT-200; BMDS, Seaford, DE) will be injected for ID and temperature measurements to lessen animal stress during the observation period. See Appendix I for a detailed description of the subcutaneous microchip implantation using a needle (12 Gauge) and syringe like device. The endpoint currently mandated by the FDA for approval of radiation countermeasures is mortality. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress, using an extensive set of criteria. See Appendix G for a detailed description of the clinical observations with criteria for euthanasia.

V.3.5.2. Reduction

A total of 36 NHPs will be used in this protocol. We have made significant efforts to maximize research use of this model. Non-irradiated animals in Experiments #2 and #3 will be reused in the subsequent Experiment #4. We plan to consider tissue sharing and collaborations, via IACUC protocol amendments, to enhance the benefits of the study. At every stage of the protocol, plans will be reevaluated in light of incoming data in an attempt to reduce the number of experiments. If it is determined at any point that the proof of principal studies are inconclusive, then the rest of the experiments using other NHPs will not be performed.

V.3.5.3. Replacement

None.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C ___0__ (# of animals)

V.4.1.1.1.2. Column D ___0__ (# of animals)

V.4.1.1.1.3. Column E ___36__ (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
3,1,4	NIO-1: Donor => PRE=> Dose Algorithm (8.5 Gy)			1	1
3,1,4	NIO-2: Donor=> PRE=> Dose Algorithm (8.5 Gy)			1	1
3,1,4	NIO-3: Donor => PRE=> Dose Algorithm (1 Gy)			1	1
3,1,4	NIO-4: Donor => PRE=> Dose Algorithm (1 Gy)			1	1
3,1,4	NIO-5: Donor => PRE=> Dose Algorithm (3.5 Gy)			1	1
3,1,4	NIO-6: Donor => PRE =>Dose Algorithm (3.5 Gy)			1	1
1,2	NIO-7: PRE=> HD study (6.5 Gy)			1	1
1,2,1,4	NIO-8: PRE=>HD study (0 Gy) =>PRE=> Dose Algorithm (3.5 Gy)			1	1
1,2	NIO-9: PRE=> HD study (6.5 Gy)			1	1
1,2,1,2	NIO-10: PRE=>HD study (0 Gy)=>PRE=> Dose Algorithm ~~(6.5 Gy)~~ 3.5 Gy			1	1
1,3	NIO-11: PRE=>Treatment (6.5 Gy)			1	1
1,3,1,4	NIO-12: PRE=> Treatment (0 Gy)=> PRE=>Dose Algorithm ~~(3.5 Gy)~~ 8.5 Gy			1	1
1,3,1,4	NIO-13: PRE=>Treatment (0 Gy)=>PRE=>Dose Algorithm (8.5 Gy)			1	1
1,3	NIO-14: PRE=>Treatment (6.5 Gy)			1	1
1,3,1,4	NIO-15: PRE=>Treatment (0 Gy)=>PRE=>Dose Algorithm (6.5 Gy)			1	1
1,3	NIO-16: PRE=>Treatment (6.5 Gy)			1	1
1,3,1,4	NIO-17: PRE=>Treatment (0 Gy)=>PRE=>Dose Algorithm (6.5 Gy)			1	1
1,3	NIO-18: PRE=>Treatment (6.5 Gy)			1	1
1,4,1,4	NIO-19: PRE=> Dose Algorithm (0 Gy)=>PRE=>Dose Algorithm (3.5 Gy)			1	1
1,4,1,4	NIO-20: PRE=> Dose Algorithm (0 Gy)=> PRE=>Dose Algorithm (3.5 Gy)			1	1
1,4	NIO-21: PRE=> Dose Algorithm (8.5 Gy)			1	1
1,4	NIO-22: PRE=> Dose Algorithm (8.5 Gy)			1	1
1,4	NIO-23: PRE=> Dose Algorithm (6.5 Gy)			1	1
1,4	NIO-24: PRE=> Dose Algorithm (6.5 Gy)			1	1
1,4	NIO-25: PRE=> Dose Algorithm (5 Gy)			1	1
1,4	NIO-26: PRE=> Dose Algorithm (5 Gy)			1	1
1,4	NIO-27: PRE=> Dose Algorithm (1 Gy)			1	1
1,4	NIO-28: PRE=> Dose Algorithm (1 Gy)			1	1
1,4,1,4	NIO-29: PRE=> Dose Algorithm (0 Gy)=> PRE=> Dose Algorithm (1 Gy)			1	1
1,4,1,4	NIO-30: PRE=> Dose Algorithm (0 Gy)=> PRE=> Dose Algorithm (1 Gy)			1	1
1,4	NIO-31: PRE=> Dose Algorithm (5 Gy)			1	1
1,4	NIO-32: PRE=> Dose Algorithm (5 Gy)			1	1
1,4	NIO-33: PRE=> Dose Algorithm (6.5 Gy)			1	1
1,4	NIO-34: PRE=> Dose Algorithm (6.5 Gy)			1	1
1,4,1,4	NIO-35: PRE=> Dose Algorithm (0 Gy)=> PRE=>Dose Algorithm (5 Gy)			1	1
1,4,1,4	NIO-36: PRE=> Dose Algorithm (0 Gy)=>PRE=> Dose Algorithm (5 Gy)			1	1
Totals:		0	0	36	36

Note. Experiment 1 = preliminary (PRE) baseline study; Experiment 2: high dose (HD) study; Experiment 3: Conventional treatment (Treatment) with selected animals used as blood donors (Donor); Experiment 4: Dose Algorithm Study.

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

A. Anesthesia for Subdermal Implantable Microchip Temperature Monitor Insertion

This procedure will be performed by a veterinarian or a trained veterinary technician with the primate not fed in the morning.

1. The AFRRI veterinarians will access the general health of the NHPs prior to anesthesia to ensure that each animal is healthy enough to undergo the procedure.

2. Fast 12 hours prior to anesthesia (water OK).

3. The primate is sedated with ketamine 10mg/kg via intramuscular (IM) injection to sedate.

4. See Table entitled "Provisions for Both Pre- and Post-Procedural Care" below and Appendix D and E for additional details.

B. Tranquilization for loading into radiation boxes, transport to ^{60}Co facility, measurement of mid-body widths, irradiation of NHPs, and transport back to VSD facility.

This procedure will be performed by a veterinarian or a trained veterinary technician with the primate fasted over night prior to procedure.

1. The AFRRI veterinarians will access the general health of the NHPs prior to anesthesia to ensure that each animal is healthy enough to undergo the procedure.

2. Fast 12 hours prior to anesthesia (water OK).

3. The primate is sedated with ketamine 10mg/kg IM and positioned in the radiation box.

4. Additional ketamine 10mg/kg IM is used to sedate animal as necessary during irradiation and transit to and return from the VSD vivarium to the ^{60}Co facility.

5. See Table entitled "Provisions for Both Pre- and Post-Procedural Care" below Appendix D and E for additional details.

C. Antipyretic Therapy for Fever

1. If body temperature rises above 104^0 F, NHP will be administered Rimadyl 2.2mg/kg BID or 4.4mg/kg QD,IM,IV,PO or 5-10mg/kg PO TID).

2. See Table entitled "Provisions for Both Pre- and Post-Procedural Care" below and Appendix D and E for additional details.

D. Analgesic for Radiation-Induced Distress

This procedure will he performed by a veterinarian or a trained veterinary technician with the primate.

1. The analgesic, Buprenorphrine HCL (0.01 mg/kg up to 0.02 mg/kg BID will he administered to all irradiated (1, 3.5, 5, 6.5, 8.5 Gy) animals from 5 to 35 days after radiation exposure; See Appendix D and E.

2. The protocol staff will daily monitor the animals for clinical signs and symptoms including using an ARS severity scoring system; See Appendix B. Animals demonstating an ARS severity level of 3 will he monitored more frequently as described in the protocol monitoring plan; See Appendix F. Protocol staff will notify the Protocol PIs and AFRRI Veterinarian of the health status of these animals.

3. The protocol staff will monitor animals for clinical observations identified as criteria for euthanasia consideration; see Appendix G.

4. The AFRRI veterinarians will access the general health of the NHPs and order analagesic (Buprenorphrine HCL, 0.01 mg/kg up to 0.02 mg/kg BID, IM 22-25 G needle) for distress if not aleviated by treatment.

4. See Table entitled "Provisions for Both Pre- and Post-Procedural Care" helow and Appendix D and E for additional details.

E. Analgesic for Mouth Ulcers or Bloody Stools

This procedure will he performed by a veterinarian or a trained veterinary technician with the primate.

1. The protocol staff will daily monitor the animals for clinical signs and symptoms including using an ARS severity scoring system; See Appendix B. Animals demonstrating amouth ulcers or bloody stools will be identified and reported to the Protocol PIs and AFRRI veterinarians.

2. *The* AFRRI veterinarians will access the general health of the NHPs and order analagesic (Bupivacaine HCL, 0.1 ml of 25% solution with a dab of surgical lubricant and administered locally using a cotton-tipped applicator to a restrained animal.

3. See Table entitled "Provisions for Both Pre- and Post-Procedural Care" below and Appendix D and E for additional details

F. Anesthesia to calm NHP while in restraint during prolonged IV fluid treatments, blood donation, and whole blood transfusion

This procedure will he performed by a veterinarian or a trained veterinary technician with the primate.

1. Criteria to identify animals for treatment by prolonged IV fluid are described in Appendix G. Criteria to identify animal selection for blood donation and whole blood transfusion are described in Appendix H.

2. The AFRRI veterinarians will access the general health of the NHPs prior to anesthesia to ensure that each animal is healthy enough to undergo the procedure.

3. Fast 12 hours prior to anesthesia (water OK).

4. The primate is sedated with ketamine 10mg/kg via intramuscular (IM) injection to sedate.

5. Isoflurane gas (2% in O2) will be administered via mask as determined by the AFRRI veterinarian.

6. See Table entitled "Provisions for Both Pre- and Post-Procedural Care" below and Appendix E for additional details.

V.4.1.2.2. Pre- and Post-procedural Provisions

Provisions for Both Pre- and Post-Procedural Care		
Procedures*/Location	**Anesthesia/Analgesic***	**Criteria to Assess for Pain or Distress**
Microchip injection/ (b)(6)	Ketamine#/NA	NA
Placement of NHP in Rad Box (b)(6) Procedural Room; Transport to ^{60}Co Facility (b)(6) Measurement of mid-body widths and irradiation/^{60}Co Facility; Transport to NHP holding residence (b)(6)	Ketamine#/NA	NA
Distress from radiation exposure/animal room/NHP residence holding room	NA/Buprenorphine HCL	Observation of any criteria shown in Appendix G – Clinical Observations with Criteria for Euthanasia
Mouth ulcers or bloody stools/animal room/NHP residence holding room	NA/Bupivacaine HCL	Observation of mouth ulcers or bloody stools
Calm NHP while in restraint during prolonged IV fluid treatments, blood donation, and blood transfusion (b)(6) (b)(6)	Ketamine and 2% Isoflurane in O_2/NA	NA
*See Appendices D, E, and F for additional details regarding clinical observation that trigger procedures, drug dosages, route of administration, protocol monitoring plan, etc. # Ketamine 10 mg/kg, IM, will be the anesthetic of choice and additional doses of ketamine are given as required based on assessment of VSD veterinarian.		

V.4.1.2.3. Paralytics N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

Because the **principal purpose** of this study is to evaluate dosimetric technology to determine the radiation dose, there are no alternatives to the irradiation. Supporting information such as selection of hematological, proteomic analysis in blood measurement, etc. will help identify the usefulness or problems associated with the technology going to be tested for detection of early response after mass-casualty irradiation scenarios.

V.4.1.3.1. Sources Searched

Johns Hopkins Center for Alternatives to Animal Testing (Altweb): http://altweb.jhsph.edu/

AGRICOLA: http://agricola.nal.usda.gov/cgi-bin/Pwebrecon.cgi?RESET=bbSearch&PID=uiYCM3-dk9182GlSnrKeAxFRy7C

BIOSIS:http://roger.ucsd.edu/search/X?&undefined=&Da=1926&Db=&undefined=&searchscope=9&p=&SORT=D

PubMed: http://www.ncbi.nlm.nih.gov.lre1.usuhs.edu/pubmed

V.4.1.3.2. Date of Search September 11, 2012

V.4.1.3.3. Period of Search

AltWeb: Up to 9/2012 [whole database available]
AGRICOLA: 1950 to 9/2012
BIOSIS: 1926 to 9/2012
PubMed: 1950-10/26/2012

V.4.1.3.4. Key Words of Search

Bioassay: Altweb (54), AGRICOLA (5232), BIOSIS (375), PubMed (110177).
Biological and dosimetry: Altweb (0), AGRICOLA (8), BIOSIS (38), PubMed (22354).
Biological and dosimetry and blood: Altweb (7), AGRICOLA (0), BIOSIS (3), PubMed (3058).
Biological and dosimetry and blood and radiation: Altweb (2), AGRICOLA (0), BIOSIS (1), PubMed (1615).
Biological and dosimetry and blood and radiation and non-human primate: Altweb (0), AGRICOLA (0), BIOSIS (0), PubMed (1).
Biological and dosimetry and blood and radiation and non-human primate and analgesic: Altweb (0), AGRICOLA (0), BIOSIS (0), PubMed (0).
Biological and dosimetry and blood and radiation and non-human primate and pain: Altweb (0), AGRICOLA (0), BIOSIS (0), PubMed (0).
Biological and dosimetry and blood and radiation and non-human primate and pain and relief: Altweb (0), AGRICOLA (0), BIOSIS (0), PubMed (0).
Biological and dosimetry and blood and radiation and non-human primate and pain and G-CSF: Altweb (0), AGRICOLA (0), BIOSIS (0), PubMed (0).
Biological and dosimetry and blood and radiation and non-human primate and G-CSF: Altweb (0), AGRICOLA (0), BIOSIS (0), PubMed (0).

Biological and dosimetry and hair-follicle and radiation and non-human primate and pain: Altweb (0), AGRICOLA (0), BIOSIS (0), PubMed (0).
Biological and dosimetry and urine and radiation and non-human primate and pain: Altweb (0), AGRICOLA (0), BIOSIS (0), PubMed (0).
Biological and dosimetry and nail-clipping and radiation and non-human primate and pain: Altweb (0), AGRICOLA (0), BIOSIS (0), PubMed (0).

V.4.1.3.5. Results of Search
A thorough search of databases using keywords listed revealed no animal alternatives for the investigation involving total body irradiation.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification
One needs to irradiate animals to study biodosimetric endpoints using a NHP radiation dose-response model, and to investigate the correlation between these endpoints and dose, ARS response severity response, and survival. *There are no alternative procedures for irradiation because it is a unique stimulus that cannot be otherwise duplicated.*

Irradiation itself is not a painful process but it induces various changes in the body (i.e., vomiting and nausea, changes in hematology cells numbers, etc.). Although radiation does not induce pain, animals in these experiments might experience pain and distress prior to death because of sequelae. Radiation compromises the immune system. As a result of a compromised immune response, various types of infections can initiate and become painful. The sequelae of nausea, vomiting, and diarrhea may cause pain and distress in humans observed in the early post-irradiation period, when lethal doses are used. All study animals that show any pain from radiation-induced distress as judged by exhibition of any euthanasia criteria as listed in Appendix G will be administered analgesic as described above and in Appendices D and E. In addition, analgesics (acetaminophen/Rimadyl) are considered as a way of minimizing discomfort during the onset of opportunistic infections as judged by temperature changes as described in Appendices D and E. *We will humanely euthanize animals when unrelieved pain and severe distress occurs as described in Appendix G.*

See Section IV. Military Relevance for additional details. *These studies are scientifically justified based on the national interest to identify, optimize, and validate FDA-approved biodosimetry devices for potential radiological threats including mass-casualty incidences.*

V.4.2. Prolonged Restraint N/A

V.4.3. Surgery N/A
V.4.3.1. Pre-surgical Provisions N/A
V.4.3.2. Procedure(s) N/A
V.4.3.3. Post-surgical Provisions N/A
V.4.3.4. Location N/A
V.4.3.5. Surgeon N/A

V.4.3.6. Multiple Major Survival Operative Procedures
V.4.3.6.1. Procedures N/A
V.4.3.6.2 Scientific Justification N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections

Microchips. The animals will be anesthetized at the beginning of the study for microchip implantation, see V.4.1.2.1; see Appendix I for additonal details and illustrations. Briefly the microchip is located inside of a needle (12 Gauge) and syringe like device. The animal skin between the shoulder blades is lifted up and the microchip is injected subcutaneously. A small amount of tissue glue may be applied to the exit area, in order to ensure that the microchip stays in the place as the animal begins to move around. These devices facilitate monitoring from a distance, the subcutaneous body temperature without having to anesthetize the primates. The use of microchip is a further refinement in that it does reduce stress and provides improvement diagnosis without the intervention of staff to handle the animals. IM injections will be administered in the cranial or caudal thigh muscles using a 25 – 22 gauge needle.

Minimum vs. Conventional Treatment (G-CSF) Supportive Care Therapy. The Table below illustrates the study design relative to use of "Minimum" vs. Conventional Treatment Supportive Care Therapy.

Minimum vs. Conventional Treatment (G-CSF +) Supportive Care Therapy Study Design			
Experiment		Supportive Care	
	Doses (Gy)	Minimum	G-CSF and Conventional
		Number of animals	
1 PRE Study			
n=36	NA	–	–
2 High Dose Study			
	0	n= 2	–
	6.5	n=2	
3a Treatment Study			
	0		n=4
	6.5		n=4
3b Blood donation			
n=6	NA		
4 Dose Algorithm Study			
	0	n=6	
	1	n=6	
	3.5	n=6	
	5	n=6	
	6.5	n=6	
	8.5	n=6	

Minimum Supportive Care Therapy. Experiments 2 and 4 involve use of "Minimum Supportive Care Therapy". The scope of this approach is described in detail in Appendix D and Section V.4.2. (Pain Relief/Prevention). Appendix D provides information about the indication, drug, dosage, route of administration, etc. for nutritional support, anesthesia and analgesic, antiemetics and antigastric reflux, antigas, antidiarrheal, antipyretic, antiseptic, dehydration, and antiulcerative therapy.

Conventional Treatment (G-CSF) Supportive Care Therapy. Experiment #3 involves use of conventional supportive-care therapy with use of cytokine treatment (G-CSF) as a countermeasure used as a mitigator, as required IV saline fluids for dehydration, systematic antibiotics, and whole blood transfusions. The scope of this approach is described in detail in Appendix E and Section V.4.2 (Pain Relief/Prevention). Appendix E provides information about the indication, drug, dosage, route of administration, etc. for nutritional support, anesthesia and analgesic, antiemetics and antigastric reflux, cytokine treatment, antigas, antidiarrheal, antipyretic, antiseptic, dehydration, antiulcerative, and blood product (whole blood transfusion) support therapy. Conventional treatment supportive care therapy evokes use of components of "minimum supportive care therapy" but supplemented with cytokine treatment and as needed whole-blood transfusion, systemic antibiotic, and more aggressive dehydration therapy.

G-CSF. In the "*Conventional Treatment Supportive Care Therapy*" Experiment #3 animals are scheduled for G-CSF treatment (10 µg/kg, once a day; 22-25 G needle, 1ml subcutaneous bolus) will start one day after irradiation and continue until recovery of absolute neutrophil counts (ANC) $\geq$ 2,000 μL^{-1} for 2 consecutive days, anticipated to last up to 23 days after irradiation. Injection sites will vary starting initially in the lower extremities and then sequentially move up the limb. See Appendix E for additional details.

IV-Fluids for Dehydration. In the "*Conventional Treatment Supportive Care Therapy*" Experiment #3 dehydration will be treated more aggressively that in the "Minimum Supportive Care Therapy" described above by use of slow IV push-fluids (isotonic saline). See Appendix E for additional details including indications for treatment, isotonic fluid volumes, and needle gauge.

Whole-Blood Transfusions. In the "*Conventional Treatment Supportive Care Therapy*" Experiment #3 whole blood transfusions will be performed when platelet counts drop below 75-100 x 10^9/L. If an anaphylactic reaction is observed, then transfusion is stopped until resolution/improvement of signs for an anaphylactic reaction. See Appendices E and H for additional details including indications for treatment, blood volumes, needle gauge, etc.

The animal is monitored for anaphylactic reaction (swelling, urticarial, hives, rash, difficult breathing). If reaction occurs, the transfusion is stopped (at least temporarily), and corticosteroids (dexamethasone at 1 mg/kg IV, 19-23 gauge needle) and /or histamine (diphenhydramine 2.2 mg/kg, IM, 19-23 gauge needle) are given. After resolution/improvement of signs, the transfusion may be re-started at ½ to 1/3 the original rate.

V.4.4.1.1. Pharmaceutical Grade Drugs

Amgen Pharmaceuticals - Neupogen® (Filgrastin) - recombinant methionyl human granulocyte colony-stimulating factor (r-metHuG-CSF).

The same pbarmaceutical grade formulation of G-CSF will be used for the current study as has been used previously in nonhuman primate studies conducted at AFRRI[(b)(4)] [(b)(4)] Filgrastim is the common trade name (brand name NEUPOGEN), manufactured by Amgen Pharmaceuticals. The drug is provided as sterile vials of 480 µg of human recombinant methionyl human G-CSF in 1.5 ml aqueous solution (Amgen G-CSF Product Insert). The solution also contains sorbitol.

Neupogen is typically used to treat myelosuppression resulting from hematologic disease or as a side effect of chemotherapy regimens (Amgen G-CSF Product Insert).

G-CSF is provided as a 300 µg/ml stock and will be diluted in sterile 5% dextrose solution prior to administration. NEUPOGEN® will be stored in the refrigerator at 2° to 8°C (36° to 46°F). Avoid shaking. Prior to injection, NEUPOGEN® may be allowed to reach room temperature for a maximum of 24 hours. Any vial or prefilled syringe left at room temperature for greater than 24 hours will be discarded. Parenteral drug products will be inspected visually for particulate matter and discoloration prior to administration. If particulates or discoloration are observed, the container will be discarded.

All other pharmaceutical grade drugs will be obtained from VSD.

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

V.4.4.2. Biosamples

Blood will be collected under appropriate SOPs by following the procedure described with sterile 20-23 gauge needle attached to a needle holder or sterile syringe from saphenous, cephalic or any alternative vein as selected by the AFRRI vet; see Appendices C and H. Manual pressure will be used to reduce the chances of hematoma formation after blood collection. Typically blood volumes 0.8 to ≥4 ml are taken routinely for study purposes. Larger blood volumes are taken in the case of blood donation as described in Appendix H. For specific animals the blood volume collected will vary depending on the health status of the animal, animal weight, and guidance from the VSD veterinarians. Blood collection schedule is shown in the Appendices C and H.

V.4.4.3. Adjuvants N/A

V.4.4.4. Monoclonal Antibody (MAb) Production N/A

V.4.4.5. Animal Identification

Animals are identified by the ID number that is tattooed on the chest or inner thigh and by implantable microchips.

V.4.4.6. Behavioral Studies N/A

V.4.4.7. Other Procedures

A) Transport Plan: The animals for irradiation will be moved through the first floor hallway to the High Level Gamma Radiation (HLGR) facility. At no time will the animals be removed from the irradiation boxes and secured while outside of the VSD vivarium. Once within the HLGR facility the animals will be moved (still sedated in the irradiation box) down to the radiation source area. The animals in the irradiation boxes will be lifted onto the irradiation platform. The AFRRI dosimetrists will assure that the irradiation boxes are placed accurately in the radiation field. During irradiation, the irradiation box will be secured to prevent movement. The NHP's arms and legs will be secured to limit the ability to turn in the collar. Animals are expected to be in the irradiation box for no more than 60 minutes. The animals will be closely monitored by remote TV during the irradiation. After irradiation, the NHPs in their irradiation boxes will be removed from the platform and returned to the vivarium along the same route.

B) Blood Collection: Peripheral blood collection from NHP will be performed using standard procedures. VSD staff will assist, as able, the PI and PI staff to obtain blood samples if advanced preparations are made and sufficient VSD personnel are available.

C) ARS response severity scoring system (includes hematology, body temperatures, and body weights)

Clinical signs and symptoms for ARS subsyndromes including body weights and hematology changes will be assessed prior to and following irradiation to characterize the time course and dose response in the NHP radiation model (see Appendix C for a detailed schedule for blood draws, etc.). We plan to use a rhesus macaque ARS response severity scoring system (see Appendix B) in these studies. Typically blood draws and body weights can be obtained using NHP collar and chair without anesthesia. Blood drawas and body weights can also be obtained with NHP under anesthesia for another reason as determined by the Attending Veterarian. Several of the GI system dehydration measurements are only measured if the NHPs are planned to be placed under anesthesia for another reason. Subdermal body temperatures will be measured using the radiotelemetry detector.

The schedule for animal monitoring is shown in Appendix F. Once an animal has been determined to be in a critical period or morbid (See Appendix G) that animal will be monitored late at night on all days, for a minimum of 3 times per day including weekends. Night checks are the responsibility of the lab unless provisions are made in special circumstances for VSD to cover (if available).

D) Procedures for Selection and Preparing Animals for Blood Donation & Procuring, Processing, and Administration of NHP Donor Blood for Whole Blood Transfusions

Procedures for selection and preparing animals for blood donation as well as procuring, processing and administration of NHP donor blood for whole blood transfusion are described in Appendix H.

E) Criteria for euthanasia:

Criteria for euthanasia are described in Appendix G.

F) Histopathology:

In selected animals in dose/drug cohorts, histopathology will be requested to contribute to the assessment of radiation and treatment effects; see table below.

Exp. #	Experimental/Control Group	Histopathology
1	Pre	NA
2	High dose pilot (sham)	NA
2	High dose pilot (6.5 Gy)	NA
3	Treatment (0 Gy -> G-CSF/treatment)	NA
3	Treatment (6.5 Gy -> G-CSF/treatment)	n=2

Exp. #	Experimental/Control Group	Histopathology
4	Dose response (sham)	NA
4	Dose response (1 Gy)	n=2
4	Dose response (3.5 Gy)	n=2
4	Dose response (5 Gy)	n=2
4	Dose response (6.5 Gy)	NA
4	Dose response (8.5 Gy)	NA
Totals:		8

V.4.4.8. Tissue Sharing

Tissue sharing will be considered and be allowed with the submission of appropriate IACUC forms and approvals.

V.4.4.9. Animal By-Products

V.4.5. Study Endpoint

Surviving animals will be monitored up to 60 days after irradiation. Expected outcome for animals in the experiments are shown in the Table below. Animals will be monitored using the NHP ARS severity scoring system to support assessment of our defined euthanasia criteria. All moribund animals will be humanely euthanized.

Exp. #	Experimental/Control Group	Outcome expected	Totals
1	Pre	Recovery	36
2	High dose pilot (sham)	Recovery	2
2	High dose pilot (6.5 Gy)	60% risk for euthanasia/death	2
3	Treatment (0 Gy -> G-CSF/treatment)	Recovery	4
3	Treatment (6.5 Gy -> G-CSF/treatment)	70% recovery; 30% euthanasia/death	4
4	Dose response (sham)	Recovery	6
4	Dose response (1 Gy)	Recovery; of these 2 animals will be euthanized for histopathology	6
4	Dose response (3.5 Gy)	Recovery; of these 2 animals will be euthanized for histopathology	6
4	Dose response (5 Gy)	70% recovery; 30% euthanasia/death; 2 animals will be euthanized for histopathology	6
4	Dose response (6.5 Gy)	60% risk of euthanasia/death	6
4	Dose response (8.5 Gy)	Euthanasia/death	6

Exp. #	Experimental/Control Group	Outcome expected	Totals
Totals:			36
Note. NHP (non-irradiated) from Experiments #2, 3, and 4 will be used in Experiment #4.			

V.4.6. Euthanasia

Animals will be euthanized according to current American Veterinary Medical Association (AVMA) Euthanasia Guidelines. Animals will first be anesthetized with ketamine (IM, 10 mg/kg). Then the animal will be euthanized with an appropriate dose of Beuthanasia-D or other commercial euthanasia solutions (IV, 1ml/4.5 kg, 390 mg Pentobarbital/ml). For additional details regarding criteria for euthanasia, see Appendix G.

V.5. Veterinary Care

V.5.1. Husbandry Considerations Appropriate VSD SOPs will be followed.

V.5.1.1. Study Room

NHPs will be located for the duration of the study in AFRRI VSD vivarium. NHP waiting to be used in baseline experiment (PRE) will be paired housed, if the wait period is ≥4 weeks. NHP selected for baseline experiment (PRE), usually less than 4-5 weeks prior to irradiation, will be individually housed but with visual and/or tactile contact with other animals in stainless-steel cages in an environmentally controlled and continuously monitored animal rooms, as assigned by VSD per guidance from the AFRRI IACUC Chair (23 Nov 2012 MEMO).

V.5.1.2. Special Husbandry Provisions

Water will be provided to all irradiated NHPs via water sipping tubes.

NHP will be fed commercial primate biscuits ad libitum supplemented with fresh fruits (i.e., apples, bananas, oranges, etc.). Following irradiation NHP food will include soft food and non-citrus fruits. (b)(6) and other experts recommended that citrus fruits be restricted in the NHP radiation model (b)(6) It is thought that acid from the citrus fruits can cause irritation of the intestinal system (b)(6) In addition citrus fruits in the diet can interfere with candidate radiation biomarkers (i.e., citrulline, citrullinated proteins) under study.

V.5.1.3. Exceptions

NA

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care

The animals in this study will undergo an additional quarantine at AFRRI (45 d) using VSD's NHP quarantine standard operating procedures.

Each animal will be observed for evidence of pain or distress; see Appendix F – Monitoring Plan. Body weights will be determined prior to and after exposure; see Appendix C. Body temperature will be determined using subcutaneously implanted chips that will also serve to identify individual animals; see Appendix C for schedule. The time of each temperature

collection will be recorded for each NHP. The general health of experimental animals will be qualitatively assessed by the research staff twice daily (a.m. and p.m.) for general behavioral status and food consumption, in addition to VSD's normal health check procedures. If animals show evidence of severe clinical signs and symptoms of ARS, then VSD staff will be alerted to check the animal and to assess the NHPs. Appropriate standard operating procedures will be followed by staff veterinarians in the event of pain or distress.

The planned number and blood volume draws scheduled will be reviewed by the AFRRI veterinarians and staff and if determined to place the individual animals under severe health risk will be drastically reduced or suspended until the animal shows adequate recovery.

The 5-HT3 receptor antagonists: Ondansetron (1 or 2 mg/kg) or Granisetron (0.25 mg/kg) can be administered either orally, intravenous (IV), or subcutaneous (SC) after observation of vomiting or signs of emesis to decrease subsequent radiation-induced vomiting in NHPs as described in paper published by Martin et al. "Anti-emetic Effect of Ondansetron and Granisetron after Exposure to Mixed Neutron and Gamma Irradiation" (Radiation Research 149: 631-636 (1998). Authors demonstrated that two deliveries of 5-HT3 receptor antagonists seem to disrupt serotonergic transmission at the brain stem structures and to affect the peripheral release of serotonin from the gut, thus completely preventing radiation-induced vomiting".

In the case of Experiments # 2 and 4 irradiated animals, additional supportive care should be minimal; platelet counts may dip below 20,000/µl for several days, but whole blood will not be transfused as this would negate the purpose of the study. The extent of dehydration and diarrhea will be monitored for each animal. Based on the ARS response severity score animals' status will be graded as: normal, mild, moderate, or severe. In the case of mild dehydration and diarrhea, supplemental oral fluids (type and volume as judged appropriate by the veterinarian) will be offered when not counter-indicated due to severe drops in platelet counts. In the case of a moderate dehydration and diarrhea subcutaneous fluids and /or over the counter (OCT) anti-diarrheal (e.g., loperamide 1mg PO BID, bismuth subsalicylate 40mg/kg PO BID), anti-emesis (Cerenia, 1mg/kg, SC; 2mg/kg PO), anti-gastric reflux (e.g., Famotidine 0.5mg/kg PO or IM SID) or anti-gas (e.g., "TUMS" 1 tablet PO PRN) medications will be administered by VSD staff. If body temperature rises above 103 F, NHP will be administered Carprofen (Rimadyl 2.2 mg/kg, BID; QD/IM/IV/PO); see Appendices D and E.

V.5.2.2. Emergency Veterinary Medical Care

Experiments #2 and 4 in this study design involve use of "minimal supportive care" treatment. In the case of severe dehydration and or diarrhea or if pain /distress are noted, clinical staff veterinarian will be notified immediately and to evaluate the animal to determine the best course of medical care under the broad scope of a study design involving minimum treatment. VSD will provide a veterinarian availability 24/7 to provide emergency care as care is needed. There will be a veterinarian available to provide back-up to the on call veterinarian if necessary. Additional medical diagnostic beyond the described ARS response severity scoring system (i.e., body temperature, hematology, etc.) may be performed as judged by the AFRRI veterinarian. Symptomatic therapy will be provided with the development of abnormal clinical signs. For example, fruit or treats may be given in case of anorexia, topical antiseptics may be used for open wounds with significant defects following skin necrosis due to subcutaneous infections.

Inappetence or dehydration with or without signs of gastrointestinal distress will be treated with either fluid supplementation or Liquid Primate Diet at the request of the staff veterinarian. Antibiotic cream may be administered topically in order to ameliorate discomfort due to skin lesions. Euthanasia will be carried out according to pre-defined criteria as determined by veterinarians.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

Forms of psychological and environmental enrichment will include fruit, vegetable or additional supplements (that do not require analyses) and various cage-enrichment devices as well as contact with staff personnel. Fruits and vegetables that are not root vegetables will be disinfected by washing with a fruit and vegetable disinfectant solution, recommended by AFRRI veterinarians. If root vegetables such as sweet potatoes are given, the vegetable skin will be removed to reduce exposure to microorganisms.

V.5.3.2. Enrichment Restrictions

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				Code 7b, 7c, 7f	(b)(6)
				Code 7b, 7c, 7f	
				Code 7b, 7c, 7f; Code 1a, 1b, 3a, 3b, 4, 5, 7a upon VSD confirmation of completion of training	

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)					(b)(6)
				Code 1b, 5, 4b, 7b, 7c, 7d, 7e, 7f; Code 1a, 3a, 3b, 4a, 5, 7a upon VSD confirmation of completion of training	
				Code 7b, 7d, 7f	
				Code 1a, 1b,3a,3b,4a, 5, 7a, 7b, 7c, 7f upon VSD confirmation of completion of training	
				Code 1a, 1b, 3a, 3b, 4b, 7a, 7c, 7f; Code4a, 5 and 7b upon VSD confirmation of completion of training	

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)					(b)(6)
				Code 5, 7b, 7f upon VSD confirmation of completion of training	
				Code: 1, 2, 3, 4, 5, 6, 7a, 7b, 7c upon VSD confirmation of completion of training	
Vet Tech 1 (TBN new hire; will be added by amendment)	(b)(6)		TBD	Code: 1, 2, 3, 4, 5, 6, 7a, 7b, 7c upon VSD confirmation of completion of training	TBD
Vet Tech 2 (TBN new hire; will be added by amendment)			TBD	Code: 1, 2, 3, 4, 5, 6, 7a, 7b, 7c upon VSD confirmation of completion of training	TBD

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
		(b)(6)			
(b)(6)					

Procedure and manipulation codes:
Code 1= Animal handling (1a), chairing (1b), and euthanasia (1c)
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a), when chaired (4b), when pinned with cage (4c)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)
Code 7a = Anesthesia injection
Code 7b = ARS signs (ARS Severity Response)
Code 7c = Transport (From VSD to cobalt and return)
Code 7d = Urine collection (4a- from clean tray, 4b- from bladder)
Code 7e = Hair collection (from eye brow, whiskers or head)
Code 7f = Blood processing

VII. BIOHAZARDS/SAFETY

AFRRI VSD Policies and procedure, including the SOP for dealing with the possibility of Herpes B-infected animals will be followed. All animals are presumed to be Herpes B-infected.

VIII. ENCLOSURES

Label	Title	Pages
Appendix A.	Hematology and Body Weights in NHP TBI Radiation Model	41-43
Appendix B.	NHP Procedures. "NHP ARS-Response Severity Scoring System (ARS-RS^3) PROTOCOL"	44-47
Appendix C.	Study schedule: Blood draw schedule and blood volumes.	48-49
Appendix D.	Minimum Supportive Care Therapy	50-51
Appendix E.	Conventional Treatment Supportive Care Therapy	52-55
Appendix F.	Protocol Monitoring Plan	56
Appendix G.	Clinical Observations with Criteria for Euthanasia	57
Appendix H.	Procedures for Selection and Preparing Animals for Blood Donation & Procuring, Processing, and Administration of NHP Donor Blood for Whole Blood Transfusions	58-62
	Attachment A (List of Supplies)	63
	Attachment B. NHP Donor Study Schedule: Blood draw schedule and blood volumes	64
	Attachment C. Platelet counts in NHPs TBI to 6.5 and 8.5 Gy	65

X. ASSURANCES

Protocol Title: Evaluation of novel biodosimetry technologies using non-human primates (NHP; *Macaca mulatta*) radiation model

As the Principal Investigator on this protocol, **I** acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that **I** have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: **I** acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and **I** assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

Appendix A. Hematology and Body Weights in NHP TBI Radiation Model

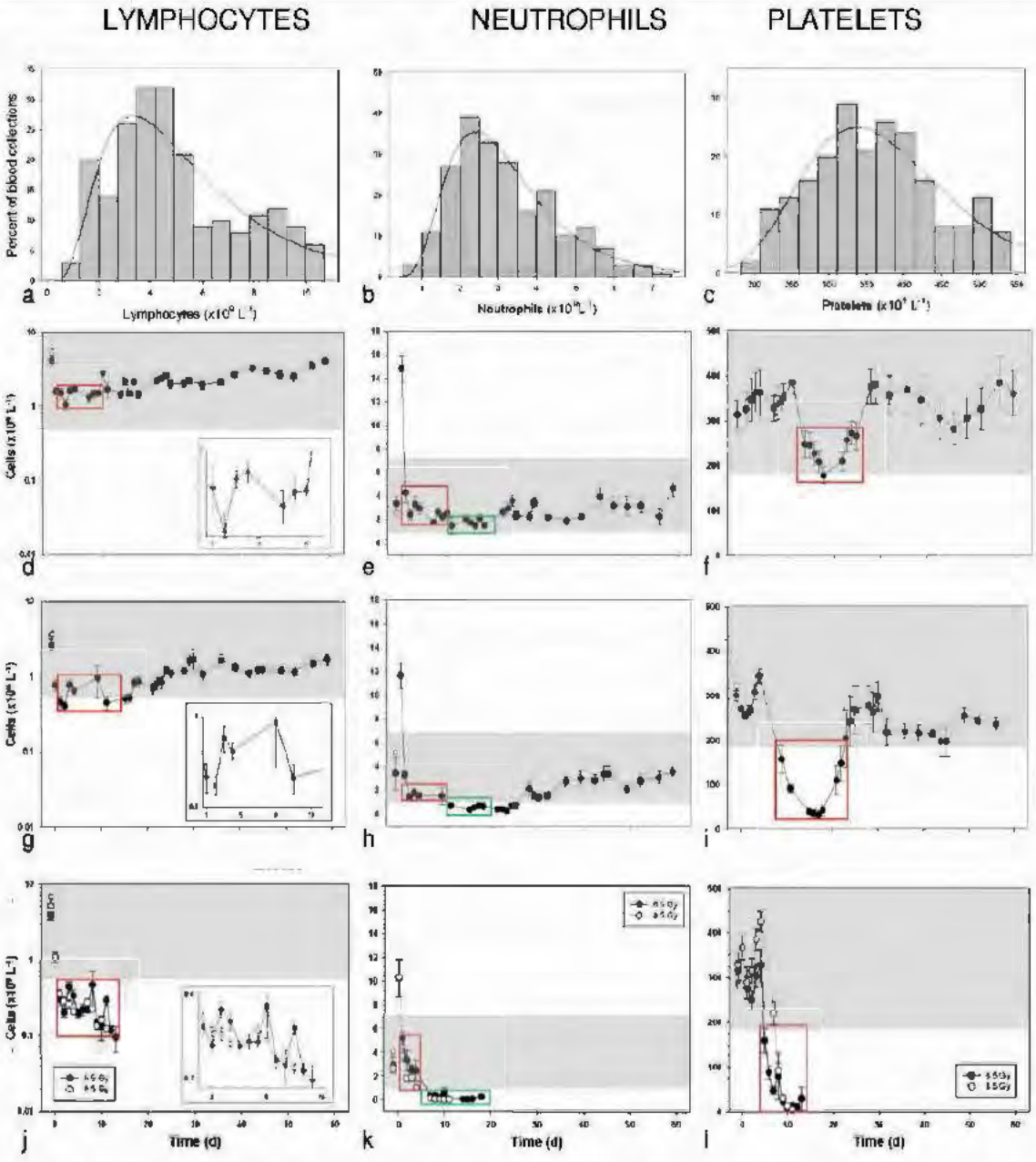


Figure A-1. Hematology parameters in NHP radiation model. Baseline distributions of lymphocytes (a), neutrophils (b), and platelets (c) are shown. The 95% confidence levels are shown in the shaded area in respective panels following exposure to 1 Gy (d, e, f), 3.5 Gy (g, h, i), and 6.5 (closed symbols) and 8.5 Gy (open circles) (j, k, l).

Continue Appendix A.

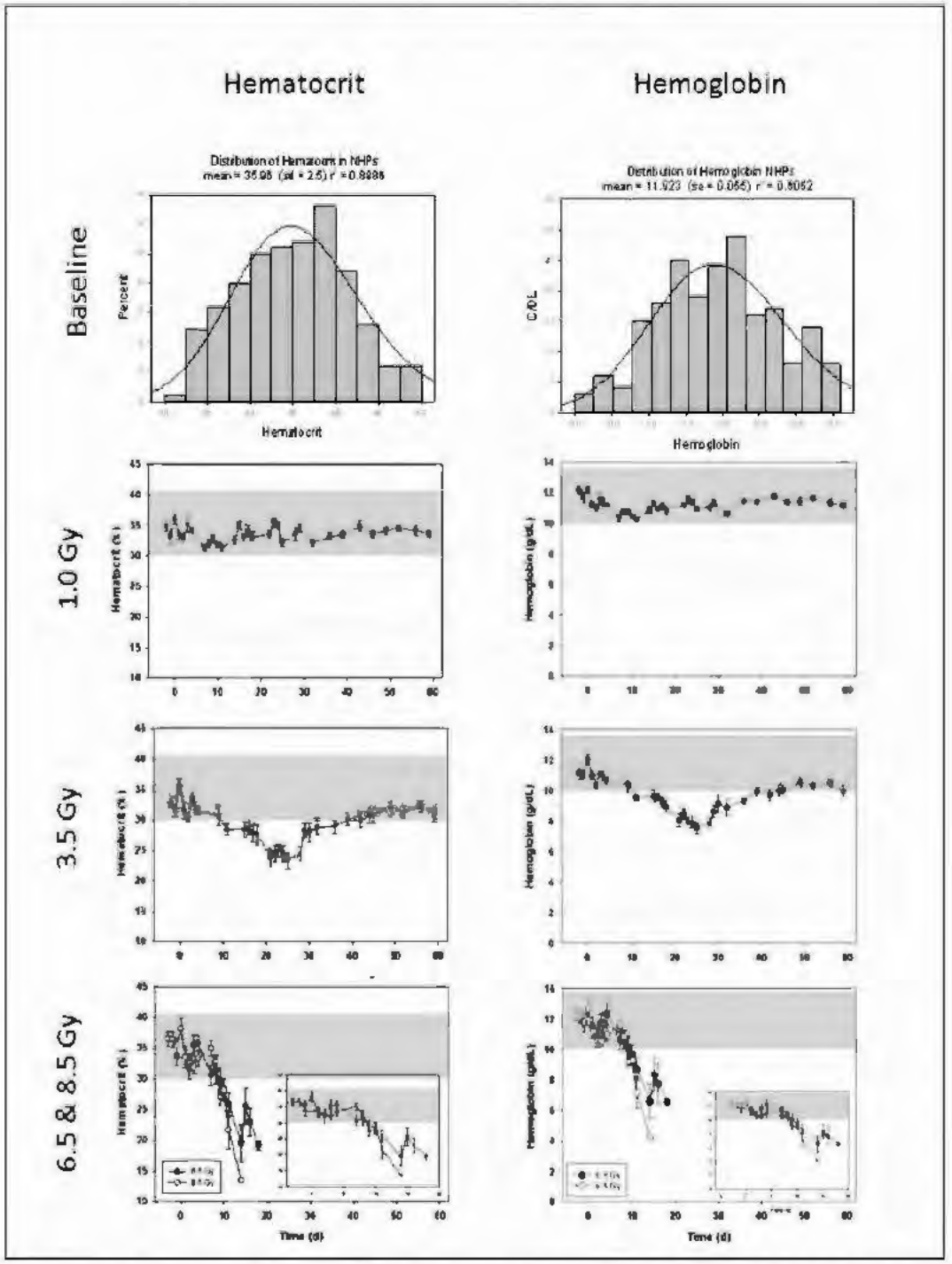


Figure A-2. Time course of hematocrits and hemoglobin in a NHP radiation model. Data are normalized to initial body weights. The hematocrits are following exposure to 0 Gy, 1 Gy, 3.5 Gy 6.5 Gy, and 8.5 Gy. Symbols represent mean values (n = 6) with error bars the SEM.

Continue Appendix A.

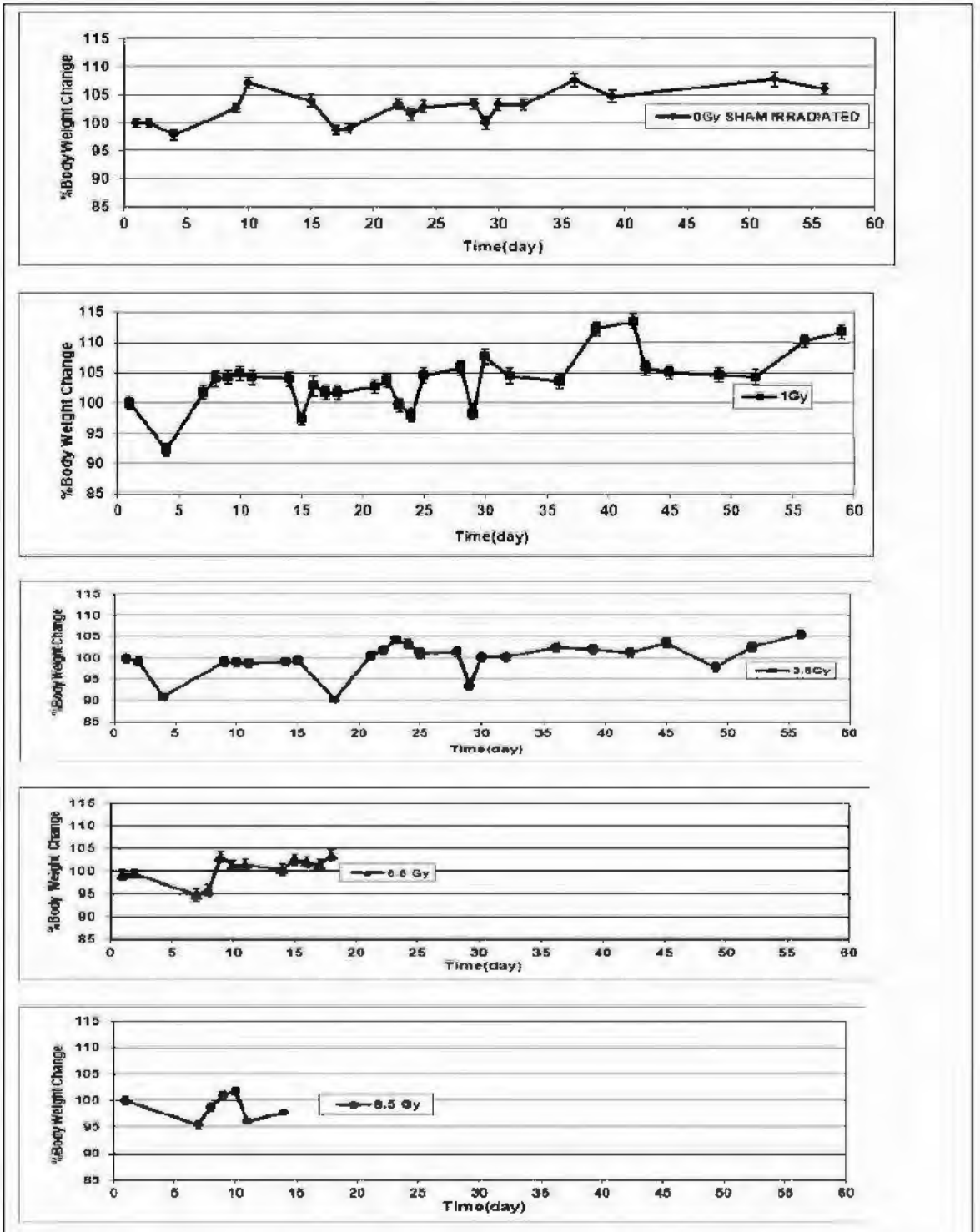


Figure A-3. Time course of body weights changes in NHP radiation model. Data are normalized to initial body weights. The time course of body weights are following exposure to 0 Gy, 1 Gy, 3.5 Gy 6.5 Gy, and 8.5 Gy. Symbols represent mean values (n = 6) with error bars the SEM.

Appendix B: NHP Procedures

"NHP ARS-Response Severity Scoring System (ARS-RS^3) PROTOCOL"

Note. Protocol PI (NIO) and co-PI (WFB) will meet with staff individuals to discuss the ARS-RS^3 protocol and assessment criteria prior to initiation of the study.

Sample Requirements.
Annotated ARS-RS^3 scoring sheet for experimental animals 3-4 times per work week including a measurement on Fridays. [Note. In cases of animal under crisis due to severe ARS, this frequency may be increased to include weekends, because it contributes to an assessment for euthanasia decision.]

Prior to Measurements.

1. ARS-R^3 form copies are made available in the individual NHP cage records.
2. Pre-labeled tubes (Animal ID, Date, Time) for collection of feces.
3. Pre-labeled tubes (Animal ID, Date, and Time) for collection of blood into serum separator tubes, and EDTA vacucontainers.
4. Measure weights for chairs; label chair weights.

During Rounds by Vet. Tech. and Animal Care Taker (twice a day/3-4 times a week) and by Research Staff (once a day/3-4 times a week).

1. At any time the animal is observed to be retching./.vomiting, record date and time.
2. Before any replacement of the cage tray for urine collection, if appropriate, check for vomitus.
3. Assess for shivering, fatigue/lethargy, response to stimuli, and piloerection; then record,
4. Count respiratory rate for a full minute and record.
5. Observe for abdominal distension and wounds; record.
6. Observe and record for consistency and color of feces; record.

During window of the workday by Research Staff (once a day/3-4 times a week).

1. At any time the animal is observed to be retching/vomiting or there is evidence of vomitus, record date and time.
2. Observe and record for consistency and color of feces; record.

During blood draw without anesthesia [NHP will be collared and chaired; frequency as described in the individual NHP schedule for biosampling].

1. Collect blood into appropriate tubes and volume as shown on the schedule for each specific NHP and record date and time.
2. If NHP defecates during blood draw, record consistency and color of feces.
3. Weight animal in chair and record NHP body weight.
4. Observe and record NHP SC temperature.

Procedures done under anesthesia [frequency as described in the individual NHP schedule for biosampling:

1. Collect blood into appropriate tubes and volume as shown on the schedule for each specific NHP and record date and time
2. Weight animal and record NHP body weight.
3. Observe and record both SC and rectal temperatures.
4. Measure dehydration status (skin turgor, gum capillary refill, buccal mucosal / tongue hydration).

Grading System for Rhesus Macaque ARS Response of Gastrointestinal, Neurovascular, Respiratory, Hematopoietic Systems				
	NORMAL (DEGREE 0)	MILD (DEGREE 1)	MODERATE (DEGREE 2)	SEVERE (DEGREE 3)
Gastrointestinal system				
- Dehydration				
% of weight loss	± 5	10	15	20
Skin turgor (anesthesia)	1 sec	2 sec, < 3 sec	3 sec	> 3 sec
Gum capillary refill (anesthesia)	1 sec	2 sec, < 3 sec	3 sec	> 3 sec
Buccal mucosal / tongue hydration (anesthesia)	Normal	< Normal amount of saliva, yet still some shine to membrane, tacky	Dry, no saliva, mucus membranes not shiny, tacky, sunken eyes	Dry and inflamed? Tongue parched
Inappetance	Normal eating habits, consumes all or most of food	Moderately slow to respond to meals & treat	Portions of meal & treat remain uneaten, picks up food but may abandon it	Ignores food when delivered, all (or most) of food remains uneaten
Retching / Vomiting	None	Mild – some evidence of vomitus on cage floor	Moderate – much evidence of vomitus on cage floor	Severe – actively R/V upon inspection
- Diarrhea				
Abdominal distension	None	Mild	Moderate	Severe
Stool consistency	Formed	Soft	Loose/watery	Bloody watery
Stool color	Normal	Other (eg, green, yellow)	Black	Bright red
Neurovascular system				
Response to stimuli	Vigilant and reactive; stands on perch inside cage; looks around room; follows personnel and procedures	Vigilant and mildly reactive; stands on perch; looks around room; follows personnel and procedures	Generally sits on the perch; appears depressed; moves with difficulty when provoked	Always sits on the perch or floor of cage, even with personnel in the room; appears depressed or 3++ (unresponsive; lies on floor or perch)
- Fever				
Rectal body temperature (°C) with anesthesia	~ 101.5	~ 103.5	~ 105	> 107
Subcutaneous body temperature (°C) / with anesthesia	TBD	TBD	TBD	TBD
Subcutaneous body temperature (°C) / without anesthesia	TBD	TBD	TBD	TBD
Shivering	None	Mild	Moderate	Severe
Piloerection (unrelated to fear or aggression)	None	Any	NA	NA
Respiratory system				
Respiratory rate	RR = 35 – 50	RR > 60	RR > 60 + shallow	Labored or gasping
Behavioural				
Fur Grooming	Good (groomed)	2 areas not groomed	3-4 areas not groomed	Not groomed
Aggression/Vocalization	No sounds/Calm	Makes sound 1-2 times	Sounds 2 or more times	Frequent sounds
Hematopoietic system				
Lymphocytes (reference value, X 10^3 cells/L)	± 10 % of baseline value: 0.06 – 9.78	1-14d: 1.0 - 2.0	1-14d: 0.4 - 1.0	1-14d: < 0.5
Neutrophils (reference value, X 10^3 cells/L)	± 10 % of baseline value: 0.47 – 5.83	< 1d: 13 - 18	< 1d: 10 - 13	< 1d: 8 - 10
		10-20d: 1.2 - 2.2	10-20d: < 1.2	5-20d: < 1.0
Platelets (reference value, X 10^3 cells/L)	± 10 % of baseline value: 194 – 525	≥ 12d: < 300	≥ 8d: < 180	≤ 8d: < 180
Hemoglobin (reference value: G/DL)	± 10 % of baseline value: 10.3 – 13.5	> 20d: 8 - 10	10-20d: 8 - 10	≤ 10d: < 10
			≥ 20d: < 8	10-20d: < 8
Hematocrit (reference value, %)	± 10 % of baseline value: 30.96 – 40.96	> 20d: 25 - 30	10-20d: 25 - 30	≤ 10d: < 30
			≥ 20d: ≤ 25	10-20d: ≤ 25

Animal ID

Rhesus Macaque ARS-Response Severity-Scoring System								
User's initials								
Day assessed (yymmdd)								
Time assessed								
Gastrointestinal system	**Degree of severity none = 0; 1 (mild) to 3 (severe)**							
- Dehydration								
Drooling (Y/N)								
Baseline mean body weight, kg								
Daily body weight, kg								
Daily body weight, lb								
% of weight loss								
Skin turgor (anesthesia)								
Gum capillary refill (anesthesia)								
Buccal mucosal / tongue hydration (anesthesia)								
Vomitus (Y/N)								
Retching / vomiting								
Inappetance								
- Diarrhea								
Abdominal distension								
Stool consistency								
Stool color								
Maximum grading:								
Neurovascular system	**Degree of severity none = 0; 1 (mild) to 3 (severe)**							
Response to stimuli								
- Fever								
Body temperature (°C) Subcutaneous (SC) or Rectal (R)	SC: R:	SC: R:	SC: R:	SC: R:	SC: R:	SC: R:	SC: R:	SC: R:
Anesthesia at time of temperature measurement (Y/N)								
Shivering								
Piloerection (unrelated to fear or aggression)								
Maximum grading:								
Respiratory system	**Degree of severity none = 0; 1 (mild) to 3 (severe)**							
Respiratory rate (breaths/min)								
Respiratory rate - degree								
Maximum grading:								
Cutaneous system	**Degree of severity none = 0; 1 (mild) to 3 (severe)**							
Wounds								
Local infection								
Maximum grading:								
Behavioural	**Degree of severity none = 0; 1 (mild) to 3 (severe)**							
Fur Grooming								
Curiousity (Y/N)								
Aggression/Vocalization								
Watching TV (Y/N)								
Hematopoietic system	**Blood cell counts (C) and degree of severity (S) none = 0; 1 (mild) to 3 (severe)**							
Lymphocytes (X 10^9 cells/L)	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:
Neutrophils (X 10^9 cells/L)	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:
NIL								
Platelets (X 10^9 cells/L)	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:
Hemoglobin	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:
Hematocrit	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:	C: S:
Maximum grading:								
Response category (RC) = (0-3)								

Appendix C. Study schedule: Blood draw schedule and blood volumes										
	Blood Chemistry	CBC+diff and Proteomic Biomarker Studies								
Time, wk/d/h	Blood volume in Micro SS Tube, ml	Blood volume in an EDTA tube, ml								
NHP body weights, kg		3	3.5	4	4.5	5	5.5	6	6.5	7
Maximum daily blood draw volume, ml*		1.6	1.9	2.2	2.45	2.7	2.95	3.2	3.5	3.8
Baseline studies										
1st week baseline	0.225	1.4	1.7	2.0	2.25	2.5	2.5	2.5	2.5	2.5
		Microchip injection (IM): Ketamine (10mg/kg; IM or SC) anesthesia after 12h fasting								
2nd week baseline	--	1.4	1.7	2.0	2.25	2.5	2.5	2.5	2.5	2.5
3rd week baseline	0.225	1.4	1.7	2.0	2.25	2.5	2.5	2.5	2.5	2.5
		Measurement of mid-body widths: Ketamine (10mg/kg; IM or SC) anesthesia after 12 h fasting								
Irradiation studies										
		Irradiation: Ketamine (10 mg/kg; IM or SC) tranquilization after 12 h fasting for loading into radiation boxes, transport to 60Co facility, irradiation, and transport back to VSD facility								
5 h	0.225	1.5	1.8	2.1	2.4	2.6	2.9	3.1	3.4	3.7
1d	0.225	1.5	1.8	2.1	2.4	2.6	2.9	3.1	3.4	3.7
2d	0.225	1.5	1.8	2.1	2.4	2.6	2.9	3.1	3.4	3.7
3d	0.225	1.5	1.8	2.1	2.4	2.6	2.9	3.1	3.4	3.7
4d	--	1.6	1.8	2.1	2.4	2.6	2.9	3.1	3.4	3.7
5d	0.225	1.5	1.8	2.1	2.4	2.6	2.9	3.1	3.4	3.7
6d	--	1.6	1.9	2.2	2.45	2.7	2.95	3.2	3.5	3.7
7d	0.225	1.5	1.8	2.1	2.4	2.6	2.9	3.1	3.4	3.7
10d	0.225	1.5	1.98	2.1	2.4	2.6	2.9	3.1	3.4	3.7
13d	--	1.6	1.9	2.2	2.45	2.7	2.9	3.2	3.4	3.7

3rd wk (3X)	0.225 (15d); 0.225 (~20d)	1.2	1.35	1.5	1.65	1.8	2.0	2.2	2.35	2.5
4th wk (3X)	--	1.2	1.35	1.5	1.65	1.8	2.0	2.2	2.35	2.5
5th wk (3X)	0.225 (~30d)	1.2	1.35	1.5	1.65	1.8	2.0	2.2	2.35	2.5
6th wk (2X)	0.225 (~40d)	1.2	1.35	1.5	1.65	1.8	2.0	2.2	2.35	2.5
7th wk (2X)	--	1.2	1.35	1.5	1.65	1.8	2.0	2.2	2.35	2.5
8th wk (2X)	0.225 (~50d)	1.2	1.35	1.5	1.65	1.8	2.0	2.2	2.35	2.5
60d	0.225	1.2	1.35	1.5	1.65	1.8	2.0	2.2	2.35	2.5
*Note. For repeated daily blood draws the maximum daily amount of blood volume should be ≤1% of total blood volume, typically estimated based on the animal body weight. For Rhesus the blood volume per body weight is 54 ml/kg (Laboratory Animals 27:1-22, 1993). Sample calculation: Assume a 4.5 kg NHP this represents a daily blood draw of 2.4 ml (4.5 kg x 54 ml/kg x 0.01).										

Appendix D. Minimum Supportive Care Therapy					
Nutritional Support					
Fluids	Unlimited access to filtered water; Following irradiation animals are provided flavored fluids.				
Food	Commercial primate biscuits ad libitum supplemented with fresh fruits (i.e., apples, bananas, oranges, etc.); Following irradiation all citrus removed from their diets, diets include soft food				
Indication	**Drug**	**Dosage**	**Route**	**Needle gauge**	**Syringe size**
Anesthesia					
Placement of NHP in rad box; measurement of mid-body widths; microchip injection	Ketamine	10mg/kg	IM or SC	22-25 G	3 ml
Analgesic					
Distress from radiation exposure (5 to 35 days after radiation exposure)	Buprenorphine HCL	0.01 mg/kg up to 0.02 mg/kg BID	IM	22-25 G	variable
Mouth ulcers or bloody stools	Bupivacaine HCL	0.1 ml of 25% bupivacaine HCL with a dab of surgical lubricant)	Locally using cotton-tipped applicator	NA	NA
Antiemetic/AntiGastric Reflux					
Gastric reflux	famotidine	0.5mg/kg	PO or IM SID	22-25 G	variable
Emesis	Cerenia	1mg/kg (SC); 2mg/kg (PO)	PO or SC	22-25	variable
Emesis	Ondansetron Granisetron	1 or 2 mg/kg 0.25 mg/kg	PO or SC or IV	22-25	variable
Antigas					
gas	TUMS	tablet	PO PRN	NA	NA
Antidiarrheal					
Observation of diarrhea	Loperamide	0.1 – 0.2 mg/kg	PO BID, SC, IM	NA; 22-	1-3 ml

	Hydrochloride (Imodium)			25 G	
Persistent diarrhea (3 successive days with Imodium treatment) or if watery stool without any signs of formed stool	Diphenoxylate hydrochloride	0.1 mg/kg	PO BID for 3 d	NA	NA
Diarrhea >3 d	Loperamide Hydrochloride (Imodium)	0.1 – 0.2 mg/kg	PO BID, SC, IM	NA; 22-25 G	1-3 ml
Antipyretic					
Body temperature >104 °F	Carprofen (Rimadyl)	2.2 mg/kg; 4.4 mg/kg	BID; QD,/IM/IV/PO		
Continued for 48 h after the 1st d body temperature <104°F	Carprofen (Rimadyl)	2.2 mg/kg; 4.4 mg/kg for 2 days	BID; QD/IM/IV/PO		
Antiseptic (Local)					
Local infection	Neobacimyn	NA	Topical	NA	As clinically determined
Dehydration					
Mild – presence of tacky mucus membranes without signs of gastrointestinal (GI) distress	Flavored fluid supplementation	NA	Use of water bottles	NA	As needed
Moderate- mild criteria plus dry mucous, sunken eyes with signs of GI distress	Flavored fluid supplementation along with antidiarrheal treatments	NA	Use of water bottles	NA	As needed
Severe – Any of mild and or moderate criteria plus pale mucous membranes, rapid and weak pulse, cold extremities, lethargy, or rapid breaching	Flavored fluid supplementation along with antidiarrheal treatments	NA	Use of water bottles; See above for anti-diarrheal treatments	NA	As needed
Antiulcerative					
Bloody stool	Sucralfate (Carafate)	1g/day	BID	NA	

Appendix E. Conventional Treatment Supportive Care Therapy					
Nutritional Support					
Fluids	Unlimited access to filtered water; Following irradiation animals are provided flavored fluids.				
Food	Commercial primate biscuits ad libitum supplemented with fresh fruits (i.e., apples, bananas, oranges, etc.); Following irradiation all citrus removed from their diets, diets include soft food.				
Indication	**Drug**	**Dosage**	**Route**	**Needle gauge**	**Syringe size**
Anesthesia					
Placement of NHP in rad box; measurement of mid-body widths; microchip injection	Ketamine	10mg/kg	IM or SC	22-25 G	3 ml
Analgesic					
Calm NHPs while in restraint during prolonged IV fluid treatments	Midazolam HCL	0.2 mg/kg	IM	22-25 G	variable
Distress from radiation exposure (5 to 35 days after radiation exposure)	Buprenorphine HCL	0.01 mg/kg up to 0.02 mg/kg BID	IM	22-25 G	variable
Mouth ulcers or bloody stools	Bupivacaine HCL	0.1 ml of 25% bupivacaine HCL with a dab of surgical lubricant)	Locally using cotton-tipped applicator	NA	NA
Antiemetic/AntiGastric Reflux					
Gastric reflux	famotidine	0.5mg/kg	PO or IM SID	22-25 G	variable
Emesis	Cerenia	1mg/kg (SC); 2mg/kg (PO)	PO or IM	22-25 G	variable

Emesis	Ondansetron Granisetron	1 or 2 mg/kg 0.25 mg/kg	PO or SC or IV	22-25	variable
Cytokine Treatment					
Treatment group 1 d after irradiation or sham exposure until the post-irradiation recovery of ANC ≥2,000 μL^{-1} for 2 consecutive days	G-CSF	10µg/kg; once a day until no indication (recovery of ANC, ≥2,000 μL^{-1}	SC	22-25 G	variable
Antigas					
gas	TUMS	tablet	PO PRN	NA	NA
Antidiarrheal					
Observation of diarrhea	Loperamide Hydrochloride (Imodium)	0.1 – 0.2 mg/kg	PO BID, SC, IM	NA; 22-25 G	1-3 ml
Persistent diarrhea (3 successive days with Imodium treatment) or if watery stool without any signs of formed stool	Diphenoxylate hydrochloride	0.1 mg/kg	PO BID for 3 d	NA	NA
Diarrhea >3 d	Loperamide Hydrochloride (Imodium)	0.1 – 0.2 mg/kg	PO BID, SC, IM	NA; 22-25 G	1-3 ml
Antipyretic					
Body temperature >104 °F	Carprofen (Rimadyl)	2.2 mg/kg; 4.4 mg/kg	BID; QD,/IM/IV/PO		
Continued for 48 h after the 1st d body temperature <104°F	Carprofen (Rimadyl)	2.2 mg/kg; 4.4 mg/kg for 2 days	BID; QD/IM/IV/PO		
Antiseptic (Local)					

Local infection	Neobacimyn	As clinically determined	Topical	NA	NA
Antibiotic Therapy					
ANC <500 μL^{-1} and continued until animal maintained ANC >500 μL^{-1} for 48 h.	Enrofloxacin (Baytril)	5mg/kg	QD: PO, IV, IM	22-25G	variable
ANC <500 μL^{-1} and continued until animal maintained ANC >500 μL^{-1} for 48 h (Baytril) and 24 h (GentaMax) with body temperature ≥103°F	Enrofloxacin (Baytril)	5mg/kg	QD: PO, IV, IM	22-25G	variable
	Gentamicin sulfate (GentaMax)	4 mg/kg	QD: IM or IV	22-25G	variable
ANC <500 μL^{-1} and continued until animal maintained ANC >500 μL^{-1} with body temperature ≥103°F when microbial resistance demonstrated to enrofloxacin or gentamicin	Primaxin	5-10 mg/kg:	BID: IM	22-25 G	variable
Dehydration					
Mild – presence of tacky mucus membranes without signs of gastrointestinal (GI) distress: Use of isotonic saline provided only if platelets >10 x 10^9/L	Flavored fluid supplementation	NA	Use of water bottles	NA	NA
	Isotonic saline	10-15ml/kg	Slow IV push	18-23 G	variable

Moderate- mild criteria plus dry mucous, sunken eyes with signs of GI distress; Use of isotonic saline provided platelets >10 x 10^9/L	Flavored fluid supplementation along with antidiarrheal treatments	NA	Use of water bottles	NA	NA	
	Isotonic saline	20-39 ml/kg	Slow IV push	18-23 G	Variable	
Severe – Any of mild and or moderate criteria plus pale mucous membranes, rapid and weak pulse, cold extremities, lethargy, or rapid breaching; Use of isotonic saline provided platelets >10 x 10^9/L	Flavored fluid supplementation along with antidiarrheal treatments	15±5 ml/kg/h over a period of 2-4 h	Slow IV push	18-23 G	Variable	
	Isotonic saline; animals in a restraint device, allowed to awaken, and sedated (Midazolam HCL)					
Antiulcerative						
Bloody stool	Sucralfate (Carafate)	1g/day	BID	NA	NA	
Blood Product Support*						
Platelets less than 70-100 x10^9/L or uncontrolled hemorrhage	Whole blood (exposed to 20 Gy) transfusion	7-14 ml/kg	IV using an 18µ blood filter (Hemo Nat Filter, Utah Medical Products)	18-23 G	variable	

*See Appendix H for additional details regarding whole blood transfusions.

Appendix F. Protocol Monitoring Plan						
Time Window	Animal Care Takers (VSD)	Vet. Tech (VSD)	Vets (VSD)	Research Techs (PI)		Principal Investigators
				ARS Severity Assessments (± blood draws)	Careside Observations *	Careside Observations*
Prior to irradiation exposure						
M->F	Twice a day (<6:30 am; ~1pm)	Twice a day (~9am; 2-3pm)	As needed	Once a day (AM)	Once a day (Afternoon)	Once a day (TBD)
Sat, Sun, Holidays	Once a day (6:30am-Noon)	Once a day (afternoon)	On call	NA	Once on SAT or SUN	Once on Sun or SAT and Holidays
Post-radiation exposure						
M->F	Twice a day (<6:30 am; ~1pm)	Twice a day (~9am; 2-3pm) or as needed	As needed or on call	Once a day (AM)	Once a day (Late afternoon) or ass needed	Once a day (TBD) or as needed
Sat, Sun, Holidays [Routine]	Once a day (6:30am-Noon)	Once a day (afternoon)	On call as needed	Once a day (TBD)	Once on SAT or SUN (TBD)	Once on SUN or SAT (TBD)
Sat, Sun, Holidays [NHPs with ANC <500 μL^{-1}; NHPs given whole blood transfusions (24h after transfusion)]	Once a day (6:30am-Noon)	At least once a day (afternoon)	On call as needed	Once a day (AM)	Once a day (Afternoon/Early Evening) or as needed	Once a day (TBD) or as needed
*Note. If a NHP exbibits clinical observations with criteria of "morbid", then Researeh Teehs & PIs will monitor animal late at night, for a minimum of 3 to 4 times a day, to include weekends.						

Appendix G. Clinical Observations with Criteria for Euthanasia (NHPs)		
PARAMETER	**OBSERVATIONS**	
	COMBINATION CRITERIA (two or more of the following combined is sufficient to consider euthanasia)	**ABSOLUTE CRITERIA** (any of these signs alone is criteria for euthanasia)
General appearance	Ocular discharge; Mildly to moderate dehydrated (aggressively treat)*; Hair shedding	Paralysis of one or more extremities; Cyanosis (bluish color to skin or mucus membranes); Moderate to severe emaciated/dehydrated (skin tent) (uncontrolled by treatment)
Stool appearance	Mild to moderate loose stools (treat)*; Mild blood present in the stools (treat)*	Uncontrolled loose stools with frank blood in the stools even after treatment
Bleeding	Mild generalized petechiation on the body with no signs of active bleeding*; Platelets < $50x10^3$/uL; Hematocrit <30%	Uncontrolled bleeding Precipitous drop of hematocrit (>20% in 48 hours) Hematocrit <15% and platelets <$3x10^3$/uL with lethargy
Respiratory rate	Mild dyspnea (shortness of breath with rapid and shallow breathing; Mild abdominal breathing	Gasping/open mouth breathing
General behavior	Decreased mobility; Mild inappetence (eats treats, eats parts of meals), assuming animal has recovered from anesthesia	Weakness/inability to obtain food or water; Inappetence for 3 meals, assuming animal has recovered from anesthesia; Repeated and uncontrollable self trauma; Seizures
Provoked behavior	Subdued responsive to gentle prodding, assuming the animal has recovered from anesthesia	Decreased or absent response to stimulation, assuming the animal has recovered from anesthesia;
Weight loss	10-20 % of baseline body weight	More than or equal to 20% from the baseline weight
Core body temperature, °C	Uncontrollable fever (≥4°C of baseline) (treat)*	<35.5 °C following a period of febrile neutropenia
Note	***If any one of the designated parameters is exhibited, notify responsible personnel immediately for treatment of NHPs.**	

Appendix H.

Date last updated: Dec 7, 2012 (b)(6)

(SOP. NIO-XXX)

Procedures for Selection and Preparing Animals for Blood Donation & Procuring, Processing, and Administration of NHP Donor Blood for Whole Blood Transfusions

Ref:

(a) Farese AM, Cohen MV, Katz BP, Smith CP, Jackson III W, Cohen DM, and MacVittie TJ. A nonhuman primate model of the hematopoietic acute radiation syndrome plus medical management. Health Physics 103(4): 367-382.

(b) (b)(6)

(c) NHP Whole Blood Transfusion, Standard Operating Procedures 5011, Armed Forces Radiobiology Research Institute, Veterinary Science Department, Oct 2009.

(d) Diehl K-H, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, Vidal J-M, van de Vorstenbosch C. A good practice guide to the administration of substances and removal of blood, including routes and volumes.

I. Purpose. The purpose of this protocol specific Standard Operating Procedures (SOP, NIO-XXX) is to specify the required procedures to be used for the proper procuring, processing, and transfusion of donor irradiated whole blood to recipient animals.

II. Scope. This SOP applies to planned studies at AFRRI by staff personnel in support of BARDA funded studies awarded to Dr. (b)(6) and under the co-direction of Dr. (b)(6) (b)(6) for selection of animals for blood donation, preparing animals for blood transfusion.

III. Responsibility. Drs (b)(6) are responsible for reviewing and updating this SOP as needed.

IV. Procedures.

A. Supplies. See Appendix H - Attachment A.

B. Identify Donor Animals. Ideally, animals with high body weight (~≥5 kg) should be used for blood donation. Select from pre-approved healthy individuals with matched infectious disease status.

C. General Monitoring of Donor Animals. Animals on this protocol will be monitored for general health including: body weight, body temperature, hydration status, hematology

and chemistry parameters. AFRRI's Veterinarian will be notified if any unusual weight tends, body temperature, abnormal complete blood counts (CBC) or abnormal serum chemistries are observed. See Appendix H - Attachment B for the conditions and blood volumes withdraw for this purpose.

D. Anesthesia. Animals will be anesthetized with ketamine (10 mg/kg, IM in the quadriceps muscle using a 22-25 gauge needle) for transport to the treatment room, clinical observations requiring animal compliance, blood donation, and blood transfusion. Isoflurane gas (2% in O2) will be administered via mask as determined by the AFRRI Veterinarian, per the request of AFRRI's Veterinarian.

E. Physiologic Measurements. Body temperatures will be monitored using the injected microchips on days when the animals are biosampled or assessed for clinical observations. Body weights will be obtained each day the animals are anesthetized for other procedures. The body weight is obtained prior to administration of any fluids support including fluid replacement at time of blood donation. Protocol staff will notify AFRRI's Veterinarian of any abnormal body temperatures or weights.

F. Phlebotomy.

a) Peripheral Blood for Routine Health Monitoring. VSD staff will assist, as able, the PI and PI staff to obtain blood samples if advanced preparations are made and sufficient VSD personnel are available. Blood will be collected under appropriate SOPs by following the procedure described with sterile 20-23 gauge needle attached to a needle holder or sterile syringe from saphenous, cephalic or any alternative vein as selected by the AFRRI Veterinarian. Manual pressure will be used to reduce the chances of hematoma formation after blood collection. Typically blood volumes ≤1 ml are taken routinely. For specific animals the blood volume collected will vary depending on the health status of the animal, animal weight, and guidance from the VSD veterinarians. Blood collection schedule for donor animals is shown in Appendix H - Attachment B.

b) Peripheral Blood for Blood Donation and LRS infusion. Put on sterile gloves. Venipuncture site is prepared by cleansing with Betadine, allowed to dry, then clean the area with an alcohol swab and allow it to dry. Repeat this process for a total of 3 scrubs. Blood will be collected under appropriate SOPs by following the procedure described with sterile 20-23 gauge needle attached to a needle holder or sterile syringe from saphenous, cephalic or any alternative vein as selected by the AFRRI Veterinarian. Manual pressure will be used to reduce the chances of hematoma formation after blood collection. Label a 30ml syringe containing 3ml of citrate phosphate dextrose (CDP-a) anticoagulant "NHP blood: with the animal ID, date

collected, time of donation, and expiration date. Then withdraw no more than 27 ml of blood into a 30-ml syringe. The filled syringe will be removed from the infusion set and capped. Prepare a syringe with lactated Ringer's Solution (LRS) with volumes as described in Appendix H - Attachment B. Attach the syringe containing the LRS to the infusion set and infuse this solution. The infusion set will be removed and pressure will be applied over the venipuncture site with gauze until bleeding stops.

G. Blood Donation.

Healthy animals will be selected from the donor pool. Donor animal will be anesthetized with ketamine (10 mg/kg, IM in the quadriceps muscle using a 22-25 gauge needle). The body temperature and weight will be obtained. Blood will be collected (19-23 gauge needle) for a CBC (0.4 ml). If CBC levels are within acceptable limits, then withdraw whole blood volumes from the animals and infuse LRS volumes as described in Attachment B from the animals as described in "Peripheral Blood for Blood Donation" section above. The date and amount of blood withdrawn during each phlebotomy procedure is recorded. As donations are required, the donor animals will be selected on a rotating basis (e.g., animals who have donated most recently will be at the end of the rotation) and evaluated as a possible donor according to the date and amount of previous donations and established blood draw volume limits.

H. Processing and Storage of Donor Blood.

Typically whole blood is administered within 48 hours of donation. Donor blood will be irradiated prior to transfusion into the recipient NHP or before refrigeration storage. Research staff will use all appropriate personal protective equipment (gloves, mask, goggles, lab coat, etc.) and the blood will be processed under a biosafety cabinet prior to and following irradiation (25 Gy).

The50-ml tube(s) is labeled "NHP blood" with the animal's identification number, date, and time of donation, and expiration date. Using sterile technique a cell strainer (70 micro [µm]) will be opened and placed on an open 50ml centrifuge tube and the donor blood from the syringe will be slowly transferred into the 50ml tube. The tube is capped and the top is wrapped with parafilm and placed on a mechanical mixer until it is irradiated. Whole blood expires 48 hours after collection and will be discarded if not used using biohazard safety guidelines. If whole blood is not needed immediately, then it is stored in a chilled centrifuge tube.

The NHP donor blood in the 50ml centrifuge tube will be placed into a secondary 250ml tube and then into a spill proof transport container for transport to and return from the Irradiation Facility. Irradiation will be to a dose of 25 Gy to eliminated donor stem cells and lymphocytes that could result in donor cell-initiated hematopoietic engraftment.

cellular sensitization and graft-versus-host disease. No cross-typing or blood-typing will be performed.

If whole blood is to be administered, the unit of blood is placed on a rocker for continuous mixing at room temperature in order to preserve platelet function and administered with 48 hours of donation.

I. Justification and Criteria for Selection of Animal for Whole Blood Transfusion.
In this model we know the radiation dose (6.5 Gy) and also assume that adequate biodosimetry assessments will support medical treatment decisions. In our previous study NHP were euthanized 16 ±2.5d (range: 14 to 20d) after exposure to 6.5 Gy. In this treatment study arm of the protocol, our goal is to rescue the 6.5 Gy NHPs. Dr. (b)(6) used a whole-blood transfusion trigger of <25% hematocrit that was reached ~10 d after exposure in our previous study of 6.5 Gy irradiated NHPs (See Appendix A). We think that platelet levels were critical and plan to rely on low platelet levels as the trigger for whole-blood transfusions to provide donor platelets to allow the animals to bridge over until recovery. We are concerned about bleed out when performing whole blood transfusions due to low platelets so have elected to trigger whole blood transfusion a little before the precipitous decline in platelets (See Appendix H). At AFRRI there is limited experience in whole blood transfusions with NHPs, so we are being conservative in using a trigger of platelet level of 70 to 100 x 10^9/L would occur as early as 6-7 d.

Whole blood transfusions will be performed on appropriate protocol study irradiated (6.5 Gy) animals when platelet counts drop below 75 to 100 x 10^9/L. Based on prior studies we expected to need to transfuse whole blood to 6.5 Gy irradiated animals as early as 8 days after irradiation and likely a second time several days later while the animals are undergoing radiation-induced thrombocytopenia; see Appendix H - Attachment C.

J. Whole Blood Transfusion.
The site of venipuncture on anesthetized animals will be cleansed with betadine, and then alcohol swabs X 2 prior to the infusion of whole blood.

Animals will not be give antihistamine or corticosteroid before transfusion of whole blood. Whole blood is usually administered slowly (approximately 30 ml over 5 minutes, 19-23 gauge needle, using a sterile disposable 18 micron (µm) mesh filter).

The animal will be monitored for anaphylactic reaction (swelling, urticarial, hives, rash, difficult breathing). If reaction occurs, the transfusion will be stopped (at least temporarily), and corticosteroids (dexamethasone at 1 mg/kg IV, 19-23 gauge needle) and /or histamine (diphenhydramine 2.2 mg/kg, IM, 19-23 gauge needle) will be given.

After resolution/improvement of signs, the transfusion may be re-started at ½ to 1/3 the original rate.

Appendix H – Attachment A (List of Supplies)

Equipment

1. VSD scale for measurement of animal body weight
2. Temperature wand
3. Temperature controlled centrifuge with appropriately sized rotors
4. Mechanical mixer
5. Refrigerator

Personal Protection Equipment

1. Gloves
2. Masks
3. Goggles
4. Lab coat
5. Sleeves
6. Hairnets
7. Foot booties

Supplies

1. Hazardous waste containers
2. Preprinted labels for syringes and centrifuge tubes
3. Ice buckets
4. Secondary transport container

Chemicals

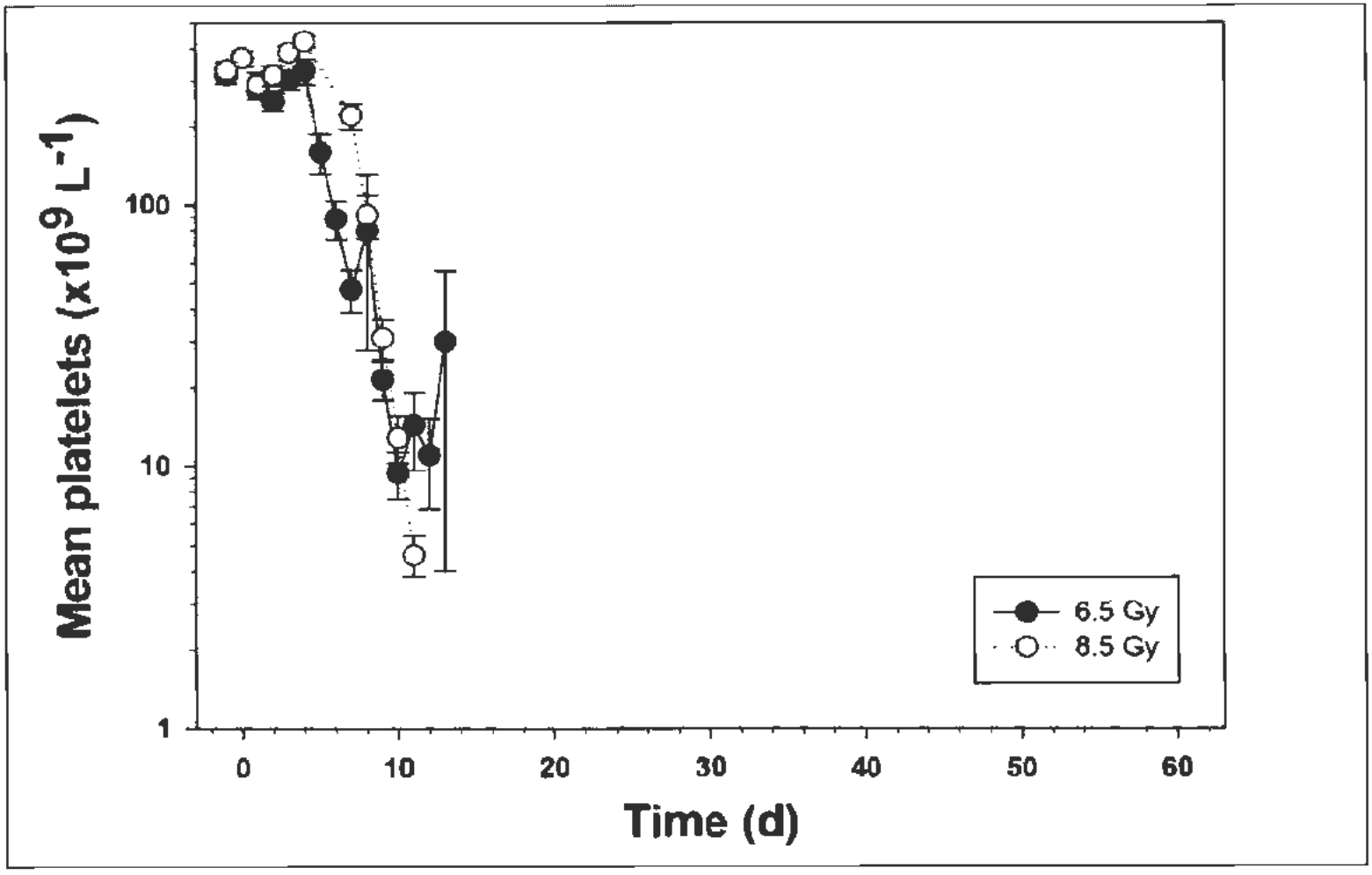
1. Xylazine
2. Ketamine
3. Medical grade lactated Ringers Solution
4. Sterile saline
5. Dexamethasone (corticosteroids)
6. Diphenhydramine (antihistamine)
7. Citrate-phosphate-dextrose with adenine (CPD-A) [Sigma]

Blood Collection Supplies

1. 19-25 Gauge needles
2. Winged catheter infusion set
3. EDTA tubes
4. Serum separator tubes
5. 1 mL syringes
6. 30 mL syringes
7. Syringe caps
8. 50 mL tubes
9. 250 mL tubes (secondary containers) [Denville Scientific C-3411. (http://www.denvillescientific.com/node/950]
10. Parafilm
11. 70 micron cell strainer [BD Biosciences]
12. 18 micron blood filter [Utah Medical Products]

Appendix H - Attachment B. NHP Donor Study Schedule: Blood draw schedule and blood volumes										
Time, wk/d/h	Blood volume in Micro SS Tube for chemistries, ml	Blood volume in an EDTA tube for CBC, ml Syringe containing Citrate Phosphate Dextrose, ml & Lactate Ringers Solution Replacement Volumes Required, ml (per 30 d period)								
NHP body weights, kg		3	3.5	4.0	4.5	5	5.5	6	6.5	7
Maximum daily blood draw volume, ml*		1.6	1.9	2.2	2.45	2.7	2.95	3.2	3.5	3.8
Maximum blood draw volume in 3-4 wk period#, ml		16	19	22	24.5	27	29.5	32	35	38
LRS fluid replacement, ml		8	10	11	12.5	14	15	16	18	19
At time of blood donation										
CBC	--	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Blood donation maximum volume, ml		16	19	22	24.5	27	29.5	32	35	38
During periods of blood donation										
Once every 2 weeks	0.225	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
During periods when animal is not used for blood donation										
Blood for CBC and chemistries once every 6±2wk	0.225	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
*Note. For repeated daily blood draws the maximum daily amount of blood volume should be ≤1% of total blood volume, typically estimated based on the animal body weight. For Rhesus the blood volume per body weight is 54 ml/kg (Laboratory Animals 27:1-22, 1993). Sample calculation: Assume a 4.5 kg NHP this represents a daily blood draw of 2.4 ml (4.5 kg x 54 ml/kg x 0.01). #Whole blood transfusion volume based on 7-14 ml per kg.										

Appendix H – Attachment C. Platelet counts in NHPs TBI to 6.5 and 8.5 Gy with ^{60}Co γ-rays (DARPA study results)



Appendix I – Implantable Microchips

Microchips. The animals will be anesthetized at the beginning of the study for microchip implantation, see V.4.1.2.1. The microchip is located inside of a needle (12 Guage) and syringe like device (Figure 1).

Figure 1. Pre-sterilized, disposable, needle assemblies.

The animal skin between the shoulder blades is lifted up and the microchip is injected subcutaneously. A small amount of tissue glue may be applied to the exit area, in order to ensure that the microchip stays in the place as the animal begins to move around (Figure 2).

Figure 2. Implantable microchip - anchors securely to tissue.

These devices facilitate monitoring from a distance, the subcutaneous body temperature without having to anesthetize the primates (Figure 3).

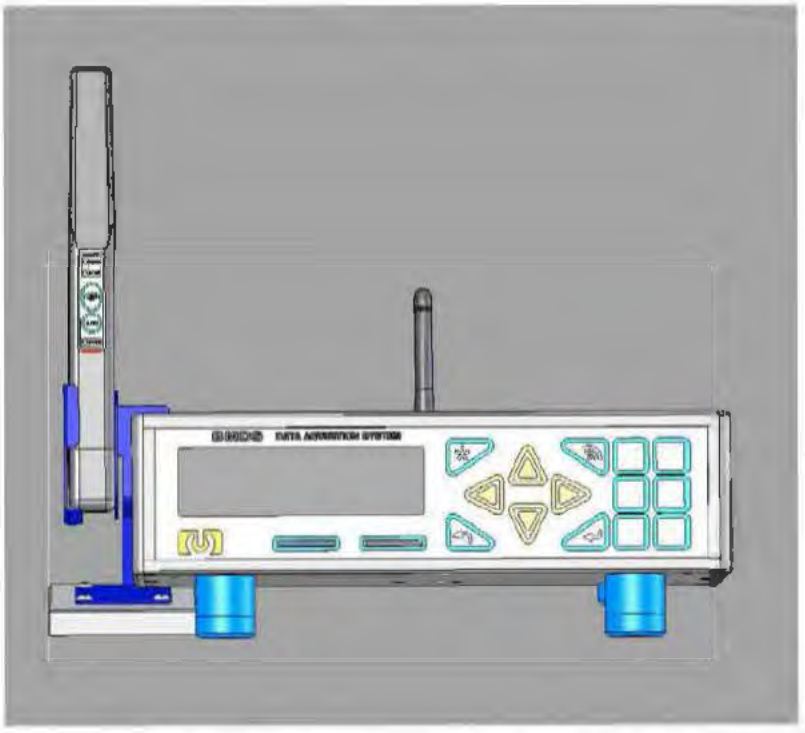


Figure 3. Bio Medic Data System DAS-6001 Data Acquisition System.

The use of microchip is a further refinement in that it does reduce stress and provides improvement diagnosis without the intervention of staff to handle the animals. IM injections will be administered in the cranial or caudal thigh muscles using a 25 – 22 gauge needle.

Appendix J – References

Balcombe JP, Barnard ND, Sandusky C. 2004. Laboratory routines cause animal stress. Contemp Top Lab Anim Sci 43: 42–51.

(b)(6),(b)(4)

Diehl K-H, Hull R, Morton D, Pfister R, Rabemamplanina Y, Smith D, Vidal J-M, van de Vorstenbosch C. A good practice guide to the administration of substances and removal of blood, including routes and volumes.

(b)(4)

MacVittie TJ, Farese AM, Jackson WE. 2005. Defining the full therapeutic potential of recombinant growth factors in the post radiation-accident environment: the effect of supportive care plus administration of G-CSF. Health Physics 89(5):546-555.

Martin et al. "Anti-emetic Effect of Ondansetron and Granisetron after Exposure to Mixed Neutron and Gamma Irradiation" (Radiation Research 149: 631-636 (1998).

(b)(6),(b)(4)

NHP Whole Blood Transfusion, Standard Operating Procedures 5011, Armed Forces Radiobiology Research Institute, Veterinary Science Department, Oct 2009.

(b)(6),(b)(4)

(b)(6),(b)(4)

Waselenko JK, MacVittie TJ, Blakely WF, Pesik N, Wiley AL, Dickerson WE, Tsu H, Confer DL, Coleman N, Seed T, Lowry P, Armitage, JO, Dainiak N: Strategic National Stockpile Radiation Working Group. 2004. Medical management of the acute radiation syndrome: Recommendations of the Strategic National Stockpile Working Group. Annals of Internal Medicine 140(12): 1037-1051.

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (October 2012)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	3/1/2013	2nd Resubmission Date:	
Approved/**Returned for Revision**:	3/18/2013	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	3/28/2013	**EXPIRATION DATE:**	4/4/2016
Approved/Returned for Revision:	4/5/2013	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Endothelial Cell Regulation of Hematopoiesis after Radiation Injury in the Laboratory Mouse (Mus musculus)

IV. PRINCIPAL INVESTIGATOR:

(b)(6) **PhD** Date
(b)(6) AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date
Head, Scientific Research Department, AFRRI
Telephone: (b)(6) Fax (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date
Statistician
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date
Head, Veterinary Sciences Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date
Environmental Health and Safety Department, USUHS
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **VC, USA**
IACUC Chair, (b)(6) AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: Endothelial Cell Regulation of Hematopoiesis after Radiation Injury in the Laboratory Mouse (Mus musculus)

PRINCIPAL INVESTIGATOR:

(b)(6) **PhD** Date
Program Advisor, Radiation Countermeasures, AFRRI
Telephone (b)(6) Fax: (b)(6)
(b)(6)

CO-INVESTIGATOR(S): N/A
AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)
This proposal corresponds to part of Aim 2 in AFRRI intramural research project (b)(6)

I. NON-TECHNICAL SYNOPSIS

Mortality after exposure to radiation from nuclear devices or dirty bombs results to a large extent from failure of blood-forming tissue to produce sufficient quantities of platelets and white blood cells, causing hemorrhage and infection. This is referred to as the hematopoietic subsyndrome of the Acute Radiation Syndrome (ARS). There is a vital need for effective treatment against radiation targeting blood-forming tissue, both in response to potential nuclear attacks and in the clinical setting. Precursors of blood cells and platelets are termed hematopoietic stem and progenitor cells (HSPC). In bone marrow (BM), HSPC are in close association with cells that form blood vessels, i.e., endothelial cells (EC). There is evidence that the maintenance and healthy functioning of HSPC require interaction with EC; however, the mechanisms by which EC support HSPC are little-investigated and poorly understood. Our hypothesis is that EC signals are important in promoting recovery of HSPC after radiation injury. Our long-term goal is to identify important EC signals that support HSPC in injured hematopoietic tissue, with a view toward developing radiation countermeasures. The roles of EC and their signals are being tested in an in vitro model system comprising coculture of EC and HSPC. We showed that EC stimulate functional recovery of HSPC after radiation injury. Specific signaling pathways are being investigated to determine which signals might be modulated in EC to improve their ability to promote HSPC production and function. In this project, we will test the hypothesis that signals in EC prolong animal survival after ionizing radiation in mice by injecting EC into mice exposed to irradiation. This has been demonstrated in one report from 2007 using brain EC, but we hypothesize that BM EC will be superior in this regard. After irradiation of mice and subsequent injection of EC, mice will be monitored for 30-day survival (hematopoietic syndrome), and BM functions will be analyzed. The work is preparatory to future work where specific signaling pathways will be altered in EC by genetic engineering before administration to mice.

II. BACKGROUND

II.1. Background

Radiation Injury and Radiation Countermeasures

There is an increasing threat of nuclear attacks.[1-3] No ARS countermeasures have

been approved by the FDA, partly due to the fact that the mechanisms of radiation injury and recovery are poorly understood. Although elements of the hematopoietic lineage have been studied in isolation,[5] another approach is to study hematopoietic signals expressed by other cell types; this resulted in a successful FDA IND application for CBLB502, based on a protein made by microorganisms.[6] The approach of identifying potential drug targets based on signals in non-hematopoietic cells can be applied to cells making up the BM microenvironment, which regulates survival, proliferation, and differentiation of HSPC.[7] Hematopoietic factors are induced in BM in response to radiation injury,[8] and one of these, G-CSF, is now the standard treatment for radiation casualties.[9] We examined interactions between irradiated HSPC and osteoblasts and discovered radiation induces hematopoietic factors, including G-CSF, in these cells. Another important component of the microenvironment is the microvasculature, because of hematopoietic signals expressed by EC.[7,11-14] These are important in normal hematopoiesis, and EC enhance recovery of HSPC from radiation,[15,16] but the specific signals are not known. The overall goal of our research program is to understand the signals expressed in EC, before and after radiation, and to determine which ones promote recovery of hematopoiesis.

(b)(4),(b)(6)

(b)(4),(b)(6)

HSPC and EC in the Perivascular Niche

HSPC comprise self-renewing, relatively quiescent stem cells that give rise to progenitors of all blood lineages, and progenitor cells, which are more rapidly dividing, and lose self-renewal capacity as they become more differentiated along specific lineages.[17] They reside within specific BM niches that support maintenance, differentiation, and proliferation:[7] the osteoblastic or "bone" niche, and the perivascular niche. The perivascular niche comprises mesenchymal progenitor cells, reticular cells, megakaryocytes, and EC. These cells regulate HSPC survival by secreting factors, and/or expressing receptors and/or ligands that signal upon cell-cell contact.[18] In addition to osteoblasts,[19-21] recent work suggests that EC[7,18,22,23] play critical roles.[24] The perivascular niche is characterized by discrete areas of endothelium that express high levels of EC adhesion molecules (CAMs) such as E-selectin, as well as growth factors and chemokines, such as SCF, TGF-β, IL-8, IL-11, CXCL12, FGF-4, and Ang1/2 (see Appendix 1 for list of acronyms).[25] Mice deficient in CXCL12 display a defect in the hematopoietic colonization of the BM during embryogenesis,[26] a defect that could be rescued by enforced expression of SDF-1 in vascular EC, suggesting EC in BM are essential for colonization of fetal BM by HSPC.[27] Isolated BMEC contribute to hematopoiesis and support the proliferation and differentiation of HSPC via cytokines and possibly by physical contact.[28-30] BM sinusoidal EC provide a platform for differentiation of progenitors, such as megakaryocytic progenitor cells.[31] Recent work showed the activation state of EC upregulates factors which support HSPC expansion via Akt or maintenance and differentiation via p44/42.[32] HSPC may also exert direct effects on EC. This is supported by the observation that HSPC VEGF-A stimulates EC proliferation.[33]

Effects of EC on hematopoiesis after radiation injury

Radiation exposure leads to myelosuppression via apoptosis of hematopoietic cells as well as effects on the BM vasculature. There is no hematopoietic regeneration without

vascular reconstitution of BM.[7,34] Damage to EC within BM is followed by incomplete repair and cell death.[35] In addition to endothelial apoptosis, radiation induces endothelial NF-κB activation, alters chemokines and adhesion molecules, tissue factor, and thrombomodulin,[36] and induces release of von Willebrand factor, promoting a pro-inflammatory response. Radiation upregulates PDGF (A-D) in EC.[37] VCAM-1 upregulation and P-selectin downregulation in BM microvessels were observed after TBI, and this led to increases in hematopoietic progenitors adhering to vessels.[38] Damage to EC causes an increase in circulating EC after irradiation.[39] Reestablishment of EC activity improves hematopoietic recovery and survival of irradiated mice.[25] Transplantation of primary vascular EC enhanced survival of irradiated mice, and resulted in increased numbers of BM HSPC and recovery of circulating white blood cells and platelets.[15] Pleiotrophin expressed in human brain EC is a hematopoietic factor and its administration results in expansion of HSPC in vivo in mice.[40] Endogenous pleiotrophin in BM EC regulated retention and self-renewal of HSPC in BM.[12] We believe identifying roles of EC-derived factors that enhance survival of a functional hematopoietic niche will facilitate identification of drug targets and lead to development of safe, effective radiation countermeasures.

II.2. Literature Search for Duplication
II.2.1. Literature Sources Searched: BRD, NIH Reporter, PubMed
II.2.2. Date of Search: January 18-20, 2013

II.2.3. Period of Search All available years:
BRD: FY2005-FY2009
NIH Reporter: 1988-2013 (Projects and Publications)
PubMed: includes MEDLINE (1966-present) and OLDMEDLINE (1950-1965).

II.2.4. Key Words of Search Hematopoietic AND Endothelial AND Radiation AND Transplantation

Subsets of these key words produce many projects and papers with which we are familiar, and which are relevant to our program. However, all keywords together are necessary to find possible projects and papers that are duplicative of the proposed work.
II.2.5. Results of Search

Most of the projects revealed in the searches of the three databases did not deal with transplantation of EC for addressing ARS. They addressed use of EC to expand HSPC in vitro for subsequent transplantation of HSPC, other types of transplants, and radiation injury to EC.
BRD: 220 references were found. None were relevant to the issue of duplication.

NIH Reporter: 70 results were found. Two relevant funded programs were found. They are parts of NIAID-funded Centers for Medical Countermeasures against Radiation (CMCRs) at:

1) Duke University, where a project headed by John Chute is exploring transplantation of EC as a radiation countermeasure. Papers from this program are cited in the Background section.[12,15,40] Endothelial progenitor cells (EPC) were shown previously by this group to promote hematopoietic reconstitution in vivo after total-body irradiation.[41]

2) Albert Einstein College of Medicine of Yeshiva University, where a project headed by Chandan Guha is testing transplantation of EPC as a radiation countermeasure. EPC were shown by this group to mitigate gastrointestinal radiation injury.[42]

Our proposed protocol is not duplicative of the two cited projects because:

1. The project at Duke is using EC from tissues other than BM. EC from different tissues are known to have widely different properties. In fact, it is known that some types of EC from tissues other than BM have no effect on hematopoiesis, while others do.[15] Therefore, it is important to test EC from the tissue being affected by radiation injury during the hematopoietic syndrome: BM. These are the endogenous EC that can potentially mediate recovery from hematopoietic injury.

2. The Guha project is not using EC, but EPC, as was done previously by the Chute group.[41] Although these may have some beneficial effect, it is important to test the ability of mature, differentiated EC to promote hematopoiesis and survival after radiation injury, as our hypothesis, supported by the literature, is that it is the EC that have these beneficial effects.

3. The Guha group is studying gastrointestinal injury, whereas we propose to study the hematopoietic syndrome.

4. In addition to the previous three issues, the current project is part of a long-term research program at AFRRI that aims to identify important signaling molecules in EC and to genetically engineer EC to produce a radiation countermeasure superior to native EC. It is important for us to establish EC transplantation in our laboratory, and to verify their beneficial effects, as part of this larger program.

5. To date, only one publication showed administration of EC (from brain) mitigates the hematopoietic syndrome.[15] This needs to be confirmed by an independent group, as we propose here. This needs to be confirmed not only for EC in general, but for EC from BM. This confirmation is required to set the stage for our subsequent work.

PubMed: 111 papers were found. In addition to the work cited above, a recent relevant study on EPC transplantation by Zeng et al.[43] was discovered in this search. Our proposal is not duplicative of the Zeng project because we are using EC, not EPC. Moreover, the Zeng paper only tracked migration of injected EPC rather than studying functions or effects of the transplanted cells, as we propose here. Other papers in the search results used EPC to treat nonradiation injuries.

III. OBJECTIVE / HYPOTHESIS 1. The primary hypothesis to be tested in this protocol is that postexposure administration of mouse BM EC will enhance survival of mice irradiated at doses that cause the hematopoietic syndrome.
2. A secondary, related hypothesis is that postexposure administration of mouse BM EC will improve bone marrow function as assessed by peripheral blood counts and clonogenic quantification of hematopoietic progenitors in BM.

IV. MILITARY RELEVANCE Currently, there is no safe and non-toxic radiation countermeasure available. Military personnel are potentially exposed to radiation in various scenarios, some of which include exposure to neutrons. Both early and late health effects of radiation exposure are major concerns for the military. Thus there is an urgent need to develop clinically safe, potent and non-toxic radiation countermeasures. Developing countermeasures to radiation exposure is a top priority for the US Department of Defense.

With the deployment of radiation countermeasures, forces exposed to moderate doses of ionizing radiation will exhibit enhanced survivability, expanding the range of operable threat environments. The options available to field commanders will increase, and the morale of personnel at risk of exposure to ionizing radiation will improve. DoD Defense Technology Objective MD.18 listed the military payoffs of radiation countermeasure deployment as follows: "Effective mitigation of health consequences will (1) reduce casualty load at medical treatment facilities, (2) sustain a more effective operational force after a radiation exposure event, (3) allow commanders to conduct operations in radiation field environments without undue risk of decremented performance of personnel due to acute tissue injury ..., and (4) reduce the negative psychological impact on personnel tasked to operate in contaminated environments."

V. MATERIALS AND METHODS
V.1. Experimental Design and General Procedures
General:
Male CD2F1 mice will be obtained from an approved vendor (Harlan preferred), quarantined upon arrival, and representative animals screened for evidence of disease before irradiation and experimentation. Total-body irradiations of 280 mice (64 from Expt. 1, 120 from Expt. 2, and 96 from Expt. 3) at 0.6 Gy/min in the high-level Cobalt-60 facility will be performed according to previously published methods, following AFRRI (b)(4),(b)(6) standard operating procedures [redacted] Animals will be placed into well-ventilated Plexiglas boxes and irradiated bilaterally. All irradiations will be performed before 12 noon. 112 mice (64 from Expt.1, 96 from Expt. 3) will be sham-irradiated. Sham-irradiated mice will undergo the same procedures as the irradiated mice (transport to radiation facility, placement into Plexiglas box), minus the radiation exposure.

Isolation of BM EC from donor mice

BM aspirates from femurs of male CD2F1 mice will be washed and seeded in gelatin-coated flasks containing EC media, and cells in suspension will be separated 4-6 h later. This time allows adherent cells to stick and any cells in suspension, including cells that may express the EC marker CD31, to be removed with the

medium. After adherent cells are near confluence, they will be trypsinized, and incubated with Dynabeads® (Invitrogen, Inc.) CD31 EC isolation beads following the manufacturer's protocol. The identify of the CD31-enriched cells will be verified, cells expanded and tested for possible *Mycoplasma* contamination in-house using a commercially available kit (MycoAlert, Lonza, Inc.), and used in *in vivo* experiments.

V.1.1. Experiment 1 Determine the maximum number of BMEC efficiently administered intravenously via tail vein injection.

This experiment will involve technical training to gain competence in IV injections, and to specifically determine the maximum dose and injection schedule of BMEC. Three different doses of BMEC ($2.5X10^5$, $4X10^5$, $5X10^5$ cells/mouse) diluted in Hanks Balanced Salt Solution (HBSS) + 1% BSA , along with HBSS/BSA as a vehicle control will be infused iv 5 times (1 time/day) into male CD2F1 mice (8 mice per group) that are non-irradiated or irradiated at an LD 70/30 (currently 9.25 Gy) to ensure the technical accuracy of injecting cells and the effect of irradiation on cell infusion. Mice will be monitored for 10 days to ensure that there is no reaction to the cell injection. Based on the results of this study, the highest dose efficiently administered will be selected for remaining studies.

Number of mice required for Experiment 1:
Radiation doses: 2 (0 Gy, 9.25 Gy)
Treatment groups: 4 (1=HBSS, 2=$2.5X10^5$ BMEC, 3=$4X10^5$ BMEC, 4=$5X10^5$ BMEC)
Mice/Group: 8
Iterations: 2
Training mice (for perfecting iv injections): 50
Note: training mice will also be used for donor mice.
Total number calculated by multiplying cells:

Radiation Doses	Treatment Groups	Mice/ Group	Iterations	Total
2	4	8	2	**128**

Total mice for Experiment 1: 128 + 50 = **178**

V.1.1. Experiment 2 Effect of administered EC on survival (moribundity)

All mice (except donor mice) will be exposed to radiation at a dose that causes 70% mortality in our laboratory, currently 9.25 Gy. There will be three experimental groups (b)(4),(b)(6) differing by what will be administered after irradiation: 1) nothing, 2) HBSS+ 1% bovine serum albumen, and 3) BM EC. After irradiation, mice will be given BM EC with a preliminary injection schedule based on results from Experiment 1, but not to exceed 5x105 cells daily for five days by iv tail vein injection, with the first injection at 2 h post-irradiation. The HBSS group will receive injections with the same schedule. This initial dosing schedule and administration route is based on the work of Chute at al.[15] but may be altered based on preliminary results. Following Chute et al, [15] EC will be used after

no more than 5 passages, and washed twice and resuspended in HBSS + BSA just before injection.
The experimental endpoint will be 30-day mouse survival (number of mice reaching 30 days without dying or displaying signs of moribundity requiring euthanasia).

Number of mice required for Experiment 2:
Radiation doses: 1
Treatment groups (1. no treatment, 2. HBSS, 3. EC): 3
Mice/Group: 20
Iterations: 2

Total number calculated by multiplying cells:

Radiation Doses	Treatment Groups	Mice/ Group	Iterations	Total
1	3	20	2	120

Donor mice for EC for Group 3 (2 iterations): 40
Total mice for Experiment 1 = 120 + 40 = 160

V.1.2. Experiment 3 Effect of administered EC on hematology (peripheral blood elements and BM HSPC)
Mice (except donor mice) will be exposed to a sublethal dose of radiation low enough to allow sufficient cells in circulation and BM for analysis during the first two weeks after exposure: 3 Gy[redacted] To assess the effect of injections on unirradiated mice, other mice will be subjected to sham-irradiation. Treatment groups will be as in Experiment 1, differing by what will be administered after irradiation: 1) nothing, 2) HBSS, and 3) BM EC. After irradiation, mice will be given BM EC or HBSS (or nothing) as described for Experiment 2.
The experimental endpoints will be 1) quantitation of peripheral blood elements using CBC-diffs, and 2) quantitation of BM progenitors using clonogenic assays standard in our laboratory.[44] Mice will be euthanized on days 7 and 14 (8 mice/group) after radiation to perform these procedures.

(b)(4),(b)(6)

Number of mice required for Experiment 3:
Radiation doses (1. sham-irradiation, 2. 3 Gy): 2
Treatment groups (1. no treatment, 2. HBSS, 3. EC): 3
Mice/Group: 16
Iterations: 2
Total number calculated by multiplying cells:

Radiation Doses	Treatment Groups	Mice/ Group	Iterations	Total
2	3	16	2	192

Donor mice for EC for Group 3 (2 iterations): 40

Total mice for Experiment 3 = 192 + 40 = 232

Grand Total of mice needed for Experiments 1, 2, and 3: 178 + 160 + 232 = 570

V.2. Data Analysis Means with standard errors, or percentages, will be reported if applicable. The significance level will be set at 5% for each test. All statistical tests will be two-sided. Multiple comparison tests will be used when appropriate. Drugs will be compared to vehicle injections, and irradiation will be compared to sham irradiation where appropriate. Statistical software, PC SAS, will be used for statistical analyses.

Experiment 2: Log-rank tests will be used in comparing survival curves for each experiment. If those are significant, pair-wise comparisons will be done by Fisher's exact tests.

Experiment 3:
Analysis of variance (ANOVA) will be used to detect significant differences among groups. If significant, then Tukey-Kramer pair-wise comparisons will be used to identify which groups are different from each other.

Sample size justification for Experiment 2:
The 20 mice per group could have 80% power to detect a significant difference between two groups, given type I error of 5% and a treatment group survival rate of at least 73%, if the control group is 25%. Similar statements would apply, provided the treatment groups display survival of at least 86%, 83%, or 78%, if the respective control groups would be 40%, 35%, and 30% respectively. Note: power analysis may suggest smaller groups are adequate. However, radiobiologists at AFRRI and elsewhere observe considerable variability from experiment to experiment in mouse survival protocols that is not reflected in the power analysis. In fact, some institutions have gone to a group size of 30 in these types of experiments for this reason (b)(6) (b)(6). We have found over the last six years that 20 mice per group are sufficient to obtain results that are interpretable and repeatable.

Sample size justification for Experiment 3:
The sample size per group (N=8) would have 80% power to detect a significant difference between two groups, given type I error of 5% and delta/sigma of at least 1.4, where the delta is the estimated mean differences between the two groups and sigma is the standard deviation of the ANOVA model.[47]

Note on requirement to repeat experiments: It is a generally accepted principle of the scientific method that results must be repeated to evaluate a hypothesis.[48] Scientific journals (e.g., Endocrinology) state that experiments must be repeated to qualify for publication.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

V.3.2. Animal Model and Species Justification We considered computer models and tissue culture as alternatives to animals. Both alternatives lack the knowledge base and complexity needed to evaluate the effects of radiation and countermeasures on the hematological and immune systems. The phenomena under study involve complex information-processing networks comprising hundreds of cell types and biological signaling molecules. Responses to irradiation and drugs involve interactions between the central nervous system, the peripheral nervous system including the enteric nervous system, endocrine glands, the immune and inflammatory systems, hematopoietic organs, etc. We do not know all of the cell types and tissues involved and many of the signaling molecules have not been discovered yet. Even if all of the tissues, cell types and signaling molecules were known, the present state of the art of tissue culture would be incapable of reproducing their in vivo relationships. One would have to reproduce much of an entire mammalian organism in culture to study these phenomena in vitro, a scenario that would have its own ethical issues and would blur the distinction between "in vitro" and "in vivo." As for computer models, the most powerful supercomputers available in the foreseeable future would be incapable of analyzing interactions between so many elements in the network. These drugs and formulations will have to be evaluated in whole animal models. The purpose of the proposed research is to begin to identify the cells and signaling molecules necessary to construct a model of radiation injury and countermeasure mechanisms. It is also important to understand that it is a fallacy to assume that high level "emergent" phenomena can be understood by the reductionist approach of studying each basic component in isolation, and then synthesizing the results.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species: Mice (Mus musculus)

V.3.3.2. Strain / Stock: CD2F1. Origin: female BALB/c x male DBA/2

V.3.3.3. Source / Vendor: VSD approved vendor (Harlan preferred)

V.3.3.4. Age: 10-14 weeks

V.3.3.5. Weight: 24-32 g

V.3.3.6. Sex Male. To avoid influences of hormonal cycles in these proof-of-principle experiments, females are not included. Effects of sex differences will be explored in the future if funding is obtained.

V.3.3.7. Special Considerations The vendor's animal husbandry, breeding, and shipping practices should be in accordance with the Animal Welfare Act P.L. 89-544 and The Guide For The Care and Use of Laboratory Animals.

The animals must be adventitious disease-free. All animals should be raised with restricted access and strict entry and exit policy in caging that reduces the exposure to

pathogens such as in barrier condition, microisolation caging or isolators. They must be free of pathological lesions as assessed by gross exam.

Health surveillance monitoring must be performed regularly on the colonies and verified by an outside source. Health Surveillance reports should be sent with the animal shipments and must be current as well as accurate.

The mice must be pathogen-free, specifically for the following agents: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus morgani* and *Pasteurella*. In addition, all mice need to be free of the following viral agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epizootic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus, *Mycoplasma pulmonis*, and *Clostridium piliforme*. Endoparasite- and ectoparasite-free.

Genetic monitoring should be used to detect possible contamination and policies and procedures must be in place to reduce genetic drift.

Diet and Bedding Monitoring:
Standard laboratory animal diet should be used, Teklad preferred. Diet should be manufactured under ISO 9000 procedures with careful ingredient sourcing and monitoring. High quality bedding and bedding enrichment products should be manufactured following appropriate quality standards and screened periodically for contaminants, with certification available.

V.3.4. Number of Animals Required (by species) Mice: 570

V.3.5. Refinement, Reduction, Replacement (3 R's)
V.3.5.1. Refinement The radiation dose for Experiment 2 is sublethal, minimizing discomfort due to ARS. In the mortality studies, in order to minimize discomfort when animals become morbid, investigators and animal care staff will examine the mice at least twice daily, including in the early morning and late afternoon. Moribund animals will be considered to have arrived at the study endpoint and will be euthanized. Analgesics were considered as a way of minimizing discomfort during the onset of opportunistic infections, which will occur during the survival experiments. However, analgesics and anesthetics are well-known to alter the functional properties of the immune system and resistance to infection,[49-73] which would defeat the purpose of the study.

V.3.5.2. Reduction We will use training animals in Experiment 1 as donor animals for BM EC. We will use the same mice for peripheral blood counts and clonogenic studies of BM progenitors. We will use only one dose of radiation for the survival experiment, and one for the hematology experiment, to avoid gathering data that does not add

substantially to the main points being tested. We considered reducing the number of mice per group, but decided we could not in light of previous results [redacted] (b)(4),(b)(6) Although some institutions have gone to a group size of 30 in these types of experiments because of variability [redacted] we have found over the last six years that 20 mice per group is sufficient to obtain results that are interpretable and repeatable.

V.3.5.3. Replacement None (see section V.3.2 for explanation)

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C 290 (# of animals)

V.4.1.1.1.2. Column D 0 (# of animals)

V.4.1.1.1.3. Column E 400 (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1	Training/Donor animals (for BM EC)	50			50
1	All groups (non-irradiated and irradiated at 9.25 Gy)	64		64	128
2	Donor animals (for BM EC)	40			40
2	All groups (irradiated at 9.25 Gy)			120	120
3	Donor animals (for BM EC)	40			40
3	All groups (non-irradiated and irradiated at 3.0 Gy)*	96		96	192
Totals:		290	0	280	570

* The 3.0 Gy dose of total-body gamma radiation in CD2F1 mice (LD50 ~8.75 Gy) corresponds to a dose of approximately 1.3 Gy in humans (LD50 ~3.75 Gy). ARS is not clinically significant below 2.0 Gy in humans (corresponding to 4.7 Gy in CD2F1 mice) [redacted] (b)(4)

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization Mice will be anesthetized as part of euthanasia (in cases of moribundity or for sample collection) using a portable metered Isoflurane system. There will be no recovery from anesthesia in this case, and no prolonged period of anesthesia, hence hypothermia is not a concern. All euthanasia will be according to AVMA guidelines and performed by trained and technically proficient personnel.[80] For injection of EC into the tail vein, the same Isoflurane system will be used, but animals will revive after the procedure. Body temperature will be maintained using a heated stage or heating lamp.

V.4.1.2.2. Pre- and Post-procedural Provisions: N/A

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched: Agricola and PubMed
V.4.1.3.2. Date of Search: January 22, 2013

V.4.1.3.3. Period of Search: All available years:
Agricola: 1600s to present (according to Wikipedia)
PubMed: includes MEDLINE (1966-present) and OLDMEDLINE (1950-1965).

V.4.1.3.4. Key Words of Search: The aspects of the study relevant to alternatives to painful or distressful procedures are exposure to 9.25 Gy total-body ionizing irradiation causing ARS, and iv injections of EC. Therefore, the following key words were used: ionizing radiation AND pain, ionizing radiation AND alternatives, cell transplants AND pain, cell transplants AND alternatives.

V.4.1.3.5. Results of Search: Agricola: no hits.
PubMed:
ionizing radiation AND pain: 170 hits
ionizing radiation AND alternatives: 27 hits
cell transplants AND pain: 145 hits
cell transplants AND alternatives: 61 hits

The only relevant reference found was the work by Teskey et al. showing that total-body irradiation can produce analgesic effects.[81] For a discussion of alternatives considered, please see section V.3.2.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification Irradiated animals die due to compromised immune responses and opportunistic infections. The percentage of surviving animals is the indicator of the efficacy of a countermeasure. As discussed above, we cannot give anesthetic and/or analgesic agents to animals since they are known to interact with the immune system, and would confound the interpretation, resulting in a waste of animals. See references in section V.3.5.1.

V.4.2. Prolonged Restraint N/A

V.4.3. Surgery N/A

V.4.3.1. Pre-surgical Provisions N/A

V.4.3.2. Procedure(s) N/A

V.4.3.3. Post-surgical Provisions N/A

V.4.3.4. Location N/A

V.4.3.5. Surgeon N/A

V.4.3.6. Multiple Major Survival Operative Procedures

V.4.3.6.1. Procedures N/A

V.4.3.6.2 Scientific Justification N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections Personnel will be trained in tail vein injections by VSD. Guidance on injections of cell suspensions into mouse tail veins from the Animal Care and Use Committee of NCI in Frederick[82] and AAALAS[83] will also be followed. For injections of EC (or equivalent volume of HBSS) into tail veins, animals will be anesthetized as described in section V.4.1.2.1. An alcohol swab will be used to remove the scale from the animal's tail. This will increase the visibility of the vein, as well as make entry into the tail easier. If the vein is not easily visualized, the tail will be warmed by placing it in warm water (about 40 °C) for several minutes and/or placing the mouse at least 12 inches from a heat lamp for no longer than 3 minutes[84]. Preparation, dosages of cells and frequency will be as described in section V.1.1. Experiment 1. A 1.0 cc syringe will be loaded by drawing cells up with a 20 gauge needle. The needle will then be replaced with a 27 gauge needle for injection. A different injection needle will be used for each mouse. All syringes and needles will be sterile. Cell suspensions will be agitated just prior to withdrawal from the vial and injection, to avoid cell clumping. The 1% bovine serum albumen added to the HBSS will also help to reduce clumping. The cell suspension will be at room temperature. The tail will be held so that the lateral tail vein is uppermost. The tail will be held taut with one hand, near the tip of the tail, and the needle inserted into the vein at a minimal angle, in the proximal 1/3 of the tail. The needle will be advanced to approximately ½ (~5 mm) the length, feeding the needle into the vein, being careful not to perforate the vein. The syringe will be drawn back slightly to detect traces of blood flow into the needle hub, which will indicates the needle was successfully inserted into the vein. The plunger will be pushed in slowly, to inject all material into the vein. The cell suspension injected will be 200 µL in volume or less and will be delivered at a rate of less than 6 µL/sec. There should be minimal resistance when injecting, and the dark vein should turn clear as the HBSS or cell suspension displaces the venous blood. The needle will be removed and gentle pressure using sterile gauze will be applied directly to the injection site (approximately 5-10 seconds) to stop any bleeding.

In most cases an injection volume of 0.2-0.5 ml can be safely given to an adult mouse.[82] Most users try to limit the injection to 0.25 ml, a standard veterinary recommendation, but for cell injections, over-concentration may lead to embolism in lung capillaries and death of the recipient.[82] If moderate to severe dyspnea is observed immediately following injection (uncommon), the animals will be euthanized. Most mice show only reduced activity levels. Animals will be segregated and observed for the first hour following injection. If no problems are observed other than reduced activity, the mice will be returned to standard housing; normal behavior should be evident the next morning.

V.4.4.1.1. Pharmaceutical Grade Drugs No pharmaceutical grade drugs are used in this protocol.

V.4.4.1.2. Non-Pharmaceutical Grade Drugs
The cells will be grown in endothelial growth medium (Lonza, Inc.) supplemented with 5% FBS, EGF, and VEGF to support EC growth. The medium is not FDA-approved but it has been used to grow cells for implantation in *in vivo* models[85]. The recombinant cytokines (mouse) will be purchased from R&D systems and Invitrogen. We will wash cells with HBSS and inject cells suspended in HBSS + 1% BSA. The pH of the HBSS is 7.4. Sterile solutions of culture grade will be used and handled under aseptic procedures.

V.4.4.2. Biosamples Blood will be drawn from the caudal vena cava or by cardiac puncture in mice anesthetized by Isoflurane inhalation as a terminal procedure. After Isoflurane anesthesia and blood collection, animals immediately will be euthanized by cervical dislocation. Femur marrow for clonogenic assays or EC preparation, or bodies for disposal, will be collected after euthanasia as described under Euthanasia (V.4.6). All euthanasia will be according to AVMA guidelines by trained and technically proficient personnel.[80]

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAb) Production: N/A

V.4.4.5. Animal Identification: Cage cards. Mice will be held in groups of 4 per cage. Each card states the investigator's name, protocol number, experiment number, cage number, start date, end date, species, item number (animal lot), birthdate, gender, strain, number of animals in the cage, vendor, arrival date, treatments, and dates of death, including the initials of the staff person who found and removed the dead animals.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: mice will be weighed prior to radiation. This weight will provide a baseline for monitoring weight loss. Mice will only be weighed after radiation if weight loss is suspected by visual inspection. Weight loss (up to 10%) is a concern that will be reported to VSD.

V.4.4.8. Tissue Sharing: N/A

V.4.4.9. Animal By-Products: Culture-derived mouse BM EC will be used in this study. BM samples will be taken from CD2F1 donor mice post-mortem while in VSD and brought to our culture laboratory (b)(6) for isolation and expansion of EC. These cells will be washed in HBSS + 1% BSA and injected into mice through the tail vein. Testing for infectious adventitious agents is not required as the cells will be from the same lot of mice (VSD "item") as the recipient mice.

V.4.5. Study Endpoint In Experiment 2, the endpoint is euthanasia of moribund animals. In Experiment 3, the endpoints are peripheral blood counts (CBC/diffs) and clonogenic quantification of hematopoietic progenitors in femur. A score sheet is now provided for criteria for euthanasia in the 30-day survival experiments, according to IACUC Policy #10 (see Table 1, next page).

V.4.6. Euthanasia For sample collection, mice first will be anesthetized with Isoflurane and then cervical dislocation will be performed to cause death under a portable fume hood with charcoal filter. Mice to be euthanized at the end of the experiment will be exposed to CO2 by investigative or VSD staff followed by cervical dislocation.

V.5. Veterinary Care

V.5.1. Husbandry Considerations
No more than 4 mice will be placed in a cage during experiments. Animals will receive Harlan Teklad Rodent Diet and water *ad libitum*. Acidified water (pH 2.5 – 3.0) is routinely provided to AFRRI mice. Cages will be regularly changed and cleaned by VSD staff in accordance with Standard Operating Procedures and the most recent edition of the *Guide for the Care and Use of Laboratory Animals*. Animal rooms will be maintained under a 12 h light/dark cycle.

V.5.1.1. Study Room
As assigned by VSD within the vivarium.

V.5.1.2. Special Husbandry Provisions N/A
Table 1: Score Sheet for Euthanasia Criteria (taken from IACUC Policy Letter #10)

Appearance:		
	Normal (smooth coat, clear eyes/nose)	0
	Hunched and/or fluffed	1
	Ocular discharge, and/or edema	3
	Emaciated, dehydrated (skin tent) **	5
	Soft stools (fecal matter around anus)	5
	Bloody diarrhea	9
Respiratory rate:		
	Normal breathing	0
	Increased breathing (double normal rate, rapid, shallow)	6
	Abdominal breathing (gasping +/- open mouth breathing)*	12
General Behavior:		
	Normal (based on baseline observations)	0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)	1
	Decreased mobility	2
	Ataxia, wobbly, weak**	6
	Inability to stand*	12
Provoked Behavior:		
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))	0
	Subdued; responds to stimulation (moves away briskly)	1
	Subdued even to stimulation (moves away slowly)	3
	Unresponsive to gentle prodding **	6
	Does not right when placed gently on side within 5 seconds*	12
TOTAL		_____

Definitive criteria for morbidity:
Weight Loss up to 10% baseline (if available)**
Pale, white mucous membranes/ skin * *
Fever >104F/40C (if available)**

Definitive criteria for moribundity:
Weight Loss > 20% OR <15g (adult mouse)*
Blue mucous membranes/ skin (cyanosis)*
Cool to the touch or <86F/30C (if available)*

**** Regardless of score, notify appropriate person immediately.**

*** Regardless of score, immediately euthanize (death is imminent)**

Score:
< 6 Normal
6 - 9 Morbid: Monitor at least 3 times per day; notify appropriate personnel immediately
> 10 Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*)
Any single criteria of 12* euthanize immediately; consider as 'found dead'

(Note: This form does not have to filled out for every individual at every observation, so long as the criteria are used in informing decisions on increased monitoring and/or euthanasia. This form may be used to make a spreadsheet for greater ease of multiple animal observations, as long as the criteria and numbering system are exactly copied.)

V.5.1.3. Exceptions

Mice will be socially (group-) housed. There is a possibility that during survival studies cagemates may be lost through attrition and for a period of time the sole surviving mouse may remain singly housed until the end of the 30-day study. In addition, fighting among cagemates may require removal and single housing of the aggressor(s).

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care The animals will be observed daily by research staff of this protocol. Morbid mice will be observed twice a day (early morning

and late afternoon). Any moribund mice will be euthanized as soon as possible. If needed, a VSD veterinarian will be contacted for decision to euthanize.

V.5.2.2. Emergency Veterinary Medical Care
Emergency veterinary medical care is available 24 hours a day, seven days a week by VSD veterinarians and technicians and will include immediate euthanasia of any moribund mice.

V.5.3. Environmental EnrichmentV.5.3.1. Enrichment Strategy The animals will be housed in standard/conventional rodent caging. Mice will be group-housed, 4 mice per cage, with cotton bedding nestlets. Plastic tubes, hutches etc. will be introduced once mice have been declared non-radioactive by Safety.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				3	(b)(6)
				4a	
				5	
				3	
				4a	
				5	
				1	
				3a,b	
				4a	
				1	
				3	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY N/A (Cell suspensions will be from the same lot of mice (VSD "item") as the recipient mice.)

VIII. ENCLOSURES

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X. ASSURANCES

Protocol Title: Endothelial Cell Regulation of Hematopoiesis after Radiation Injury in the Laboratory Mouse (Mus musculus)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM** / **AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

APPENDIX 1.

ACRONYMS

ARS = acute radiation syndrome
HSPC = hematopoietic stem and progenitor cells
BM = bone marrow
EC = endothelial cells
G-CSF = granulocyte colony stimulating factor
CAM = cell adhesion molecule
SCF = stem cell factor
TGF-b = transforming growth factor
IL = interleukin
SDF-1 = stromal derived factor
FGF = fibroblast growth factor
Ang = Angiopoietin
PDGF = platelet derived growth factor
VCAM = vascular cell adhesion molecule
EPC = endothelial progenitor cells
HBSS = Hanks balanced salt solution

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	3/28/2013	2nd Resubmission Date:	
Approved/**Returned for Revision**:	4/19/2013	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	4/25/2013	**EXPIRATION DATE:**	5/5/2016
Approved/**Returned for Revision**:	5/6/2013	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER (b)(6)

III. PROTOCOL TITLE: Radioprotective efficacy of a novel formulation of GT3-Lipid nanoparticle in a mouse (*Mus musculus*) model

IV. PRINCIPAL INVESTIGATOR:

(b)(6) Ph. D
AFRRI phone: (b)(6) Fax (b)(6)
(b)(6)

CO-INVESTIGATOR(S):

(b)(6) Ph. D
AFRRI phone: (b)(6)
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date
Head, Scientific Research Department, AFRRI
Telephone (b)(6) Fax: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date
Statistician
Telephone (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date
Head, Veterinary Sciences Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date
Environmental Health and Safety Department, USUHS
Telephone (b)(6) Fax: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **, Ph.D.**
IACUC Chair, (b)(6) Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: Radioprotective efficacy of a novel formulation of GT3-Lipid nanoparticle in a mouse (*Mus musculus*) model

PRINCIPAL INVESTIGATOR: (b)(6) **Ph. D**
CO-INVESTIGATOR(S): (b)(6) **Ph. D**

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS Gamma-tocotrienol (GT3), one of the eight vitamin E isoforms that is not a pharmaceutical grade drug and is known for its robust antioxidant capabilities with little to no known toxicity (Berbee *et al.*, 2009). We have shown previously that (1) GT3 is a radiation prophylactic drug with a dose reduction factor (DRF) of 1.28-1.32, (2) GT3 ameliorates radiation-induced pancytopenia (reduction in all types of blood cells) in peripheral blood and protects bone marrow cells suggesting the sustenance of innate and adaptive immunity, and (3) GT3 protects crypt cells from injury and accelerates post-radiation regeneration of the gastro-intestinal (GI) mucosa when GT3 was administered via SC route. These results indicate that GT3 prevents lethality due to radiation-induced infection, hemorrhage and opportunistic infection by translocation of intestinal bacterial flora into systemic circulation, and provides medical options against acute radiation syndrome (ARS). Technological advantages of lipid based nanoparticle formulations include biodegradability, no or little toxicity, high stability at ambient temperature, and significantly high cellular uptake following administration. (b)(4)

(b)(4)

Additionally, we will assess the efficacy of the nano-GT3 formulation via two different administration routes (SC and Oral) to determine if it is effective, regardless of administration route. A mouse model was selected because we are going to compare the efficacy of the nano-GT3 formulation compared to the parent compound (IACUC Protocol (b)(6) in the same strain of CD2F1 mice after whole body gamma radiation.

II. BACKGROUND

II.1. Background Overview of the subject, issue, and/or problem.

The issue and/or the problem that we will address in this proposal is the urgent need to protect first responders deployed in a radiation field for rescue and recovery military operations resulting from accidental or intentional nuclear/radiological detonations. Currently there is no FDA approved drug to protect first responders deployed in a radiation field for military operations. This is a serious gap in medical capabilities that needs to be addressed. Drugs under various stages of development in small and large animals are only effective in large doses or are toxic at effective doses. Therefore, there is an immediate need for non-toxic radioprotectants (prophylactic

agents that can prevent radiation induced tissue injuries) useful for military personnel and civilians who may be exposed to a nuclear/radiological scenario. The present proposal directly addresses this need by moving forward with the development of a promising drug delivery approach to increase radioprotective efficacy of subcutaneous (SC) or oral (PO) delivery of the gamma-tocotrienol (GT3).

In an effort to identify suitable radioprotectants, a number of compounds of diverse chemical structures were considered. These compounds include thiols (Davidson *et al.*,1980), cytokines (Waddick *et al.*, 1991), steroids (b)(4) prostaglandin analogs (Hanson *et al.*, 1988), antioxidants (b)(4) (b)(4) and nutraceuticals (b)(4) (b)(4) The search for radioprotectants has been dominated by the study of sulphydryl compounds such as Amifostine (Davidson *et al.*, 1980). Amifostine has been approved for clinical use in conjunction with cisplatin for patients undergoing radiotherapy for head and neck cancer (Capizzi and Oster, 1995). Despite its effectiveness, Amifostine has not been accepted and approved as a suitable radioprotectant for military personnel. Moreover, it may not be useful for first responders in response to nuclear threat due to its performance- degrading toxicity (b)(4) and resultant hypocalcaemia (Glover *et al.*,1983).

Available Information for GT3

We reported that subcutaneously (SC) administered microsuspension formulation of gamma-tocotrienol (GT3) in 5% Tween-80, can prevent pancytopenia and reduce lethality in the short-term period following the adminstration of a lethal radiation dose in mice (b)(4),(b)(6) oral GT3 was minimally effective (preliminary study by us). GT3 and other potential drugs for radiation countermeasures being developed are used subcutaneously. This proposal directly addresses the need to develop an oral formulation of GT3 that is equally effective. We propose to accomplish this by using recently developed advanced concepts of nanotechnology for drug delivery to enhance the bioavailability of GT3 by oral administration, which is the preferred route of drug administration for emergency personnel involved in rescue operations. We also propose to increase radioprotective efficacy of GT3 by SC administration using this advanced formulation in nanoparticles.

Lipid Nanoparticles: For the GT3 radioprotectant, a series of formulations will be prepared by incorporating varying amounts of the drug and lipid modifier by our collaborators (b)(4)

(b)(4)

Nanoemulsion:	(b)(4)
(b)(4)	

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched:
1) PubMed, 2) RePORT(Research Portfolio Online Reporting Tools)er (Expenditure and Results) (formerly CRISP), 3) Biomedical Research Database (BRD)

II.2.2. Date of Search

PubMed	**February 4, 2013**
RePORTer	**February 5, 2013**
BRD	**February 5, 2013**

II.2.3. Period of Search

PubMed	**1974-2013**
RePORTer	**1980-2013**
BRD	**1998-2013**

II.2.4. Key Words of Search
Gamma-tocotrienol, radiation, radioprotection, mice, toxicity, nanoformulation, microsuspension formulations, free radicals, oral, subcutaneous

II.2.5. Results of Search Table 1 includes a summary of the keywords and keyword combinations used for searching the RePORTer, BRD, and PubMed databases.

Table 1: Summary of Database Keyword Searches			
keywords	RePORTer (CRISP)	BRD	PubMed
GT3	1	9	252
GT3 and radiation	1	9	21
GT3 and radioprotection	0	4	3
GT3 and toxicity	1	2	25
GT3 and toxicity and oral	0	0	2
GT3 and toxicity and subcutaneous	1	0	0
GT3 and mice and radiation	1	7	12
GT3 and mice and radioprotection	0	4	1
GT3 and nanoformulation	0	0	0
GT3 and free radicals	1	0	20
GT3 and hematopoietic cytokines	0	0	3
GT3 and G-CSF	0	0	4
Microsuspension formulation	0	0	15
Microsuspension formulation and radioprotection	0	0	0

Although there have been several studies performed using GT3 as a radioprotectant, the searches indicated that there have been no previous performed utilizing a nanoformulation of GT3 as a radioprotectant. Therefore, the proposed study to determine the optimal method of drug delivery (SC or PO) and the dose required for effective radioprotection is not a duplication of a previous experiment. Several groups have explored GT3 for its anti-inflammatory properties, however only our colleagues have explored the efficacy of GT3 as a radioprotectant (b)(4) For example, *in vitro* studies using a murine macrophage cell line (RAW264.7) determined that treatment with GT3 has anti-inflammatory properties by inhibiting NF-κB and C/EBP activation (Wang and Jiang, 2012). Reporter database revealed only one ongoing project with GT3 as a radioprotectant, but this study was not utilizing the novel nanoformulation of GT3 that we have proposed.

III. OBJECTIVE / HYPOTHESIS

In this proposal, we hypothesize that (b)(4) (b)(4) administered via SC or PO will provide (b)(4) (b)(4) Our specific aim for this pilot project is to assess the radioprotective efficacy of nano-GT3 administered via oral and SC routes.

IV. MILITARY RELEVANCE

Military operations are seriously impaired by radiation exposures depending on the dose and dose rate. Currently, there is no FDA approved preventive measure available for military men and women to protect them from the hazards of radiation exposure. The problem is more acute in recent times due to the unpredictable nature of terrorist attacks. Recognizing this urgent need, the United States Department of Defense has assigned top priority in "Defense Technology Objectives (DTO)" to the "development of medical countermeasures to radiation exposure" against both early effects and late

arising health effects. These concerns address the urgent need to develop an appropriate radiation protective agent (prophylactic or therapeutic) to sustain immediate warfighter capabilities and to enable the commanders to make judicious decisions in troop deployment. Currently, there are several drugs at various stages of development. This study will focus on revealing the mechanism of action of a prophylactic radiation countermeasure, gamma tocotrienol (GT3) using a promising new lipid based nanoparticle formulation to increase the radioprotective efficacy of GT3 following SC or PO administration.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

We propose to do the following experiments to assess the improved radioprotective efficacy of nano-GT3 given both orally and subcutaneously over microsuspension formulations of GT3.

Animals.

Male CD2F1 mice will be used throughout this study. Mice between the ages of 8 and 10 weeks, weighing approximately 22 to 25 g, will be purchased from Harlan Laboratories, IN, held in quarantine for a minimum of 10 days, tested for *Pseudomonas*, and maintained in an AAALACi (Association for Assessment and Accreditation of Laboratory Animal Care)-International accredited facility at the Armed Forces Radiobiology Research Institute (AFRRI) before use in the experiment. Animals will be kept in polycarbonate micro-isolator cages (eight per cage) on sani-chip contact bedding with free access to food and acidified water in an environmentally controlled room.

Irradiation.

All irradiations of mice will be carried out in well-ventilated plexiglass boxes that are compartmentalized to accommodate eight un-anaesthetized mice per box. Radiation administration will occur via bilateral irradiation in the cobalt-60 facility of AFRRI at a dose rate of 0.6 Gy/minute. All mice with drug administered SC will be irradiated at doses of 11, 11.5 and 12 Gy while mice with drug administered PO will be irradiated at doses of 9 and 9.5 Gy.

Administration of drug or vehicle :

The microsuspension formulation of GT3 or vehicle (5% Tween-80) will be supplied by (b)(4) We are using the same formulation for three other protocols at AFRRI. 100 mg/kg GT3 (optimum dose) or vehicle will be injected subcutaneously (SC) 24 h prior to radiation (0.1 ml per mouse) at the nape of the neck with a 23-25 G needle. The nanoformulation of GT3 will be supplied by (b)(4) (b)(4) Two drug doses of the nanoformulation of GT3 (dose1 and dose 2), with dose 1 being 1/4 of the maximum tolerated dose (as determined by ODDS method, please see experimental design V.1.1) and dose 2 being 2 times of dose 1, will be used for the initial screening. Thirty day survival will be determined in drug treated and control

mice after exposure to radiation (Cobalt-60 gamma at 0.6 Gy/min). Drug-treated mice will be compared to a vehicle-treated control group. V.1.1.

V.1.1. Experiment 1. Determination of the maximum tolerated dose of nano-GT3 (SC and PO). We will use the general guidelines adopted in our screening protocols (# (b)(6) . We will adopt the modified toxicity protocol Optimal (non-toxic) Drug Dose for screening (ODDs) which has been used in evaluating several drugs in other IACUC protocols at AFRRI. The purpose of using the ODDs method is that it allows testing in one gender (males in the present protocol) and requires fewer animals when compared to the FDA protocol. Drugs will be administered by two routes, subcutaneously (SC) and orally (PO).

We will start with 300 mg/kg as starting dose (a single administration) which is the maximum tolerated dose (MTD) for GT3 parent microsuspension formulation for SC administration as we have determined from our previous study. A maximum of six animals per drug dose (maximum 5 doses per drug) will be used to evaluate toxicity. Animals will be monitored daily for acute (1 to 4 h) and chronic (up to 14 days) signs of toxicity. Signs of acute toxicity include: decreased activity, squinting eyes, hunching, labored breathing or injection site swelling. If animals display any signs/symptoms of being adversely effected by the drug (i.e. toxicity symptoms previously described) they will be euthanized CO_2 overdose from a compressed gas cylinder followed by cervical dislocation in accordance with the current American Veterinary Medical Association (AVMA) Guidelines for Euthanasia. Death will be confirmed by cessation of heart function. All observations will be done by a trained technician including PI and Investigators. Animals will be observed twice daily for 14 days for morbidity (chronic sign of toxicity such as weight loss (>10%), decreased activity, hunched posture, labored breathing or any other abnormal clinical signs of toxicity). Moribund mice will be euthanized and necropsy will be performed (see section V.4.5), and all surviving mice will be transferred to another protocol. If a drug dose shows no initial toxicity in mice with a test dose of the drug, then the dose will be escalated approximately 3-fold and evaluated. If there is still no toxicity, we will continue the 3-fold increase until either maximum tolerated dose is reached or issues with solubility of drug become apparent. Conversely, if toxicity is found, the dose will be stepped down approximately 3-fold until no adverse effect is found.

For PO formulation, we will start with 600 mg/kg dose (a single gavage, 0.2 mL dose) as an estimated safe dose (300 mg/kg was found to be the MTD for SC administration for the parent GT3 formulation, (b)(6) Again, a maximum of six animals per drug dose (maximum 5 doses per drug) will be used to evaluate toxicity. Animals will be monitored daily for acute (1 to 4 h) and chronic (up to 14 days) signs of toxicity using the same signs/symptoms mentioned in the previous paragraph for the SC administration. If a drug dose shows no initial toxicity in mice with a test dose of the drug, then the dose will be escalated approximately 3-fold and evaluated. If there is still no toxicity, we will continue the 3-fold increase until either maximum tolerated dose is reached or issues with solubility of drug become apparent. Conversely, if toxicity is found, the dose will be stepped down approximately 3-fold until no adverse effect is found. The mice will be restrained by hand to insure minimal head movement. The drug

will be administered according to AAALAC guidelines (WRAIR Investigators' Handbook – Section 1; Injection routes, sites and needle sizes for common laboratory animals) for mice: 20 gauge needle and disposable syringe with a curved shaft that is 1.5 inches in length and contains a 2.25 mm ball to minimize trauma to oral and esophageal tissue.

In conclusion, if a drug dose shows no initial toxicity in mice with a test dose of the drug, then the dose will be escalated approximately 3-fold and evaluated. Conversely, if toxicity is found, the dose will be stepped down approximately 3-fold until no adverse effect is found. This experiment will only be used to determine the MTD that does not result in overt toxicity in mice. If we have to use more drug doses to achieve MTD, we will use fewer animals to do a pilot study until we achieve a dose with no observed adverse effect in animals.

Mice needed:
No of groups: 7 (1 vehicle/ blank nanoparticle and 3 drug doses each for both SC and PO) No of mice per group: 6
Total mice for this experiment: 7x6 = 42

V.1.2. Experiment 2. Compare the survival efficacy of nano-GT3 with the microsuspension formulation of GT3 administered SC 24 h prior to radiation.
CD2F1 mice will be irradiated at doses of 11, 11.5 and 12 Gy and monitored for 30 days for any observed weight loss, ruffled fur, behavioral changes and any other signs of morbidity or moribundity. If animals show signs or symptoms of morbidity/moribundity, they will be euthanized either by (1) cervical dislocation under isoflurane anesthesia or (2) CO_2 overdose from a compressed gas cylinder followed by cervical dislocation in accordance with the current American Veterinary Medical Association (AVMA) Guidelines for Euthanasia. At the end of experiments, surviving unused animals will be transferred to other protocols. Death will be confirmed by cessation of heart function. Two doses of nano-GT3 will be used; one is ¼ of MTD as determined from expt 1 and one dose higher (i.e. ½ of MTD).

Mice needed:
No of groups: 4 (vehicle/ blank nanoparticle, nano-GT3 dose 1, nano-GT3 dose 2, and microsuspension formulation of GT3, 100 mg/kg)
Radiation doses: 3 (11, 11.5 and 12 Gy)
No of mice per each exposure: 16
Number of repeats: 2
Total mice for this experiment: 3x4x16x2 = 384

V.1.3. Experiment 3. Compare the survival efficacy of nano-GT3 with the microsuspension formulation of GT3 administered PO pre- and post-radiation.
CD2F1 mice will be irradiated at doses of 9 and 9.5 Gy and monitored for 30 days for any observed weight loss, ruffled fur, behavioral changes and any other signs of morbidity or moribundity. Two doses of nano-GT3 will be used; one is ¼ of MTD as determined from expt 1 and one dose higher (i.e. ½ of MTD).

Mice needed:
No of groups: 4 (vehicle/ blank nanoparticle, nano-GT3 dose 1, nano-GT3 dose 2, and microsuspension formulation of GT3, 100 mg/kg)
Radiation doses: 2 (9 and 9.5 Gy)
Time of administration: 4 (-24, -12, +6, and +12
h) No of mice per each exposure: 16
No. of repeats = 2
Total mice for this experiment: 4x2x4x16x2 = 1024

V.2. Data Analysis
Toxicity Study: We will follow the same guideline as we used in our Intramural screening protocol to find the optimal drug dose for screening (ODDS) (Protocol# (b)(6) In this method, six mice per group will be used per drug dose. Since we will be using fewer mice, this procedure will be strictly observational and no statistical significance will be derived from this study.

For the survival data, Fisher's exact test will be used to compare survival at 30 days and a log-rank test will be used to compare survival curves. Analysis of variance (ANOVA) will be used to determine if significant differences exists among different groups. For a given day, if a significant difference among groups will be detected, a pair wise comparison will be performed using the Turkey-Kramer method. A significance level will be set at 5% for each test. All statistical analyses described in this protocol will be approved by a qualified statistician.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered
Our ultimate goal is to find a suitable radioprotectant for human use. We have already established that the microsuspension formulation GT3 is a good prophylactic radiation countermeasure in male CD2F1 mice, protecting both the hematopoietic and GI systems from radiation injury when injected SC. In order to increase the radioprotective efficacy of GT3 and reduce the dose required, the nano-GT3 drugs must be tested in an animal model. Additionally, we hope to improve the efficacy of GT3 as an oral prophylactic which we hope to demonstrate with our nano-GT3 formulation. Structure based drug design employing computer modeling and *in vitro* screening in cell cultures are used by drug manufacturers during the development of candidate drugs. However, this method cannot tell us whether a given regimen, prophylactic or therapeutic, is effective in promoting survival or not. No *in vivo* data are currently available that can address the questions we are addressing in this protocol.

V.3.2. Animal Model and Species Justification
As discussed in the previous section, animals must be used to study the radioprotective efficacy of a drug *in vivo*. We want to use the mouse as the animal model because we share 97.5 - 99% of our DNA with mice, and mouse models are well characterized to study the mechanism of action of a radioprotectant in a whole body system (b)(4)

(b)(4) Zebrafish and Drosophila are not considered for this study because their physiology is largely different from mammalian systems.
The use of the mouse model is necessary to determine if a prophylactic or therapeutic agent can protect against acute radiation effects and prevent lethality. It is not an option to use non-animal systems in place of animal models to address the research questions in this proposal (please see section V.3.1 for justification).

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species
Mus musculus

V.3.3.2. Strain / Stock
CD2F1

V.3.3.3. Source / Vendor
Harlan Labs, Indianapolis, IN or similar approved vendors.

V.3.3.4. Age
8-10 wks old

V.3.3.5. Weight
22-25 g

V.3.3.6. Sex
Male

Justification of using only male mice: Previous studies have been conducted with male mice, so male mice are needed for data comparison. Although, it is important to confirm observations in females, such studies may be carried out at a later time.

V.3.3.7. Special Considerations
Commercially procured animals must be adventitious, disease free, and pathogen-free—including *Pseudomonas aeruginosa*, and *Pasteurella*. All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus, *Mycoplasma pulmonis*, and *Clostridium piliforme*. They must also be endoparasite and ectoparasite-free.

V.3.4. Number of Animals Required (by species): 1450

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement
Moribund animals (as discussed in V.4.1) will be euthanized to minimize the magnitude and duration of suffering these animals might experience when they are subjected to toxic effects of ionizing radiation and/or high doses of the test drug. In such cases moribundity (please see V.4.1) will be used as a surrogate for death in animals and will be considered to have arrived at an early endpoint.

V.3.5.2. Reduction
Previously published and unpublished data have established which radiation doses are required for each method of drug delivery to accurately assess radioprotective efficacy. Results from the toxicity study will allow us to utilize 2 doses of the drug to minimize the number of animals required. Based on our previous experiments on survival study, we will use the minimum required number of animals for statistical validity. This will reduce the number of animals to be used in this protocol.

V.3.5.3. Replacement
N/A

V.4. Technical Methods

V.4.1. Pain / Distress Assessment
According to the IACUC Operating Policy Letter #10 "Guidelines for establishment of early endpoints in experiments with expected mortality", examples of clinical signs of morbidity and moribundity are listed below in Appendix A: Clinical Observations with Criteria for Euthanasia (Rodent):

VIII. Appendix A: Clinical Observations with Criteria for Euthanasia (Rodent)

DATE:	Time:	Animal ID#	Score
Appearance:			
	Normal (smooth coat, clear eyes/nose)		0
	Hunched and/or fluffed		1
	Ocular discharge, and/or edema		3
	Emaciated, dehydrated (skin tent) **		5
	Soft stools (fecal matter around anus)		5
	Bloody diarrhea		9
Respiratory rate:			
	Normal breathing		0
	Increased breathing (double normal rate, rapid, shallow)		6
	Abdominal breathing (gasping +/- open mouth breathing)*		12
General Behavior:			
	Normal (based on baseline observations)		0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)		1
	Decreased mobility		2
	Ataxia, wobbly, weak**		6
	Inability to stand*		12
Provoked Behavior:			
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))		0
	Subdued; responds to stimulation (moves away briskly)		1
	Subdued even to stimulation (moves away slowly)		3
	Unresponsive to gentle prodding **		6
	Does not right when placed gently on side within 5 seconds*		12
TOTAL			_____

Definitive criteria for morbidity:	**Definitive criteria for moribundity:**
Weight Loss up to 10% baseline (if available)**	Weight Loss > 20% OR <15g (adult mouse)*
Pale, white mucous membranes/ skin * *	Blue mucous membranes/ skin (cyanosis)*
Fever >104F/40C (if available)**	Cool to the touch or <86F/30C (if available)*
**** Regardless of score, notify appropriate person immediately.**	***Regardless of score, immediately euthanize (death is imminent)**

Score:
< 6 Normal
6 - 9 Morbid: Monitor at least 3 times per day; notify appropriate personnel immediately
> 10 Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*)
Any single criteria of 12* euthanize immediately; consider as 'found dead'

(Note: This form does not have to filled out for every individual at every observation, so long as the criteria are used in informing decisions on increased monitoring and/or euthanasia. This form may be used to make a spreadsheet for greater ease of multiple animal observations, as long as the criteria and numbering system are exactly copied.)

The moribund condition is defined as a clinically irreversible condition leading inevitably to death. Animals involved in experiments that may lead to moribundity or death will be monitored daily by personnel experienced in recognizing signs of moribundity (Tomasivic, 1988, Montgomery, 1990). Experimental mice that are found morbid (affected with disease or illness), but not moribund, will be allowed to continue in the experimental protocol, but no supportive care is proposed. The morbid mice will be examined at least twice daily, in the early morning and late afternoon, by the research

staff (including weekends and holidays) in addition to the regular VSD health checks. VSD veterinarians will be consulted for all matters regarding animal welfare. Animals receiving a score ranging from 6-9 on the IACUC Policy Letter #10, Appendix A: Clinical Observations with Criteria for Euthanasia (Rodent) will be considered morbid and subjected to 3 health checks per day. Any animals receiving a score of >10 will be considered morbid and immediately euthanized.

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C ___42__ (# of animals)

V.4.1.1.1.2. Column D ___0__ (# of animals)

V.4.1.1.1.3. Column E ___*1408__ (# of animals)*

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
V.1.1	Experiment 1	42			42
V.1.2	Experiment 2			384	384
V.1.3	Experiment 3			1024	1024
Totals:		42		1408	1450

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization
Radiation might cause momentary pain in animals. We placed the animals which will be irradiated or drug treated in the E category. We will not be using any anesthesia/analgesia in this protocol as a measure of pain relief.

V.4.1.2.2. Pre- and Post-procedural Provisions
Mice will be housed and monitored in accordance with VSD standard operating procedures both before and after irradiation (see Section V.5."Veterinary Care")

V.4.1.2.3. Paralytics
No paralytic agents will be administered

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched
Altweb
AGRICOLA
PubMed

V.4.1.3.2. Date of Search
Altweb – 02/13/2012
AGRICOLA – 02/13/2013
PubMed – 02/13/2013

V.4.1.3.3. Period of Search
1970-2013

V.4.1.3.4. Key Words of Search
Pain, toxicity, analgesia, anesthesia, radiation, radioprotection, mouse, humane, alternative, gamma-tocotrienol, nano-GT3, micro-GT3.

V.4.1.3.5. Results of Search
We are studying a new formulation of GT3 in this protocol. We will be using lethal doses of radiation (9, 9.5, 11, 11.5, and 12 Gy) in this study based on results from our previous studies to assess the improved radioprotective efficacy of nano-GT3 given both orally and subcutaneously over microsuspension formulations of GT3. Radiation sickness is an acute illness caused by high doses of penetrating ionizing radiation to most or all of the body in a relatively short time period which can be painful. Literature search verifies that developing symptoms due to hematopoietic and gastrointestinal damage appears to be painful and distressful to humans and animals. Table 2 includes a summary of the keyword combinations used for searching and the results of the search. We are using nanoparticles to minimize the dose of drug that we must administer to the animals prior to radiation exposure. The literature suggests that nanoparticles are now commonly used to treat many forms of cancer. Although they are not toxic to the animals' system, the drug preparations in this formulation are highly toxic to cancer cells and therefore very effective. A nanoparticle formulation of GT3 was highly efficacious in increasing intestinal permeability of GT3 and therefore enhancing its oral bioavailability (Abuasal *et al.*, 2012). While the literature indicates that cell models have been used to study radiation damage, such models cannot alone predict mechanism of radioprotection of a specific drug. Although radiation-induced pain was alleviated during some studies (Ciezki and Macklis, 1995, Desai *et al.*, 1997, Sonoo *et al.*, 1995), such pain relief was the aim of the studies in question, which focused on patients receiving radiotherapy for cancer treatment. We do not have the option of using alternatives such as a cell culture model. We do expect to see clinical signs of pain in mice since we are using a lethal doses of 9, 9.5, 11, 11.5, and 12 Gy. Therefore, if animals show signs of pain or distress (please see section V.4.1), all moribund animals will be euthanized immediately to minimize potential pain or distress.

Table 2: Summary of Database Keyword Searches			
keywords	Hits		
	AGRICOLA (NAL catalog)	AltWeb	PubMed
Nanoparticles and toxicity	23	30	5819
Gammatocotrienol AND toxicity	11	0	25
Analgesia AND radiation	3	4	507
Pain AND radiation	93	7	9503
Pain AND analgesia AND radiation	23	4	335
Gammatocotrienol AND pain	0	0	0
Nano-GT3	0	0	0
Micro-GT3	0	0	0
Radioprotection AND pain	0	0	5
Radiation AND humane	0	28	8
Pain AND analgesia AND mice	0	21	1656
Radiation AND mouse AND pain	0	6	207
Radiation AND mouse AND pain AND alternative	0	5	3

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification
Since the purpose of these studies is to determine the radioprotective efficacy of nano-GT3, we will be unable to use analgesics and anesthetics because they may interfere with the innate system (Galley *et al.*, 1998, Galley and Webster, 1997). However, if we see any morbidity or moribundity (as described in section V.4.1) frequency of examination will increase. All moribund (see section V.4.1) animals will be euthanized by overdose of CO_2 inhalation and cervical dislocation as described in section V.4.6 rather than allowing them to progress to death.

V.4.2. Prolonged Restraint
N/A

V.4.3. Surgery
N/A

V.4.3.1. Pre-surgical Provisions N/A

V.4.3.2. Procedure(s) N/A

V.4.3.3. Post-surgical Provisions N/A

V.4.3.4. Location
N/A

V.4.3.5. Surgeon
N/A

V.4.3.6. Multiple Major Survival Operative Procedures N/A

V.4.3.6.1. Procedures
N/A

V.4.3.6.2 Scientific Justification
N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections
Microsuspension formulation of GT3 (200 mg/kg) will be injected subcutaneously (SC) (0.1 ml) using a sterile 23-25 G needle and syringe at the nape of the neck (b)(4) (b)(4) Nanoparticle formulations of GT3 will be injected (dose to be determined following toxicity study) SC (0.1 ml, maximum volume 0.25 ml is approximately equal to 1% body weight for 25 g mouse) using a sterile 23-25 G needle and syringe at the nape of the neck. Oral gavage will be performed using 20 G disposable gavage feeding syringes, with an approximate gavage volume of 0.2 ml (maximum volume 0.3 ml/mouse). Mice will be restrained with hand restraint for both the SC injection as well as the oral gavage.

V.4.4.1.1. Pharmaceutical Grade Drugs N/A

V.4.4.1.2. Non-Pharmaceutical Grade Drugs
GT3 is a non-pharmaceutical grade drug. It had been tested endotoxin free for another protocol (Protocol# (b)(6) , and was found to have no adverse reactions. GT3 will be tested for endotoxin by Charles River Laboratory before using in the animals. We will be using the same excipient (5% Tween-80 in saline, pH 6.8, osmolality 290 mOsm) as we used for GT3 in the other protocol (b)(6) as a SC injection in a volume of 0.1 ml per mouse. (b)(4)

(b)(4)

(b)(4) So, we are not expecting any toxic effects in animals.

V.4.4.2. Biosamples
N/A

V.4.4.3. Adjuvants
N/A

V.4.4.4. Monoclonal Antibody (MAb) Production
N/A

V.4.4.5. Animal Identification
Different groups of mice for a particular treatment group will be identified in cage cards. However, for toxicology experiments, ear punching will be used to identify individual animals in a cage. Three animals (in a cage) will be identified as L (as left ear punched), R (as right ear punched) and N (as no ear punched). This will help us monitor each animal for drug toxicity. The individuals who have the training and are responsible for ear punching have been indicated in the table on qualification and animal handling experience described in section VI. Animals will be restrained (hand restraint) during the ear punching procedure, ears will be cleaned with alcohol swabs prior to performing the punch, and the instrument will be cleaned with an alcohol swab in between animals. The punch will be placed on the pinna of the ear in a standard location (outermost point). After firmly pressing on the punch device and verifying that the procedure left a circular hole through the ear, the ear will be gently separated from the punch device to avoid additional injury to the ear.

V.4.4.6. Behavioral Studies
None

V.4.4.7. Other Procedures
None

V.4.4.8. Tissue Sharing
At present there are no plans for tissue sharing.

V.4.4.9. Animal By-Products
N/A

V.4.5. Study Endpoint

For the toxicity study, the endpoint will be a 14-day survival with any surviving animals euthanized. For the radioprotective efficacy study, the endpoint will be 30-day post-irradiation survival and the surviving mice will be transferred to another protocol.

If we see any moribund animals beyond access to food and water, we will humanly euthanize the animals. It is critical to define criteria for euthanizing mice humanely without compromising the study objective and complying with the IACUC recommendations stipulated in The IACUC Operating Policy Letter #10 "Guidelines for establishment of early endpoints in experiments with expected mortality" (please see V.4.1).

Experimental mice that are found morbid, but not moribund, will be allowed to continue in the experimental protocol, but no supportive care is proposed. The morbid mice will be examined at least twice daily, in the early morning and late afternoon, by the research staff (including weekends and holidays) in addition to the regular VSD health checks. VSD veterinarians will be consulted for all matters regarding animal welfare. However, an extra arm involving histopathology of animals that survive 14 days using the "pluck" method for collection of tissues will be used. For this purpose, animals

will be euthanized by CO2 inhalation and cervical dislocation prior to harvesting the following tissues: (Heart; tongue; lung; esophagus; trachea; kidney; thymus; liver; spleen; stomach; colon; cecum; duodenum; jejunum; pancreas; ileum; seminal vesicle; testis; epididymus; urinary bladder; haired skin; skeletal muscle; eye; cerebrum; cerebellum; bone marrow; tibia; femur) will be collected. Tissues such as those described above will also be collected in animals that are euthanized due to a moribund condition (as described in section V.4.1). Histopathology will include evaluation of all major organ systems and the data will be used to characterize tissue-specific responses.

V.4.6. Euthanasia All moribund animals (see section V.4.5) as well as surviving animals at the end of the study will be euthanized by overdose of CO_2 inhalation and cervical dislocation as described by VSD SOP, by trained investigators or technicians in accordance with current AVMA Guidelines on Euthanasia. Compressed CO_2 gas in a cylinder with a regulator will be used for euthanasia in a euthanasia chamber.

V.5. Veterinary Care

V.5.1. Husbandry Considerations
Animal husbandry procedures will be followed according to the VSD SOPs.
Mice will be group housed in polycarbonate micro-isolator cages (eight per cage) on sanichip contact bedding with free access to food and acidified water in an environmentally controlled room.

V.5.1.1. Study Room
Mice after quarantine will be housed in designated rooms and used when they are ready. For brief periods, mice will be transported (slowly to minimize vibration and/or bumpiness) to the cobalt irradiation facility in well ventilated Plexiglas boxes that are completely covered by an opaque lab coat to minimize transportation stress.

V.5.1.2. Special Husbandry Provisions
None

V.5.1.3. Exceptions
None

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care
Experimental animals will be observed at least once a day by one of the investigators or technicians. As described in section V.4.1, morbid animals will be monitored at least twice daily, early morning and late afternoon by the research staff. In addition, VSD personnel will also monitor the experimental animals during their routine twice daily rounds. The experimental design does not contemplate supportive therapy.

V.5.2.2. Emergency Veterinary Medical Care

Emergency veterinary medical care is available 24 hrs and 7 days a week. Veterinary staff will be contacted as needed.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

The enrichment strategy for mice will be according to VSD Standard Operating Procedure, which involves socially grouped housing and providing nestlets and huts/igloos in the cage.

V.5.3.2. Enrichment Restrictions N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1,3,4,5	(b)(6)
				1,3,4,5	
				1,3b,4	
				1, 3b, 4, 5	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a-ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY

An AFRRI Safety and Health Department protocol Hazard Analysis was approved on January 2, 1997 for the general research program of the Radiation Casualty Management team (now Radiation Countermeasures Program). All personnel listed in the present IACUC protocol have been trained either as radioactive users or as general radiation users on a regular basis. All investigators and technicians while in the vivarium will use appropriate procedures as described by VSD. This will include wearing of mask and gloves during observation of mice. All investigators and technicians are current in safety training and medical surveillance.

VIII. ENCLOSURES

References

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X. ASSURANCES

Protocol Title: Radioprotective efficacy of a novel formulation of GT3-Lipid nanoparticle in a mouse model

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)		4-29-2013
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (October 2012)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	**4/19/2013**	2nd Resubmission Date:	
Approved/Returned for Revision:	**5/6/2013**	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	**5/22/2013**	**EXPIRATION DATE:**	**6/24/2016**
Approved/Returned for Revision:	**6/25/2013**	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

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- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. **NAME OF FACILITY: Armed Forces Radiobiology Research Institute**

II. **PROTOCOL NUMBER:** (b)(6)

III. **PROTOCOL TITLE:** Effect of supportive care on irradiated Gottingen minipigs (*Sus scrofa domestica*)

IV. **PRINCIPAL INVESTIGATOR:** (b)(6)

(b)(6) PhD Date
Principal Investigator, (b)(6) Scientific Research Department
Tel: (b)(6) Fax (b)(6) E-mail: (b)(6)

V. **DEPARTMENT HEAD:** This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date
Head, Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)

(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician

Telephone (b)(6) Fax (b)(6)

(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI

Telephone (b)(6) Fax (b)(6)

(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department, USUHS

Telephone (b)(6) Fax (b)(6)

(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**

IACUC Chair (b)(6) Scientific Research Department, AFRRI

Telephone (b)(6) Fax: (b)(6)

(b)(6)

PROTOCOL TITLE: Effect of supportive care on irradiated Gottingen minipigs (*Sus scrofa domestica*)

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6) PhD Date
Principal investigator (b)(6) Scientific Research Department
Tel (b)(6) Fax (b)(6) E-mail (b)(6)

CO-INVESTIGATOR(S): Dr (b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS

The Acute Radiation Syndrome (ARS) develops over time, depending on the amount of radiation absorbed. The ARS is classically divided into 4 sub-syndromes, based on the organ with the most severe damage: hematopoietic (H), gastrointestinal (GI), cutaneous (C) and neurovascular (N). Evidence suggests that these syndromes have some overlap in time and dose range. Efficacy of clinical management should therefore be assessed on multiple organs involved in concurrent sub-sequelae.

The GI syndrome progresses at the same time as the hematopoietic syndrome starts to develop. The GI-ARS is expected to be affected by H-ARS bone marrow aplasia; similarly, changes in the intestinal tract may influence development of H-ARS. Lethality from the GI develops fast (days), and involves desquamation of epithelial mucosa in the intestine, leading to loss of fluids and electrolytes, decreased absorption of nutrients and bacterial translocation. Lethality from H-ARS happens within months, and it is speculated to be due mainly to bone marrow aplasia, susceptibility to sepsis and low platelets count. Some delayed GI damage may be expected. Because of the difference in the mechanism of lethality, recovery from GI-ARS does not guarantee survival from H-ARS. Development of animal models to study the natural history of ARS and test radiation countermeasures remains one of the main bottlenecks for drug development. So far, a very limited number of large animal models are available for countermeasures testing (NHP, dogs and swine); understanding of the natural history of radiation sickness and development of multi-organ failure is still largely incomplete.

We are developing a minipig model of ARS. So far we have optimized implantation and patency of a vascular access port for blood sampling, characterized the H-ARS, and tested the efficacy of G-CSF on survival and bone marrow recovery in the irradiated Gottingen minipig. Signs and symptoms, kinetics of blood cell depletion and recovery, multi-organ dysfunction and histological findings resembled closely what observed in human victims of radiation accidents and in other large animal models (b)(4)

In the G. minipig, the H-ARS occurs between 1.6 and 2.0 Gy. (b)(4)
(b)(4)

Supportive care has been shown to profoundly alter and extend the survival period of human victims and animal models irradiated at hematopoietic doses (4). Standard of care consist of antibiotics, analgesics, antipyretics, fluids/ nutritional support and blood products (5). Because of the integrity of the GI tract at (b)(4) doses, we expect that administration of antibiotics, analgesics and fluids/ nutritional support will greatly improve survival at these doses, although blood products may still be necessary.

The purpose of the current study is to (i) use supportive care to extend the survival to a period of time sufficient to study the potential effects of countermeasures on recovery of bone marrow and the GI

system; (ii) characterize the natural history (NHx) of ARS at those doses; and (iii) to establish a probit curve to allow for future countermeasure drug testing.

The primary data points of the study will be mortality; secondary data points will be standard biomarkers of CBC counts, histology of the GI tract (primarily the small bowel) and bone marrow, and blood and tissue microbiology for broad cultures of suspected infections at necropsy.

II. BACKGROUND

II.1. Military personnel are potentially exposed to radiation during their duty (assembling, disassembling, stockpiling of nuclear weapons, cleanup of facilities, disposal of radioactive materials, civilian defense, etc). So far, only four drugs specifically designated for use in radiation emergency are present in the Strategic National Stockpile. Limited availability of a sufficient number of well-characterized animal models to test radiation countermeasures had been indicated as one of the main bottlenecks for advanced drug development. Developing large animal models for testing of countermeasures to radiation exposure and identification of the patho-physiological mechanisms underlying the development of the acute radiation syndrome (ARS) are fundamental to clinical management and one of the primary objectives of the Department of Defense.

Development of the acute radiation syndrome is dose and time dependent. A continuum in the development of symptoms and pathologies has been proposed over the years, and covers bone marrow (BM), gastrointestinal (GI), cutaneous and CNS syndrome. Although developing concomitantly, GI- and H-ARS have different mechanisms of action and time course. It is expected that mitigation of the GI syndrome will NOT waive the treatment for the hematopoietic syndrome if left untreated. Effects of standard drug treatment for the H-ARS must therefore be determined on the irradiated GI tract.

We recently developed a minipig model for the hematopoietic syndrome, characterized by signs and symptoms, bone marrow aplasia, kinetics of blood cell depletion and recovery similar to what observed in humans, canines and NHPs (b)(4) Sequence of signs and symptoms development, dynamics of hematological cell loss, susceptibility to infection and occurrence of multi-organ dysfunction leading to death, all confirm the validity of this model to study the ARS. Implantation of a subcutaneous vascular access port (VAP) (b)(4) allows for longitudinal sampling of large amounts of blood for CBC/diff counts, clinical chemistry parameters and in vitro assays that could be correlated to survival, gross and histo-pathology and vital signs.

(b)(4)

(b)(4)

The primary data point of the study will be mortality; secondary data-points will be standard biomarkers of CBC counts, histology of the GI tract (primarily the small bowel) and bone marrow (sternum, L1 vertebrae and femur or humerus, and blood and tissue microbiology.

II.2. Literature Search for Duplication
II.2.1. Literature Sources Searched BRD, RePORTER, PubMed
II.2.2. Date of Search February 24, 2013
II.2.3. Period of Search
BRD: 1998 – 2009
RePORTER: 1998-2012
Pubmed: 1998-2012

II.2.4. Key Words of Search
Swine OR minipig
AND Radiation
AND supportive care
AND total body

II.2.5. Results of Search

BRD database:
Keyword Swine .....776 results
Keyword Swine+ radiation: ..... 4 results

1) Implantation Techniques and Specific Absorption Rates for Swine (Sus scrofa) in Directed Energy Health and Safety Studies
2) Pilot study: Development of Gottingen minipig (Sus scrofa domestica) as radiation injury model
3) NIRVANA: Non-Ionizing Radiation Vision for a New Army
4) Extended Electro Magnetic Medical Advisor: A Diagnostic Tool for Casualty Triage by Combat Medics

Keyword Swine + supportive care: 129 results (note relevant for this study)
For these projects, animals are used to train medical practitioners in skills (surgical emergency care, catheter implantation, etc) vital for peacetime medicine, battlefield operations, combat casualties, trauma/trauma-hemorrhagic patients.. Alternatively, animals are used for training of new veterinary staff

Keyword Swine+ radiation+ supportive care: ..... 1 results (not relevant for this study)

1) Extended Electro Magnetic Medical Advisor: A Diagnostic Tool for Casualty Triage by Combat Medics

Keyword Minipig: .....25 results
Keyword Minipig + radiation: .....4 results

1) Pilot study: Development of Gottingen minipig (Sus scrofa domestica) as radiation injury model
2) Determination of Thermal Injury Thresholds in the Yucatan Minipig Using Radio Frequency Radiation
3) Determination of Thermal Injury Thresholds in the Yucatan Minipig Using Radio Frequency Radiation
4) Determination of Thermal Injury Thresholds in the Yucatan Minipig Using Radio Frequency Radiation

Keyword Minipig + supportive care 1 results (not relevant for this study)

1) Exploratory Assessment of Immediate Behavioral Effects Of TASER-Like Devices On Swine

Keyword Minipig + radiation + supportive care.....0 results

RePORTER database:
Keyword swine: .....838 Results
Keyword swine+radiation: ....108 Results
Keyword swine+ supportive care: 3 results (not relevant to this study)

1) Modulating secondary damage following traumatic brain injury in the child
2) See, reach, treat tumor-optimized transarterial chemoembolization drug delivery.

3) A multidisciplinary NMR center for biomedical research

Keyword swine+radiation+supportive care: .....0 Result

Keyword Minipig: .....10 result (6 not relevant to this project)

1. DEVELOP RADIATION INJURY MODEL: GOTTINGEN MINIPIG (SUS SCROFA DOMESTICA)
2. DEVELOP RADIATION INJURY MODEL USING THE GOTTINGEN MINIPIG
3. COUNTERMEASURES AGAINST RADIOLOGICAL AND NUCLEAR ATTACK - AFRRI/IAA
4. COUNTERMEASURES AGAINST RADIOLOGICAL AND NUCLEAR ATTACK

Keyword Minipig+radiation: .....4 results

1. DEVELOP RADIATION INJURY MODEL: GOTTINGEN MINIPIG (SUS SCROFA DOMESTICA)
2. DEVELOP RADIATION INJURY MODEL USING THE GOTTINGEN MINIPIG
3. COUNTERMEASURES AGAINST RADIOLOGICAL AND NUCLEAR ATTACK - AFRRI/IAA
4. COUNTERMEASURES AGAINST RADIOLOGICAL AND NUCLEAR ATTACK

Keyword Minipig+ supportive care 0 results

Keyword Minipig + radiation + supportive care.....0 results

PUBMED database:
Keyword Swine ..... 175608 results
Keyword Swine + supportive care.....30 results (not relevant to this study)

Publications focused on brain death, kidney/liver/lung injury (non radiation-induced), thoracic trauma, animal welfare, H1N1, sepsis/infections (non radiation-induced), influenza, poisoning from ingested agents, septic shock, respirators/surgical masks, ischemia/oxygenation, burn care, hypenatremia, fetal surgical protocols.

Keyword Swine + radiation: ..... 4257 results
Keyword swine + radiation + intestine + supportive care 0

Keyword Minipig:.....5952 results

Keyword minipig+ supportive care 2 results (not relevant)
1) High-sodium crystalloid solution for treatment of hypernatremia in a Vietnamese pot-bellied pig.
2) Fetal surgical protocols in Yucatan miniature swine.

Keyword Minipig+radiation.....229 results

Keyword minipig+radiation+ supportive care.....0

III. OBJECTIVE / HYPOTHESIS

Our preliminary data show that doses ≥ 2 Gy are 100% lethal and that morbidity was reached ≤14 days after exposure to radiation. In the GI tract, villar blunting and fusion are observed, but no loss of crypts or decline in plasma citrulline levels are observed at doses 2.0-3.0 Gy.

Our hypothesis is that use of supportive care at doses 2.0 – 3.0 Gy will increase the survival of irradiated minipigs and that a 30-day survival probit curve in the presence of supportive care can be generated for estimation of lethality and for medical countermeasure (MCM) testing.

We plan to investigate the effect of additive elements of supportive care on 30-day survival starting with doses in the 2.0 - 3.0 Gy range, and to build a probit curve in the presence of an optimized supportive care regimen that will encompass the LD0/30 to the LD100/30.

IV. MILITARY RELEVANCE

According to the DoD Chemical and Biological Defense Program 2009 Annual Report to Congress (6): "Although the overall number of nuclear weapons continues to decline because of Russian and U.S. treaty commitments, the United States anticipates an increase in weapon numbers in China, India, and Pakistan. Motivated by economic and strategic interests, Russia and China (or political entities in each) and North Korea continue to supply technologies and components that are dual use and could support weapons of mass destruction and missile programs, especially in the Middle East and South Asia. Iran continues to develop its enrichment program in defiance of United Nations (U.N.) Security Council resolutions and also continues to build a heavy water reactor in Arak, which will be capable of producing plutonium that could be weaponized. While North Korea has halted portions of its nuclear program, it is possible that it has stockpiled several nuclear weapons from plutonium produced at Yongbyon. Non-nuclear radiological dispersal devices (RDD) and radiological exposure devices (RED) pose a significant potential threat especially in the hands of non-state groups ... Contamination and recovery issues associated with operating in a radiological environment remain significant concerns for military operations and underscore the need for robust detection, protection, and decontamination systems."

Currently, there is no safe and non-toxic radiation countermeasure available. Military personnel are potentially exposed to radiation in various scenarios. Both early and late health effects of radiation exposure are major concerns for the military. Developing countermeasures to radiation exposure is a top priority for the US Department of Defense. Effective countermeasures would expand the options available to field commanders operating Ionizing Radiation (here defined as IR) threat environments, and improve the morale of personnel at risk of exposure to IR. DoD Defense Technology Objective MD.18 states: "Effective mitigation of health consequences [of IR] will (1) reduce casualty load at medical treatment facilities, (2) sustain a more effective operational force after a radiation exposure event, (3) allow commanders to conduct operations in radiation field environments without undue risk of decremented performance of personnel due to acute tissue injury, and (4) reduce the negative psychological impact on personnel tasked to operate in contaminated environments."

An important barrier to advanced development of radiation countermeasures is the paucity of large, long-lived, non-rodent animal models of radiation injury. The only choices at present are NHPs and canines. The advanced evolutionary stage of NHPs, and the expense and danger of working with NHPs, are also disadvantages. The addition of miniature swine to available large animal radiation injury models would facilitate development of promising countermeasures.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

The purpose of this study is to determine the ability of levels of supportive care to extend the survival of minipigs irradiated at doses 2.0-3.0 Gy to 30 days. Primary data-points of the study will be mortality;

secondary data-points will be CBC counts, histology of the GI tract and bone marrow, tissues and blood microbiology at necropsy.
Male Gottingen minipigs will be obtained from Marshall BioResources, North Rose, NY, at age of 4 months, and at a weight appropriate for healthy animals of that age range. Minipigs will be single-housed but kept in visual contact with each other, and fed twice a day according to their weight, following recommendations from the vendor (Marshall Bioresources), and IAW VSD SOP 2019, Care and Maintenance of Minipigs. Minipigs may be group-housed during quarantine. Age and weight will be strictly controlled, which will avoid possible complications related to increasing weight and fatty mass.

Animal care and use procedures will only be pertormed by trained and certified personnel. Secondary data-points will be standard hematology measurements in peripheral blood (numbers of neutrophils, platelets, erythrocytes, lymphocytes, mean corpuscular hemoglobin, etc.), body temperature, body weight, and occurrence of bloody stools. On day 30-33, each surviving minipig will be euthanized, blood taken for CBC/differentials, and tissues collected for histopathology and tissue repository.

An implantable Micro Identification transponder with programmable Temperature Transponder (Bio Med Data System) will be implanted subcutaneously to monitor body temperature (see section **V.4.3.1. Pre-surgical Provisions).** A Vascular Access Port (VAP) PMID or MID model (Instech Solomon, PA), will be used to improve ease of blood draws and facilitate administration of supportive care (fluids and blood products) (see section **V.4.3.2. Procedure(s)**). In all experiments listed below, VAPs will be implanted 7-14 days after arrival of animals at AFRRI. In case of VAP malfunction, we will access alternative veins (see list of potential sites under section V.4.4.2.Biosamples "Collection of blood samples").

Health of animals will be monitored twice-daily, CBC/differential will be monitored on the days indicated below (except in emergency cases, where blood for CBC counts will be taken by VSD request). These experiments will utilize the husbandry, irradiation, and VAP procedures perfected in the studies performed under previously approved protocols. Animals will be euthanized on day 30-33. Tissues and blood samples may be taken at the time of euthanasia and shared with other investigators for biodosimetric and mechanistic studies.

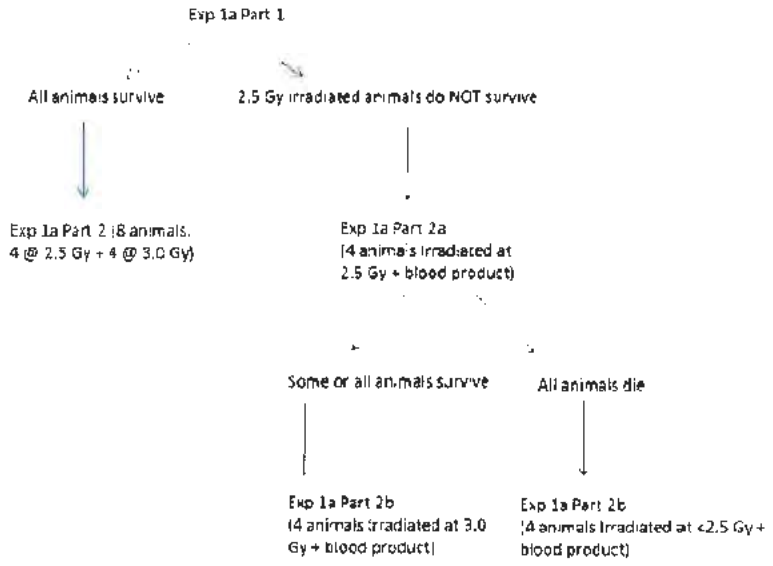
V.1.1. Experiment 1a - Pilot study: preliminary estimate of dose-survival relationship for whole-body gamma-irradiation at GI-light doses (2.0 – 3.0 Gy) in the presence of supportive care (n=16 animals).

Radiation doses of 2 Gy and higher are 100% lethal in the absence of supportive care. This experiment will allow us to obtain data on 30-d survival in the dose range 2.0 – 3.0 Gy in the presence of two levels of supportive care.

We will implant VAPs in male Gottingen minipigs to facilitate blood sampling and administration of supportive care. Three weeks after VAP implantation, we will expose minipigs to irradiation (Cobalt-60, total body, bilateral, 0.6 Gy/min). Two cohorts of 8 animals each will be used for Experiment 1; animals will be irradiated and administered supportive care, as described in Table 1. Survival will be followed for 30 days.

Table 1: study design	Supportive care	Radiation levels			Total no. animals
		2.0 Gy	2.5 Gy	3.0 Gy	
Exp 1a. Part 1	Per harmonized protocol, including VAPs with antibiotics, IV fluids.	4	4		8
Exp 1a. Part 2	As in Part 1, with blood products added		4	4	8
			Total no. of animals Exp 1a		16

Experiment 1a Part 2 is contingent upon results from Experiment 1a Part 1.
We expect that the use of supportive care will extend the survival of all 2.0 Gy irradiated animals to >14 days. If animals irradiated with 2.5 Gy (Experiment 1a Part 1) do not survive >14 days, we will break down to Exp 1a Part 2 into 2 sequential experiments (Exp 1a Part 2a and Exp 1a Part 2b). We will first irradiated 4 animals at 2.5 Gy and we will administered supportive care PLUS blood products added. If these animals survive >14 days, we will proceed to irradiating at 3.0 Gy (Exp 1a. Part 2); if the 2.5 Gy irradiated animals, receiving supportive care and blood, do not survive more than 14 days, the remaining 4 animals in the study will be irradiated at a dose lower than 2.5 Gy and will still be given supportive care WITH blood (see scheme below).



The regimen of supportive care is described in Table 2 (see section **V.4.4. Animal Manipulations**); it follows (b)(4) harmonized minipig protocol guidelines. Whole, anti-coagulated (ADC), blood will be purchased from commercial companies, shipped overnight on wet ice, irradiated, and filtered again prior to administration (see section **V.4.4.9. Animal By-Products**). ADC blood will be infused at a rate of 100 ml/hour, unless otherwise directed by VSD veterinarians.

TABLE 2: Regimen for supportive care

Treatment		Regimen	Admin route	Start/end
Antibiotics	Enrofloxacin (Baytril)	5-7 mg/kg *SID*	*PO* (or *IM* or *IV* if *PO* not possible)	3-30 d
	Amoxicillin	10 mg/kg *BID*	*PO* (or *IM* or *IV* if *PO* not possible)	3-30 d
Antipyretic/anti-inflammatory	Carprofen (Rimadyl)	2.2–4.4 mg/kg *BID*	*IV*, (or *PO* or *IM* if *PO* not possible	3-30 d
Dietary Supplementation	Moistened/liquid food, Metamucil, pumpkin mash			3-30 d
IV fluids	Lactate Ringers Solution	10-30 mL/kg	slow *IV* push (10-20 min)	Start day 3, every 1-3 days*

Blood product support	Whole blood	Up to 7-14 ml/kg, IV**		Once a week, starting on day 7-8.
* Depending on the hydration state of the animal, in consultation with VSD ** Total volume will depend upon HCT and platelets count, in consultation with VSD				

Multiple blood draws may be performed, with the following schedule (with day of irradiation or sham irradiation as "day 0"): blood will be collected -14, -7, -1 day (before irradiation) and on days 0, 1, 2, 3, 7, 10, 14, 17, 20, 23, and 30 (after irradiation). Shifts in blood draw days and reduction in the number of bleeds may be made as contingencies arise. Time points for single blood draws after day 17 after IR may be subject to changes of up to +/-48 hours. Volume of blood taken will not exceed 1% of total blood volume (65ml/kg in case of swine) or 6 ml, whichever is less.

ANIMALS FOR EXPERIMENT 1: 16 animals

V.1.2. Experiment 2 – Mortality probit curve study.

Based on results in Experiment 1, AFRRI will perform a 30-day survival probit study over several radiation levels in the presence of supportive care, as suggested by results from Experiment 1a and in consultation with (b)(4)

The probit curve will be constructed from 7 separate groups of 8 animals each (56 animals), at doses (TBI, 0.6 Gy/min) to be determined based on results of experiment 1a, in the AFRRI cobalt facility. The primary data-points of the study will be 30-day survival; secondary data-points will be CBC counts, histology of the GI tract (small bowel representative areas) and bone marrow (sites noted above), and tissue and blood microbiology at necropsy. The blood draw schedule and supportive care regimen will be those established in pilot study 1.

TOTAL ANIMALS FOR EXPERIMENT 2: 7 doses x 8 animals/dose = 56

TOTAL ANIMALS FOR EXPERIMENT 1+2: 56+16 = 72

V.2. Data Analysis

Survival in Exp 1a Part 1 with supportive care at 2.0 Gy will be compared against historical controls with no supportive care.
Survival in Part 1a with supportive care at 2.5 Gy will be compared against supportive care at 2.0 Gy.
Survival in Part 2 with supportive care and blood products at 2.5 Gy will be compared against supportive care only at 2.5 Gy.
Dose-survival relationship in Part 2 (supportive care and blood products) will be compared across a dose range to be determined based on results of Exp1a Part 1. We will compare groups using the ANOVA test and we will obtain preliminary information on the effect of supportive care and blood products on survival. This information will be used to establish the regimen for the generation of the survival curve.

For the generation of the survival curve, the LD30, 50 and 70 will be estimated using the probit method. This method allows for construction of confidence intervals to describe the precision of the estimated lethal doses. The width of the confidence interval depends on the number of doses, number of animals per dose, and the slope of the dose-response curve, and a narrow interval indicates a more reliable estimate. We propose to use seven doses that are equally spaced, and eight animals per dose. For a dose-response curve with mortality rates of 5, 20, 35, 50, 65, 80 and 95 percent for the seven doses, the expected width of the 95% confidence interval for the LD50 will be 0.57 Gy, and the expected width of the 95% confidence interval for the LD30 and LD70 will be .72 Gy. We anticipate that most dose-response curves will be steeper and therefore will yield even more precise estimates of the LD30, 50 and

70. Expected confidence interval widths are based on the average of 1,000 simulated data sets using the study design and mortality parameters indicated.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered.

The purpose of this project is to determine the effect of supportive care on survival. There is no computer model or tissue culture that can mimic the effect of radiation on the whole organism and predict lethality. Survival depends on an intricate network of organ cross-talks and cellular microenvironment that cannot be recreated *in vitro*. Consequently, administration of supportive care to cellular components grown *in vitro* will not allow any assessment on survival.

We do not know all of the cell types and tissues involved in radiation injury or how radiation injury causes lethality. The concept of multi-organ failure has been suggested, but how the injured organs affect each other's vital functions is unknown. The present state of the art of tissue culture is still incapable of reproducing the *in vivo* relationships. Computer modeling is similarly limited by lack of full characterization of the biological elements and interactions under study.

These considerations are consistent with the FDA requirement for preclinical research in both small and large mammals before granting approval for drug testing in humans.

V.3.2. Animal Model and Species Justification
We have been developing the Gottingen minipig as an additional large animal model for the study of the ARS and advanced development of radiation countermeasures. Large, long-lived, non-rodent animal models are required for drug studies submitted to the FDA for licensure applications. The current study will help characterize the model in terms of natural history of ARS. The close similarity to humans in anatomy and physiology of organs such as the liver, pancreas, kidney and heart has made the pig the primary species of interest as organ donors for xenographic procedures. Minipigs are routinely used for toxicity testing. As such, further and rapid characterization at physiological, molecular and genetic level of the minipig is likely to occur, thus increasing acceptance of this model and facilitating advanced drug development. Our previous studies have employed male Gottingen minipigs to develop a probit curve; female minipigs of a corresponding age (approximately 4 months old) are more sensitive to irradiation, based on data from other laboratories. Since we are testing the effect of supportive care on survival and we are using our own historical data as one of the terms of comparison, we will continue to use male minipigs for this study.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species *Sus scrofa domestica*

V.3.3.2. Strain / Stock Gottingen

V.3.3.3. Source / Vendor Marshall BioResources/Marshall Farms Group Ltd

V.3.3.4. Age 4-6 months

V.3.3.5. Weight 7-13 kg

V.3.3.6. Sex Male

V.3.3.7. Special Considerations NA

V.3.4. Number of Animals Required (by species)

Exp 1+2: 72 animals

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement
The study data-points currently mandated by the FDA for approval of radiation countermeasures under the Animal Efficacy Rule is mortality. Hence the primary study data-point in our development of Gottingen minipigs as a model for ARS is survival. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress, using an extensive set of criteria (see animal endpoints, paragraph V.4.5 – Lethargy, weight loss, temperature, respiratory distress, food intake, bleeding etc). Animal care and use procedures will only be performed by trained personnel. We have considerable experience in the care and handling of minipigs. Extensive human interaction every weekday will reduce stress in the animals. Use of slings, topical anesthetics, and the VAPs will minimize distress during blood collections and the animals will be gradually acclimated to the slings for these procedures. VAP design and the blood collection have been refined during multiple iterations of previous studies to minimize discomfort to the animals.

V.3.5.2. Reduction Blood samples obtained from minipigs before irradiation will serve as controls, thus reducing the number of animals required for this study. Use of historical controls will reduce the number of animals needed for this study.

V.3.5.3. Replacement NONE

V.4. Technical Methods
V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C ___ __ (# of animals)

V.4.1.1.1.2. Column D ___72__ (# of animals)

V.4.1.1.1.3. Column E ___0_ (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1	Pilot study: estimate of dose-survival relationship with supportive care			16	16
2	Mortality probit curve study with supportive care			56	56
Totals:				72	72

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

SURGERY
To prepare animals for intubation and VAP implantation, pigs will be sedated with intramuscular tiletamine/zolazepam (Telazol®, 6-8 mg/kg IM; 100 mg/ml, Fort Dodge Laboratories, IA) or ketamine (20 mg/kg *IM*)/xylazine (2 mg/kg *IM*) will be considered as an additional anesthetic option for sedation prior to intubation. Atropine (0.05 mg/kg *IM, SC, IV*; atropine sulfate, Butler, Columbus, OH) may be given to reduce mucosal secretions. Cetacain topical spray will be used to enhance tracheal intubation success

rate. Following tracheal intubation, pigs will be maintained under general anesthesia using isoflurane gas (Abbott Laboratories, North Chicago, *IL*) at a maintenance rate of 1% to 3% and oxygen flow rate of 1 to 2 l/min.

IRRADIATION
Prior to irradiation, animals will be sedated with Telazol® (Telazol, 6-8 mg/kg *IM*; 100 mg/ml, Fort Dodge Laboratories, IA). Alternatively, Telazol® (4.4 mg/kg *IM*), in combination with dexmedtomidine (0.05-0.1 mg/kg *IM*) or ketamine (20 mg/kg *IM*)/xylazine (2 mg/kg *IM*) can also be used for sedation. Atropine (0.05 mg/kg *SC*, *IM* or *IV*; atropine sulfate, Butler, Columbus, OH) or glycopyrrolate (0.01 mg.kg) may be given to reduce mucosal secretions. At the end of the irradiation procedure, once the animals are back in their quarters, dexmedetomidine can be reversed by atipamezole *IM* (same volume as dexmedotmidine) to facilitate recovery.

V.4.1.2.2. Pre- and Post-procedural Provisions

Body weights will be determined at least once prior to treatment, on the first day of treatment, and on days of blood collection. Body temperature will be determined daily between 8 am and 12 noon. The time of each temperature collection will be recorded for each minipig.

IRRADIATION

Pre-Irradiation: Animals will be fasted overnight (approximately 16 – 18 h) and sedated as described above. Once sedated in the VSD animal facility, minipigs will be transported to the AFRRI cobalt facility in an approved, covered transport cage. Heart rate and temperature will be measured prior to irradiation. At least one vet tech or one veterinarian will be present at the time of irradiation. Immediately before irradiation, the vet or vet tech will determine whether or not to administer additional sedative to keep the animal asleep during irradiation.

Irradiation procedure: animals will be strapped in place into slings in polyvinylchloride PVC frames for irradiation. Effect of anesthesia will be monitored in real time through video cameras positioned in the Cobalt waiting area. Minipigs will be removed from the slings once irradiation is completed. Temperature and heart rate will be taken before returning the minipigs to their home cages.

Post-Irradiation: After irradiation, animals will be transported back to their cages, where they will be allowed to recover from sedation under the careful surveillance of VSD and/or research staff who will monitor vital signs and provide thermal support (e.g. Bair Hugger blanket) if necessary. Atipamezole *IM* (same volume as dexmedotmidine) may be used to reverse the anesthetic effect of dexmedetomidine.

BLOOD SAMPLING

Preparation of the animals: for blood draws, lidocain (5%) will be applied topically to the VAP site to minimize discomfort to the animals about 15 minutes before starting the procedure. Animal may be sedated at the time of blood sampling and administration of supportive care with Midazolam (0.1-0.5 mg/kg s.c.or *IM*), or acepromazine (1.1 mg/kg *IM*),10 minutes prior to venous access

After blood draw, animals will be returned to their cages and checked to ensure that the needle puncture wound is closed and the blood has clotted.

V.4.1.2.3. Paralytics N/A

V.4.1.3. Lightrature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched Agricola, Pubmed

V.4.1.3.2. Date of Search 04/12/2013

V.4.1.3.3. Period of Search All available

V.4.1.3.4. Key Words of Search The aspects of the study relevant to alternatives to painful or distressful procedures are the sequelae to exposure to TBI at doses causing ARS. We used the following key words in the search for alternatives:
swine, radiation, ARS, sequelae, pain, distress, alternatives,

V.4.1.3.5. Results of Search

AGRICOLA
Swine AND radiation AND pain:.....0 results found

Swine AND radiation AND distress:..... 0 results found

Swine AND radiation AND alternatives.....5 results (not relevant)
1) Energy for swine facilities II Alternative sources of energy
Fehr, Rl NAL Catalog (AGRICOLA)
2) Evaluation of a porcine lens and fluorescence assay approach for in vitro ocular toxicological investigations
Oriowo, Om NAL Catalog (AGRICOLA)
3) Molecular characterization, chromosomal location, alternative splicing and polymorphism of porcine GFAT1 gene
Liu, K. NAL Catalog (AGRICOLA)
4) Evaluation of alternative techniques to determine pork carcass value
Akridge, Jt NAL Catalog (AGRICOLA)
5) Genomic structure, chromosomal localization and expression profile of a porcine long non-coding RNA isolated from long SAGE libraries
Ren, H. NAL Catalog (AGRICOLA)

Swine AND ARS AND pain:.....0 results found

Swine AND ARS AND distress:..... 0 results found

Swine AND ARS AND alternatives.....2 results (not relevant)

1) Environmental Prevalence and Persistence of Salmonella spp in Outdoor SwineWallows
Callaway, Tr NAL Catalog (AGRICOLA)
2) Factors affecting cellular outgrowth from porcine inner cell masses in vitro
Schilperoort-Haun, Kr NAL Catalog (AGRICOLA)

Swine AND radiation sequelae AND pain:.....0 results found

Swine AND radiation sequelae AND distress:.....0 results found

Swine AND radiation sequelae AND alternatives:.....0 results found

Pubmed
Swine AND radiation AND pain:.....264 results found

Swine AND radiation AND distress:..... 115 results found

Swine AND radiation AND alternatives.....0 results (not relevant)

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(b)(4),(b)(6)

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7) Therapeutic Effects of Xanthine Oxidase Inhibitors: Renaissance Half a Century after the Discovery of Allopurinol. PÁL PACHER, ALEX NIVOROZHKIN, CSABA SZABÓ. Pharmacol Rev. 2006 March; 58(1): 87–114. doi: 10.1124/pr.58.1.6
8) HUMAN HEALTH RISK ASSESSMENT FOR ALUMINIUM, ALUMINIUM OXIDE, AND ALUMINIUM HYDROXIDE Daniel Krewski, Robert A Yokel, Evert Nieboer, David Borchelt, Joshua Cohen, Jean Harry, Sam Kacew, Joan Lindsay, Amal M Mahfouz, Virginie Rondeau
J Toxicol Environ Health B Crit Rev. Author manuscript; available in PMC 2009 November 25.
Published in final edited form as: J Toxicol Environ Health B Crit Rev. 2007; 10(Suppl 1): 1–269.

Swine AND radiation sequelae AND alternatives:.....47 results found, not related to this work (mainly related to swine flu, medical imaging, mice models, ablation techniques, medical surgeries, bone marrow transplantation, cancer treatment

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification
Depending upon the irradiation dose irradiated animals die due to compromised immune responses and opportunistic infections. The percentage of surviving animals is the indicator of the efficacy of a countermeasure. We cannot give systemic anesthetic agents to animals after the irradiation procedures, since they are known to interact with the immune system (see references in Jacobsen, K. O., V. Villa, V. L. Miner, and M. H. Whitnall. 2004. Effects of anesthesia and vehicle injection on circulating blood elements in C3H/HeN male mice. *Contemp Top Lab Anim Sci* 43:8-12.), and would confound the correlation of radiation dose with incidence of moribundity, resulting in a waste of animals. However, we are providing supportive care in terms of antibiotics, analgesics, antipyretics, fluids/ nutritional support and blood products to increase survival. Use of supportive care is expected to mitigate the pain associated to the sequelae of irradiation.

V.4.2. Prolonged Restraint
Only short periods of restraint in the sling (<30 minutes) will be necessary for blood collections and the irradiation procedure. To minimize discomfort, we have designed a new type of sling that uses softer, elastic material to support the animal, and utilizes a more efficient restraint design that maintains the animal in place while reducing manipulation by technicians and consequently stress to the animals. After the quarantine period, animals are acclimated to the sling for 5-10 minutes, 2-3 times before initiating blood draws. Acclimation to the sling is very rapid and non-stressful; animals are petted and gently spoken to while restrained. It has been our experience that resting on the sling does not agitate the animal at all.
Animals are anesthetized and transported to the Cobalt Facility holding area before being put into slings for irradiation (<20 minutes). Animals will be kept under continuous observation while in the sling. Slings will be cleaned with a towel after each use, and washed weekly and sanitized.

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions
Animals will be cared for according to section V.5.1 and IAW VSD SOP 2019, Care and Maintenance of Minipigs. They will be fasted overnight (approximately 16 – 18 h) prior to VAP implantation surgery.
For antibiotic, Cefazolin sodium 20 mg/kg *IV* or ampicillin sodium 10-20 mg/kg *IV* will be given just prior to the surgery, or intra-operatively, for prophylaxis. At the time of surgery, a temperature microtransponder (Implantable Electronic ID Transponder IPPT-300, Bio Medic Data Systems, Seaford, DE) will be

implanted subcutaneously behind the neck, over the shoulder opposite to the site of VAP surgery, to facilitate body temperature measurements.

V.4.3.2. Procedure(s)

VAP SURGERY

VAP catheters (SoloPort subcutaneous access port, either PMID or MID model; Instech Solomon, PA) will be surgically implanted in the right external jugular veins under general anesthesia according to the principles of aseptic surgery. Animals will be lying on their back; vital signs will be taken at regular intervals by vet techs. The catheter tip will be introduced through an 2-3 mm incision in the right jugular vein and advanced to the junction of the external jugular vein and vena cava. Prior to insertion of the VAP catheter, it will be flushed with locking solution (heparin lock flush solution, 100 IU/ml). Prior to securing the VAP catheter within the vein, 1 preplaced 3-0 polydioxanone (PDS) suture (Ethicon, Somerville, NJ) will be used to ligate the vessel just cranial to the insertion point of the catheter tip. A second and third PDS ligature will be placed around the caudal portion of the vessel containing the VAP catheter and retention bead, with 1 ligature on either side of the bead. The port will be secured within the scapular incision site to the underlying musculature by use of 3-0 PDS (Ethicon) at a minimum of 2 of the 5 anchor holes on the port. Catheter function will be confirmed intraoperatively through aspiration of locking solution and withdrawal of a blood sample via the septum. The port reservoir and catheter will be flushed again with 6 ml saline and locked with 3 ml of a heparin lock flush solution (100 IU/ml) all subsequent lock solutions will be heparin (100 IU/ml).

During closure of the surgical sites, special attention will be given to eliminating dead space around the port and around the catheter loop on the neck. The muscle and subcutaneous layers will be closed with 3-0 PDS II (polydioxanone suture; Ethicon). Skin incisions will be closed with 2-0 PDS II (Ethicon) in a continuous intradermal pattern. Tissue glue (VetBond, 3M, St Paul, MN) may be used to help seal surgical sites as necessary to help prevent contamination. Cefazolin sodium 20 mg/kg *IV* or ampicillin sodium 10-20 mg/kg *IV* will be given just prior to the surgery, or intra-operatively, for prophylaxis.

V.4.3.3. Post-surgical Provisions

VAP SURGERY

Postoperative intramuscular buprenorphine (0.01 to 0.02 mg/kg; Buprenex, Reckitt Benckiser, Richmond, VA) or Carprofen (2.2 mg/kg) will be provided foranalgesia before full recovery from anesthesia (12). Additional post-operative analgesia will be given as Carprofen (2.2 mg/kg *PO* BID) for three days starting the day after surgery, or as recommended by an AFRRI veterinarian in consultation with the PI. Alternatively, fentanyl transdermal patch at 5mcg/kg/hr or buprenorphine sustained release 0.12 – 0.27 mg/kg SC for analgesia can be used as determined by the veterinarian. The fentanyl patch, if used, will be applied to a shaved portion of skin and secured. It takes approximately 12 hrs for the fentanyl patch to take effect. Animals will continue to be monitored until fully recovered from anesthesia; they will be returned to their home cage when ambulatory. Supplemental heat may be provided in the home cage post-operatively (e.g. Bair Hugger warm air blanket

After surgery, 25-50 mg/kg trimethoprim-sulfa *PO* (500 mg *PO* for 10 kg pig or 5mg/hg *IM*), *SID*, will be given for 5 days or more as determined by veterinarian to lessen the risk of post-op infection. Alternatively, enrofloxacin 5-10 mg/kg *IV/PO* can be given every 24 hrs for 3-5 days or more if needed as determined by the veterinarian. Convenia 5-10 mg/kg SC may be used as an antibiotic as determined by the veterinarian. The advantage of this antibiotic is that the single injection provides protection against the bacterial infection for several days.
Postsurgical recovery and pain will be monitored twice daily by visual examination of the incisions and overall conditions of the animals. Parameters monitored will be food and water intake, activity, alertness, vocalization, guarding, and response to human contact.

V.4.3.4. Location VSD surgical suite

V.4.3.5. Surgeon
Surgeons will be AFRRI veterinarians.

V.4.3.6. Multiple Major Survival Operative Procedures

V.4.3.6.1. Procedures NA

V.4.3.6.2 Scientific Justification NA

V.4.4. Animal Manipulations

V.4.4.1. Injections
All injections listed in section V.4.1.2.1 will be administered *IM*, sc, using sterile needles (23 – 21 gauge) and syringes, or IV through the VAP using Huber needles or *PO*.

V.4.4.1.1. Pharmaceutical Grade Drugs

Lidocain (5%), topical
Telazol®
Ketamine/xylazine
Atropine atropine sulfate
Cefazolin sodium
Ampicillin sodium
Heparin lock, IV
Saline
Ibuprenorphine
Carprofen
Fentanyl transdermal patch, topical
Metamucil
Enroflaxacin
Baytril
Amoxicillin
Carprofen
Rimadyl
Midazolam
Acepromazine
Cetacain topical spray
All the drugs listed above are ordered from veterinary product distributors by VSD and they all are pharmaceutical grade drugs.
Atipamezole (Antisedan, Pfizer)
Dexmedtomidine (Dexdomitor, Pfizer)
Convenia (Cefovecin sodium, Pfizer)

V.4.4.1.2. Non-Pharmaceutical Grade Drugs NA

V.4.4.2. Biosamples

Collection of blood samples. This has been done routinely in the previous minipig protocols. Each sample will be a maximum of 6 ml in volume or less than 1% total blood volume (whichever is less) and will be collected from the VAP if the device is available and functional. Mean blood volume for pigs is 65-67 ml/kg. At the time of blood draws, our animals are about 10-14 kg in weight. Up to 1% of total blood will be drawn per time point, depending upon the frequency of previous bleeds ((Walter Reed Army Institute

of Research. 1997. Investigators' Handbook. Section I: Handling Techniques and Noninvasive Procedures). We will obtain blood samples using Solomon Instech VAPs; sedation may be used. Collection of samples will be performed with the minipig restrained in a sling; at the same time, we will take vitals. The entire procedure takes less than 30 min. Two types of non-coring Huber needles will be used to access the port through the skin: straight or a right-angled (the needles will be of appropriate length to facilitate uncomplicated blood withdrawal from the VAP reservoir).

Before accessing the VAP site, 5% lidocaine ointment USP will be applied topically 15 min prior to sampling, to minimize animal discomfort Before placement of a non-coring needle, the port injection site will be scrubbed with povidone iodine and 70% isopropyl alcohol until all gross debris is removed (3 to 5 scrubs). To collect a sample, approximately 1 ml fluid will be withdrawn from the catheter and port until blood no longer appears to be mixed with saline. This fluid will be discarded. The blood sample will be collected into a new syringe and placed in appropriate vials for analysis. Next, 3 ml heparin lock flush solution (100 IU/ml) will be infused into the port and catheter as a locking solution. A standard protocol will be followed for obtaining a sample from a problematic catheter. First, external pressure will be applied to the non-coring needle where it entered the port to ensure the tip of the needle is all the way through the septum and into the reservoir. Second, high-pressure turbulent flushing will be attempted with a change in the animal's position. A second person will be needed to extend the neck of the animal by raising it or flexing it away from the side that contains the VAP. Third, the non-coring needle will be replaced; at times the needle may be clotted. Fourth, if the described series of interventions fails to return the VAP to functioning, the sample will be collected from accessible peripheral vessels (e.g., the jugular, saphenous, femoral or ear vein). If required during collection from alternate vessels (peripheral vessels, marginal ear veins, possibly femoral if animals have recovered from irradiation), the animals may be anesthetized for restraint following the recommendations of an AFRRI veterinarian. All procedures will be entered into the experimental record. The next team would repeat the procedures at the next time point in hopes of restoring the VAP to use. Three sequential attempts will be made to rescue the VAP before it is considered nonfunctional. In the previous pilot protocol, collections from the VAPs have been 99% successful.

Animal may be sedated at the time of blood sampling and administration of supportive care with Midazolam (0.1-0.5 mg/kg s.c.or *IM*), or acepromazine (1.1 mg/kg *IM*),10 minutes prior to venous access, or Telazol if necessary

Tissue and histological samples:
Tissue samples, to include organs, blood and hairs, may be taken at the time of euthanasia for slide preparation as well as for frozen tissue repository, and may be shared with other investigators. The cadaver of a few animals may be kept intact and used to establish dosimetry for LINAC for future experiments requiring the use of the LINAC to irradiate minipigs.

V.4.4.3. Adjuvants NA

V.4.4.4. Monoclonal Antibody (MAb) Production NA

V.4.4.5. Animal Identification
Tattoos or ear tags and cage cards/tags

V.4.4.6. Behavioral Studies NA

V.4.4.7. Other Procedures

V.4.4.8. Tissue Sharing Blood, tissues, plasma or urine samples may be provided to other investigators for assays, as experiments permit

V.4.4.9. Animal By-Products

Anticoagulated whole blood will be obtained from Gottingen Minipigs from Marshall Bioresources and shipped overnight to AFRRI on wet ice. When available, blood from siblings of animals that have been irradiated at AFRRI will be provided by Marshall Bioresources. Donors will be tested for the pathogens listed in the Report shown below. These are the same organisms for which the minipigs that enter AFRRI VSD vivarium are being tested. Blood will be irradiated and filtered before transfusion.

Health Monitoring Report According to FELASA Recommendations

Name and address of the breeder: *Marshall Farms USA, Galen Rd North Rose, NY*

Date of issue: September 2012	**Unit N°:** *Marshall Farms, Galen Rd – PI*	**Examination date :** August 2012
Species: Porcine	**Strain:** Gottingen Minipig	**Rederivation:**
		Populated: August 2003

	HISTORICAL RESULTS	CURRENT TEST RESULTS	LABORATORY	METHOD
VIRAL INFECTIONS				
Aujeszky's Disease (Pseudorabies)	0/250	0/10	PU	ELISA
Classical Swine Fever (hog cholera)	NA	NT	NA	NA
Porcine Epidemic Diarrhea	NA	NT	NA	NA
Encephalomyocarditis Virus	0/160	0/10	UM	SN
Haemagglutinating Encephalomyelitis	0/160	0/10	UM	HI
Porcine Circovirus II	0/160	0/10	PU	IFA
Porcine Influenza				
H1N1	0/160	0/10	PU	ELISA
H3N2	0/160	0/10	PU	ELISA
Porcine Parvovirus	0/160	0/10	PU	IFA
Porcine Reproduct. & Resp. Syndrome	0/160	0/10	PU	ELISA
Porcine Respiratory Coronavirus	0/160	0/10	PU	ELISA
Porcine Rotavirus	88/160	6/10	PU	IFA
Transmissible Gastroenteritis	0/160	0/10	PU	ELISA
BACTERIAL INFECTIONS				
Actinobacillus pleuropneumoniae				
Serotypes 1, 5, 7	0/160	0/10	UM	ELISA
Bordetella bronchiseptica	1/160	0/10	PU	Culture
Brachyspira (Serpulina) hyodysenteriae	0/160	0/10	PU	PCR
Brucella abortus	0/150	0/10	PU	Agglutination
Campylobacter spp.	2/160	0/10	PL	Culture
Clostridium perfringens Type C	0/160	0/10	PL	Culture
Erysipelothrix rhusiopathiae	0/160	0/10	PL	Culture
Fusobacterium spp.	0/160	0/10	PL	Culture
Haemophilus parasuis	0/160	0/10	PL	Culture
Lawsonia intracellularis	0/160	0/10	PU	PCR
Leptospira spp. (pomona, grippotyphosa, hardjo, canicola, icterohaemorrhagiae bratislava)	0/160	0/10	PL	MA
Mycoplasma hyopneumoniae	0/160	0/10	PU	ELISA
P. multocida (toxin producing)	0/160	0/10	PU	Culture
P. haemolytica	0/160	0/10	PL	Culture
P. pneumotropica	0/160	0/10	PU	Culture
other pasteurellae	0/160	0/10		
Salmonella spp.	0/160	0/10	PU	Culture
Staphylococcus hyicus	3/160	0/10	PU	Culture
β-haemolytic Streptococci	0/160	0/10	PU	Culture
Streptococcus suis type 2	0/160	0/10	PU	Culture
Streptococcus suis other	3/160	0/10	PU	Culture
Yersinia enterocolitica	0/160	0/10	PL	Culture
FUNGAL INFECTIONS				
Candida albicans	4/160	0/10	PU	Culture
Microsporum spp.	0/160	0/10	PU	Culture
Trichophyton spp.	0/160	0/10	PL	Culture
PARASITOLOGICAL INFECTIONS				
Arthropods	0/160	0/10	In-house	Micr. Insp.
Helminths	0/160	0/10	In-house	Flotation
Coccidia (Eimeria, Isospora)	0/160	0/10	In-house	Flotation
Toxoplasma gondii	0/160	0/10	PU	IFA

Sample Size: 10

NA: not applicable
NE=not examined

The VAP be used as the primary access for blood support. If the VAP fails, it will be to the discretion of the veterinarians how to best proceed, given the fact that the animals are thrombocytopenic and the superficial veins are not easily accessible. If blood products cannot be administered, animal will still be maintained in the study and will serve as control for survival in the absence of blood products.

V.4.5. Study Endpoint The data-point currently mandated by the FDA for approval of radiation countermeasures under the Animal Efficacy Rule is moribundity or mortality. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress. Euthanasia will be carried out when any one or combination of the following signs of moribundity are observed and in consultation with AFRRI veterinarians.

Absolute

1. Non responsive, assuming the animal has recovered from anesthesia.
2. Dyspnea
3. Loss of 20% of expected weight (based on Marshall Bio-resources growth chart –weight vs age- and day 0 baseline percentile).

Non-absolute

1. Hyper/hypothermia
2. Anorexia (skip 3 consecutive BID meals)
3. Anemia/pallor, CRT >2 seconds. CRT will be performed behind the ears.
4. Petechiae/ecchymosis
5. Vomiting/diarrhea
6. Lethargy
7. Seizures or vestibular signs (falling, circling or head tilt)
8. Uncontrollable hemorrhage

V.4.6. Euthanasia

Animals will be euthanized according to current American Veterinary Medical Association (AVMA) guidelines. Animals will be anesthetized with Telazol® (6-8 mg/kg *IM*) or ketamine (20 mg/kg *IM*)/xylazine (2 mg/kg *IM*). Animals will then be euthanized with and injected IV Euthasol® (sodium pentobarbital, (1 ml/4.5 kg IV or IC) with Euthasol® (sodium pentobarbital) or another commercial euthanasia solution. Death will be confirmed by VSD veterinarian or veterinary technician via auscultation of the heart, with cessation of sounds for at least 2 minutes or longer as per current SOP. See item V.4.5 for euthanasia criteria before the end of the study

V.5. Veterinary Care

V.5.1. Husbandry Considerations

Minipigs will be housed in stainless steel cages/runs, in tactile, visual and olfactory contact with adjacent animals. During quarantine/acclimation period, up to day of surgery, minipigs may be group-housed. After surgery, they will be singly housed to prevent damage to the VAPs and to allow individual assessment of feed consumption and fecal/urine/blood production. Rooms will be maintained on a 12:12 h light (0600 to 1800) dark cycle with the temperature set to 27 to 29°C and 30% to 70% relative humidity. Commercial diet (Mini-Swine Diet 8753, Harlan Teklad Diets, Madison, WI) will be provided. Water will be provided ad libitum.

V.5.1.1. Study Room

Minipigs will be located at AFRRI for the duration of the study. They will either be in a VSD surgical suite for VAP implantation, in transit to and from the cobalt facility in a covered transport cage, in slings for irradiation in the cobalt facility, or in their housing cage or a VSD prep room for blood collections or euthanasia. They will be housed in stainless-steel cages in an environmentally controlled and continuously monitored animal room as described in Section V.5.1

V.5.1.2. Special Husbandry Provisions
Tap water will be provided ad libitum. Diet will be controlled in accordance with vendor's recommendations so as to prevent excessive weight gain. Metamucil and pumpkin mash will be provided starting at day 7 after irradiation to all animals. Liquid food (i.e. ProMod Liquid Protein) and/or moistened pellet will be offered if observed that the sick animal prefers it to dry food. No treats in the form of marshmallow, peanut butter, crackers will be offered to this animal, since they are on a calorie-restricted diet. Instead, fresh fruits, vegetables, and/or yogurt will be offered daily.

V.5.1.3. Exceptions NA

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care
Animals will be acclimated for approximately 1-2 weeks prior to VAP implantation. Each animal will be observed at least twice daily for evidence of pain or distress. Body weights will be determined at least once prior to implantation, on the day of implantation, and on days of blood collection. The time of each daily temperature collection will be recorded for each animal. Animals will be qualitatively assessed at least twice daily (a.m. and p.m.) for general behavioral status and food consumption by VSD techs and/or research techs. The heart rate will be measured at the time of each blood collection. Stools consistency, left-over food, demeanor and overall animal appearance. If animals look dehydrated, supplemental oral fluids (Pedialyte, etc.) will be provided. Diarrhea has not been observed in minipigs irradiated at doses <8 Gy. Appropriate standard operating procedures will be followed by staff veterinarians in the event of pain or distress.

V.5.2.2. Emergency Veterinary Medical Care Emergency veterinary medical care is available 24/7 and will be provided based on the restrictions approved in earlier sections of this protocol

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy
Toys and balls will be provided to the minipigs and they will be housed in the same room as conspecifics as per VSD SOPs on enrichment of minipigs. Single housing justifications are filed under IACUC memo regarding animals undergoing surgery and irradiation.

V.5.3.2. Enrichment Restrictions
Minipigs will be single-housed after surgery and after irradiation until completion of the study, to avoid mechanical and infectious complications related to implanted VAPs, but they will be in visual, olfactory and auditory contact with other minipigs in the same room.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

Trained personnel are certified by VSD.

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3b, 4, 5, 7 (drug delivery)	(b)(6)
				1 through 5	
				1, 3b, 4, 5, 7 (drug	

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
		(b)(6)	2011	delivery)	(b)(6)

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY NA

VIII. ENCLOSURES

Bibliography

(b)(4),(b)(6)

4. MacVittie TJ, Farese AM, Jackson WI. Defining the full therapeutic potential of recombinant growth factors in the post radiation-accident environment: the effect of supportive care plus administration of G-CSF. ***Health Phys*** 89:546Y555; 2005.
5. Waselenko JK, MacVittie TJ, Blakely WF, Pesik N, Wiley AL, Dickerson WE, Tsu H, Confer DL, Coleman CN, Seed T, Lowry P, Armitage JO, Dainiak N. Medical management of the acute radiation syndrome: recommendations of the Strategic National Stockpile Radiation Working Group. ***Annals Internal Med*** 140:1037Y1051; 2004
6. Defense, D. o. 2009. Chemical and Biological Defense Program: Annual Report to Congress.

X. ASSURANCES

Protocol Title: Effect of supportive care on irradiated Gottingen minipigs (*Sus scrofa domestica*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (October 2012)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD Instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	**4/19/2013**	2nd Resubmission Date:	
Approved/Returned for Revision:	**5/6/2013**	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	**5/22/2013**	**EXPIRATION DATE:**	**6/24/2016**
Approved/Returned for Revision:	**6/25/2013**	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

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- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. **NAME OF FACILITY: Armed Forces Radiobiology Research Institute**

II. **PROTOCOL NUMBER:** (b)(6)

III. **PROTOCOL TITLE:** Effect of supportive care on irradiated Gottingen minipigs (*Sus scrofa domestica*)

IV. **PRINCIPAL INVESTIGATOR:** (b)(6)

(b)(6) PhD Date
Principal Investigator, (b)(6) Scientific Research Department
Tel (b)(6) Fax (b)(6) E-mail: (b)(6)

V. **DEPARTMENT HEAD:** This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date
Head, Scientific Research Department, AFRRI
Telephone (b)(6) Fax: (b)(6)

(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician

Telephone (b)(6) Fax: (b)(6)

(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department, USUHS

Telephone (b)(6) Fax: (b)(6)

(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**

IACUC Chair (b)(6) Scientific Research Department, AFRRI

Telephone: (b)(6) Fax (b)(6)

(b)(6)

PROTOCOL TITLE: Effect of supportive care on irradiated Gottingen minipigs (*Sus scrofa domestica*)

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6) PhD Date
Principal investigator (b)(6) Scientific Research Department
Tel: (b)(6) Fax: (b)(6) E-mail: (b)(6)

CO-INVESTIGATOR(S): Dr (b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS

The Acute Radiation Syndrome (ARS) develops over time, depending on the amount of radiation absorbed. The ARS is classically divided into 4 sub-syndromes, based on the organ with the most severe damage: hematopoietic (H), gastrointestinal (GI), cutaneous (C) and neurovascular (N). Evidence suggests that these syndromes have some overlap in time and dose range. Efficacy of clinical management should therefore be assessed on multiple organs involved in concurrent sub-sequelae.

The GI syndrome progresses at the same time as the hematopoietic syndrome starts to develop. The GI-ARS is expected to be affected by H-ARS bone marrow aplasia; similarly, changes in the intestinal tract may influence development of H-ARS. Lethality from the GI develops fast (days), and involves desquamation of epithelial mucosa in the intestine, leading to loss of fluids and electrolytes, decreased absorption of nutrients and bacterial translocation. Lethality from H-ARS happens within months, and it is speculated to be due mainly to bone marrow aplasia, susceptibility to sepsis and low platelets count. Some delayed GI damage may be expected. Because of the difference in the mechanism of lethality, recovery from GI-ARS does not guarantee survival from H-ARS. Development of animal models to study the natural history of ARS and test radiation countermeasures remains one of the main bottlenecks for drug development. So far, a very limited number of large animal models are available for countermeasures testing (NHP, dogs and swine); understanding of the natural history of radiation sickness and development of multi-organ failure is still largely incomplete.

We are developing a minipig model of ARS. So far we have optimized implantation and patency of a vascular access port for blood sampling, characterized the H-ARS, and tested the efficacy of G-CSF on survival and bone marrow recovery in the irradiated Gottingen minipig. Signs and symptoms, kinetics of blood cell depletion and recovery, multi-organ dysfunction and histological findings resembled closely what observed in human victims of radiation accidents and in other large animal models (b)(4)

In the G. minipig, the H-ARS occurs between 1.6 and 2.0 Gy (b)(4)
(b)(4)

Supportive care has been shown to profoundly alter and extend the survival period of human victims and animal models irradiated at hematopoietic doses (4). Standard of care consist of antibiotics, analgesics, antipyretics, fluids/ nutritional support and blood products (5). Because of the integrity of the GI tract at (b)(4) doses, we expect that administration of antibiotics, analgesics and fluids/ nutritional support will greatly improve survival at these doses, although blood products may still be necessary.

The purpose of the current study is to (i) use supportive care to extend the survival to a period of time sufficient to study the potential effects of countermeasures on recovery of bone marrow and the GI

system; (ii) characterize the natural history (NHx) of ARS at those doses; and (iii) to establish a probit curve to allow for future countermeasure drug testing.

The primary data points of the study will be mortality; secondary data points will be standard biomarkers of CBC counts, histology of the GI tract (primarily the small bowel) and bone marrow, and blood and tissue microbiology for broad cultures of suspected infections at necropsy.

II. BACKGROUND

II.1. Military personnel are potentially exposed to radiation during their duty (assembling, disassembling, stockpiling of nuclear weapons, cleanup of facilities, disposal of radioactive materials, civilian defense, etc). So far, only four drugs specifically designated for use in radiation emergency are present in the Strategic National Stockpile. Limited availability of a sufficient number of well-characterized animal models to test radiation countermeasures had been indicated as one of the main bottlenecks for advanced drug development. Developing large animal models for testing of countermeasures to radiation exposure and identification of the patho-physiological mechanisms underlying the development of the acute radiation syndrome (ARS) are fundamental to clinical management and one of the primary objectives of the Department of Defense.

Development of the acute radiation syndrome is dose and time dependent. A continuum in the development of symptoms and pathologies has been proposed over the years, and covers bone marrow (BM), gastrointestinal (GI), cutaneous and CNS syndrome. Although developing concomitantly, GI- and H-ARS have different mechanisms of action and time course. It is expected that mitigation of the GI syndrome will NOT waive the treatment for the hematopoietic syndrome if left untreated. Effects of standard drug treatment for the H-ARS must therefore be determined on the irradiated GI tract.

We recently developed a minipig model for the hematopoietic syndrome, characterized by signs and symptoms, bone marrow aplasia, kinetics of blood cell depletion and recovery similar to what observed in humans, canines and NHPs (b)(4) Sequence of signs and symptoms development, dynamics of hematological cell loss, susceptibility to infection and occurrence of multi-organ dysfunction leading to death, all confirm the validity of this model to study the ARS. Implantation of a subcutaneous vascular (b)(4) access port (VAP) allows for longitudinal sampling of large amounts of blood for CBC/diff counts, clinical chemistry parameters and in vitro assays that could be correlated to survival, gross and histo-pathology and vital signs.

(b)(4)

The primary data point of the study will be mortality; secondary data-points will be standard biomarkers of CBC counts, histology of the GI tract (primarily the small bowel) and bone marrow (sternum, L1 vertebrae and femur or humerus, and blood and tissue microbiology.

II.2. Literature Search for Duplication
II.2.1. Literature Sources Searched BRD, RePORTER, PubMed
II.2.2. Date of Search February 24, 2013
II.2.3. Period of Search
BRD: 1998 – 2009
RePORTER: 1998-2012
Pubmed: 1998-2012

II.2.4. Key Words of Search
Swine OR minipig
AND Radiation
AND supportive care
AND total body

II.2.5. Results of Search

BRD database:
Keyword Swine .....776 results
Keyword Swine+ radiation: ..... 4 results

1) Implantation Techniques and Specific Absorption Rates for Swine (Sus scrofa) in Directed Energy Health and Safety Studies
2) Pilot study: Development of Gottingen minipig (Sus scrofa domestica) as radiation injury model
3) NIRVANA: Non-Ionizing Radiation Vision for a New Army
4) Extended Electro Magnetic Medical Advisor: A Diagnostic Tool for Casualty Triage by Combat Medics

Keyword Swine + supportive care: 129 results (note relevant for this study)
For these projects, animals are used to train medical practitioners in skills (surgical, emergency care, catheter implantation, etc) vital for peacetime medicine, battlefield operations, combat casualties, trauma/trauma-hemorrhagic patients,. Alternatively, animals are used for training of new veterinary staff

Keyword Swine+ radiation+ supportive care: ..... 1 results (not relevant for this study)

1) Extended Electro Magnetic Medical Advisor: A Diagnostic Tool for Casualty Triage by Combat Medics

Keyword Minipig: .....25 results
Keyword Minipig + radiation: .....4 results

1) Pilot study: Development of Gottingen minipig (Sus scrofa domestica) as radiation injury model
2) Determination of Thermal Injury Thresholds in the Yucatan Minipig Using Radio Frequency Radiation
3) Determination of Thermal Injury Thresholds in the Yucatan Minipig Using Radio Frequency Radiation
4) Determination of Thermal Injury Thresholds in the Yucatan Minipig Using Radio Frequency Radiation

Keyword Minipig + supportive care 1 results (not relevant for this study)

1) Exploratory Assessment of Immediate Behavioral Effects Of TASER-Like Devices On Swine

Keyword Minipig + radiation + supportive care.....0 results

RePORTER database:
Keyword swine: .....838 Results
Keyword swine+radiation: .....108 Results
Keyword swine+ supportive care: 3 results (not relevant to this study)

1) Modulating secondary damage following traumatic brain injury in the child
2) See, reach, treat tumor-optimized transarterial chemoembolization drug delivery.

3) A multidisciplinary NMR center for biomedical research

Keyword swine+radiation+supportive care: .....0 Result

Keyword Minipig: .....10 result (6 not relevant to this project)

1. DEVELOP RADIATION INJURY MODEL: GOTTINGEN MINIPIG (SUS SCROFA DOMESTICA)
2. DEVELOP RADIATION INJURY MODEL USING THE GOTTINGEN MINIPIG
3. COUNTERMEASURES AGAINST RADIOLOGICAL AND NUCLEAR ATTACK - AFRRI/IAA
4. COUNTERMEASURES AGAINST RADIOLOGICAL AND NUCLEAR ATTACK

Keyword Minipig+radiation: .....4 results

1. DEVELOP RADIATION INJURY MODEL: GOTTINGEN MINIPIG (SUS SCROFA DOMESTICA)
2. DEVELOP RADIATION INJURY MODEL USING THE GOTTINGEN MINIPIG
3. COUNTERMEASURES AGAINST RADIOLOGICAL AND NUCLEAR ATTACK - AFRRI/IAA
4. COUNTERMEASURES AGAINST RADIOLOGICAL AND NUCLEAR ATTACK

Keyword Minipig+ supportive care 0 results

Keyword Minipig + radiation + supportive care.....0 results

PUBMED database:
Keyword Swine ..... 175608 results
Keyword Swine + supportive care.....30 results (not relevant to this study)

Publications focused on brain death, kidney/liver/lung injury (non radiation-induced), thoracic trauma, animal welfare, H1N1, sepsis/infections (non radiation-induced), influenza, poisoning from ingested agents, septic shock, respirators/surgical masks, ischemia/oxygenation, burn care, hypenatremia, fetal surgical protocols.

Keyword Swine + radiation: ..... 4257 results
Keyword swine + radiation + intestine + supportive care 0

Keyword Minipig:.....5952 results

Keyword minipig+ supportive care 2 results (not relevant)
1) High-sodium crystalloid solution for treatment of hypernatremia in a Vietnamese pot-bellied pig.
2) Fetal surgical protocols in Yucatan miniature swine.

Keyword Minipig+radiation.....229 results

Keyword minipig+radiation+ supportive care.....0

III. OBJECTIVE / HYPOTHESIS

Our preliminary data show that doses ≥ 2 Gy are 100% lethal and that morbidity was reached ≤14 days after exposure to radiation. In the GI tract, villar blunting and fusion are observed, but no loss of crypts or decline in plasma citrulline levels are observed at doses 2.0-3.0 Gy.

Our hypothesis is that use of supportive care at doses 2.0 – 3.0 Gy will increase the survival of irradiated minipigs and that a 30-day survival probit curve in the presence of supportive care can be generated for estimation of lethality and for medical countermeasure (MCM) testing.

We plan to investigate the effect of additive elements of supportive care on 30-day survival starting with doses in the 2.0 - 3.0 Gy range, and to build a probit curve in the presence of an optimized supportive care regimen that will encompass the LD0/30 to the LD100/30.

IV. MILITARY RELEVANCE

According to the DoD Chemical and Biological Defense Program 2009 Annual Report to Congress (6): "Although the overall number of nuclear weapons continues to decline because of Russian and U.S. treaty commitments, the United States anticipates an increase in weapon numbers in China, India, and Pakistan. Motivated by economic and strategic interests, Russia and China (or political entities in each) and North Korea continue to supply technologies and components that are dual use and could support weapons of mass destruction and missile programs, especially in the Middle East and South Asia. Iran continues to develop its enrichment program in defiance of United Nations (U.N.) Security Council resolutions and also continues to build a heavy water reactor in Arak, which will be capable of producing plutonium that could be weaponized. While North Korea has halted portions of its nuclear program, it is possible that it has stockpiled several nuclear weapons from plutonium produced at Yongbyon. Non-nuclear radiological dispersal devices (RDD) and radiological exposure devices (RED) pose a significant potential threat especially in the hands of non-state groups ... Contamination and recovery issues associated with operating in a radiological environment remain significant concerns for military operations and underscore the need for robust detection, protection, and decontamination systems."

Currently, there is no safe and non-toxic radiation countermeasure available. Military personnel are potentially exposed to radiation in various scenarios. Both early and late health effects of radiation exposure are major concerns for the military. Developing countermeasures to radiation exposure is a top priority for the US Department of Defense. Effective countermeasures would expand the options available to field commanders operating Ionizing Radiation (here defined as IR) threat environments, and improve the morale of personnel at risk of exposure to IR. DoD Defense Technology Objective MD.18 states: "Effective mitigation of health consequences [of IR] will (1) reduce casualty load at medical treatment facilities, (2) sustain a more effective operational force after a radiation exposure event, (3) allow commanders to conduct operations in radiation field environments without undue risk of decremented performance of personnel due to acute tissue injury, and (4) reduce the negative psychological impact on personnel tasked to operate in contaminated environments."

An important barrier to advanced development of radiation countermeasures is the paucity of large, long-lived, non-rodent animal models of radiation injury. The only choices at present are NHPs and canines. The advanced evolutionary stage of NHPs, and the expense and danger of working with NHPs, are also disadvantages. The addition of miniature swine to available large animal radiation injury models would facilitate development of promising countermeasures.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

The purpose of this study is to determine the ability of levels of supportive care to extend the survival of minipigs irradiated at doses 2.0-3.0 Gy to 30 days. Primary data-points of the study will be mortality;

secondary data-points will be CBC counts, histology of the GI tract and bone marrow, tissues and blood microbiology at necropsy.
Male Gottingen minipigs will be obtained from Marshall BioResources, North Rose, NY, at age of 4 months, and at a weight appropriate for healthy animals of that age range. Minipigs will be single-housed but kept in visual contact with each other, and fed twice a day according to their weight, following recommendations from the vendor (Marshall Bioresources), and IAW VSD SOP 2019, Care and Maintenance of Minipigs. Minipigs may be group-housed during quarantine. Age and weight will be strictly controlled, which will avoid possible complications related to increasing weight and fatty mass.

Animal care and use procedures will only be pertormed by trained and certified personnel. Secondary data-points will be standard hematology measurements in peripheral blood (numbers of neutrophils, platelets, erythrocytes, lymphocytes, mean corpuscular hemoglobin, etc.), body temperature, body weight, and occurrence of bloody stools. On day 30-33, each surviving minipig will be euthanized, blood taken for CBC/differentials, and tissues collected for histopathology and tissue repository.

An implantable Micro Identification transponder with programmable Temperature Transponder (Bio Med Data System) will be implanted subcutaneously to monitor body temperature (see section **V.4.3.1. Pre-surgical Provisions).** A Vascular Access Port (VAP) PMID or MID model (Instech Solomon, PA), will be used to improve ease of blood draws and facilitate administration of supportive care (fluids and blood products) (see section **V.4.3.2. Procedure(s)**). In all experiments listed below, VAPs will be implanted 7-14 days after arrival of animals at AFRRI. In case of VAP malfunction, we will access alternative veins (see list of potential sites under section V.4.4.2.Biosamples "Collection of blood samples").

Health of animals will be monitored twice-daily, CBC/differential will be monitored on the days indicated below (except in emergency cases, where blood for CBC counts will be taken by VSD request). These experiments will utilize the husbandry, irradiation, and VAP procedures perfected in the studies performed under previously approved protocols. Animals will be euthanized on day 30-33. Tissues and blood samples may be taken at the time of euthanasia and shared with other investigators for biodosimetric and mechanistic studies.

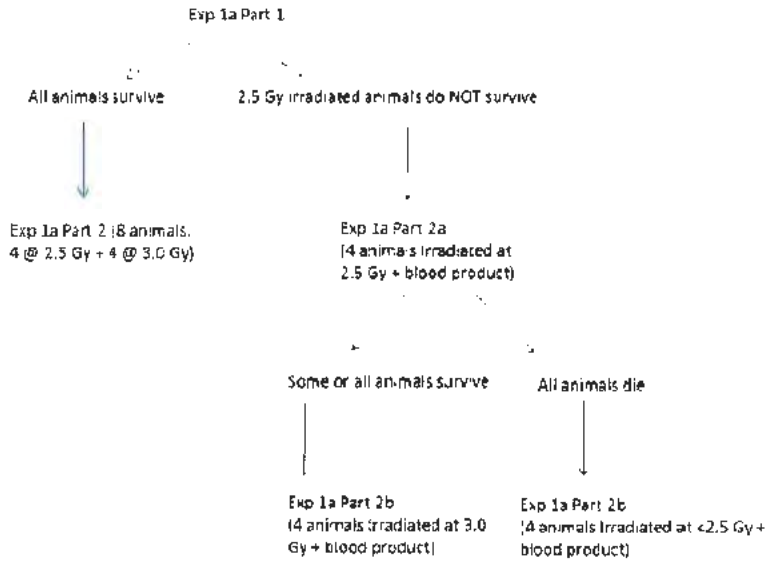
V.1.1. Experiment 1a - Pilot study: preliminary estimate of dose-survival relationship for whole-body gamma-irradiation at GI-light doses (2.0 – 3.0 Gy) in the presence of supportive care (n=16 animals).

Radiation doses of 2 Gy and higher are 100% lethal in the absence of supportive care. This experiment will allow us to obtain data on 30-d survival in the dose range 2.0 – 3.0 Gy in the presence of two levels of supportive care.

We will implant VAPs in male Gottingen minipigs to facilitate blood sampling and administration of supportive care. Three weeks after VAP implantation, we will expose minipigs to irradiation (Cobalt-60, total body, bilateral, 0.6 Gy/min). Two cohorts of 8 animals each will be used for Experiment 1; animals will be irradiated and administered supportive care, as described in Table 1. Survival will be followed for 30 days.

Table 1: study design	Supportive care	Radiation levels			Total no. animals
		2.0 Gy	2.5 Gy	3.0 Gy	
Exp 1a. Part 1	Per harmonized protocol, including VAPs with antibiotics, IV fluids.	4	4		8
Exp 1a. Part 2	As in Part 1, with blood products added		4	4	8
			Total no. of animals Exp 1a		16

Experiment 1a Part 2 is contingent upon results from Experiment 1a Part 1.
We expect that the use of supportive care will extend the survival of all 2.0 Gy irradiated animals to >14 days. If animals irradiated with 2.5 Gy (Experiment 1a Part 1) do not survive >14 days, we will break down to Exp 1a Part 2 into 2 sequential experiments (Exp 1a Part 2a and Exp 1a Part 2b). We will first irradiated 4 animals at 2.5 Gy and we will administered supportive care PLUS blood products added. If these animals survive >14 days, we will proceed to irradiating at 3.0 Gy (Exp 1a. Part 2); if the 2.5 Gy irradiated animals, receiving supportive care and blood, do not survive more than 14 days, the remaining 4 animals in the study will be irradiated at a dose lower than 2.5 Gy and will still be given supportive care WITH blood (see scheme below).



The regimen of supportive care is described in Table 2 (see section **V.4.4. Animal Manipulations**); it follows (b)(4) harmonized minipig protocol guidelines. Whole, anti-coagulated (ADC), blood will be purchased from commercial companies, shipped overnight on wet ice, irradiated, and filtered again prior to administration (see section **V.4.4.9. Animal By-Products**). ADC blood will be infused at a rate of 100 ml/hour, unless otherwise directed by VSD veterinarians.

TABLE 2: Regimen for supportive care

Treatment		Regimen	Admin route	Start/end
Antibiotics	Enrofloxacin (Baytril)	5-7 mg/kg *SID*	*PO* (or *IM* or *IV* if *PO* not possible)	3-30 d
	Amoxicillin	10 mg/kg *BID*	*PO* (or *IM* or *IV* if *PO* not possible)	3-30 d
Antipyretic/anti-inflammatory	Carprofen (Rimadyl)	2.2–4.4 mg/kg *BID*	*IV*, (or *PO* or *IM* if *PO* not possible	3-30 d
Dietary Supplementation	Moistened/liquid food, Metamucil, pumpkin mash			3-30 d
IV fluids	Lactate Ringers Solution	10-30 mL/kg	slow *IV* push (10-20 min)	Start day 3, every 1-3 days*

Blood product support	Whole blood	Up to 7-14 ml/kg, IV**		Once a week, starting on day 7-8.
* Depending on the hydration state of the animal, in consultation with VSD ** Total volume will depend upon HCT and platelets count, in consultation with VSD				

Multiple blood draws may be performed, with the following schedule (with day of irradiation or sham irradiation as "day 0"): blood will be collected -14, -7, -1 day (before irradiation) and on days 0, 1, 2, 3, 7, 10, 14, 17, 20, 23, and 30 (after irradiation). Shifts in blood draw days and reduction in the number of bleeds may be made as contingencies arise. Time points for single blood draws after day 17 after IR may be subject to changes of up to +/-48 hours. Volume of blood taken will not exceed 1% of total blood volume (65ml/kg in case of swine) or 6 ml, whichever is less.

ANIMALS FOR EXPERIMENT 1: 16 animals

V.1.2. Experiment 2 – Mortality probit curve study.

Based on results in Experiment 1, AFRRI will perform a 30-day survival probit study over several radiation levels in the presence of supportive care, as suggested by results from Experiment 1a and in consultation with (b)(4)

The probit curve will be constructed from 7 separate groups of 8 animals each (56 animals), at doses (TBI, 0.6 Gy/min) to be determined based on results of experiment 1a, in the AFRRI cobalt facility. The primary data-points of the study will be 30-day survival; secondary data-points will be CBC counts, histology of the GI tract (small bowel representative areas) and bone marrow (sites noted above), and tissue and blood microbiology at necropsy. The blood draw schedule and supportive care regimen will be those established in pilot study 1.

TOTAL ANIMALS FOR EXPERIMENT 2: 7 doses x 8 animals/dose = 56

TOTAL ANIMALS FOR EXPERIMENT 1+2: 56+16 = 72

V.2. Data Analysis

Survival in Exp 1a Part 1 with supportive care at 2.0 Gy will be compared against historical controls with no supportive care.
Survival in Part 1a with supportive care at 2.5 Gy will be compared against supportive care at 2.0 Gy.
Survival in Part 2 with supportive care and blood products at 2.5 Gy will be compared against supportive care only at 2.5 Gy.
Dose-survival relationship in Part 2 (supportive care and blood products) will be compared across a dose range to be determined based on results of Exp1a Part 1. We will compare groups using the ANOVA test and we will obtain preliminary information on the effect of supportive care and blood products on survival. This information will be used to establish the regimen for the generation of the survival curve.

For the generation of the survival curve, the LD30, 50 and 70 will be estimated using the probit method. This method allows for construction of confidence intervals to describe the precision of the estimated lethal doses. The width of the confidence interval depends on the number of doses, number of animals per dose, and the slope of the dose-response curve, and a narrow interval indicates a more reliable estimate. We propose to use seven doses that are equally spaced, and eight animals per dose. For a dose-response curve with mortality rates of 5, 20, 35, 50, 65, 80 and 95 percent for the seven doses, the expected width of the 95% confidence interval for the LD50 will be 0.57 Gy, and the expected width of the 95% confidence interval for the LD30 and LD70 will be .72 Gy. We anticipate that most dose-response curves will be steeper and therefore will yield even more precise estimates of the LD30, 50 and

70. Expected confidence interval widths are based on the average of 1,000 simulated data sets using the study design and mortality parameters indicated.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered.

The purpose of this project is to determine the effect of supportive care on survival. There is no computer model or tissue culture that can mimic the effect of radiation on the whole organism and predict lethality. Survival depends on an intricate network of organ cross-talks and cellular microenvironment that cannot be recreated *in vitro*. Consequently, administration of supportive care to cellular components grown *in vitro* will not allow any assessment on survival.

We do not know all of the cell types and tissues involved in radiation injury or how radiation injury causes lethality. The concept of multi-organ failure has been suggested, but how the injured organs affect each other's vital functions is unknown. The present state of the art of tissue culture is still incapable of reproducing the *in vivo* relationships. Computer modeling is similarly limited by lack of full characterization of the biological elements and interactions under study.

These considerations are consistent with the FDA requirement for preclinical research in both small and large mammals before granting approval for drug testing in humans.

V.3.2. Animal Model and Species Justification
We have been developing the Gottingen minipig as an additional large animal model for the study of the ARS and advanced development of radiation countermeasures. Large, long-lived, non-rodent animal models are required for drug studies submitted to the FDA for licensure applications. The current study will help characterize the model in terms of natural history of ARS. The close similarity to humans in anatomy and physiology of organs such as the liver, pancreas, kidney and heart has made the pig the primary species of interest as organ donors for xenographic procedures. Minipigs are routinely used for toxicity testing. As such, further and rapid characterization at physiological, molecular and genetic level of the minipig is likely to occur, thus increasing acceptance of this model and facilitating advanced drug development. Our previous studies have employed male Gottingen minipigs to develop a probit curve; female minipigs of a corresponding age (approximately 4 months old) are more sensitive to irradiation, based on data from other laboratories. Since we are testing the effect of supportive care on survival and we are using our own historical data as one of the terms of comparison, we will continue to use male minipigs for this study.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species *Sus scrofa domestica*

V.3.3.2. Strain / Stock Gottingen

V.3.3.3. Source / Vendor Marshall BioResources/Marshall Farms Group Ltd

V.3.3.4. Age 4-6 months

V.3.3.5. Weight 7-13 kg

V.3.3.6. Sex Male

V.3.3.7. Special Considerations NA

V.3.4. Number of Animals Required (by species)

Exp 1+2: 72 animals

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement
The study data-points currently mandated by the FDA for approval of radiation countermeasures under the Animal Efficacy Rule is mortality. Hence the primary study data-point in our development of Gottingen minipigs as a model for ARS is survival. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress, using an extensive set of criteria (see animal endpoints, paragraph V.4.5 – Lethargy, weight loss, temperature, respiratory distress, food intake, bleeding etc). Animal care and use procedures will only be performed by trained personnel. We have considerable experience in the care and handling of minipigs. Extensive human interaction every weekday will reduce stress in the animals. Use of slings, topical anesthetics, and the VAPs will minimize distress during blood collections and the animals will be gradually acclimated to the slings for these procedures. VAP design and the blood collection have been refined during multiple iterations of previous studies to minimize discomfort to the animals.

V.3.5.2. Reduction Blood samples obtained from minipigs before irradiation will serve as controls, thus reducing the number of animals required for this study. Use of historical controls will reduce the number of animals needed for this study.

V.3.5.3. Replacement NONE

V.4. Technical Methods
V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C ___ __ (# of animals)

V.4.1.1.1.2. Column D ___72__ (# of animals)

V.4.1.1.1.3. Column E ___0_ (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1	Pilot study: estimate of dose-survival relationship with supportive care			16	16
2	Mortality probit curve study with supportive care			56	56
Totals:				72	72

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

SURGERY
To prepare animals for intubation and VAP implantation, pigs will be sedated with intramuscular tiletamine/zolazepam (Telazol®, 6-8 mg/kg IM; 100 mg/ml, Fort Dodge Laboratories, IA) or ketamine (20 mg/kg *IM*)/xylazine (2 mg/kg *IM*) will be considered as an additional anesthetic option for sedation prior to intubation. Atropine (0.05 mg/kg *IM, SC, IV*; atropine sulfate, Butler, Columbus, OH) may be given to reduce mucosal secretions. Cetacain topical spray will be used to enhance tracheal intubation success

rate. Following tracheal intubation, pigs will be maintained under general anesthesia using isoflurane gas (Abbott Laboratories, North Chicago, *IL*) at a maintenance rate of 1% to 3% and oxygen flow rate of 1 to 2 l/min.

IRRADIATION
Prior to irradiation, animals will be sedated with Telazol® (Telazol, 6-8 mg/kg *IM*; 100 mg/ml, Fort Dodge Laboratories, IA). Alternatively, Telazol® (4.4 mg/kg *IM*), in combination with dexmedtomidine (0.05-0.1 mg/kg *IM*) or ketamine (20 mg/kg *IM*)/xylazine (2 mg/kg *IM*) can also be used for sedation. Atropine (0.05 mg/kg *SC*, *IM* or *IV*; atropine sulfate, Butler, Columbus, OH) or glycopyrrolate (0.01 mg.kg) may be given to reduce mucosal secretions. At the end of the irradiation procedure, once the animals are back in their quarters, dexmedetomidine can be reversed by atipamezole *IM* (same volume as dexmedotmidine) to facilitate recovery.

V.4.1.2.2. Pre- and Post-procedural Provisions

Body weights will be determined at least once prior to treatment, on the first day of treatment, and on days of blood collection. Body temperature will be determined daily between 8 am and 12 noon. The time of each temperature collection will be recorded for each minipig.

IRRADIATION

Pre-Irradiation: Animals will be fasted overnight (approximately 16 – 18 h) and sedated as described above. Once sedated in the VSD animal facility, minipigs will be transported to the AFRRI cobalt facility in an approved, covered transport cage. Heart rate and temperature will be measured prior to irradiation. At least one vet tech or one veterinarian will be present at the time of irradiation. Immediately before irradiation, the vet or vet tech will determine whether or not to administer additional sedative to keep the animal asleep during irradiation.

Irradiation procedure: animals will be strapped in place into slings in polyvinylchloride PVC frames for irradiation. Effect of anesthesia will be monitored in real time through video cameras positioned in the Cobalt waiting area. Minipigs will be removed from the slings once irradiation is completed. Temperature and heart rate will be taken before returning the minipigs to their home cages.

Post-Irradiation: After irradiation, animals will be transported back to their cages, where they will be allowed to recover from sedation under the careful surveillance of VSD and/or research staff who will monitor vital signs and provide thermal support (e.g. Bair Hugger blanket) if necessary. Atipamezole *IM* (same volume as dexmedotmidine) may be used to reverse the anesthetic effect of dexmedetomidine.

BLOOD SAMPLING

Preparation of the animals: for blood draws, lidocain (5%) will be applied topically to the VAP site to minimize discomfort to the animals about 15 minutes before starting the procedure. Animal may be sedated at the time of blood sampling and administration of supportive care with Midazolam (0.1-0.5 mg/kg s.c.or *IM*), or acepromazine (1.1 mg/kg *IM*),10 minutes prior to venous access

After blood draw, animals will be returned to their cages and checked to ensure that the needle puncture wound is closed and the blood has clotted.

V.4.1.2.3. Paralytics N/A

V.4.1.3. Lightrature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched Agricola, Pubmed

V.4.1.3.2. Date of Search 04/12/2013

V.4.1.3.3. Period of Search All available

V.4.1.3.4. Key Words of Search The aspects of the study relevant to alternatives to painful or distressful procedures are the sequelae to exposure to TBI at doses causing ARS. We used the following key words in the search for alternatives:
swine, radiation, ARS, sequelae, pain, distress, alternatives,

V.4.1.3.5. Results of Search

AGRICOLA
Swine AND radiation AND pain:.....0 results found

Swine AND radiation AND distress:..... 0 results found

Swine AND radiation AND alternatives.....5 results (not relevant)
1) Energy for swine facilities II Alternative sources of energy
Fehr, Rl NAL Catalog (AGRICOLA)
2) Evaluation of a porcine lens and fluorescence assay approach for in vitro ocular toxicological investigations
Oriowo, Om NAL Catalog (AGRICOLA)
3) Molecular characterization, chromosomal location, alternative splicing and polymorphism of porcine GFAT1 gene
Liu, K. NAL Catalog (AGRICOLA)
4) Evaluation of alternative techniques to determine pork carcass value
Akridge, Jt NAL Catalog (AGRICOLA)
5) Genomic structure, chromosomal localization and expression profile of a porcine long non-coding RNA isolated from long SAGE libraries
Ren, H. NAL Catalog (AGRICOLA)

Swine AND ARS AND pain:.....0 results found

Swine AND ARS AND distress:..... 0 results found

Swine AND ARS AND alternatives.....2 results (not relevant)

1) Environmental Prevalence and Persistence of Salmonella spp in Outdoor SwineWallows
Callaway, Tr NAL Catalog (AGRICOLA)
2) Factors affecting cellular outgrowth from porcine inner cell masses in vitro
Schilperoort-Haun, Kr NAL Catalog (AGRICOLA)

Swine AND radiation sequelae AND pain:.....0 results found

Swine AND radiation sequelae AND distress:.....0 results found

Swine AND radiation sequelae AND alternatives:.....0 results found

Pubmed
Swine AND radiation AND pain:.....264 results found

Swine AND radiation AND distress:..... 115 results found

Swine AND radiation AND alternatives.....0 results (not relevant)

Swine AND ARS AND pain:.....16 results found (same as those found for

Swine AND acute radiation syndrome AND pain.....16 results (b)(4)

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Swine AND radiation sequelae AND alternatives:.....47 results found, not related to this work (mainly related to swine flu, medical imaging, mice models, ablation techniques, medical surgeries, bone marrow transplantation, cancer treatment

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification
Depending upon the irradiation dose irradiated animals die due to compromised immune responses and opportunistic infections. The percentage of surviving animals is the indicator of the efficacy of a countermeasure. We cannot give systemic anesthetic agents to animals after the irradiation procedures, since they are known to interact with the immune system (see references in Jacobsen, K. O., V. Villa, V. L. Miner, and M. H. Whitnall. 2004. Effects of anesthesia and vehicle injection on circulating blood elements in C3H/HeN male mice. *Contemp Top Lab Anim Sci* 43:8-12.), and would confound the correlation of radiation dose with incidence of moribundity, resulting in a waste of animals. However, we are providing supportive care in terms of antibiotics, analgesics, antipyretics, fluids/ nutritional support and blood products to increase survival. Use of supportive care is expected to mitigate the pain associated to the sequelae of irradiation.

V.4.2. Prolonged Restraint
Only short periods of restraint in the sling (<30 minutes) will be necessary for blood collections and the irradiation procedure. To minimize discomfort, we have designed a new type of sling that uses softer, elastic material to support the animal, and utilizes a more efficient restraint design that maintains the animal in place while reducing manipulation by technicians and consequently stress to the animals. After the quarantine period, animals are acclimated to the sling for 5-10 minutes, 2-3 times before initiating blood draws. Acclimation to the sling is very rapid and non-stressful; animals are petted and gently spoken to while restrained. It has been our experience that resting on the sling does not agitate the animal at all.
Animals are anesthetized and transported to the Cobalt Facility holding area before being put into slings for irradiation (<20 minutes). Animals will be kept under continuous observation while in the sling. Slings will be cleaned with a towel after each use, and washed weekly and sanitized.

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions
Animals will be cared for according to section V.5.1 and IAW VSD SOP 2019, Care and Maintenance of Minipigs. They will be fasted overnight (approximately 16 – 18 h) prior to VAP implantation surgery.
For antibiotic, Cefazolin sodium 20 mg/kg *IV* or ampicillin sodium 10-20 mg/kg *IV* will be given just prior to the surgery, or intra-operatively, for prophylaxis. At the time of surgery, a temperature microtransponder (Implantable Electronic ID Transponder IPPT-300, Bio Medic Data Systems, Seaford, DE) will be

implanted subcutaneously behind the neck, over the shoulder opposite to the site of VAP surgery, to facilitate body temperature measurements.

V.4.3.2. Procedure(s)

VAP SURGERY

VAP catheters (SoloPort subcutaneous access port, either PMID or MID model; Instech Solomon, PA) will be surgically implanted in the right external jugular veins under general anesthesia according to the principles of aseptic surgery. Animals will be lying on their back; vital signs will be taken at regular intervals by vet techs. The catheter tip will be introduced through an 2-3 mm incision in the right jugular vein and advanced to the junction of the external jugular vein and vena cava. Prior to insertion of the VAP catheter, it will be flushed with locking solution (heparin lock flush solution, 100 IU/ml). Prior to securing the VAP catheter within the vein, 1 preplaced 3-0 polydioxanone (PDS) suture (Ethicon, Somerville, NJ) will be used to ligate the vessel just cranial to the insertion point of the catheter tip. A second and third PDS ligature will be placed around the caudal portion of the vessel containing the VAP catheter and retention bead, with 1 ligature on either side of the bead. The port will be secured within the scapular incision site to the underlying musculature by use of 3-0 PDS (Ethicon) at a minimum of 2 of the 5 anchor holes on the port. Catheter function will be confirmed intraoperatively through aspiration of locking solution and withdrawal of a blood sample via the septum. The port reservoir and catheter will be flushed again with 6 ml saline and locked with 3 ml of a heparin lock flush solution (100 IU/ml) all subsequent lock solutions will be heparin (100 IU/ml).

During closure of the surgical sites, special attention will be given to eliminating dead space around the port and around the catheter loop on the neck. The muscle and subcutaneous layers will be closed with 3-0 PDS II (polydioxanone suture; Ethicon). Skin incisions will be closed with 2-0 PDS II (Ethicon) in a continuous intradermal pattern. Tissue glue (VetBond, 3M, St Paul, MN) may be used to help seal surgical sites as necessary to help prevent contamination. Cefazolin sodium 20 mg/kg *IV* or ampicillin sodium 10-20 mg/kg *IV* will be given just prior to the surgery, or intra-operatively, for prophylaxis.

V.4.3.3. Post-surgical Provisions

VAP SURGERY

Postoperative intramuscular buprenorphine (0.01 to 0.02 mg/kg; Buprenex, Reckitt Benckiser, Richmond, VA) or Carprofen (2.2 mg/kg) will be provided foranalgesia before full recovery from anesthesia (12). Additional post-operative analgesia will be given as Carprofen (2.2 mg/kg *PO* BID) for three days starting the day after surgery, or as recommended by an AFRRI veterinarian in consultation with the PI. Alternatively, fentanyl transdermal patch at 5mcg/kg/hr or buprenorphine sustained release 0.12 – 0.27 mg/kg SC for analgesia can be used as determined by the veterinarian. The fentanyl patch, if used, will be applied to a shaved portion of skin and secured. It takes approximately 12 hrs for the fentanyl patch to take effect. Animals will continue to be monitored until fully recovered from anesthesia; they will be returned to their home cage when ambulatory. Supplemental heat may be provided in the home cage post-operatively (e.g. Bair Hugger warm air blanket

After surgery, 25-50 mg/kg trimethoprim-sulfa *PO* (500 mg *PO* for 10 kg pig or 5mg/hg *IM*), *SID*, will be given for 5 days or more as determined by veterinarian to lessen the risk of post-op infection. Alternatively, enrofloxacin 5-10 mg/kg *IV/PO* can be given every 24 hrs for 3-5 days or more if needed as determined by the veterinarian. Convenia 5-10 mg/kg SC may be used as an antibiotic as determined by the veterinarian. The advantage of this antibiotic is that the single injection provides protection against the bacterial infection for several days.
Postsurgical recovery and pain will be monitored twice daily by visual examination of the incisions and overall conditions of the animals. Parameters monitored will be food and water intake, activity, alertness, vocalization, guarding, and response to human contact.

V.4.3.4. Location VSD surgical suite

V.4.3.5. Surgeon
Surgeons will be AFRRI veterinarians.

V.4.3.6. Multiple Major Survival Operative Procedures

V.4.3.6.1. Procedures NA

V.4.3.6.2 Scientific Justification NA

V.4.4. Animal Manipulations

V.4.4.1. Injections
All injections listed in section V.4.1.2.1 will be administered *IM*, sc, using sterile needles (23 – 21 gauge) and syringes, or IV through the VAP using Huber needles or *PO*.

V.4.4.1.1. Pharmaceutical Grade Drugs

Lidocain (5%), topical
Telazol®
Ketamine/xylazine
Atropine atropine sulfate
Cefazolin sodium
Ampicillin sodium
Heparin lock, IV
Saline
Ibuprenorphine
Carprofen
Fentanyl transdermal patch, topical
Metamucil
Enroflaxacin
Baytril
Amoxicillin
Carprofen
Rimadyl
Midazolam
Acepromazine
Cetacain topical spray
All the drugs listed above are ordered from veterinary product distributors by VSD and they all are pharmaceutical grade drugs.
Atipamezole (Antisedan, Pfizer)
Dexmedtomidine (Dexdomitor, Pfizer)
Convenia (Cefovecin sodium, Pfizer)

V.4.4.1.2. Non-Pharmaceutical Grade Drugs NA

V.4.4.2. Biosamples

Collection of blood samples. This has been done routinely in the previous minipig protocols. Each sample will be a maximum of 6 ml in volume or less than 1% total blood volume (whichever is less) and will be collected from the VAP if the device is available and functional. Mean blood volume for pigs is 65-67 ml/kg. At the time of blood draws, our animals are about 10-14 kg in weight. Up to 1% of total blood will be drawn per time point, depending upon the frequency of previous bleeds ((Walter Reed Army Institute

of Research. 1997. Investigators' Handbook. Section I: Handling Techniques and Noninvasive Procedures). We will obtain blood samples using Solomon Instech VAPs; sedation may be used. Collection of samples will be performed with the minipig restrained in a sling; at the same time, we will take vitals. The entire procedure takes less than 30 min. Two types of non-coring Huber needles will be used to access the port through the skin: straight or a right-angled (the needles will be of appropriate length to facilitate uncomplicated blood withdrawal from the VAP reservoir).

Before accessing the VAP site, 5% lidocaine ointment USP will be applied topically 15 min prior to sampling, to minimize animal discomfort Before placement of a non-coring needle, the port injection site will be scrubbed with povidone iodine and 70% isopropyl alcohol until all gross debris is removed (3 to 5 scrubs). To collect a sample, approximately 1 ml fluid will be withdrawn from the catheter and port until blood no longer appears to be mixed with saline. This fluid will be discarded. The blood sample will be collected into a new syringe and placed in appropriate vials for analysis. Next, 3 ml heparin lock flush solution (100 IU/ml) will be infused into the port and catheter as a locking solution. A standard protocol will be followed for obtaining a sample from a problematic catheter. First, external pressure will be applied to the non-coring needle where it entered the port to ensure the tip of the needle is all the way through the septum and into the reservoir. Second, high-pressure turbulent flushing will be attempted with a change in the animal's position. A second person will be needed to extend the neck of the animal by raising it or flexing it away from the side that contains the VAP. Third, the non-coring needle will be replaced; at times the needle may be clotted. Fourth, if the described series of interventions fails to return the VAP to functioning, the sample will be collected from accessible peripheral vessels (e.g., the jugular, saphenous, femoral or ear vein). If required during collection from alternate vessels (peripheral vessels, marginal ear veins, possibly femoral if animals have recovered from irradiation), the animals may be anesthetized for restraint following the recommendations of an AFRRI veterinarian. All procedures will be entered into the experimental record. The next team would repeat the procedures at the next time point in hopes of restoring the VAP to use. Three sequential attempts will be made to rescue the VAP before it is considered nonfunctional. In the previous pilot protocol, collections from the VAPs have been 99% successful.

Animal may be sedated at the time of blood sampling and administration of supportive care with Midazolam (0.1-0.5 mg/kg s.c.or *IM*), or acepromazine (1.1 mg/kg *IM*),10 minutes prior to venous access, or Telazol if necessary

Tissue and histological samples:
Tissue samples, to include organs, blood and hairs, may be taken at the time of euthanasia for slide preparation as well as for frozen tissue repository, and may be shared with other investigators. The cadaver of a few animals may be kept intact and used to establish dosimetry for LINAC for future experiments requiring the use of the LINAC to irradiate minipigs.

V.4.4.3. Adjuvants NA

V.4.4.4. Monoclonal Antibody (MAb) Production NA

V.4.4.5. Animal Identification
Tattoos or ear tags and cage cards/tags

V.4.4.6. Behavioral Studies NA

V.4.4.7. Other Procedures

V.4.4.8. Tissue Sharing Blood, tissues, plasma or urine samples may be provided to other investigators for assays, as experiments permit

V.4.4.9. Animal By-Products

Anticoagulated whole blood will be obtained from Gottingen Minipigs from Marshall Bioresources and shipped overnight to AFRRI on wet ice. When available, blood from siblings of animals that have been irradiated at AFRRI will be provided by Marshall Bioresources. Donors will be tested for the pathogens listed in the Report shown below. These are the same organisms for which the minipigs that enter AFRRI VSD vivarium are being tested. Blood will be irradiated and filtered before transfusion.

Health Monitoring Report According to FELASA Recommendations

Name and address of the breeder: *Marshall Farms USA, Galen Rd North Rose, NY*

Date of issue: September 2012	**Unit N°:** *Marshall Farms, Galen Rd - PI*	**Examination date :** August 2012
Species: Porcine	**Strain:** Gottingen Minipig	**Rederivation:**
		Populated: August 2003

	HISTORICAL RESULTS	CURRENT TEST RESULTS	LABORATORY	METHOD
VIRAL INFECTIONS				
Aujeszky's Disease (Pseudorabies)	0/250	0/10	PU	ELISA
Classical Swine Fever (hog cholera)	NA	NT	NA	NA
Porcine Epidemic Diarrhea	NA	NT	NA	NA
Encephalomyocarditis Virus	0/160	0/10	UM	SN
Haemagglutinating Encephalomyelitis	0/160	0/10	UM	HI
Porcine Circovirus II	0/160	0/10	PU	IFA
Porcine Influenza				
H1N1	0/160	0/10	PU	ELISA
H3N2	0/160	0/10	PU	ELISA
Porcine Parvovirus	0/160	0/10	PU	IFA
Porcine Reproduct. & Resp. Syndrome	0/160	0/10	PU	ELISA
Porcine Respiratory Coronavirus	0/160	0/10	PU	ELISA
Porcine Rotavirus	88/160	6/10	PU	IFA
Transmissible Gastroenteritis	0/160	0/10	PU	ELISA
BACTERIAL INFECTIONS				
Actinobacillus pleuropneumoniae				
Serotypes 1, 5, 7	0/160	0/10	UM	ELISA
Bordetella bronchiseptica	1/160	0/10	PU	Culture
Brachyspira (Serpulina) hyodysenteriae	0/160	0/10	PU	PCR
Brucella abortus	0/150	0/10	PU	Agglutination
Campylobacter spp.	2/160	0/10	PL	Culture
Clostridium perfringens Type C	0/160	0/10	PL	Culture
Erysipelothrix rhusiopathiae	0/160	0/10	PL	Culture
Fusobacterium nuis	0/160	0/10	PL	Culture
Haemophilus parasuis	0/160	0/10	PL	Culture
Lawsonia intracellularis	0/160	0/10	PU	PCR
Leptospira spp. (pomona, grippotyphosa, hardjo, canicola, icterohemorrhagiae bratislava)	0/160	0/10	PL	MA
Mycoplasma hyopneumoniae	0/160	0/10	PU	ELISA
P. multocida (toxin producing)	0/160	0/10	PU	Culture
P. haemolytica	0/160	0/10	PL	Culture
P. pneumotropica	0/160	0/10	PU	Culture
other pasteurellae	0/160	0/10		
Salmonella spp.	0/160	0/10	PU	Culture
Staphylococcus hyicus	0/160	0/10	PU	Culture
β-haemolytic Streptococci	0/160	0/10	PU	Culture
Streptococcus suis type 2	0/160	0/10	PU	Culture
Streptococcus suis other	3/160	0/10	PU	Culture
Yersinia enterocolitica	0/160	0/10	PL	Culture
FUNGAL INFECTIONS				
Candida albicans	4/160	0/10	PU	Culture
Microsporum spp.	0/160	0/10	PU	Culture
Trichophyton spp.	0/160	0/10	PL	Culture
PARASITOLOGICAL INFECTIONS				
Arthropods	0/160	0/10	In-house	Micr. Insp.
Helminths	0/160	0/10	In-house	Flotation
Coccidia (Eimeria, Isospora)	0/160	0/10	In-house	Flotation
Toxoplasma gondii	0/160	0/10	PU	IFA

Sample Size: 10

NA: not applicable
NE=not examined

The VAP be used as the primary access for blood support. If the VAP fails, it will be to the discretion of the veterinarians how to best proceed, given the fact that the animals are thrombocytopenic and the superficial veins are not easily accessible. If blood products cannot be administered, animal will still be maintained in the study and will serve as control for survival in the absence of blood products.

V.4.5. Study Endpoint The data-point currently mandated by the FDA for approval of radiation countermeasures under the Animal Efficacy Rule is moribundity or mortality. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress. Euthanasia will be carried out when any one or combination of the following signs of moribundity are observed and in consultation with AFRRI veterinarians.

Absolute

1. Non responsive, assuming the animal has recovered from anesthesia.
2. Dyspnea
3. Loss of 20% of expected weight (based on Marshall Bio-resources growth chart –weight vs age- and day 0 baseline percentile).

Non-absolute

1. Hyper/hypothermia
2. Anorexia (skip 3 consecutive BID meals)
3. Anemia/pallor, CRT >2 seconds. CRT will be performed behind the ears.
4. Petechiae/ecchymosis
5. Vomiting/diarrhea
6. Lethargy
7. Seizures or vestibular signs (falling, circling or head tilt)
8. Uncontrollable hemorrhage

V.4.6. Euthanasia

Animals will be euthanized according to current American Veterinary Medical Association (AVMA) guidelines. Animals will be anesthetized with Telazol® (6-8 mg/kg *IM*) or ketamine (20 mg/kg *IM*)/xylazine (2 mg/kg *IM*). Animals will then be euthanized with and injected IV Euthasol® (sodium pentobarbital, (1 ml/4.5 kg IV or IC) with Euthasol® (sodium pentobarbital) or another commercial euthanasia solution. Death will be confirmed by VSD veterinarian or veterinary technician via auscultation of the heart, with cessation of sounds for at least 2 minutes or longer as per current SOP. See item V.4.5 for euthanasia criteria before the end of the study

V.5. Veterinary Care

V.5.1. Husbandry Considerations

Minipigs will be housed in stainless steel cages/runs, in tactile, visual and olfactory contact with adjacent animals. During quarantine/acclimation period, up to day of surgery, minipigs may be group-housed. After surgery, they will be singly housed to prevent damage to the VAPs and to allow individual assessment of feed consumption and fecal/urine/blood production. Rooms will be maintained on a 12:12 h light (0600 to 1800) dark cycle with the temperature set to 27 to 29°C and 30% to 70% relative humidity. Commercial diet (Mini-Swine Diet 8753, Harlan Teklad Diets, Madison, WI) will be provided. Water will be provided ad libitum.

V.5.1.1. Study Room

Minipigs will be located at AFRRI for the duration of the study. They will either be in a VSD surgical suite for VAP implantation, in transit to and from the cobalt facility in a covered transport cage, in slings for irradiation in the cobalt facility, or in their housing cage or a VSD prep room for blood collections or euthanasia. They will be housed in stainless-steel cages in an environmentally controlled and continuously monitored animal room as described in Section V.5.1

V.5.1.2. Special Husbandry Provisions
Tap water will be provided ad libitum. Diet will be controlled in accordance with vendor's recommendations so as to prevent excessive weight gain. Metamucil and pumpkin mash will be provided starting at day 7 after irradiation to all animals. Liquid food (i.e. ProMod Liquid Protein) and/or moistened pellet will be offered if observed that the sick animal prefers it to dry food. No treats in the form of marshmallow, peanut butter, crackers will be offered to this animal, since they are on a calorie-restricted diet. Instead, fresh fruits, vegetables, and/or yogurt will be offered daily.

V.5.1.3. Exceptions NA

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care
Animals will be acclimated for approximately 1-2 weeks prior to VAP implantation. Each animal will be observed at least twice daily for evidence of pain or distress. Body weights will be determined at least once prior to implantation, on the day of implantation, and on days of blood collection. The time of each daily temperature collection will be recorded for each animal. Animals will be qualitatively assessed at least twice daily (a.m. and p.m.) for general behavioral status and food consumption by VSD techs and/or research techs. The heart rate will be measured at the time of each blood collection. Stools consistency, left-over food, demeanor and overall animal appearance. If animals look dehydrated, supplemental oral fluids (Pedialyte, etc.) will be provided. Diarrhea has not been observed in minipigs irradiated at doses <8 Gy. Appropriate standard operating procedures will be followed by staff veterinarians in the event of pain or distress.

V.5.2.2. Emergency Veterinary Medical Care Emergency veterinary medical care is available 24/7 and will be provided based on the restrictions approved in earlier sections of this protocol

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy
Toys and balls will be provided to the minipigs and they will be housed in the same room as conspecifics as per VSD SOPs on enrichment of minipigs. Single housing justifications are filed under IACUC memo regarding animals undergoing surgery and irradiation.

V.5.3.2. Enrichment Restrictions
Minipigs will be single-housed after surgery and after irradiation until completion of the study, to avoid mechanical and infectious complications related to implanted VAPs, but they will be in visual, olfactory and auditory contact with other minipigs in the same room.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

Trained personnel are certified by VSD.

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3b, 4, 5, 7 (drug delivery)	(b)(6)
				1 through 5	
				1, 3b, 4, 5, 7 (drug	

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
		(b)(6)	2011	delivery)	21 mos

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY NA

VIII. ENCLOSURES

Bibliography

(b)(4),(b)(6)

4. MacVittie TJ, Farese AM, Jackson WI. Defining the full therapeutic potential of recombinant growth factors in the post radiation-accident environment: the effect of supportive care plus administration of G-CSF. ***Health Phys*** 89:546Y555; 2005.
5. Waselenko JK, MacVittie TJ, Blakely WF, Pesik N, Wiley AL, Dickerson WE, Tsu H, Confer DL, Coleman CN, Seed T, Lowry P, Armitage JO, Dainiak N. Medical management of the acute radiation syndrome: recommendations of the Strategic National Stockpile Radiation Working Group. ***Annals Internal Med*** 140:1037Y1051; 2004
6. Defense, D. o. 2009. Chemical and Biological Defense Program: Annual Report to Congress.

X. ASSURANCES

Protocol Title: Effect of supportive care on irradiated Gottingen minipigs (*Sus scrofa domestica*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)		
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (October 2012)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD Instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

Department of Defense Sponsored Animal Research Proposal Signature Coordination Sheet

Submitted: 6-13-2013
Revised: 7-30-2013

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	6/13/2013	2nd Resubmission Date:	
Approved/**Returned for Revision**:	7/18/2013	**Approved**/Approval Withheld by IACUC:	Final – 8/30/13
1st Resubmission Date:	7/30/2013	**EXPIRATION DATE:**	8/29/2016
Approved/Returned for Revision:	8/21/2013	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:	8/21/2013	Approved/**Returned for Revision**:	8/27/2013
Approved/**Returned for Revision**:	8/23/2013	2nd Resubmission Date:	8/28/2013
1st Resubmission Date:	8/27/2013	**Approved**/Approval Withheld:	8/29/2013

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Evaluation of (b)(4) as a radiation mitigator in nonhuman primates, rhesus macaque

IV. PRINCIPAL INVESTIGATOR:

(b)(6) **PhD** Date

Scientific Research Department
AFRRI (b)(6) Fax: (b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date

Head, Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) **PhD** Date

Statistician
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. (Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)

(b)(6) Date

Environmental Health and Safety Department, USUHS
Telephone (b)(6) Fax (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **PhD**

IACUC Chair, (b)(6) Scientific Research Department, AFRRI
Telephone (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: Evaluation of (b)(4) as a radiation mitigator in nonhuman primates, rhesus macaque

PRINCIPAL INVESTIGATOR:
(b)(6) **PhD**
Scientific Research Department
AFRRI (b)(6) Fax: (b)(6)

CO-INVESTIGATOR(S):
(b)(6) **PhD**
Scientific Research Department
Staff Scientist
(b)(6)

(b)(6) **PhD**
Scientific Research Department
Research Associate
(b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS: Hematopoietic syndrome resulting from exposure to ionizing radiation is characterized by loss of neutrophils, an important white blood cell component, as well as significant suppression to overall immunity and increased susceptibility to opportunistic infection with high potential for mortality. Failure to recover from infection can be attributed to a lack of circulating white blood cells and reduced proliferative capacity of the bone marrow to compensate this loss by increasing progenitors. In addition, thrombocytopenia (decreased platelet counts) resulting from radiation exposure may lead to uncontrolled internal bleeding. Recently, (b)(4) (b)(4) (b)(4) that specifically increased platelets five-fold compared to normal untreated mice. Further, the drug significantly increased 30-day survival in mice exposed to a lethal dose of ionizing radiation. We have an ongoing IACUC protocol (b)(6) that evaluates the drug (b)(4) as a radiation countermeasure in a large mammal model, specifically, nonhuman primates (NHP). We have shown that various drug doses (b)(4) injected subcutaneously increased platelet levels in NHPs and caused no drug-related deleterious effects. Finally, in a recent ongoing experiment a dose of (b)(4) of the drug injected as a single subcutaneous administration significantly increased platelets by two-fold in unirradiated animals without causing any obvious toxicity. Therefore, survival studies at this highest dose are in progress.

The present protocol involves evaluating this drug (b)(4) in lethally irradiated NHPs that are provided supportive care such as antibiotics and blood transfusions in order to increase post-irradiation survival. We hypothesize that administration of the drug (b)(4) being specific to control of thrombocytopenia will enhance survival significantly. For example, if without supportive care animals survive 7 Gy, then with support the survivable dose may increase to 8 Gy and with support care plus (b)(4) it may increase to 9 Gy.

II. BACKGROUND

II.1. Background

Increased mortality due to bone marrow failure: Because of increasing terrorist activity and dissemination of nuclear materials, the chances of military or civilian personnel being exposed to dirty bombs or improvised nuclear devices have risen dramatically in recent years. The prevailing sentiment of U.S. government officials is that it is a matter of when, not if, an attack with radiological or nuclear weapons will take place. The nation needs to be prepared for this eventuality. Acute radiation syndrome results when whole-body ionizing radiation causes defective hematopoiesis as a function of the radiation dose, dose-rate, and radiation quality. Defective hematopoiesis due to ionizing radiation exposure results in a decline in all blood cell counts for several weeks postirradiation. The primary cause of death during the early phase of the radiation-induced hematopoietic syndrome is multi-organ failure (MoF) resulting from opportunistic infection due to a decline in the numbers of neutrophils (neutropenia) and increased translocation of bacteria across the damaged gastrointestinal mucosa. Sepsis and shock result in eventual multiple organ failure and death. Acute radiation syndrome is often complicated by hemorrhage as a consequence of thrombocytopenia resulting in reduced circulating platelets (Stickney et al., 2007).

Current treatment strategies: The current treatment practice for radiation victims is the off-label administration of marrow growth factors such as G-CSF, which are not yet approved for this purpose by the Food and Drug Administration (FDA), and must be used under the constant supervision of physicians because of possible adverse effects. Other agents that may be used for radiation victims, like potassium iodide (KI), Prussian Blue, Calcium DTPA, and Zinc DTPA, address the internalization of specific radioactive isotopes, not the general injury caused by external exposure to ionizing radiation. For example, KI is used to prevent thyroid cancers by competing with internalized radioactive iodine for binding sites. Other potential radiation countermeasures still are in the pre-clinical research stage. There is a pressing need to develop countermeasures to ionizing radiation that will have a general beneficial effect on blood-forming tissues and immune competence. In order to expedite such studies that cannot be undertaken in humans due to ethical reasons, the FDA has established "the animal efficacy rule" (FDA 2002). Under this rule, pivotal efficacy studies in large-animal models, such as use of rhesus macaques exposed to lethal doses of ionizing radiation, are appropriate to supportive approval of novel radiation countermeasure agents for human use.

Role of Thrombocytopenia: The effectiveness of drugs to reduce or eliminate deleterious biological effects of ionizing radiation depends on their effectiveness in controlling neutropenia, thrombocytopenia, and possibly anemia. Off-label drugs such as growth factors (GFs), G-CSF, and GM-CSF are available to treat neutropenia but no such treatment exists for thrombocytopenia (although thrombopoietic GFs interleukin 11 (IL-11) and thrombopoietin (TPO) have been studied). However, administration of (b)(4) (b)(4) formulations resulted in adverse effects in clinical trials (Kuter 2002, 2007, 2008) although they were effective in increasing circulating platelets. (b)(4) (b)(4) have been successful in clinics for treating idiopathic thrombocytopenia purpura (ITP).

Brief background information on (b)(4) as a radiation countermeasure agent:

The drug for the present study will be synthesized by incorporation of a 14-amino-acid peptide into a fully human antibody (PA83) scaffold at both the end region of the light chains and the hinge region of the heavy chain. Therefore, control antibody without the 14-amino-acid ligand will be used as vehicle or PBS vehicle used for suspending (b)(4) in the present experiment.

We evaluated (b)(4) (b)(4) as a radiation countermeasure in mice. (b)(4) (b)(4) (b)(4),(b)(6) Briefly, thrombopoietin (TPO) receptor agonists lacking sequence homology to TPO were designed by grafting a known peptide sequence into the hinge and/or kappa constant regions of a human anti-anthrax antibody. Some of these proteins were equipotent to TPO in stimulating cMpl-r activity *in vitro*, and in increasing platelet levels *in vivo*. (b)(4) (b)(4) (b)(4) (2 mg/kg, SC) administered once either 24 h pre- or 6 h post-TBI showed superior protection to five once daily doses given pre- or post-TBI. Prophylactic administration (69 to 94% 30-day survival) was superior to therapeutic schedules (60% 30-day survival). (b)(4) conferred significant survival benefit ($p<0.01$) when administered 4 days before or even 12 h after exposure, and across a dose range of 0.1 to 8 mg/kg. The dose reduction factors (DRFs) with a single dose of 1 mg/kg (b)(4) 24 h pre- or 12 h post-TBI were 1.32 and 1.11, respectively ($p<0.0001$). Furthermore, (b)(4) increased bone marrow cellularity, megakaryocytic development, and accelerated multi-lineage hematopoietic recovery in irradiated mice demonstrating the potential of (b)(4) as both protectant and mitigator in the event of a radiological incident.

Ongoing nonhuman primate studies using (b)(4) Currently, we are evaluating the (b)(4) as a radiation countermeasure in the rhesus macaque. This project is different from the current protocol because approved protocol animals are provided minimal supportive care. They are not provided antibiotics or blood products for enhancing their postirradiation survival; instead, they will be humanely euthanized on meeting appropriate observational criteria after irradiation. Preliminary results show that the drug (b)(4) is effective in enhancing platelets although to a lesser degree than seen in mice (see figures in preliminary section).

Impact of the present protocol: The use of antibiotics, fluids, blood products, analgesics, and nutrition is the "standard of care" for patients exposed to cytotoxic chemotherapy and cytotoxic "conditioning" for stem cell transplant (Smith, et al. 2006, Timmer-Bonte et al. 2005). These measures will be similar for personnel exposed to myelosuppressive and potentially lethal doses of radiation (as reviewed in Farese 2013). It is known that medical management alone can significantly enhance survival of animals exposed to lethal doses of uniform total-body irradiation (TBI) (Jackson et al. 1959; MacVittie et al. 2005).

(b)(4) proposed a pilot 60-day efficacy study of subcutaneous administration of a radiation countermeasure agent (b)(4) to treat hematopoietic syndrome of the Acute Radiation Syndrome (H-ARS) following a lethal dose (70/60) of ionizing radiation in rhesus macaques. The relationship between

supportive care and lethality under H-ARS has been shown previously in canines (MacVittie et al. 1991; MacVittie et al. 2005). Recently, Farese et al. (2012) have established a NHP model with supportive care. The model defines radiation dose- and time-dependent relationships for mortality and major signs of morbidity to include other organ damage that may contribute to morbidity and mortality in nonhuman primate exposed to total-body radiation and with supportive care, which has also been defined as medical management.

Drug to be tested in the current protocol: (b)(4) (b)(4),(b)(6)

Broad scope of the current protocol: The current protocol will use both males and females and the proportion will be dependent on availability. The design will consist of exposure of both control- (vehicle-PBS) and (b)(4) PBS) treated groups to a LD70/60 dose of cobalt-60 gamma radiation with supportive care, including blood transfusion and standard care for neutropenic and thrombocytopenic events in irradiated NHPs. We will be using PBS vehicle instead of the control antibody because in our ongoing NHP project we have shown that control antibody has no effect on circulating platelets and the fact that the (b)(4) formulation is in PBS.

Sixty (60) day post-irradiation survival will be the primary endpoint, with several secondary end points: Neutrophil-related parameters: absolute neutrophil counts (ANC) nadir, duration of neutropenia (days), ANC <500/μL and <100/μL, and time to recovery to ANC ≥500/μL and ≥1,000/μL, for three consecutive days. It also will include platelet (PLT)-related parameters such as PLT nadir, duration of thrombocytopenia (days), PLT <20,000/μL, time to recovery to PLT count ≥20,000/μL, for three consecutive days. Additional parameters that will be evaluated but not restricted to are: a. Number of whole-blood transfusions; b. Number of days with fever (temp ≥103 °F); c. Incidence of documented infection (confirmed by aerobic culture of blood or tissues or histopathology); and d. Incidence of febrile neutropenia (FN).

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched: PubMed, NIH Reporter, BRD and Embase

II.2.2. Date of Search: 3/3/2013

II.2.3. Period of Search: 1946 to current for PubMed, 1988 to current for NIH Reporter, 1987-current for BRD, and 2006-current for Embase.

II.2.4. Key Words of Search: See table for single and multiple keyword combinations.

II.2.5. Results of Search

Keywords	PubMed 1946-present	NIH Reporter FY 1988-present	BRD 1987-active	Embase 2006-present
Nonhuman primate + rhesus macaque	1658	792	13	3044
Thromobopoietin + (b)(4) (b)(4) + platelet + megakaryocyte	9	3	0	14
Ionizing radiation + hematopoietic + hematopoiesis + neutropenia +	0	0	0	0

thrombocytopenia				
Radiation protection + mitigation + therapy + (b)(4)	0	0	0	0
Support care + antibiotic + blood transfusion + blood products + cytokine	1	0	0	279
Nonhuman primate + rhesus macaque + thromobopoietin + (b)(4) (b)(4) + platelet + megakaryocyte	0	0	0	0
Nonhuman primate + rhesus macaque + ionizing radiation + hematopoietic + hematopoiesis + neutropenia + thrombocytopenia	0	0	0	0
Nonhuman primate + rhesus macaque + radiation protection + mitigation + therapy + (b)(4)	0	0	0	0
Nonhuman primate + rhesus macaque + support care + antibiotic + blood transfusion + blood products + cytokine	0	0	0	0
Nonhuman primate + rhesus macaque + thromobopoietin + (b)(4) (b)(4) + platelet + megakaryocyte + ionizing radiation + hematopoietic + hematopoiesis + neutropenia + thrombocytopenia	0	0	0	0
Nonhuman primate + rhesus macaque + thromobopoietin + (b)(4) (b)(4) + platelet + megakaryocyte + radiation protection + mitigation + therapy + (b)(4)	0	0	0	0
Nonhuman primate + rhesus macaque + ionizing radiation + hematopoietic + hematopoiesis + neutropenia + thrombocytopenia + radiation protection + mitigation + therapy + (b)(4)	0	0	0	0
Nonhuman primate + rhesus macaque + ionizing radiation + hematopoietic + hematopoiesis + neutropenia + thrombocytopenia + support care + antibiotic + blood transfusion + blood products + cytokine	0	0	0	0
Nonhuman primate + rhesus macaque + thromobopoietin + (b)(4) (b)(4) + platelet + megakaryocyte + ionizing radiation + hematopoietic + hematopoiesis + neutropenia + thrombocytopenia + radiation protection + mitigation + therapy + (b)(4) + support care	0	0	0	0

+ antibiotic + blood transfusion + blood products + cytokine				

Summary: Results reveal that although there are specific hits in certain combinations, there is no duplication in terms of the work that is being proposed. References such as those from Farese, McVittie as well as Graham and others have been incorporated in specific sections of the present protocol.

III. OBJECTIVE/HYPOTHESIS: The primary objective of the present study is to evaluate the beneficial effects radiation countermeasures (such as [(b)(4)] as measured by significant increase in 60-day post-irradiation survival with the drug being administered post-irradiation (as a mitigator) in conjunction with supportive care (such as intravenous fluids, blood products, nutrition and antibiotics). A significant increase is considered 30% higher than the control treatment modality.
Secondary objective(s) include studying hematopoietic recovery including neutrophil and platelet profiles, mean survival time (MST), incidence of febrile neutropenia (FN), and infection, number of blood transfusions, and incidence and severity of diarrhea in the rhesus macaque.

We hypothesize that [(b)(4)] will be an effective radiation countermeasure in NHPs and that drug administration postirradiation will enhance survival over and above the protection provided by standard supportive care for immunosuppression (such as antibiotics and blood product administration).

IV. MILITARY RELEVANCE: Nuclear proliferation, terrorist activity, and the distribution of nuclear and radioactive materials through underground networks make incidents involving radiation injuries increasingly likely. As explained in a recent Department of Defense (DOD) Report to Congress (www.acq.osd.mil/cp), Al-Qaeda is known to have ambitious plans to acquire or develop nuclear weapons and is receptive to outside nuclear assistance. That group has attempted to purchase enriched uranium. Nuclear fuel-cycle and weapons-related technologies have spread to the point that additional states may be able to produce sufficient fissile material to develop radiological weapons. In view of the increasing likelihood of radiation exposure and bioterrorism, the need for field-able radiation countermeasures has been recognized as a high priority by the DOD.

Advances in radiation countermeasure strategies may enable military forces to operate, when required, in nuclear or radioactive combat environments while minimizing both short- and long-term consequences of exposure to ionizing radiation. Accurate casualty-prediction models promote effective command decisions and force structure planning. Advanced biological dosimetry methodology is used in triage, treatment decisions, and risk assessment. Together, the results of the three research thrusts improve therapeutic strategies for the treatment and prevention of early and long-term health effects and mitigate the risks to our personnel (AFRRI mission statement).

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

The experimental design for the proposed nonhuman primate (NHP) studies will include:

1) Specific aim 1: Establish LD70/30 radiation dose with supportive care
Fliedner TM et al. (2001) describe various categories of supportive care for patients exposed to accidental irradiation or in their acute phase of treatment. Four response categories (RC) have been identified. In RC1, outpatient care is recommended and patients are allowed to recover on their own and no supportive care is deemed necessary. In RC2, patients are provided supportive care and substitution (blood component therapy). The present protocol and this specific experiment are compatible with the "RC2" clinical supportive care regimen. The RC3 group would require stimulation with growth factors in addition to whatever is applicable to be RC2 supportive. Thus, "RC3" clinical supportive could be equated to the next specific aim, which involves treatment with medical countermeasure agents (in this case, (b)(4) . In the fourth category, RC4, patients would require stem cell transplantation in addition to supportive care.

In a recent publication (Farese et al. 2012), supportive care for NHPs subjected to ionizing radiation has been reviewed extensively. The article indicates that supportive care studies such as the one being proposed here have been studied only to a limited extent. There have been no studies with cobalt-60 gamma radiation exposure and supportive care. However, based on historical data and that of Farese, et al. (2012), a dose modifying factor of 1.13 is suggested for supportive care studies. For example, if the LD70/60 dose is 7.0 Gy, then with supportive care the LD70/60 dose will be 8.0 Gy."

Farese et al. (2012) have provided LD50/60 and probit curve for supportive care in animals exposed to radiation from a 6 MV linear accelerator (LINAC). However, because the source and type of exposure differ from AFRRI's cobalt-60 pool type facility, the current protocol will test 3 radiation doses between 7.2 Gy and 8.5 Gy for establishing preliminary data on survival with supportive care. Although large numbers of animals are required to establish dose response relationship (DRR), the observations from these sets will be used to identify an initial radiation dose for studying the effect of (b)(4)

Animals required: 3 radiation doses × 3 animals per radiation = 9 animals

2) Specific aim 2: Study the effect of postirradiation drug treatment on 60-day survival in animals receiving LD70/60 gamma radiation @ 0.6 Gy/min and supportive care.

Preliminary Dose Escalation Study (DES) data with (b)(4) in NHPs at AFRRI:
The research strategy for the present study is to extend our significant observations in (b)(4) irradiated mice to a large-animal model. Use of a large-animal model is essential as per the FDA's "animal efficacy rule" for advancement of radiation countermeasures to humans. We showed recently (ongoing research at AFRRI supported by (b)(4) (b)(4) that the proposed drug (b)(4) is safe for use with NHPs. Figure 1 shows preliminary data on enhancement of platelet (1A) in unirradiated animals administered a single sc injection of 8 mg/kg (b)(4) as well as changes in neutrophil counts (1B).

Figure 2 shows that a single sc injection of (b)(4) administered either pre-irradiation (-24 h) or post-irradiation (+24 h) resulted in survival and recovery of circulating platelets by day 18. The non-drug treated animal, the control, although survived had a prolonged recovery (day 29). It is important to note that animals in these experiments received minimal supportive care which is different from the current protocol under review.

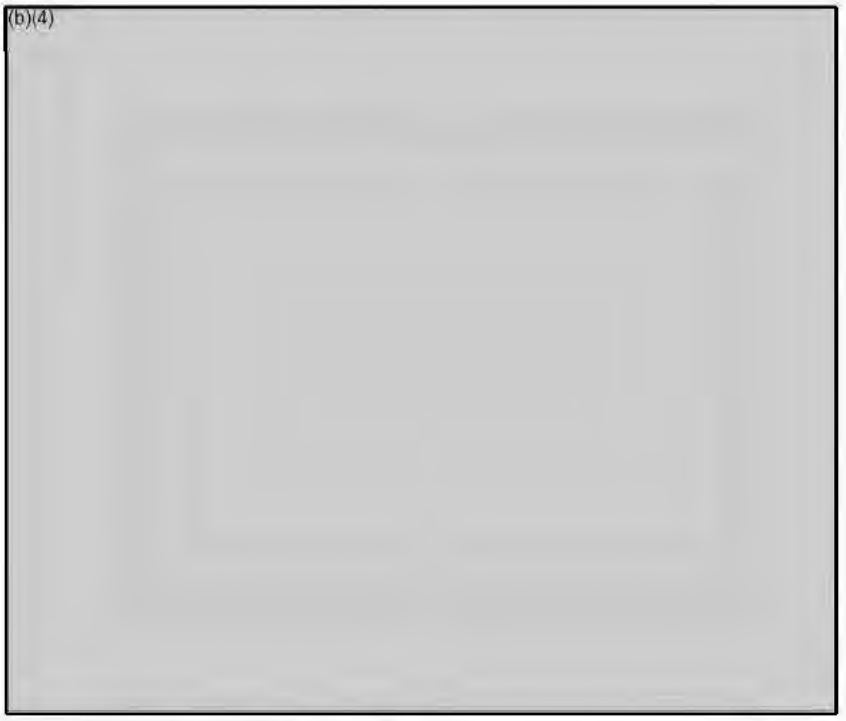

Figure 3 shows that a single sc injection of (b)(4) administered either pre-irradiation (-24 h) or post-irradiation (+24 h) resulted in recovery of circulating platelets (Figure 3A) and neutrophils (Figure 3B) in irradiated (7 Gy). The control animal did not survive.

Note: The proposed IACUC protocol differs significantly from the ongoing NHP study (Figures 1–3) because all animals other than blood donor animals in this proposed protocol will receive supportive care.

Therefore, the following survival experiments will be conducted:
Specific aim 2, Experiment 1: In this preliminary experiment, we will evaluate two time points for drug administration. The drug will be administered either +6 h or +24 h postirradiation.

PBS vehicle (used to dilute/suspend (b)(4)	3 animals
(b)(4) administered sc 6 h postirradiation	3 animals
administered sc 24 h postirradiation	3 animals

Total: 9 animals

Specific aim 2, experiment 2: Because this is a confirmatory experiment, only one time point that is optimum in response will be used (for example, either +6 h or +24 h) for drug administration.

PBS vehicle (used to dilute/suspend (b)(4)	3 animals
(b)(4) administered sc either 6 h or 24 h	6 animals

Total: 9 animals

3) Specific aim 3: Multiple-dose regimen for (b)(4)

(b)(4)
(b)(4) is effective when administered as a once-weekly injection for 8 weeks to idiopathic thrombocytopenia purpura (ITP). Our drug has not been tested as a multiple regimen although experiments are in progress at AFRRI (ongoing NHP protocol), we propose a three-dose regimen for (b)(4) Three doses to be used are based on the potential benefit to animals recovering from radiation damage. We hypothesize that the drug will be effective in enhancing postirradiation survival of irradiated NHPs in a three-drug regimen, as shown below.

Specific aim 3, experiment 1: Two-dosing regimens with three injections will be tested to evaluate improved drug efficacy using the multiple injection modality. The drug for these studies will be a nontoxic drug dose of 8 mg/kg.

The experimental groups for this experiment are as follows:

Control:	PBS vehicle administered +6 h, +4 d, and +10 d	3 animals
Regimen 1:	(b)(4) administered +6 h, +4 d, and +10 d	3 animals
Regimen 2:	administered +24 h, +4 d, and +10 d	3 animals
		Total: 9 animals

Specific aim 3, experiment 2: This is a confirmatory experiment in which one of the two regimens described above will be used.
The experimental groups are:

1. PBS vehicle administered as per one of the schedules: 3 animals
2. (b)(4) administered as regimen 1 or Regimen 2: 6 animals

(Regimen 1 = +6 h, +96 h, and +240 h. Regimen 2 = +24 h, +96 h, and +240 h)

Total: 9 animals

4) Blood donor program: There will be 10 donor animals that will provide whole blood for transfusion. Total: 10 animals

Total number of animals for all studies: specific aim 1 (9) + specific aim 2 (18) + specific aim 3 (18) + donor (10) = 55. Male donor animals will be preferred due to their larger size. Therefore, total number of males = 33 and females = 22.

Drug procurement:

(b)(4)

(b)(4) The drug has been well characterized by the company.

Irradiation

Animals will not be fed in the morning of radiation in order to control potential radiation-induced vomiting. Animals will be anesthetized with ketamine (10–15 mg/kg IM) prior to transferring them to NHP-specific radiation plexiglass boxes. Additional anesthetic will be administered at the discretion of the veterinarian or veterinary technician to keep the primates sedated during irradiation and transport. Animals will be transported to the radiation facility in covered carriers. For all radiation studies, a radiation dose rate of 0.6 Gy/min will be used. Radiation dose used will be 7.2–8.5 Gy (LD70/60), a dose that will result in mortality of 70% of the irradiated NHP over a 60-day period with supportive care (described elsewhere). It should be noted that specific LD70/60 dose for NHPs using AFRRI's cobalt-60 gamma radiation facility with supportive care as described elsewhere in the protocol has not been determined. Farese et al. (2012) have demonstrated that supportive care itself provides a certain

degree of protection and increased survival. Therefore, PI has projected a wide range of radiation doses for dose-determination studies.

Description of supportive care:

The supportive care can be divided into 4 major categories: 1) intravenous (parenteral) fluids, 2) blood products, 3) nutrition, and 4) antibiotics. Details on the indication/criteria for specific supportive care are provided in Section V-4: technical methods. A brief description of each supportive care category is described below:

Nutrition: This will consist of Bio-Serv-certified Rhesus Liquidiets or similar products and will be administered to control weight loss or anorexia.

Parenteral fluids: Ringer's lactate and Ringer's lactate with 5% dextrose in equal volumes or other products such as hetastarch will be administered to control dehydration.

Blood products: Whole blood or packed red blood cells will be obtained via a commercial source (see appendix), obtained from donor animals and irradiated at 2500 cGy with a source in USUHS prior to transfusion and will be administered when thrombocytopenia and anemia are observed.

Antibiotic(s) administration: Based on evidence of infection (neutropenia, and febrile neutronpenia), various antibiotics (a broad spectrum or those specific to Gram positive or Gram negative organisms) may be used. Blood samples will be collected prior to antibiotic treatment and 10 days after ceasing antibiotic treatment to assess bacterial load.

General: In addition to these supports, animals may receive analgesics and non-steroidal anti-inflammatory drugs as well as antidiarrheal agents.
See Table 1 for details concerning supportive care.

V.2. Data Analysis

Survival study:

For this protocol we will use the criteria of declaring a countermeasure or test agent as promising if a 30 percent increase in survival is observed over the vehicle-treated group exposed to a radiation dose producing approximately 70% (±10%) mortality over 60 days postirradiation. This criterion is based on discussions with AFRRI and (b)(4) in terms of assessing a drug for use in mass-casualty scenarios involving civilian populations, and has been used extensively in mouse studies to evaluate potential radiation countermeasures. The Exact Log Rank test (one-tailed) and the generalized Savage (Mantel-Cox) procedure (BMDP Statistical Software Inc., CA, available with an institutional statistician) will be used to compare survival of drug-treated and control groups in the radiation-survival experiments. In order to obtain the number of animals, data on human volunteers using a similar class of compound (b)(4) in terms of enhancement of platelets and variation between subjects also was considered (b)(4) (b)(4) Further, multiple blood draws from the same animal over a 60-day period also was considered. Experiments with nine NHPs per group have 80% of power to detect significant differences between the drug-treated and vehicle controls. For these calculations, we assumed that type-1 error is controlled at 5%, type-2 error is 20% and sample sizes are equal between the different groups.

Demographic, hematology, vital signs, blood counts, chemistry, antibody levels, and cytokine estimations will be compiled as summarized using mean, standard deviation, minimum, median and maximum values.

Vital signs will be summarized using descriptive statistics at each assessment time for each treatment group.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered: The effectiveness of drugs such as (b)(4) against radiation cannot be adequately assessed using current *in vitro* technologies. The U.S. FDA recognizes the ethical considerations involved in testing the efficacy of radioprotective drugs in humans. Therefore, the FDA requires preclinical drug assessments (safety and efficacy) to be performed using both small and large animal models (FDA 2002) prior to granting approval for safety testing in humans. The proposed experiments will provide efficacy data with NHPs, one of the preferred large-animal models. If these studies are successful, full-fledged GLP studies will be conducted for safety and efficacy. GLP studies are very expensive and cannot be done with the grant for this study.

V.3.2. Animal Model and Species Justification: Nonhuman primates (NHP) are necessary for the pre-clinical development of a drug candidate intended for use in humans, because drug metabolism and physiology are very similar between humans and nonhuman primates. Testing a drug with potential for human application in NHPs ensures safety and specificity prior to the drug entering into clinics. This is true especially because of safety risks and the chance of unexpected autoimmune or hyper-immune reactions. Due to the species-specific nature of immune reactions, the drugs can only be studied *in vivo* in hosts whose immune systems are very closely related to that of humans. Rhesus macaques are the model of choice for investigations of radiation injury and countermeasures because of the large database available from the existing literature that allows for robust comparison. The FDA has accepted rhesus macaques as the appropriate animal model for pilot and pivotal efficacy testing of radiation countermeasures under the Animal Efficacy Rule, where efficacy testing cannot be performed in humans. The safety and efficacy of (b)(4) was demonstrated in mice. (b)(4) (b)(4) and hence, it is appropriate to conduct the present set of experiments to advance the drug for human use.

V.3.3. Laboratory Animals: *Macaca mulatta* (rhesus macaque) will be obtained from vendors registered with the USDA.

V.3.3.1. Genus/Species: *Macaca mulatta*

V.3.3.2. Strain/Stock: Preference will be given to animals of Chinese origin so that comparisons can be made with existing data on NHPs from AFRRI. Male and female *M. mulatta* are required that have been chair-trained, have normal cardiovascular parameters, no infections or wounds or surgical traumas, and which have normal liver and kidney enzyme functions.

V.3.3.3. Source/Vendor: In general, the sources for these animals are commercial vendors registered with USDA. However, attempts will be made to obtain suitable animals from the DOD pool that would meet specifications stated in V.3.3.7.

V.3.3.4. Age: Animals of 3 to 8 years of age are requested for the present study.

V.3.3.5. Weight: Animals must weigh in the range of 4–8 kg.
V.3.3.6. Gender: Male and female

V.3.3.7. Special Considerations: In addition to the requirements listed in sections V.3.3.2 and V.3.3.3 above, nonhuman primates shall test negative for Herpes B Virus (aka, *Macacine herpesvirus*), simian T-cell leukemia virus type 1 (STLV-1), simian immunodeficiency virus (SIV), and simian retrovirus (SRV) Types 1, 2, 3, and 5. Animals shall also test negative by means of virus isolation or polymerase chain reaction (PCR) for SRV Type 2. NHPs will either be vaccinated for measles or, in the case of previously measles-vaccinated NHPs, tested for the presence of measles antibodies.

Nonhuman primates coming from the vendor colony must test negative for *Salmonella*, *Shigella* and *Campylobacter*. The animals also must test negative for *Klebsiella pneumoniae*. On the advice of the attending veterinarian, in consultation with the PI, the exclusion criteria for certain viral and bacterial pathogens may be waived.

CBC and blood chemistry will be obtained from vendors for each primate and screened for abnormalities by an AFRRI veterinarian. Any abnormal findings will be discussed with the PI and a determination of the fitness of the animal for use on the protocol will be made. Individual medical records will be maintained during the entire length of the study. Tuberculosis testing will be performed in accordance with VSD SOPs and results must be negative or the NHP will be removed from the study.

V.3.4. Number of Animals Required (by species): 55 (total), 33 males and 22 females

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement:
One of the most significant refinements in the reduction of distress and hemorrhage would be the use of a vascular access port (VAP) for blood withdrawal. Graham et al. (2009) have demonstrated that VAP implants were successful and safe for use with primates.

Chairing devices will be used as positional aids during the irradiation procedure as well as brief (<1 hour) restraint for phlebotomy procedures.

The use of chair-trained rhesus primates (preferably from vendor) will reduce stress on the animals during blood draw and injections. Acclimatization to bottle for oral supplementation such as drinking water, juices, Gatorade and electrolytes will be used to relieve discomfort from radiation-induced vomiting or diarrhea. Prior to placement of the animals within radiation-restraining chairs, they will receive light immobilizing doses of ketamine or Telazol (as determined by veterinarian) to reduce anxiety caused by movement. Time in the chair will be kept to a maximum of 1 h. In addition, the animals will be transported to the radiation facility in a covered cart to reduce the excitement of

being moved from their home cages to the radiation area. Over-the-counter medications will be provided in case of fever, diarrhea, vomiting, and bloating.

To minimize animal suffering, pain, or distress, moribund animals (section V.4.) will be considered to have arrived at the study endpoint. Such animals will be euthanized and recorded as "mortality".

Animals will be considered morbid when they display symptoms listed in V.4.5. Experimental animals that are morbid but not moribund will be allowed to continue in the experimental protocol with supportive care in order to evaluate effect of irradiation and the drug accurately.

Animals will be considered moribund when showing some or all signs of morbidity, or appear severely debilitated and in terminal distress. Animals will be monitored a minimum of twice daily, no sooner than 8 h apart, and appropriate actions taken when animals show signs of morbidity. Early endpoints (see 4.5, Study Endpoint) will be used in determining euthanasia.

V.3.5.2. Reduction: Information obtained in the dose-escalation study will allow us to determine the dose of (b)(4) to be used in radiation studies and, because we plan to conduct studies with only a limited number of animals (control/drug treated), we will halt these studies if adverse effects are seen during an experiment. Further, we propose to use multiple sampling points for blood collection and use individual animals as their own control that will lead to use of fewer animals in the protocol, in addition to appropriate study controls described in section 4.1.1.1.4)

V.3.5.3. Replacement: None

V.4. Technical Methods

Animal procedures: Rhesus macaques (*Macaca mulatta*) will be pair-housed as much as possible after observing compatibility. Animals will be quarantined for a minimum of 37 days prior to the inception of the experiment.

Surgical procedure will install vascular access port (VAP) in all experimental animals including donor animals based on veterinarian recommendation (see surgical section-V.4.3.2).

Chair training: The vendor will provide basic chair training involving the pole-and-collar technique. This will be reinforced at AFRRI with additional training by VSD staff.

General procedures:

Drug formulation: (b)(4) (Endotoxin, tested by Charles River laboratories) and the drug will be diluted appropriately in sterile PBS prior to injection (within 1–2 h after preparation). The drug will be maintained undiluted at 4 °C until injection. PBS will be used as control. Endotoxin testing results will be reported to the IACUC office.

Details on medical management of infection, thrombocytopenia, and anemia are provided below. See table 1 in section 4.3.2 for greater details on the criteria for initiating medical management procedures.

Evidence of Infection: Animals will be administered antibiotics as recommended by

the attending veterinarian or when a diagnosis of infection has been determined by blood culture or when there is evidence of neutropenia or FN that may be associated with infection. The Infectious Disease Society of America guidelines for treatment of humans are being followed for antibiotic administration in the case of neutropenia [i.e., antibiotics are recommended when prolonged and severe neutropenia (<100 cells/μL) is expected for 7 or more days]. Antibiotics will be selected by the attending veterinarian based on:

1) Institutional knowledge of potentially infecting organisms,
2) antibiotic susceptibility of pathogens isolated locally,
3) importance of broad-spectrum antibacterial activity, and
4) the animal's infection risk assessment.

Baytril® will be administered when the ANC (absolute neutrophil count) becomes <500/μL and will be continued until the ANC is ≥500/μL for two consecutive days. Blood collection will occur every day after Baytril is administered. Gentamicin will be initiated and administered for two days only, if FN (ANC <500/μL and body temperature ≥103 °F) is observed and is persistent during Baytril treatment. Gentamicin is effective against bacteremia partially because it stays in the bloodstream due to poor tissue absorption. Other possible antibiotics that will be used if resistance to Baytril has been demonstrated are third-generation cephalosporins such as Clavamox. Rectal swabs will be obtained for culture and sensitivity prior to irradiation to determine antibiotic sensitivity of normal gastrointestinal flora.

For microbial evidence of infection, blood (at least 1.0 mL) will be collected aseptically and inoculated into a BACTEC® culture vials and submitted to the testing facility. Blood culture will be performed on the first-day of FN to determine if antibiotic resistance has occurred. Blood cultures will be collected if FN (ANC <500/μL and body temperature ≥ 103 °F) is observed or any day the body temperature is ≥103 °F. Additional blood cultures will be collected 24 h after the first blood culture if FN persists and the preliminary bacteriology report of the first culture is either Gram negative or a Gram positive organism is identified.

Preparation of Blood Products to Treat Thrombocytopenia and Anemia: Whole blood for transfusions will be obtained from a pool of approximately 10 healthy adult donor rhesus macaques. Whole blood aliquots will be exposed to 2,500 cGy of ionizing radiation prior to transfusion in order to minimize rH factor complications although this is minimal in NHPs. Packed RBC will be obtained from a commercial source and kept for a minimum period based on blood bank procedures and will be administered in preference to whole blood when PLT counts are satisfactory (≥100,000/μL with no clinical bleeding) but the Hgb or Hct remain low (Hgb <7 g/dL or Hct <20%).

Blood collection: Blood samples will be collected according to appropriate VSD SOPs. See Table 3 for tentative blood collection scheme in the biosampling section. Blood (0.5 ml to 1.5 ml/day) will be collected from a peripheral vessel or femoral vein or through VAP using a 22–25 G needle attached to a 3 ml (generally) syringe to reduce the chances of bleeding after blood collection in irradiated animals. A small aliquot (0.25 ml) from each sample will be taken immediately for CBC/differential. Further, 0.35 ml will be used to prepare serum that will be frozen at –70 °C for clinical chemistry and antibody measurements.

The area for the blood draw will be swabbed with providone-iodine (left in place for 3 minutes) or isopropyl alcohol before inserting the needle. Providone-iodine will be swabbed off to reduce irritation to the tissue. The blood sample will be drawn as described above. While withdrawing needle, pressure will be applied at the same time with a clean gauze to reduce bleeding from the blood-draw site. While making routine observation of animals, the blood-draw site will be checked for bleeding. If the site becomes infected, topical antibiotics may be applied, under supervision by VSD.

Study endpoints: Experimental animals will be euthanized based on a moribundity scale and in consultation with the attending veterinarian. Euthanasia will be carried out when any one of the following signs and symptoms of moribundity are observed as determined by protocol technicians, the PI or by AFRRI veterinarians. Early end points will be used and are described in section V.4.5.

Body weights will be determined at least once prior to treatment, on the first day of treatment, and twice weekly except when inappetence sets in (daily measurements). Body temperature (implanted chip, see section V.4.1.2.1 for chip implantation) will be determined daily beginning approximately one week prior to the first treatment. The time of temperature collection will be recorded for each monkey. On day 60, after the last blood collection, all surviving monkeys may be transferred to AFRRI's NHP pool.

Identification of pain: NHPs demonstrate remarkably little reaction to surgical procedures and injury. An NHP in pain has a general appearance of misery and dejection. We will observe all irradiated animals for grimacing and glassy eyes, loud and persistent vocalization, avoidance of social interaction, and grooming. Gastrointestinal injury may be associated with abdominal pain displayed as facial contortions, teeth clenching, and restlessness. Headaches manifest by pressing the head against the enclosure surface. Self-directed injurious behavior may be a sign of more intense pain. Because most primates in pain will refuse food and water, daily food and water intake will be monitored closely. Further, response to familiar personnel and changes in willingness to cooperate may also indicate pain, which will be closely monitored and recorded.

Euthanasia: Animals will be euthanized according to current American Veterinary Medical Association (AVMA) guidelines. See sections V.4.5 and V.4.6.

Estimation of hematological indices: Blood (0.5–1.5 ml/day) will be obtained from a VAP or peripheral vessel using a heparinized 3 ml (mostly) syringe and a 22-25 G needle, generally from non-anesthetized NHP, and transferred immediately into pre-coated ethylenediaminetetra-acetic acid (EDTA, Sigma-Aldrich, St. Louis, MO, USA) tubes and mixed gently on a rotary shaker until analysis. The coded tubes will be analyzed for white blood cell (WBC), absolute neutrophil counts, monocytes, lymphocytes, reticulocytes, hemoglobin and platelet counts.

Concomitant medication and medical management will be given if clinically indicated. Table 1 summarizes the conditions under which concomitant medications and medical management will be given as well as the procedure for providing care.

Table 1. Summary of Plan for Medical Management and Medication Use

Drug class	Allowed medication or supportive care agents	Indication and/or criterion for administration
Anesthetics	Ketamine 10 ± 5 mg/kg, IM	**Anesthesia during procedures:** • Administered before animal manipulations to minimize stress and anxiety.
Sedatives	Xylazine (Rompum®) 1 ± 0.5 mg/kg, either IM or SC Diazepam 1 ± 0.25 mg/kg, IM,	**Sedation during procedures:** When animals are restrained a mild sedative may be administered as required.
Analgesics and non-steroidal anti-inflammatory drugs	Buprenorphine 0.01 mg/kg/dose BID, IM, IV, or SC Marcaine (0.25% bupivacaine), topical Carprofen (Rimadyl®) 2.04.0SID or BID, IM or IV Acetaminophen (Tylenol) 5–10 mg/kg PO BID-QID	**Pain Management:** • Rimadyl® (Carprofen) can be administered as an alternative to buprenorphine when evidence of pain is present. **Fever (temperature ≥ 104 °F):** • Carprofen or acetaminophen will be administered until the temperature remains below <104 °F for 2 consecutive days.
Parenteral fluids	Equal volume of Ringer's lactate and Ringer's lactate with 5% Dextrose; each at 5 ± 2.5 mL/kg body weight via slow IV push; twice daily depending on extent of dehydration; or Ringer's lactate at 40 ± 5 mL/kg body weight by IV drip over 2 hrs. Pedialyte® or Gastrolyte® orally 15 ± 5 mL/kg of body weight	**Dehydration:** • Mild to moderate dehydration (Grade 1). Ringer's lactate and Ringer's lactate with 5% dextrose eight via slow IV push. • Severe dehydration (Grade 2). Ringer's lactate by IV drip. • Pedialyte® or Gastrolyte® or similar commercial solutions containing sodium, potassium, and glucose may also be used if IV access is difficult or precluded. See Table footnote for definitions of severity grade. **Fever (temperature ≥103 °F):**
Anti-diarrheal	Loperamide hydrochloride (Imodium), Initial dose 0.04 mg/kg PIOID Diphenoxylate HCl/atropine sulfate (Lomotil), 0.1 mg/kg, (2.5 mg/tablet) will be administered BID, PO. The 2.5 mg tablet first will be dissolved in 5 mL of tap water. Then, 1 mL of the Lomotil solution (0.5 mg/mL) will be added to 5–10 mL tap water, with Oral Ringer's solution or liquid nutrition for 3 days.	**Diarrhea:** • At the first sign of soft to loose stools: Imodium will be administered up to twice daily and should continue for 3 days. It may be stopped earlier than 3 days if the diarrhea resolves. • Severe diarrhea or if Imodium has been administered for 3 days, Lomotil, up to BID, PO for 3 days. It may be stopped earlier than 3 days if the diarrhea resolves. If the diarrhea does not resolve the Imodium treatment will be repeated.
Anti-ulcer	Carafate 1g/day. (0.5 g BID) daily from days 5–30, PO	For treatment of possible ulcers of the stomach or proximal small intestine

Antibiotics	Enrofloxacin (Baytril®) 5 ± 2 mg/kg, IM or IV or PO, SID Gentamicin, 3 ± 1.5 mg/kg, IM or IV BID Clavamox, 6.5-13.5 mg/kg, PO, QID or 15 mg/kg, PO, BID Imipenem-cilastatin (Primaxin®) 10 ± 1 mg/kg, IV or IM, BID Cefepime hydrochloride (Maxipime®) 40 mg/kg IV QID	**Evidence of infection (microbial culture, neutropenia, and FN): Antibiotics will be administered when:** • Pre-nadir febrile neutropenia (FN) (ANC <500/µL, body temperature ≥103 °F) is observed. • When ANC <500/µL with the expectation of the development of a prolonged, severe neutropenia (ANC <100/µL). • Standard veterinary practice warrants administration, e.g., open wound. • Body temperature ≥ 103 °F following a positive blood culture. • Pending blood culture results, regardless of the ANC. **Antibiotics will cease when:** • No fever is observed and ANC increases ≥500/µL for two consecutive days • Fever is observed, blood culture for aerobic microorganism is negative and ANC increases ≥1,000/µL for two consecutive days.
Anti-emetics	Ondansetron (1-2 mg/kg), IM, IV or PO 25-90 minutes prior to irradiation and 30-45 minutes following irradiation to suppress emesis or Granisetron (0.25 mg/kg), PO or IV	• Administered pre-and post-radiation to suppress emesis
Blood products	Whole blood or packed red blood cells will be obtained from a commercial source or obtained from donor animals and blood product irradiated @ 2500 cGy with a source in USUHS prior to transfusion Whole blood at 7 – 14 mL/kg or Packed red blood cells (RBC). Fluid replacement policy: Volume replacement with fluids will be subject to clinical decision. <u>Note:</u> Repeat blood collection from donor animals will be defined on the total volume collected each time, recovery period and other criteria as described by Diehl et al 2001	Whole blood will be transfused if: • PLT counts are <20,000/□L and hemoglobin (Hgb) <7 g/dL or hematocrit (Hct) <20; • An animal experiences a 5-unit decrease in Hct in a 24-hour period that results in an Hct of ≤ 25%; • An animal that has previously received a transfusion demonstrates continued evidence of bleeding, such as bloody stools and/or any decrease in Hct relative to the most recent result obtained; or • If there is obvious evidence of uncontrolled hemorrhage. • An animal experiences a 7-10 unit decrease in Hct in a 24-hour period; or • An animal's Hct is ≤ 25% for 2 consecutive days and PLT counts are <3,000/□L. Packed RBCs will be administered in preference to whole blood when PLT counts are satisfactory (≥100,000/□L with no clinical bleeding) but the Hgb or Hct remain low (Hgb <7 g/dL or Hct <20 g/dL). • PLT counts are satisfactory (≥100,000/□L with no clinical bleeding) but the Hgb or Hct remain low (Hgb <7 g/dL or Hc <20 g/dL).

Barbiturates	0.4% pentobarbital sodium (Euthanasia III), 0.27 mL/kg IV or similar pentobarbital solution	**Euthanasia.**
Nutritional Support	BIO-SERV certified Rhesus Liquidiets	**Weight Loss (Anorexia):** Nutritional support will be administered via oral gastric tube when body weight is <85% of baseline and will be continued as long as the body weigh remains <85% of baseline, and the animal is not eating. In the case where an animal loses its ability to regain weight following irradiation despite actively eating and drinking, nutritional supplementation via oral gastric tube may be foregone in order to avoid daily anesthesia.

Definitions of severity grade of dehydration: Grade 1 and 2, mild to moderate dehydration. Symptoms include diminished skin turgor, dry mucous membranes, dry nose, increased hematocrit, and sunken eyes. Grade 3, severe dehydration. Symptoms include all of the above plus rapid and weak pulse, cold extremities, cyanosis, lethargy, rapid breathing, and comatose.

V.4.1. Pain/Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals: 55

V.4.1.1.1.1. Column C ___ (need to be naive animals) __ (# of animals)

V.4.1.1.1.2. Column D __10__ (# of animals)

V.4.1.1.1.3. Column E ___45__ (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
Exp #1	Dose finding study			9	9
Exp #2	Drug +6 or +24 h			18	18
Exp #3	Drug multiple regimen			18	18
	Donor animals		10		10
Totals:			10	45	55

V.4.1.2. Pain Relief/Prevention

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization microchips will be implanted under ketamine sedation (10–15 mg/kg, IM). Bone marrow biopsy will be done only for surviving animals subjected to ionizing radiation and only at the end of the experiment (60 days). It may be obtained under the same dose of ketamine or another suitable anesthetic determined by the veterinarian. Anesthetic and analgesics required for VAP surgery also will be procured by PI. Microchips may be placed during the initial physical

examination. If necessary, a drop of surgical glue will be placed at the chip-insertion site to ensure that the chip stays in place.

Bone marrow collection is expected to be a painful procedure. As such, the primates will be under general anesthesia at the time of marrow cell harvesting. NHPs will be sedated with ketamine IM in order to facilitate placement of an IV catheter or mask (as recommended by the veterinarian), and the animal is intubated and maintained on isoflurane at a surgical plane of anesthesia. Additional local anesthesia with Lidocaine (approximately 1 ml) will be provided at the marrow-harvesting site on recommendation of the staff veterinarian. Bone marrow sampling will be performed in the VSD surgical suites under aseptic conditions. Primates also will be administered opioids such as Buprenorphine at a dose of 0.01–0.03 mg/kg IM twice daily or more frequently if needed to relieve the stress and pain. Because bone marrow aspirates and/or core biopsy will be obtained at the end of the experiment (60 days), pain relief provided at that time will not interfere with the overall outcome of the experiment (see additional information on bone marrow collection under Surgery).

V.4.1.2.2. Pre- and Post-procedural Provisions Animals will be assessed for signs of pain and distress by either the veterinary or research staff at least twice daily or more often as needed according to appropriate VSD SOPs. Before procedures that require general anesthesia, NHPs will be fasted for 12 h prior to administration of anesthetic. Clinical signs of acute radiation sickness (e.g. diarrhea, vomiting, and fever) can be treated with over-the-counter medications on the advice of a VSD veterinarian. For any NHP not taking in sufficient food and/or water, additional foods can be offered to entice them to eat. In addition, NHPs will be prior-acclimated to feeding syringes and sipper bottles by vet techs and lab techs associated with the project to allow for the use of supplemental oral fluids if dehydration is noted. Criteria for euthanasia are listed in Section V.4.5.

V.4.1.2.3. Paralytics: No paralytics will be used in this study

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched: Agricola (1974 to present), Pubmed (1946 to present), and Altweb

V.4.1.3.2. Date of Search: April 8, 2013

V.4.1.3.3. Period of Search: See above (v.4.1.3.1)

V.4.1.3.4. Key Words of Search: See table below

V.4.1.3.5. Results of Search: See table below

Keywords	AGRICOLA 1974-present	PubMed 1946-present	Altweb
alternatives to painful procedures	2	119*	148
primates + bone marrow harvesting	0	79*	7

primates + radiation	7	39117*	6
primates + blood draw	0	697*	53
primate +pain	2	16598*	0
thrombopoietin + primate + pain	0	0*	1
vascular access port + pain	0	5*	1
vascular access port + pain + primate	0	3*	107
supportive care + pain	0	64*	1
supportive care + pain + primate	0	41*	3
blood transfusion + pain	0	52*	0
blood transfusion + pain + primate	0	37*	0

*Includes data from other species

The literature search did not indicate specific alternatives to pain other than what is described below.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification There are no alternative procedures for irradiation because it is a unique stimulus/stress that cannot be otherwise duplicated. Radiation does not cause pain or distress. Nevertheless, the sequelae of nausea, vomiting, and diarrhea cause pain and distress in humans in the early post-irradiation period, when high doses (8 Gy and above) are used. Although radiation does not induce pain, animals in these experiments might experience pain and distress prior to death because of hematological and gastro-intestinal damage. Therapeutic guidelines to address pain and distress are addressed in section V.4.1.2.2.

Bacteria translocated from the alimentary canal may cause infections that result in discomfort either locally or systemically. Many opportunistic pathogenic bacteria have unusual or even unique virulent characteristics but they have common attributes, including binary multiplication and penetration of tissues, and cause common responses and disease process in animals, which cannot be mimicked readily by substitutes. The investigators and animal-care staff will provide as much comfort and well-being to the animals as is consistent with the scientific integrity of the work plan. The principle purpose of these studies is to evaluate the efficacy of (b)(4) in enhancing survival of irradiated NHPs. As stated, bone marrow, peripheral blood, liver and kidney (liver and kidney analysis done by blood chemistry with the hematological studies) analyses also will be performed after (b)(4) administration. Moribund animals will be euthanized in consultation with VSD's veterinarian.

We expect that (b)(4) will provide some relief from pain and or discomfort due to the sequelae of irradiation by its protective effect and by the possibility that it will advance hematopoietic recovery in some or all of the (b)(4) treated and irradiated primates.

V.4.2. Prolonged Restraint The *Guide for the Care and Use of Laboratory Animals* and the AFRRI IACUC Policy Letter discuss "prolonged restraint" guidelines. The devices used during irradiation (chairs) are for positioning the animal, not for restraint. Primates will be sedated during irradiation and transport. The restraint devices will not be used as the normal method of housing.

V.4.3. Vascular access port (VAP) surgical procedure

General anesthesia:

- Premedication: Ketamine (10–15mg/kg IM + Atropine: 0.05 mg/kg IM), or Glycopyrrolate (0.005–0.01 mg/kg IM)
- Induction: 3–5% Isoflurane in 100% oxygen by mask
- Maintenance: 1–3% Isoflurane in 100% oxygen via endotracheal tube or mask
- Anesthesia monitoring: At least SpO2, heart rate, respiration rate, and temperature will be monitored and recorded throughout the procedure.
- Antibacterial prophylaxis: Cefazolin IV 20–25 mg/kg one time or Ampicillin 15–20 mg/kg IV one time.
- IV fluids: During the procedure, animals should be maintained on isotonic fluids at 5–10 ml/kg/hr via an IV catheter. If the catheter cannot be placed after 4 attempts, this step will be skipped as VAP placement is a quick procedure and involves accessing a vein so VAP should be available for intraoperative fluid administration once it is in place.
- Site of VAP catheter placement: Jugular vein or femoral vein or saphenous vein. No more than 1 site will be used per animal.
- Site of VAP port placement: Subcutaneously adjacent to vein or tunnel the port to the site that will be safe and easily accessible in a restrained animal (such as in between the shoulder blades on the back or caudal thigh or caudal lateral part of the chest).
- Preparation of the site: Hair will be clipped using a surgical #40 blade or equivalent. The site will be scrubbed at least 3 times using either betadine or chlorhexidine and 70% alcohol.
- Procedure: VAP catheters will be implanted surgically in the vein under general anesthesia according to the principles of aseptic surgery. A 5–7 cm incision will be made on the skin at the site of catheter placement. Vein will be isolated by doing a blunt dissection. The catheter tip will be introduced through a 2–3 mm incision in the vein and secured properly using absorbable or nonabsorbable sutures. Prior to insertion of the VAP catheter, it will be flushed with heparinized saline solution (100 IU of heparin/ml). VAP port will be placed subcutaneously and connected to the catheter tunneled through the subcutaneous tissue. The VAP will be checked for patency before securing it to the subcutaneous tissue. The VAP port will be flushed and locked with heparin lock solution (100IU of heparin/ml). The surgical site will be closed using the standard technique using the layer suture technique with absorbable or non-absorbable suture. Special attention will be given to eliminate any dead space.
- Post Op care: Baytril 5–10 mg/kg IM or PO bid for at least 5 days or longer as needed. Buprenorphine 0.01 mg/kg IM immediately after the procedure. Carprofen: 2.2 mg/kg IM or PO bid for at least 3 days or longer as needed.
- Restraint used to access the VAP: Either chemical (Ketamine 5–10 mg/kg IM) or chair restraint.
- Please note: Alternative anesthetics, analgesics and antibiotics may be used as deemed necessary by the veterinarian.
- Animals will be used only once for this purpose or any other survival surgical procedure unless the subsequent use is a terminal procedure.

Note: VAP installation in minipigs subjected to irradiation has been well documented (Ege et al 2006). According this article, VAP will be functional 2 weeks after surgery. The following general precautions and procedure will be followed: VAPs need to be

flushed at least weekly- blood could be drawn at this first flush but would have abnormal blood parameters and post-operative drugs (pain meds, antibiotics, etc.). The minimum time before return to normal would be 2 weeks, which would be the day -7 blood draw, so minimum 3 weeks between VAP implantation and irradiation will be the norms.

Bone marrow surgical procedure:

- General anesthesia
  - Premedication-ketamine: 5–15 mg/kg IM
  - Induction: 3–5% isoflurane in 100% oxygen by mask
  - Maintenance: 1–3 % Isoflurane in 100% oxygen by endotracheal tube or mask
- Anesthesia monitoring: At least SpO2, heart rate, respiration rate, and temperature will be monitored and recorded throughout the procedure.
- Site of bone marrow: iliac crest
- Procedure: Animal will be positioned appropriately (dorsal or ventral, or lateral recumbency). A sterile drape will be used to cover the site (survival study).
  - Biopsy: A stab incision (1-2 cm) will be made. A 13–16ga bone marrow biopsy needle will be advanced through the incision into the cortex of the bone. The stylet will be removed and the needle advanced 1–2 cm with gentle rotation and rocking of the needle. The needle is then retracted and advanced, slightly before being gently removed from the bone. The included expeller is used to remove the sample from the needle and placed in a cassette with foam insert, if necessary, which is then put in 10% formalin for fixation and processing.
- The incision will be closed with tissue glue or a suture after bone marrow collection. Either a non-absorbable nylon or absorbable monocryl suture will be used to close the skin.
- Additional precautions will include:
  - Antibacterial prophylaxis—Cefazolin intravenous (IV) or subcutaneous (SC) 20–25 mg/kg one time or Ampicillin 15–20 mg/kg IV or IM one time.
  - Animals will be used only once for this purpose.
  - Preparation of the site of bone marrow collection—The site will be prepared as a surgical procedure: hair will be clipped, the site area scrubbed 3 times with either betadine or chlorhexidine and 70% alcohol.

V.4.3.1. Pre-surgical Provisions Prior to general anesthesia for all surgical and procedures, all NHPs will be fasted for 12–18 h.

V.4.3.2. Procedure(s)

Microchips are inserted under ketamine anesthetic after animals clear quarantine. The chips are used to monitor body temperature and are pre-loaded in a specially designed needle/syringe device that is inserted subcutaneously between the shoulder blades. Once the chip is deposited, the needle is withdrawn and the skin is pinched between 2 fingers for about 10–20 seconds to ensure that the chip itself (about the size of a grain of rice) does not dislodge out through the puncture site. If a chip fails, a second chip will be inserted. If necessary, a drop of surgical glue will be placed at the puncture site to ensure that the chip stays in place.

Bone marrow collection: Described under general surgery section

VAP installation: Described under general surgery section

V.4.3.3. Post-surgical Provisions Incision monitoring, antibiotics and pain relief will be provided at the discretion of the staff veterinarian for bone marrow collection and VAP surgery.

V.4.3.4. Location All surgical procedures will take place in the VSD surgical suite.

V.4.3.5. Surgeon AFRRI veterinarians will perform all surgical procedures.

V.4.3.6. Multiple Major Survival Operative Procedures No major multiple surgical procedures will be performed on animals used in this protocol.

V.4.3.6.1. Procedures N/A

V.4.3.6.2 Scientific Justification N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections Trained personnel will carry out Injections (subcutaneous, Intramuscular) within the scope of VSD SOPs.

Drug injection: The drug in sterile PBS will be freshly prepared from a liquid formulation on the day of injection. Depending on the drug concentration and dilution, injection volume may vary 1 to 5 ml animal/injection.

Needle: Drug will be administered to restrained animals using 22–25G needle and 2-5 ml syringes.

V.4.4.2. Biosamples Chair restraint of rhesus primates for the purposes of phlebotomies was previously discussed as a technique to obtain blood samples over a short period of time that would reduce stress to the animals. Should technical issues with chairing and blood collection arise, sedation with ketamine may be used as an alternative at PI's discretion.

Blood will be collected on days as shown in Table 2. This will include 3 pre-irradiation time points. The hematological data from these points will be averaged and used as the control data for the specific animal. Additional blood collections will provide experiment specific data on hematology, blood chemistry, cytokine profile and CFU as indicated in Table 2.

Table 2: Blood collection; time-points and volumes (SA:2)

Time of blood draw	PK (0.5 ml)	CBC (0.5 ml)	CFU/chemistry/ cytokine (0.5 ml)	Culture (1.0 ml)	Total blood, ml
–7d	+	+	+		1.5
–3d		+			0.5
–2d	+	+	+		1.5
Day –1 (pre-TBI injection)					
Irradiation (Day 0)					
Day 1 (post-TBI injection)					

Day 2	+	+	+		1.5
Day 4	+	+	+		1.5
Day 6		+		+	1.5
Day 8	+	+	+		1.5
Day 9		+	+	+	2.0
Day 10	+	+	+		1.5
Day 11		+		+	1.5
Day 12		+		+	1.5
Day 13		+		+	1.5
Day 14		+		+	1.5
Day 15		+		+	1.5
Day 16		+		+	1.5
Day 17		+		+	1.5
Day 18		+		+	1.5
Day 19		+		+	1.5
Day 21	+	+	+		1.5
Day 28		+			0.5
Day 35	+	+	+		1.5
Day 42	+	+			1.0
Day 49		+			0.5
Day 60	+	+			1.0
				TOTAL	**33.0 mls**

V.4.4.3. Adjuvants N/A

V.4.4.4. Monoclonal Antibody (MAb) Production N/A

V.4.4.5. Animal Identification The animals are identified by indelible markings on their chest area and or inner thighs. If the primate is not tattooed upon arrival, a VSD colony number will be assigned and tattooed onto the chest or inner thigh.

V.4.4.6. Behavioral Studies No behavioral studies will be performed in this experimental protocol.

V.4.4.7. Other Procedures None

V.4.4.8. Tissue Sharing: None

V.4.4.9. Animal By-Products: Blood products such as irradiated blood or packed RBCs

V.4.5. Study Endpoint

Primary endpoint: The primary study endpoint is 60-day overall survival.

Study endpoints (primary)

1. Weight loss: Loss of more than 20% body weight over baseline. Normally animals will be weighed twice a week; however, if inappetence sets in then we will monitor weight at frequent intervals (daily if required).
2. Inappetence: Complete anorexia for 2 days with deteriorating conditions based on clinical examination.

3. Weakness/inability to obtain food or water: Inability or extreme reluctance to stand that persists for 4–6 h, assuming the animal has recovered from anesthesia.
4. Minimal or absence of response to stimuli (animal does not move when prodded), assuming the animal has recovered from anesthesia.
5. Core body temperature: below 35.9 °C following a period of febrile neutropenia ($\leq 500 \times 10^3$ cells/µl), monitoring by way of microchip will be done on a daily basis.
6. Severe acute anemia: (<40 g/L hemoglobin, <13% hematocrit).
7. Infection documented by blood culture (septicemia) and accompanied by severe systemic signs of illness.
8. Other signs of severe organ system dysfunction with a poor prognosis as determined by a veterinarian that may need additional diagnostic tests:
   - 8.1 Respiratory: severe dyspnea, severe cyanosis.
   - 8.2 Gastrointestinal: severe vomiting or diarrhea, evisceration (immediate euthanasia required). **Note:** Transient vomiting and diarrhea are expected results of total-body irradiation (TBI). Therefore, as criteria for euthanasia, these symptoms should be severe and life-threatening i.e., hemorrhagic diarrhea to the point of causing anemia as listed above or severe uncompensated dehydration.
   - 8.3 Urogenital: renal failure characterized by elevated BUN, and creatinine.
   - 8.4 Neuronal: CNS depression, seizures, paralysis of one or more extremities.
   - 8.5 Integumentary: Non-healing wounds, repeated self-trauma, severe skin infections, indicating severe organ system dysfunction with a poor prognosis.

Technical note: One or more criteria will be used to euthanize animals in consultation with a veterinarian.

V.4.6. Euthanasia Animals will be euthanized according to current American Veterinary Medical Association (AVMA) guidelines. All animals first will be anesthetized with ketamine (IM, 5–10 mg/kg). Animals will be euthanized with sodium pentobarbital (IV, 100 mg/kg), under the guidance and supervision of a staff veterinarian. After euthanasia solution has been administered, the animals will be examined by VSD veterinarian or technician to confirm death. A full-body necropsy and histopathologic analysis will be performed after death has been confirmed.

V.5. Veterinary Care Animal procedures performed at AFRRI will be in compliance with the *Animal Welfare Act* and the *Guide for the Care and Use of Laboratory Animals*. The AFRRI facility is accredited by Association for Assessment and Accreditation of Laboratory of Animal Care (AAALAC) International.

V.5.1. Husbandry Considerations All NHPs will be housed according to IAW VSD SOPs.

V.5.1.1. Study Room All animals will be assigned room(s) designated by VSD staff.

V.5.1.2. Special Husbandry Provisions Routine and emergency veterinary medical care: Animals will be quarantined for 4–6 weeks on arrival and chair-trained during the quarantine period. Each animal will be observed twice daily for evidence of pain or distress. Body weights will be determined at least once prior to treatment, on the first day of treatment, and at periodical intervals or when chaired for blood collection. Body

temperature will be determined using a microchip installed in irradiated animals. Regular temperature will be measured during blood collection schedule when the animals are chaired. Animals will be qualitatively assessed twice daily (a.m. and p.m.) for general behavioral status and food consumption. Additional observation points will be added based on recommendation from attending veterinarian and will depend on the health of experimental animals. Appropriate standard operating procedures will be followed by staff veterinarians in the event of pain or distress.

V.5.1.3. Exceptions N/A

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care All primates will be quarantined for 4–6 weeks prior to any experiment. Animals will be tested for TB. Also, note the requirements given under V.3.3.2 and V.3.3.3 above.

All NHPs will be observed at least twice per day by veterinary staff (IAW VSDM SOP 2002, Animal Health Rounds). A veterinarian and a veterinary technician will provide 24 h emergency care to the animals if needed (IAW VSD SOPs). When animals become ill or debilitated, a veterinarian will be contacted immediately to assess the animal and provide adequate/emergency care. If the animal becomes moribund, it will be euthanized according to current American Veterinary Medical Association (AVMA) guidelines.

V.5.2.2. Emergency Veterinary Medical Care The on-call veterinary officer will be available via telephone 24 h daily. The on-call roster is located outside the VSD conference room (b)(6) on the bulletin board and at the security watch desk. These procedures are in accordance with VSD SOPs.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy In these studies, all primates will receive regular enrichment (IAW VSDM SOPs).

V.5.3.2. Enrichment Restrictions None

VI. Study Personnel Qualifications and Training

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1	(b)(6)
				1	
				1	
				1	
				1,2,3, 4, 5, 6,	
				1,2,3, 4, 5, 6,	

Procedure and manipulation codes:
Code 1 = Animal handling and euthanasia
Code 2 = Surgery (aseptic technique) pre- and post-operative care
Code 3 = Injections (3a–ip, 3b-sc, 3c-gavage)—Can say 3 if trained in all manipulations or add 3d-iv
Code 4 = Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5 = Tissue collection (after euthanasia)—Need not be specific

Code 6 = Implantation (provide details)
Code 7 = Others—Provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery

VII. BIOHAZARDS/SAFETY

VIII. ENCLOSURES References:

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(b)(4)

X. ASSURANCES

Protocol Title:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard/Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal-use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): **I AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator (Printed Name) | Principal Investigator (Signature) | 8/26/2013 (Date)

Abbreviations:

ALC – absolute lymphocyte count	**HPP-CFC** – high proliferative potential colony forming cells
ANC – absolute neutrophil count	**IM** – intra muscular
BFU-E – Burst forming unit-erythroid	**ITP** – idiopathic thrombocytopenia purpura
BUN – Blood Urea Nitrogen	**MNC** – mono nuclear cells
CBC – complete blood count	**MoF** – multi-organ failure
CFC – colony forming cells	**MST** – mean survival time
CFU – Colony forming unit	**NHP** – non-human primate
CFU-GEMM – granulocyte, erythrocyte, monocyte and megakaryocyto	**PAHA** – primate humoral antibody
CFU-GM – granulocyte, monocyte	**PBS** – phosohate buffer saline
CHO – Chine Hamster overy	**PCR** – polymerase chain reaction
cMpl-r – a thrombopoietin receptor	**PK** – pharmacokinetics
DES – dose escalation study	**PLT** – platelet related parameters
DRR – dose response relationship	**rhSCF** – recombinant human stem cell factor
DTPA – radioisotope chelating agent	**sc** – subcutaneous
ECL probe – electrochemicalluminescece probe	**SOP** – standard operating procedure
Fc – Fragment crystallizable, part of Fc receptor	**TBI** – total body irradiation
FN – febrile neutropenia	**TPO** – thrombopoietin
G-CSF – granulocyte-colony stimulating factor	**VAP** – vascular access port
GLP – Good Laboratory Practices	**VSD** – veterinary science department
HAHA – human humoral antibody	
H-ARS – hematopoietic acute radiation syndrome	
HPP-CFC – high proliferative potential colony-forming cells	

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (AUGUST 2011)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

Department of Defense Sponsored Animal Research Proposal
Signature Coordination Sheet

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	7/18/2013	2nd Resubmission Date:	
Approved/Returned for Revision:	8/7/2013	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	10/8/2013	**EXPIRATION DATE:**	10/22/2016
Approved/Returned for Revision:	10/23/2013	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and *subsection*, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Evaluation of tilorone as a novel radiation countermeasure candidate using laboratory mouse model (*Mus musculus*)

IV. PRINCIPAL INVESTIGATOR:

(b)(6) M.D. Date signed:
Radiation Countermeasures, SRD
Tel: (b)(6) Fax: (b)(6)
E-mail: (b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date
Head, Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) **PhD** Date

Statistician

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI

Telephone: (b)(6) Fax (b)(6)

(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department

Telephone (b)(6) Fax: (b)(6)

(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **PhD**

IACUC Chair, SRD, AFRRI

Telephone (b)(6) Fax: (b)(6)

(b)(6)

PROTOCOL TITLE: Evaluation of tilorone as a novel radiation countermeasure candidate using a laboratory mouse model (*Mus musculus*)

PRINCIPAL INVESTIGATOR:
(b)(6) M.D. Date signed:
Radiation Countermeasures, SRD
Tel: (b)(6) Fax: (b)(6) E-mail: (b)(6)

CO-INVESTIGATOR(S):

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER:

I. NON-TECHNICAL SYNOPSIS Exposure to ionizing radiation causes rapid hematopoietic cell damage and bone marrow suppression. Radiation also induces damage of the gastrointestinal (GI) system. This multiple organ failure can result in human and animal death (b)(6),(b)(4) Over several decades, many candidate drugs have been tested. However, highly effective countermeasures are still lacking. Because radiation injuries are heterogeneous disorders that influence many pathophysiological pathways, a broader approach may increase the likelihood that we can prevent damage or sustain repair in mass casualty scenarios. We propose to explore the protective and mitigative/therapeutic effects of small molecule tilorone dihydrochloride (tilorone), on mouse hematopoietic and GI system after different doses of total-body γ-irradiation (TBI).

II. BACKGROUND

II.1. Background

Introduction: Nuclear proliferation, terrorist activity, and the distribution of nuclear and radioactive materials increase the risk of radiation injury incidents. Injury after prompt radiation of hematopoietic and GI tissue occurs over a period of hours to days (2, 3). No countermeasures for acute radiation syndrome (ARS) have been approved by the FDA, partly due to the fact that the mechanisms of radiation-induced multiple organ cellular and molecule damage and mortality are extremely complex and poorly understood (4).

(b)(4)

(b)(4) Small-molecule tilorone dihydrochloride (tilorone) is one of several compounds that have been tested in a drug screening program, according to previous reports (5, 6). Preliminary data from the screening program demonstrated that tilorone at very low dosage (7.5 mg/kg) significantly enhanced 30-day survival in lethally γ-irradiated mice. Administration of tilorone as a single subcutaneous (SC) injection 24 h before or 6 h after irradiation rescued 69% and 56% of mice from 9.0 Gy irradiation-induced death compared with 25% survival for vehicle-treated mice. In addition, (b)(4)

(b)(4)

(b)(4) Consistent with AFRRI studies, Kim and colleagues recently reported a radiation-mitigation function by tilorone after high throughput screening of small molecule libraries for modifiers of radiation responses in murine T lymphocytes (7). However, the underlying mechanisms of tilorone aiding the survival of irradiated mice are unclear.

Tilorone is the first to be recognized as an orally active interferon (IFN) inducer, developed in the 1970s by Richardson Merrell, Inc. (US Patent 3592819, filed Dec. 30, 1968, Ser. No. 788,038) (8). Tilorone is a broad-spectrum antiviral agent active *in vivo* (9, 10) for viral infection therapeutics and prophylaxis (flu, hepatitis A, B, herpes simplex). Although it has been widely used in the Ukraine since March 2005, tilorone is not available in the USA as a pharmaceutical-grade drug.

Drug-toxicity assays showed that the LD_{50} for a single dose, oral administration in mice is 959 mg/kg, and intraperitoneal administration is 145 mg/kg (Krueger, Mayer). Although the drug has a high capacity to induce interferon *in vivo*, no antiviral effects were seen in cell cultures, even at high concentration, suggesting an indirect stimulation *in vivo* (8).

AFRRI toxicity studies: Limited toxicity studies were conducted at AFRRI using the up-and-down method for evaluating drug toxicity. CD2F1 mice (6/group) were SC injected with a single dose of 30–150 mg/kg tilorone in PBS and observed for adverse effects. All mice injected with 150 mg/kg of tilorone died within 5 minutes of drug administration; our Veterinary Sciences Department pathologist concluded that death was due to CNS (central nervous system) effects rather than systemic toxicity. When the tilorone dose was reduced to 50 mg/kg, no adverse effects were monitored and tilorone was considered safe for single administration.

Hypothesis of the mechanisms of increased survival in radiated animals by tilorone: It was well known that exposure to ionizing radiation leads to rapid hematopoietic cell damage and bone marrow suppression. Radiation also induces damage of the GI system, such as loss of intestinal crypts, damage of crypt stem cells, and breakdown of the GI mucosal barrier. Failure of these organs can result in human and animal death (b)(6),(b)(4) As a countermeasure candidate, tilorone significantly enhanced 30-day survival in lethally γ-irradiated mice. It may reverse the effect of radiation on these organs and protect them. In our study, the mechanisms of the effect of tilorone on these hematopoietic and GI systems will be addressed.

Studies at the molecular biological level showed that tilorone is an effective activator of the hypoxia inducible factor-1 (HIF-1) that provides prophylaxis against stroke and spinal cord injury (11). Interestingly, (b)(4)

(b)(4)

(b)(6),(b)(4) REDD1 is a target of HIF-1, which is induced strongly under hypoxic conditions (13, 14) and subsequently inhibits a downstream protein kinase, mTOR, to regulate cell growth (15). We hypothesize that tilorone-induced HIF-1 activation is associated with its radio-protective and/or mitigative effects.

In this study, we propose to evaluate the protective and mitigation/therapeutic effects of tilorone on mouse hematopoietic and GI system after different doses of γ-radiation. We expect that the preliminary data from our study could provide sufficient information to support our hypotheses that tilorone is a valuable radiation countermeasure candidate,

and guide us to further characterize the effects and mechanisms of tilorone-induced survival in γ-irradiated mice.

II.2. Literature Search for Duplication
II.2.1. Literature Sources Searched: BRD, NIH Reporter, PubMed, FEDRIP
II.2.2. Date of Search: June 20–August 27, 2013
II.2.3. Period of Search
All available years. BRD: FY1998–FY2011; NIH Reporter and PubMed: includes MEDLINE (1966–present) and OLDMEDLINE (1950–1965); FEDRIP (up to August 27, 2013)
II.2.4. Key Words of Search: tilorone and ionizing radiation
tilorone and hematopoietic cells and radiation damage
tilorone and gastrointestinal tissue and radiation damage
tilorone and radiation countermeasures
tilorone and stress signaling
II.2.5. Results of Search: Articles for all tilorone studies = 407
Articles for tilorone and radiation studies = 9
Articles for tilorone and ionizing radiation studies = 4
Articles for tilorone and hematopoietic cells studies = 10
Articles for tilorone and gastrointestinal cells studies = 2
Articles for tilorone and radiation and hematopoietic cells studies = 1
Articles for tilorone and radiation and gastrointestinal cells studies = 0
Articles for tilorone and stress signaling = 0

Most of the articles suggested that tilorone is an inducer of interferon-1 (IFN-alpha), which induces natural killer (NK) cells and lymphocyte activity. Orally administrated tilorone can induce IFN-α production in the intestine and systemically (Munakata et al. 2008). Ratan, RR et al. (11) reported that tilorone or its analogs are novel potent activators of hypoxia-inducible factor-1 (HIF-1), which provides prophylaxis against stroke and spinal cord injury. Four articles (one *in vivo* and three *in vitro* studies) reported the effects of tilorone on ionizing radiation (including 90Sr, gamma- and helium-alpha irradiation), and the *in vivo* study was focused on helium-alpha irradiation (5). Recently, Kim, K et al. (7) reviewed the data from a drug discovery high-throughput screening (HTS) *in vitro* program (assay system endpoints included radiation-induced genotoxicity and DNA damage in yeast and apoptosis in murine lymphocytes) and suggested that tilorone has radioprotection and moderate mitigating activity. Their conclusion is that in *vitro* study has limitations and is unable to fully recapitulate all aspects of the complex *in vivo* acute radiation response.

The proposed study has no overlapping with published data.

III. OBJECTIVE/HYPOTHESIS: In the present study, we propose to evaluate the radiation-protective (before irradiation) and mitigative (post-irradiation) effects and mechanisms of tilorone on survival of γ-irradiated mice.

IV. MILITARY RELEVANCE: Currently, there is no safe and non-toxic radioprotective agent available. Military personnel potentially are exposed to ionizing radiation in various scenarios. Thus, there is an urgent need to develop clinically safe, potent and non-toxic radioprotective agents. Developing countermeasures to radiation exposure is

a top priority for the U.S. Department of Defense. We propose to conduct a study to further explore the protective and mitigative/therapeutic effects of this very promising candidate of countermeasure, tilorone, on survival of mice after exposure to different doses of γ-radiation.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

Mice: Twelve- to 14-week-old male CD2F1 mice (Harlan Laboratories, Indianapolis, IN) will be used in all experiments.

Animals will be housed in plastic micro-isolator cages on autoclaved hardwood-chip contact bedding with free access to food and acidified water (pH: 2.5–3.0) in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC-I). Animals will be grouped randomly (criteria-weight within 10%, etc.) for different treatments prior to each experiment and re-housed (6–8 to animals per cage).

Tilorone drug preparation and administration in mice model: Mice will be weighed before drug administration. Tilorone dihydrochloride (tilorone) is a water-soluble, small molecule compound and will be purchased from Sigma-Aldrich (St. Louis, MO). It will be freshly prepared and administered at a single dose of 40 mg/kg subcutaneous (SC) injection in 0.1–0.25 ml volume based on data from AFRRI's screening program. Phosphate buffered saline (PBS) will be used as solvent and vehicle control in animal studies.

Radiation: Mice will be placed in ventilated Plexiglas containers and exposed bilaterally to γ-radiation in the AFRRI cobalt-60 facility at doses of 0 (sham-irradiation), 7, 9, 10, or 12 Gy at a dose-rate of 0.6 Gy/min. Sham-irradiated mice will be treated exactly the same way as the γ-irradiated animals, except the cobalt-60 sources will not be raised from the shielding water pool. After irradiation, mice will be returned to their specific cages, thereafter animals will be monitored for 30 days after 7, 9 or 10 Gy, and for 20 days after 12 Gy irradiation. For survival studies there will be 20 mice/group. To evaluate the effects of tilorone on survival of mouse hematopoietic and GI tissues, each treatment group will include 6 mice and two separate experiments will be conducted.

V.1.1. Experiment 1

Survival study

This study will consist of 5 treatment groups, naïve, vehicle, and tilorone-treatment at 24 h before (–24 h), or 6 or 24 h postirradiation (+6 h or +24 h). A single dose of tilorone (40 mg/kg) or PBS vehicle will be injected subcutaneously into mice. Drug-treated mice weighing 25 ± 2 g will receive 0.1 ml of tilorone; injection volume will be adjusted for mice weighing more than 28 g. Control mice will receive 0.1 ml of vehicle (PBS). All SC injections of tilorone and vehicle will be performed aseptically in the nape of the neck with a 23G needle. Each treatment group will receive four doses (7, 9, 10 or 12 Gy) of radiation at a dose rate of 0.6 Gy/min in the AFRRI cobalt-60 radiation facility. Animals will be irradiated in Plexiglas boxes (8 animals/box). After irradiation, the mice will be returned to their specific cages, thereafter animals will be monitored for 20–30 days.

V.1.2. Experiment 2

Evaluate the effects of tilorone on survival of mouse hematopoietic tissues after γ-irradiation

CD2F1 mice (6/group) will be given 7 or 9 Gy irradiation or sham-irradiation as control. A single SC dose of tilorone (40 mg/kg) or vehicle control will be administrated to mice 24 h before or 6, or 24 h after irradiation. At different time points (1, 7, 14, 21 and 28 days after radiation), blood and bone marrow (BM) from femurs and humerus, sternum, and spleen tissue will be collected from the sham-irradiated and γ -irradiated mice for various experimental assays. Animals will be anaesthetized prior to blood collection via cardiac puncture and then a confirmatory cervical dislocation will be performed before tissue collection. The collected blood will be analyzed for hematological study. The total myeloid cell numbers and the viability from individual mouse BM will be counted at indicated time points and the BM cell clonogenicity will be determined by colony-forming assay (16) to evaluate the effects of tilorone on survival of mouse hematopoietic stem and progenitor cells (HSPC). Cell death and apoptosis markers and cell surface phenotypes will be analyzed by flow cytometry on freshly collected BM tissue. Immuno-fluorescence staining, immunoblotting (western blot) assay for γ-H2AX, TUNEL assay and neutral comet assay will be used to evaluate radiation-induced DNA damage.

BM pathological changes will be evaluated in HE-stained mouse sternum longitudinal sections. BM structure, BM hemorrhage and cellularity (especially megakaryocyte restoration) (17) will be examined.

The production of cytokines and chemokines in mouse serum and HSPC and spleen cell lysates will be evaluated using cytokine array and ELISA assays. Levels of hematopoietic cytokines and inflammatory cytokines including G-CSF, Flt-3 ligand, IL-1, IL-4, IL-6, IL-8, IL-10, and interferon will be measured.

V.1.3. Experiment 3

Examine the radioprotective and mitigative/therapeutic effects of tilorone on mouse gastrointestinal (GI) tract

At different time points (12 h, 3.5 days, or 10 days after radiation), blood and jejunum tissue will be collected from tilorone-treated and vehicle-treated mice after sham- or γ-irradiation (10 or 12 Gy) for mechanism studies. Animals will be anaesthetized prior to blood collection via cardiac puncture and then a confirmatory cervical dislocation will be performed before tissue collection.

HE-stained mouse intestinal tissue slides will be examined for intestinal mucosal surface area, crypt cell apoptosis, intestinal crypt colony generation, and villi. We will collect segments of proximal jejunum 12 h, 3.5 days or 10–12 days after TBI (0, 10 or 12 Gy) to study the effects of tilorone on mouse intestinal crypts and GI mucosal barrier protection.

Plasma citrulline concentrations will be determined. The plasma level of citrulline is a well-validated biomarker for functional enterocyte mass. A decrease in citrulline levels is a characteristic of radiation injury. The correlation between plasma citrulline levels and more conventional markers of intestine radiation injury will be addressed (18).

Cytokine release from jejunum mucosal will be measured and compared in different treatment groups. The effects of tilorone on radiation-induced inflammatory cytokine production in mouse jejunum mucosa will be evaluated.

Radiation-induced intestinal bacterial translocation in the liver, heart blood and spleen, and radiation-induced sepsis will be quantified by bacterial translocation assay (19). Radiation-induced bacterial translocation starts around day 7 and peaks around 2 weeks after TBI. Analysis of bacterial translocation will be performed on days 10–12 after exposure to 10 or 12 Gy TBI. The samples collected from heart blood, liver, and spleen will be applied to a special medium and cultured for bacteria growth. Single colonies of isolated microorganisms will be observed for their morphological characteristics. Gramstain characteristics will be determined by Gram staining. They will be subcultured and a pure culture will be analyzed by a Vitek 2 Compact automated system to determine the type of bacteria.

Total number of animals: 1696 male CD2F1 mice will be used.

Experiment 1 Survival study:
Naïve, vehicle- and tilorone-treated (24 h before, 6 h and 24 h after TBI) = 5 groups × 7, 9, 10 or 12 Gy TBI (4 radiation doses) × 20 mice/group = 400 mice

Experiments 2 and 3: Sham-irradiated mice will be shared between Experiments 2 and 3. Experiment 3 will need to use an extra 3.5-day post-radiation group.

Sham-irradiated section = 2 groups (vehicle and drug) × 6 animals/group × 6 time points after drug treatment = 72 animals

Irradiated section for Experiment 2 = 2 groups (vehicle, drug) × 6 animals/group × 3 drug-giving times × 5 sample collection time points after irradiation × 2 radiation doses (7 and 9 Gy) = 360 animals

Irradiated section for Experiment 3 = 2 groups (vehicle and drug) × 6 animals/group × 3 drug-giving times × 3 sample collection time points after irradiation × 2 radiation doses = 216

Sum of 72 + 360 + 216 animals = 648 animals × two experiments (statistically required) = 1296

Total animals required = 400 + 1296 = 1696

V.2. Data Analysis
The differences in 20- and 30-day survival of mice will be analyzed by Kaplan-Meier analysis. Differences between means will be compared by ANOVA and by Student's *t* tests. $P<0.05$ will be considered statistically significant. Results will be presented as means ± standard deviations.

V.3. Laboratory Animals Required and Justification
V.3.1. Non-animal Alternatives Considered: In AFRRI's drug-screening program, the preliminary data of the effect of tilorone on animal survival after irradiation are based on *in vivo* studies. As a start, we would like to conduct *in vivo* study first because the

responses of various tissues and organs to the radiation and the radio-protective effect of tilorone are linked directly to that in the previous survival study. Radiation-induced adjustment in cellular tissue homeostasis triggered by various molecular responses related to inter- and intracellular signaling causes both acute and late effects depending upon the interactions in different organs and tissues in animal models. The morphological changes in tissues can be seen only in tissues obtained from the animals. The hematological analysis and cytokine and chemokine measurements can be performed only from blood collected from the animals. Therefore, the observation of changes in hematopoietic and GI systems after exposure to radiation will be more meaningful using an *in vivo* model for the proposed study.

V.3.2. Animal Model and Species Justification
The murine model is the most well-defined animal model for research. The mouse was chosen over other animal models because of extensive published data that is readily available for comparison, review and analysis. The mouse model has significant hematological and immunological similarities to higher animals and humans. AFRRI's staff has extensive experience with mice, including male CD2F1 in radiation injury and countermeasure research.

V.3.3. Laboratory Animals

V.3.3.1. Genus/Species
Mice (*Mus musculus)*

V.3.3.2. Strain/Stock
CD2F1

V.3.3.3. Source/Vendor
CD2F1 from vendor approved by VSD (Harlan-Indianapolis, IN, preferred)

V.3.3.4. Age
12–14 weeks

V.3.3.5. Weight
24–30 g

V.3.3.6. Sex
Male

V.3.3.7. Special Considerations The mice must be pathogen-free, specifically for the following agents: Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus morgani and Pasteurella. In addition, all mice need to be free of the following viral agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epizootic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), Encephalitozoon cuniculi, CAR bacillus, Mycoplasma pulmonis, and Clostridium piliforme. Endoparasite- and ectoparasite-free.

V.3.4. Number of Animals required (by species)

Animal (genus, species and common names)	Original	All previous amendments	This amendment
CD2F1mice	1696		

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement
None.
V.3.5.2. Reduction
We shall make every effort to minimize the use of non-necessary animals. Control groups will be shared between experimental groups. At every stage of the research, plans will be reevaluated in light of incoming data in an attempt to reduce the numbers of experiments and groups.

V.3.5.3. Replacement
None.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information
V.4.1.1.1. Number of Animals
V.4.1.1.1.1. Column C: 0 (# of animals)
V.4.1.1.1.2. Column D: Vehicle- or drug-injected and sham-irradiated mice 144 (# of animals)
V.4.1.1.1.3. Column E: All irradiated mice 1552 (# of animals)
The maximum experimental period is 30 days.
1696 (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1	Survival study			400	400
1	IR-induced hematopoietic syndrome		72	720	792
2	IR-induced GI syndrome		72	432	504
Totals:			144	1552	1696

Mice in pain category D (144) will be anesthetized for blood collection by cardiac stick, which causes more than momentary pain and distress. Mice in pain category E (1552) will be irradiated and they will not receive any anesthesia or analgesia to relieve them from radiation-induced pain/distress.

V.4.1.2. Pain Relief/Prevention
V.4.1.2.1. Anesthesia/Analgesia/Tranquilization
For blood sample collection, animals will be anaesthetized with isoflurane 1–3% in

100% oxygen using the anesthesia machine in the AFRRI IACUC-approved rodent procedure room. Blood will be drawn by cardiac puncture (or from the inferior vena cava if the "cardiac stick" method is unsuccessful) in mice anesthetized by isoflurane inhalation. Animals will be euthanized by cervical dislocation immediately after blood collection and then tissue samples will be taken.

V.4.1.2.2. Pre- and Post-procedural Provisions N/A
V.4.1.2.3. Paralytics: No paralytic agent will be administered.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched
AGRICOLA, PubMed

V.4.1.3.2. Date of Search
June 20–July 30, 2013

V.4.1.3.3. Period of Search: List AGRICOLA–1976–present
PubMed: includes MEDLINE (1966–present) and OLDMEDLINE (1950–1965)

V.4.1.3.4. Key Words of Search
Irradiation or Pain and Distress or Alternatives or Animal Model; Mouse or Irradiation or Pain or Alternatives; Tilorone or Treatment or Irradiation or Mouse; Blood Collection or Mouse or Cardiac Puncture or Alternative.

V.4.1.3.5. Results of Search
Irradiation with ionizing radiation is not a painful process but it induces various changes in the body and kills hematopoietic cells and gastrointestinal epithelium. In irradiated animals, the immune response is compromised and opportunistic infections may ensue. There is no less painful or distressful alternative found that can be used to develop protective and mitigating agents for irradiation. No references were found suggesting any effect of tilorone, or PBS on pain or distress. There are alternatives for blood collection by cardiac puncture in a mouse, such as tail vein and facial vein blood draw, etc. However, none of these alternatives can provide more than 0.1–0.2 ml of blood. We will need about 1 ml of blood sample to run all the tests for our experiments. Therefore, we will be using cardiac puncture for blood collection. Blood also may be drawn from the inferior vena cava if the "cardiac stick" method is unsuccessful. However, we will do so while the animal is anesthetized and as a terminal procedure. Therefore, it will not cause more than momentary pain or distress.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification
There is no alternative to the *in vitro* system and no less painful/distressful *in vivo* system is available to test new therapies against acute irradiation syndrome. Thus, we have to use this mouse model to test the protective and mitigative/therapeutic effects of tilorone on mouse hematopoietic and gastrointestinal systems after different doses of gamma-irradiation.

Furthermore, we will need to collect about 1 ml of blood sample to run all the tests for our experiments in the proposed study. However, there is no other alternative available

to obtain 1 ml of blood from a mouse other than via use of a cardiac stick. Therefore, we will be using cardiac puncture for blood collection, while the animal is anesthetized. Therefore, it will not cause more than momentary pain or distress.

V.4.2. Prolonged Restraint N/A

V.4.3. Surgery
V.4.3.1. Pre-surgical Provisions N/A

V.4.3.2. Procedure(s) N/A

V.4.3.3. Post-surgical Provisions N/A

V.4.3.4. Location N/A

V.4.3.5. Surgeon N/A

V.4.3.6. Multiple Major Survival Operative Procedures

V.4.3.6.1. Procedures N/A

V.4.3.6.2 Scientific Justification N/A

V.4.4. Animal Manipulations
Mice will be anesthetized by isoflurane. Anesthesia will be induced in a properly scavenged chamber using 2–3% Isoflurane in 100% oxygen. Once they are recumbent and start to take slow and deep breaths, they will be maintained on 1–2% isoflurane via nose cone. Mice will be placed in dorsal recumbence. Toe pinch will be used to make sure they are deeply anesthetized before doing the procedure. A 23–27G needle with bevel up will be inserted through the diaphragm lateral to the xiphoid process and will direct the needle forward and medially toward the heart. Approximately 1 ml of blood will be collected by gentle pulling of the plunger. At the end of blood collection, mice will be immediately euthanized by cervical dislocation while deeply sedated.

V.4.4.1. Injections We will use 25- to 30-gauge needles for SC injections of 0.1 ml PBS or tilorone in the nape of the neck. PBS will be used as control. Injections will be carried out in AFRRI IACUC-approved procedure rooms.

V.4.4.1.1. Pharmaceutical Grade Drugs N/A

V.4.4.1.2. Non-Pharmaceutical Grade Drugs
Tilorone is not available in the USA as a pharmaceutical-grade drug. It will be purchased from Sigma-Aldrich Inc., St. Louis, MO. Tilorone is water soluble and can be dissolved in PBS (using PBS will allow for an acceptable pH level for SC injection) for injection solution. According to standard practice in approved AFRRI protocols, there have been no injection site reactions in these mice using the proposed volume (0.1 ml) and route (SC) for tilorone in PBS.

The drug is supplied as sterile and will be dissolved in sterile filtered PBS under aseptic

conditions. A solution of 40 mg/ml tilorone in PBS has a pH of 6.6, which is well within the acceptable range for SC injections as stipulated by IACUC (pH 6–8). The endotoxin titer for the Sigma-Aldrich research grade drug was <0.068 EU/mg; the FDA limit is 0.15 EU for a 30 g mouse^{-1}. Tilorone will be freshly prepared using PBS.

V.4.4.2. Biosamples

Blood, bone marrow, spleen, and jejunum tissue will be collected from the sham-irradiated and gamma-irradiated mice. Mechanism studies will be performed using these tissue samples. Heart, liver, and jejunum will be collected for bacterial translocation study.

V.4.4.3. Adjuvants N/A
V.4.4.4. Monoclonal Antibody (MAb) Production N/A
V.4.4.5. Animal Identification N/A
V.4.4.6. Behavioral Studies N/A
V.4.4.7. Other Procedures N/A
V.4.4.8. Tissue Sharing N/A
V.4.4.9. Animal By-Products N/A

V.4.5. Study Endpoint

In the proposed study, the animals will be euthanized by a maximum of 20–30 days. We will follow the IACUC policy letter (Operating Policy #10) on early end points. We do anticipate that the animals will become morbid during this study. However, we will not consider death as an endpoint in this study. If the animals become morbid as described in the policy letter on early end points, we will increase monitoring and will euthanize the animals immediately if they are found to be in moribund condition. Our staff is experienced in evaluating the health status of mice. However, we will consult the VSD veterinary staff if we have any questions evaluating the health status of our animals. We will use the clinical signs as described in the IACUC policy letter on early end points to categorize the animal as morbid or moribund.

[1] Limit for endotoxin is 5.0 Endotoxin Units (EU) per kg for the maximum dose to be administered in a one hour period. Center for Drug Evaluation and Research, "Guideline on Validation of the Limulus Amebocyte Lysate Test as an end product Endotoxin test for human and animal parenteral drugs, biological products and medical devices", p10.

VIII. Appendix A: Clinical Observations with Criteria for Euthanasia (Rodent)

DATE:	Time:	Animal ID#	Score
Appearance:			
	Normal (smooth coat, clear eyes/nose)		0
	Hunched and/or fluffed		1
	Ocular discharge, and/or edema		3
	Emaciated, dehydrated (skin tent) **		5
	Soft stools (fecal matter around anus)		5
	Bloody diarrhea		9
Respiratory rate:			
	Normal breathing		0
	Increased breathing (double normal rate, rapid, shallow)		6
	Abdominal breathing (gasping +/- open mouth breathing)*		12
General Behavior:			
	Normal (based on baseline observations)		0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)		1
	Decreased mobility		2
	Ataxia, wobbly, weak**		6
	Inability to stand*		12
Provoked Behavior:			
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))		0
	Subdued; responds to stimulation (moves away briskly)		1
	Subdued even to stimulation (moves away slowly)		3
	Unresponsive to gentle prodding **		6
	Does not right when placed gently on side within 5 seconds*		12
TOTAL			_____

Definitive criteria for morbidity:
Weight Loss up to 10% baseline (if available)**
Pale, white mucous membranes/ skin * *
Fever >104F/40C (if available)**

Definitive criteria for moribundity:
Weight Loss > 20% OR <15g (adult mouse)*
Blue mucous membranes/ skin (cyanosis)*
Cool to the touch or <86F/30C (if available)*

**** Regardless of score, notify appropriate person immediately.**

*** Regardless of score, immediately euthanize (death is imminent)**

Score:
< 6 Normal
6 - 9 Morbid: Monitor at least 3 times per day; notify appropriate personnel immediately
> 10 Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*)
Any single criteria of 12* euthanize immediately; consider as 'found dead'

V.4.6. Euthanasia: Animals in experiments involving blood collection will be anesthetized with isoflurane before blood collection. After blood collection, confirmatory cervical dislocation will be performed while the animal is still anesthetized.

For the non-blood collection groups, before tissue collection animals will be euthanized by CO_2 inhalation as per the AVMA Guidelines on Euthanasia.

Time of euthanasia: For Experiment 1, time of euthanasia will be 30 days after animals receive 7, 9 or 10 Gy of irradiation, and 20 days after receiving 12 Gy irradiation; for Experiment 2 it will be 1, 7, 14, 21 and 28 days post-irradiation; and for Experiment 3 it will be 12 h, 3.5 and 10–12 days post-irradiation.

V.5. Veterinary Care

V.5.1. Husbandry Considerations: Husbandry care will be done as per VSD SOPs. Mice are social animals and will be housed together in groups. Nesting material/igloos/shelters will be provided in each cage. Animals will receive Harlan Teklad Rodent Diet (w) 8604 and water *ad libitum*. Acidified water (pH 2.5–3.0) will be provided. Cages will be regularly changed and cleaned by VSD staff. Animal rooms will be kept on a 12 h/12 h light/dark cycle.

V.5.1.1. Study Room As assigned by VSD

V.5.1.2. Special Husbandry Provisions N/A

V.5.1.3. Exceptions

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care
Routine veterinary care will be provided as per VSD SOPs. Experimental animals will be observed at least once daily by our staff. However, if the animals become morbid and more monitoring is needed, our staff will increase monitoring to 2–3 times daily. Our staff will monitor the animals outside normal VSD working hours (after 5 pm in the evening and before 7 am in the morning) to provide a wider range of monitoring for morbid animals.

V.5.2.2. Emergency Veterinary Medical Care
On weekends and holidays, morning rounds will be done by VSD animal caretakers and during evening rounds it will be done by VSD veterinary technicians. Animal caretakers and veterinary technicians can contact the on-call veterinarian if needed. Our (PI) staff will provide after-hours coverage (outside of the routine veterinary coverage provided by VSD staff) on an as-needed basis as determined by the VSD veterinarians. Our staff will contact the on-call veterinarian if needed.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy
Nestling pads, shelters, and/or igloos will be provided in each cage during bedding changes.

V.5.3.2. Enrichment Restrictions N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

Name	Degree	Training	Date	Procedures	Yrs/Mos Experience
(b)(6)				1, 3b, 4a, 5, and 7 (tail vein injection) anesthesia	(b)(6)
				1, 3b, 4a, and 5, anesthesia	
				1, 3b, 4a, and 5, anesthesia	
				1, 3b, 4a, and 5, anesthesia	

Procedure and manipulation codes:
Code 1 = Animal handling and euthanasia
Code 2 = Surgery (aseptic technique) pre- and post-operative care
Code 3 = Injections (3a-ip, 3b-sc, 3c-gavage)—Three if trained in all manipulations or add 3d-iv
Code 4 = Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5 = Tissue collection (after euthanasia)—doesn't need to be specific
Code 6 = Implantation (provide detail here)
Code 7 = Others — (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY

All investigators and personnel will use appropriate protective gear while in the vivarium, as described by the VSD. All listed personnel are current regarding safety training and medical surveillance. All listed personnel have completed the Animal Care and Use in Research and Education training (AALAS learning library) courses. Recently they took three refresher courses: Common Compliance Issues, 8th Edition of the Guide for the Care and Use of Laboratory Animals, Pain Recognition and Alleviation in Laboratory Animals and passed the exams.

VIII. ENCLOSURES: References

1. (b)(6),(b)(4)
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X. ASSURANCES

Protocol Title: Evaluation of tilorone as a novel radiation countermeasure candidate: *In vivo* study using laboratory mouse model (*Mus musculus*).

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures/manipulations/observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM/AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL/WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) 9-30-2013

Principal Investigator (Printed Name) (Date)

Principal Investigator (Signature)

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (March 2013)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	8/2; 8/14/13	2nd Resubmission Date:	
Approved/Returned for Revision:	9/20/2013	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	10/8/2013	**EXPIRATION DATE:**	10/22/2016
Approved/Returned for Revision:	10/23/2013	Previous Protocol Number *(if related)*	(b)(6)
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

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- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and *subsection*, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Development of anti-ceramide reagent as mitigators of acute radiation gastrointestinal syndrome in mice (*Mus musculus*)

IV. PRINCIPAL INVESTIGATOR:

(b)(6) Ph.D. Date
(b)(6)
Radiation Countermeasures Program, AFRRI, (b)(6)
Tel (b)(6) fax (b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date

Head, Scientific Research Department, AFRRI

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician

Telephone (b)(6) Fax: (b)(6)

(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department, USUHS

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.

(Date)

(b)(6) **Ph.D.**

IACUC Chair, (b)(6) Scientific Research Department, AFRRI

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

PROTOCOL TITLE: Development of anti-ceramide reagent as mitigators of acute radiation gastrointestinal syndrome in mice (*Mus musculus*)

PRINCIPAL INVESTIGATOR:

(b)(6) Ph.D. Date
(b)(6)
Radiation Countermeasures Program, AFRRI, (b)(6)
Tel (b)(6) fax (b)(6)

CO-INVESTIGATOR(S): N/A**AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER:** (b)(6)

I. NON-TECHNICAL SYNOPSIS: The threat of a limited radiological attack on American cities (i.e. a "dirty" bomb), or a nuclear accident, requires the development of a radiation countermeasure capable of protecting the gastrointestinal (GI) tract of military personnel, who might have to enter a contaminated area, or capable of mitigating GI lethality in the general population. At present there is no effective treatment for the acute radiation GI syndrome. We aim to assess anti-ceramide monoclonal antibody (mAb) as a mechanism-based approach to protect against and mitigate this potentially-lethal pathophysiology. The drug we are going to use will mimic turning off a stress signal (the asmase gene) and thereby allow cells to heal when they would otherwise die. In preliminary studies, anti-ceramide antibody significantly protected murine GI tracts up to 15 Gy total body irradiation when delivered prior to radiation exposure, and mitigated crypt lethality when delivered up to 24 h following irradiation. In the current protocol, we propose to develop anti-ceramide antibody for preclinical testing for use as a radiation protecting/mitigating therapeutic.

II. BACKGROUND

II.1. Background: The laboratories of (b)(4) (b)(4) (b)(4) proposed a physicochemical mechanism for ceramide signaling involving ceramide-driven re-organization of microscopic membrane rafts into ceramide-rich macrodomains (b)(4) (b)(4) While initial studies focused on Fas and *Pseudomonas aeruginosa* infection (b)(4) (b)(4) more recently (b)(4) focused on ultraviolet-C (UV-C) light, ionizing radiation and T cells. Their findings, described below, indicate that ceramide-rich platforms (CRPs) play a fundamental structural role in transmembrane signaling of death signals.

Extensive literature supports ceramide as an evolutionarily-conserved second messenger for stress (14, 15). This system is usually in the "off" state under basal conditions, activated upon contacting stress, and calibrates the extent of cellular stress, evoking an adaptive or apoptotic cellular response depending on strength of signal. The most widely studied ceramide-generating mechanism involves the translocation of an acid form of the enzyme sphingomyelinase (ASMase) to the outer plasma membrane,

where it hydrolyses its substrate sphingomyelin, generating ceramide therein (b)(4) While the mechanism by which diverse stresses (UV-C, heat, mechanical stress etc.) are registered at the plasma membrane is uncertain, it has been shown that such stresses induce translocation of a non-lysosomal form of ASMase, termed secretory ASMase, to the exoplasmic leaflet of the plasma membrane to initiate stress signaling (b)(4) Ceramide, once generated, has a unique biophysical property, that of self-association, forming CRPs on the cell surface, 1-5 microns in diameter, which serve as sites for protein oligomerization and transmembrane signal transduction (b)(4) 25). The basis of this protocol is that CRP formation on the surface of endothelial cells of the GI tract mediates the pathology that leads to radiation-induced GI lethality after whole body irradiation, as a result of an acute vascular dysfunction which regulates stem cell clonogens (SCC) demise and is amenable to inhibition by anti-ceramide antibody under development for this purpose.

The ability to antagonize GI toxicity using genetic and pharmacologic inhibitors of endothelial apoptosis has revealed multiple layers of complexity concomitant with ionizing radiation damage to the GI tract. Whereas mice most commonly die from the GI syndrome within days 5-10 after whole body irradiation, death from the bone marrow (BM) syndrome occurs at day 14-17 after whole body irradiation, a 10-day cutoff for assigning lethality from the whole body irradiation-induced BM versus GI syndrome has become generally accepted(21, 28). Recent evidence from some investigators challenged this notion, demonstrating that genetic inhibition of GI death at whole body irradiation doses of ≥14 Gy resulted in mice death from an accelerated form of BM aplasia at 8.2±0.9 days (24). Hence the use of a 10-day cutoff to separate the GI from BM syndromes appears inappropriate. Studies examining the impact of autologous BM transplantation (BMT) on this accelerated mode of BM aplasia were also revealing. Whereas protection of the GI tract by genetic inhibition of endothelial cell apoptosis switched animal demise to accelerated BM aplasia as the cause of animal death, only 60-70% of mice could be rescued with BMT. The 30-40% that died from accelerated marrow aplasia despite receiving syngeneic BM appeared to succumb to BMT engraftment failure, apparently due to radiation-induced stromal damage and failure of BM "take" (unpublished observation from collaborator laboratory). Consistent with the notion that BM matrix has become refractory at high radiation doses to marrow take, increasing the delivered marrow to $1x10^7$ cells or multiple doses of $5x10^6$ cells could not save these mice. Thus, the capability to selectively inhibit the GI syndrome has allowed for redefinition of the mechanism of animal demise during the acute phase response to single high dose whole body irradiation, indicating that there are at least three syndromes that need to be treated to preserve animal survival: a HSC syndrome at low radiation doses treatable with BM transplant, a coupled endothelial cell apoptosis-crypt SCC damage-regulated GI syndrome that can be impacted by agents that prevent ceramide-mediated endothelial cell apoptosis, and a BM stromal syndrome that we know little about currently. Interrogation of the biological response to radiation exposure should address these three syndromes. *Our strategy for mitigating the GI syndrome selectively using anti-ceramide antibody is based on these principles.* Earlier, anti-ceramide antibody whole molecule has been tested against whole body irradiation. This protocol is for evaluating whole molecule of anti-ceramide antibody against partial body irradiation and its fragments against total-body irradiation (TBI). Whole antibody

molecule will be used as a control in experiments with antibody fragments for comparison

Full-length IgG monoclonal antibodies are large (150 kDa), multivalent proteins. Recombinant proteins consisting of only the antigen-binding portion of monoclonal antibodies (the variable heavy (V_H) and light (V_L) chain regions) have been generated that retain antigen binding compared to the parent monoclonal antibody, while displaying, in cases where Fc portions are not required for immunomodulatory function, equivalent biologic activity. These Fab and single-chain fusion proteins (referred to as scFv proteins), contain the parent antibody V_H and V_L domains, yet display several favorable characteristics compared to full-length monoclonal antibodies, most notably: 1) Antibody fragments display significantly faster diffusion rates into the blood stream (minutes compared to hours following im or sc administration) and higher tissue permeability than immunoglobulins, due to their small size and enhanced solubility; 2) Antibody fragments can be genetically manipulated to optimize antigen-binding, stability, and delivery (scFv forms deliverable orally to gut mucosa have been proposed); and 3) Product development costs can be considerably less than for a humanized immunoglobulins. For these reasons, antibody fragments appear ideally suited for development as medical radiation countermeasures for emergency scenarios, where an anti-ceramide reagent would be administered in triage to potentially large populations following a radiation disaster.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

Best effort has been made to find any possibility for duplication. The following data bases have been searched:

a. PubMed

b. BRD – DOD Biomedical Research Database

c. CRISP - Computer Retrieval of Information on Scientific Projects, now known as 'RePORTER'.

II.2.2. Date of Search

PubMed – 6/20/2013
BRD – 6/20/2013
CRISP/ RePORTER – 6/20/2013

II.2.3. Period of Search

PubMed - 1965-2013
BRD - 1998-2013
RePORTER – 1988-2013

II.2.4. Key Words of Search

1. Anti-ceramide AND Radiation
2. Ceramide AND Radiation
3. Gastrointestinal syndrome AND Radiation
4. Anti-ceramide AND Gastrointestinal syndrome

5. Anti-ceramide AND Crypts**II.2.5. Results of Search**

PubMed

Search string 1: We found 4 references, which did not address the questions to be tested in this protocol. (b)(4)

(b)(4)

(b)(4) Current protocol proposed work is not duplication of that study.

Search string 2: We found 510 references, none of which addressed the questions to be tested in this protocol.

Search sting 3: We found 315 references, none of which addressed the questions to be tested in this protocol.

Search string 4: We found 1 reference which does not address the questions to be tested in this protocol.

Search string 5: We found 1 reference which does not address the questions to be tested in this protocol.

BRD

Search string 1: We found 0 reference.

Search string 2: We found 1 reference, none of which addressed the questions to be tested in this protocol.

Search sting 3: We found 6 references, none of which addressed the questions to be tested in this protocol.

Search string 4: We found 0 references.

Search string 5: We found 0 references.

RePORTER

Search string 1: We found 0 references.

Search string 2: We found 72 references, none of which addressed the questions to be tested in this protocol.

Search sting 3: We found 4 references, none of which addressed the questions to be tested in this protocol.

Search string 4: We found 63 references. , none of which addressed the questions to be tested in this protocol.

Search string 5: We found 36 references. , none of which addressed the questions to be tested in this protocol.

III. OBJECTIVE / HYPOTHESIS:

The objective of this protocol is to advance the pre-clinical development of anti-ceramide antibody as radioprotector (and radiomitigator) of the acute radiation GI syndrome. Here, we propose to evaluate the hypothesis that antagonism of CRP generation on endothelium within the small intestinal lamina propria with anti-ceramide

antibody will improve GI stem cell survival, protecting and mitigating GI tract damage, improving survival. (b)(4)

(b)(4)

and based on these investigations we propose that ceramide-neutralizing Abs represent a novel class of pharmacologic agents for radiological countermeasures. We propose several experiments with the intention of advancing technology development of anti-ceramide Abs as protectors and mitigators of tissue injury following acute radiation exposure.

IV. MILITARY RELEVANCE

Currently, there are no FDA approved pharmaceutical agents that can prevent or treat injury from external ionizing radiation. The problem has become more acute in recent times due to the unpredictable nature of terrorist attacks. Recognizing this urgent need, the U.S. Department of Defense has assigned top priority to the "development of medical countermeasures to radiation exposure" against both early and late arising health effects. These concerns imply the urgent need to develop an appropriate countermeasure for radiation injuries potentially sustained by war fighters during combat operations and enable commanders to make judicious decisions in troop deployment. There are several drugs at different stages of the development. This study will perform essential research and development of a promising new agent (anti-ceramide antibody) as a therapy for potential injuries sustained by military personnel who have been accidentally exposed to ionizing radiation.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

We propose to do the following experiments to evaluate anti-ceramide antibody 2A2 as a radioprotector and a mitigator of the radiation. Experiment will be done in order shown below. Details of subsequent experiments will be based on results obtained in previous experiment. Any product found ineffective in initial experiment will be eliminated in subsequent experiments.

V.1.1. Experiment 1. Determination of the gastrointestinal (GI) lethal dose of partial body (subtotal) irradiation (X-Ray – Small Animal Radiation Research Platform – SARRP): The gastrointestinal lethal dose of subtotal body irradiation will be determined in C57BL/6 mice. Groups of 8 mice will be exposed to 13-16 Gy in 0.5 Gy (13.0, 13.5, 14.0, 14.5, 15.0, 15.5, 16.0 Gy) increments, with lead shielding of the animals' head and forelimbs (approximately 20% shielding). Anticipated GI lethal dose may be 14 Gy and mice may survive for 6 - 8 d. Animals will be monitored for survival. Expected results are that animals will survive >14 days (animals should survive indefinitely) at lower doses of subtotal body irradiation, but once a GI lethal dose is reached animals will succumb to the GI Syndrome. Animals will be observed for 30 d post-irradiation.

According to the suggestions from veterinary science department, a trial run (without irradiation) will be carried out to evaluate the best method (anesthesia etc) along with the VSD veterinarians. This will be accomplished using VSD training protocol (have consent from VSD veterinarian). Once the SARRP procedure is standardized, we will make necessary adjustments in the protocol by submitting appropriate amendment before proceeding further.

Group details and mice required for experiment 1:

Dose response of radiation: Seven radiation doses (13.0, 13.5, 14.0, 14.5, 15.0, 15.5, 16.0 Gy SAARP) x 8 mice/group x 2 experiments= 112

Total mice required for experiment 1 = 112

V.1.2. Experiment 2. Assessment of the efficacy of whole antibody molecule (H2A2) and recombinant antibody fragments (Fab and scFv) as radioprotector for the GI syndrome: H2A2 (1000 μg/ml) and recombinant antibody fragments of H2A2 (Fab or scFv, 100 μg/ml) will be administered intravenously (for additional details please see section **V.4.4.1. Injections**) to C57BL/6 mice 15 min prior to the GI lethal dose of subtotal body irradiation (100% lethal dose) to assess the efficacy of recombinant antibody fragments in comparison to whole molecule. Mice will be sacrificed 3.5 d after irradiation, and jejunum will be harvested after euthanasia for analysis by microcolony assay of Withers and Elkind as a quantifiable surrogate for GI damage following irradiation (31).

Details of treatment groups and mice required for experiment 2:

Evaluation of recombinant antibody fragments as radioprotector: Four groups (control, H2A2, Fab or scFv at -15 min) x 6 mice/group x 2 experiments (one with SARRP and other with Cobalt-60) = 48 mice

Total mice required for experiment 2 = 48

V.1.3. Experiment 3. Assessment of the efficacy of whole antibody molecule (H2A2) and recombinant antibody fragments (Fab and scFv) as radiomitigator of the GI syndrome: H2A2 and recombinant antibody fragments of H2A2 (Fab or scFv) will be administered intravenously (for details please see V.4.4.1. Injections) to C57BL/6 mice 12-48 h following GI lethal dose of subtotal body irradiation (100% lethal dose) (12, 24, and 48 h). Mice will be sacrificed 3.5 days following irradiation, and jejunum will be harvested after euthanasia for analysis in microcolony assay of Withers and Elkind as a quantifiable surrogate for GI damage following whole body irradiation.

Details of treatment groups and mice required for experiment 3:

Evaluation of recombinant antibody fragments as radiomitigator: Four groups (control, H2A2, Fab or scFv) x 6 mice/group x 3 time points (12, 24 and 48 h) x 2 experiments (one with SARRP and other with Cobalt-60) = 144 mice

Total mice required for experiment 3 = 144

V.1.4. Experiment 4. Investigation for optimal dose, schedule of treatment, and route of administration of H2A2, Fab, and scFv: Dosing, treatment schedule, and route of administration of H2A2 and recombinant antibody fragments of H2A2 (Fab or scFv) will be optimized. Antibody will be administered through various routes stated below. Mice will be sacrificed 3.5 days following, and jejunum will be harvested for analysis in Microcolony Assay of Withers and Elkind as a quantifiable surrogate for GI damage following whole body irradiation. First, optimal dose of antibody will be determined and with that dose, optimal route of administration will be determined.

Details of treatment groups and mice required for experiment 4:

Dose response: Four groups (0, 500, 750, and 1000 µg for H2A2 and 0, 25, 50 and 100 µg for Fab and scFv) x 2 experiments (one set for radioprotector and another for radiomitigator) x 3 products to be tested (H2A2, Fab, and scFv) x 6 mice/group = 144 mice

*Routes of administration: Two groups (ab and control) x 3 products to be tested (H2A2, Fab, and scFv) x 4 routes (iv, im, sc, ip) x 6 mice/group x 2 experiments (one with SARRP and other with Cobalt-60) = 288 mice

Schedule of ab administration: Two groups (control and ab) x 6 time points of ab administration x 2 sets of experiments for radioprotector and mitigator (-30 min, -1 h, -2 h, -4 h, -6 h, -12 h in relation to irradiation for protector, and 12 h, 18 h, 24 h, 30 h, 36 h, 48 h in relation to irradiation for mitigator) x 6 mice/group = 144 mice

Total mice required for experiment 4 = 144 + 288 + 144 = 576

*Preliminary data with anti-ceramide IgM Ab has been generated using iv route. Hence iv route is being used in initial experiment. Keeping in mind the limitation of iv route from drug administration point of view, other routes will be tried.

Route of administration of antibody for experiments 1.5, 1.6, and 1.7, will depend on the results of experiment 1.4 (most effective route for drug efficacy). Based on our experience, we expect retro-orbital injection will be most efficacious.

V.1.5. Experiment 5. Assessment of radioprotection by H2A2 and its fragments (Fab, and scFv) in mice (survival study): H2A2 or its fragment will be administered to C57BL/6 mice 15 min prior to the GI lethal dose of subtotal body irradiation. Radiation dose will be selected based on the success in above experiments (at least 50% higher crypt numbers compared to untreated control). Animals will be monitored for 30 d after radiation exposure for survival. Moribund mice (details discussed under section V.4.5. Study Endpoint, displaying clinical signs of imminent death from radiation gastrointestinal syndrome) will be euthanized. Jejunum and sternum will be harvested after euthanasia for hematoxylin and eosin staining and analysis.

Details of treatment groups and mice required for experiment 5:

Evaluation of recombinant antibody fragments as radioprotector: Four groups (control, H2A2, Fab or scFv at optimal time point based on above experiments) x 8 mice/group x 2 experiments (one with SARRP and other with Cobalt-60)= 64 mice

Total mice required for experiment 5 = 64

V.1.6. Experiment 6. Optimization of radiomitigation by H2A2 and its fragments (Fab, and scFv) in mice (survival study): H2A2 or its fragment will be administered to C57BL/6 mice after the GI lethal dose of subtotal body irradiation. Radiation dose will be selected from above experiments (at least 50% higher crypt numbers compared to untreated control) . Animals will be monitored for 30 d after radiation exposure for survival. Moribund mice (details discussed under section V.4.5. Study Endpoint, displaying clinical signs of imminent death from radiation gastrointestinal syndrome) will be sacrificed. Jejunum and sternum will be harvested for hematoxylin and eosin staining and analysis.

Details of treatment groups and mice required for experiment 6:

Evaluation of recombinant antibody fragments as radiomitigator: Four groups (control, H2A2, Fab or scFv) x 4 time points in relation to irradiation (12, 24, 36 and 48 h after irradiation) x 8 mice/group x 2 experiments (one with SARRP and other with Cobalt-60) = 256 mice

Total mice required for experiment 6 = 256

V.1.7. Experiment 7 Optimization of mitigation by addition of supportive care (antimicrobial therapy): Survival experiments will be performed comparing animals that receive supportive care (antimicrobial therapy) in addition to H2A2 to those receiving vehicle for antibiotics plus H2A2. H2A2 or fragments will be administered to C57BL/6 mice at the latest effective time in which H2A2 improves survival post the GI lethal dose of subtotal body irradiation. Effective supportive care is expected, in combination with H2A2, to confer 30 d survival to mice exposed to 15 Gy, while a majority of mice receiving sham antibiotic will die from sepsis. All mice with unprotected GI tracts that did not receive H2A2 will die from the radiation gastrointestinal syndrome.

Antimicrobial therapy: Antimicrobial agents from different classes, including 4-fluoroquinolones and aminopenicillins, will be selected for systemic administration based upon published recommendations for treatment of sepsis following irradiation (5) and recent experimental work in the laboratory of microbiology colleague (personal communication). Doses used to evaluate efficacy against sepsis in mice have been selected based on human pediatric doses together with consideration for the high metabolic rate of mice compared to humans and the published reports by others in non-irradiated mice. In general, doses of antimicrobial agents are three to five times greater in mice than in humans to achieve comparable efficacy and concentration in blood and tissues. Doses used in this study are based on pharmacokinetics and efficacy against sepsis determined in irradiated mice.

Systemic antimicrobial agents that will be evaluated in the classes (5):

4-Fluoroquinolone: Levofloxacin (LVX) [90-100 mg/kg *p.o.* (not gavage) *q.d.*]

(Giving drug by oral route is different from giving drug by gavage: gavage means forced feeding, specially a tube passed through the stomach (Dorland's Medical Dictionary, 28th edition, 1988), p.o. (*peros*) means by mouth, orally, commonly used in medical practice, in case of mouse – feeding needle will stay in mouth and fluid will be ejected slowly).

Aminopenicillin: Amoxicillin (AMX) [300-325 mg/kg *p.o. q.d.*].

When the combination of LVX and AMX is administered to mice, the LVX is delivered first with a 20- to 30-minute interval before AMX is delivered to allow time for LVX to be absorbed because AMX is a microencapsulated suspension, which coats the lining of the gastrointestinal tract (5).

In order to anticipate bacterial translocation and moribundity, we will start antibiotic therapy on d 3 post-irradiation and continue for 21 d (last dose on 23 d post-irradiation).

Details of treatment groups and mice required for experiment 7:

Mitigation by addition of antimicrobial therapy: Four groups (control, H2A2, Fab or scFv) x 4 antimicrobial therapy (control/sham, LVX, AMX, and combination of LVX and AMX) x 8 mice/group x 2 experiments (one with SARRP and other with Cobalt-60) = 256 mice

Total mice required for experiment 7 = 256

Total C57BL/6 mice needed for this Protocol:

Experiments	Total	C	D	E
Experiment 1	112	0	0	112
Experiment 2	48	0	0	48
Experiment 3	144	0	0	144
Experiment 4	576	0	0	576
Experiment 5	64	0	0	64
Experiment 6	256	0	0	256
Experiment 7	256	0	0	256
Ten mice for trial of anesthesia regimen – section **V.4.1.2.1**	10	0	10	0
Total mice required for this protocol	1466	0	10	1456

Drugs and routes of administration:

Experiments	**Drugs**	**Route of administration**
Experiment 1	Anticeramide antibody (whole molecule- H2A2, fragments - Fab and scFv)	Retro-orbital (iv)
Experiment 2	Anticeramide antibody (whole molecule- H2A2, fragments - Fab and scFv)	Retro-orbital (iv)
Experiment 3	Anticeramide antibody (whole molecule- H2A2, fragments - Fab and scFv)	Retro-orbital (iv)
Experiment 4	Anticeramide antibody (whole molecule- H2A2, fragments - Fab and scFv)	sc, im, ip, retro-orbital (iv)
Experiment 5	Anticeramide antibody (whole molecule- H2A2, fragments - Fab and scFv)	Dependent on results of experiment 4
Experiment 6	Anticeramide antibody (whole molecule- H2A2, fragments - Fab and scFv)	Dependent on results of experiment 4

Experiment 7	Anticeramide antibody (whole molecule- H2A2, fragments - Fab and scFv)	Dependent on results of experiment 4
	Levofloxacin (LVX), Levofloxacin (LVX)	p.o.

V.2. Data Analysis

Mean with standard errors, or percentages, will be reported if applicable. The significance level will be set at 5% for each test. All statistical tests will be two-sided. Multiple comparison tests will be used when appropriate. Statistical software, PC SAS, will be used for statistical data analysis (6). Number of animals n = 6 - 8 per group (depending on dose, route, sampling time-point) will be used for the study to provide statistical power >90% for two-tailed Student's t test of less than 10% shifts in value.

Multivariate analysis of variance (MANOVA) Wilks' Lambda statistics will be used when comparing more than two groups and two-sided Student's *t* test will be used when comparing two groups to determine significant difference among sampling time and dose-points, efficacy, etc. Values of $P < 0.05$ will be considered statistically significant. Values will be expressed as means ± standard error (SE).

Comparison of survival curves will be made using the log-rank test. Fisher's exact test will be used to detect if there is a significant difference across the groups in survival rates by performing pair-wise comparisons. The 8 mice per group would provide 80% statistical power to detect a significant difference between two groups if any, given type I error of 5% and delta/sigma has at least 1.6 (where the delta is the estimated mean differences between the two groups and sigma is the pooled SD of the two groups) and a treatment group survival rate would be at least 74%, if the control group would be 20%. Similar statement would apply, provided the treatment group would be at least 88%, 85%, and 79%, if the vehicle group would be 35%, 30%, and 25% respectively (32).

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

Both computer models and tissue culture have been considered as alternatives to animals. These alternatives lack the knowledge base and complexity needed to evaluate the effects of radiation and radiation countermeasures on the immune system. The phenomena under study involve complex information-processing networks comprising large number of cell types and biological signal transduction molecules. Responses to irradiation and radioprotective agents involve interactions between the central nervous system, the peripheral nervous system including the enteric nervous system, endocrine glands, the immune and inflammatory systems, hematopoietic system, gastrointestinal system etc. We do not know all of the cell types and tissues involved and many of the signal transduction molecules have not been discovered yet. Even if all of the tissues, cell types and signaling molecules were known, the present state of tissue culture would be incapable of reproducing their *in vivo* relationships. One would have to reproduce much of an entire mammalian organism in culture to study

these phenomena *in vitro*. As for computer models, the most powerful supercomputers available in the near future would be incapable of analyzing interactions between so many elements in the network. Thus these drugs necessitate investigation in whole animal models to see overall outcome.

V.3.2. Animal Model and Species Justification Evaluation of radiation countermeasures in *in vivo* model is the only option available for identifying clinically safe and non-toxic radiation countermeasures. The murine model is immunologically the most well defined animal model for research. The murine model is also the most studied animal model, and has significant immunological similarity to higher animals and humans. This model is also preferred over any other *in vivo* model due to readily available immunological and molecular biology research reagents. AFRRI staff has extensive experience with several strains of mice in radiation injury and radioprotection research. PI has extensive experience with C57BL/6 and CD2F1 mice. (b)(4) (b)(4) (b)(4) Thus, we need to stay with this strain of mice at this stage for the proposed study.**V.3.3. Laboratory Animals**

V.3.3.1. Genus / Species

Mus musculus, Laboratory mouse

V.3.3.2. Strain / Stock

C57BL/6

V.3.3.3. Source / Vendor

C57BL/6 mice will be purchased from a VSD approved vendor (the preferred vendor is Jackson Laboratory, 600 Main St., Bar Harbor, Maine 04609-1500; Tel: 207-288-5845; State of Maine animal welfare license is R012).

V.3.3.4. Age

6-12 weeks old

V.3.3.5. Weight

20 – 25 g

V.3.3.6. Sex

Male

Justification for using only male mice: Previous studies have been performed using male mice, so males are needed for data comparison. Also there is possibility that female hormone variation may interfere with our results. To see the effect of this drug in both sexes, we need to repeat such experiments in females. To minimize the number of animals, and to keep variables minimized, it was decided that the study will be performed only in one sex (male).

V.3.3.7. Special Considerations

Commercially procured animals must be adventitious disease free, including: *Pseudomonas aeruginosa*, and *Pasteurella*. All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse

Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus, *Mycoplasma pulmonis*, *Clostridium piliforme*, *and C. difficile.* Mice should also be endoparasite- and ectoparasite-free.

V.3.4. Number of Animals Required (by species)

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement

Moribund animals will be euthanized immediately to minimize the magnitude and duration of suffering those animals might experience when they are subjected to toxic effects of ionizing radiation. Under such situation moribundity will be used as a surrogate for death in animals and will be considered to have arrived at the endpoint. Pain experienced by retro-orbital injection will be minimized by the use of higher gauge needles and controlled isoflurane administration.

V.3.5.2. Reduction

As we move forward with experiments, plans will be reevaluated at every stage of the experiments, in light of incoming data in an attempt to reduce the numbers of experiments and groups, if possible. If deemed not necessary based on incoming data, some investigations may not be carried out to optimize the use of experimental animals.**V.3.5.3. Replacement**

It is not feasible to use non-animal systems in place of *in vivo* animal models to address the research questions proposed in this IACUC protocol.**V.4. Technical Methods**

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C.....0

V.4.1.1.1.2. Column D.....10

V.4.1.1.1.3. Column E.....1,396

V.4.1.1.1.4. Pain Category Assignments

Experiments	Total	C	D	E
Experiment 1	112	0	0	112
Experiment 2	48	0	0	48
Experiment 3	48	0	0	48
Experiment 4	576	0	0	576
Experiment 5	64	0	0	64
Experiment 6	256	0	0	256

Experiment 7	256	0	0	256
Ten mice for trial of anesthesia regimen – section **V.4.1.2.1**	10	0	10	0
Total mice required for this protocol	1466	0	10	1456

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

Since anesthesia, analgesia, and tranquilizers are known to affect functions of the immune system of animals, we do not plan to use analgesic agent during course of the experiment after irradiation (1, 9, 10). However, anesthesia will be used during partial body X-ray irradiation to prevent stress from the time the mice are placed in restraint.

The anesthesia protocol for the SARRP partial body irradiation will be developed under the oversight of VSD veterinarian/s. SARRP irradiation requires anesthesia for longer period of time (30-40 minutes). No one at AFFRI has used SARRP before. Therefore, we will need to try out the following anesthetic regimens along with VSD veterinarian to determine the best option. Ten mice have been requested for this purpose. If none of these anesthetic regimens are able to achieve our goal then we will submit an amendment to our protocol to add an alternate anesthetic as per the recommendation from VSD veterinarian.

1. Ketamine 75-150 mg/kg + Xylazine 5-10 mg/kg via ip administration. Total volume of injection will not exceed 0.5 ml/mouse.
2. Ketamine 50-100 mg/kg + Xylazine 5-10 mg/kg + Acepromazine 1-2 mg/kg. Total volume of injection will not exceed 0.5 ml/mouse.
3. Isoflurane 3-4% in 100% oxygen for induction in a chamber. 1 - 3% Isoflurane for maintenance via a nose cone.

Animals in experiments involving blood (collection/storage for future)/tissue collection (different organs) will be euthanized immediately before tissue collection by overdose of isoflurane followed by cervical dislocation. We will use isoflurane to anesthetize mice for retro-orbital injection. This allows more controlled and precise delivery of the anesthetic. We regularly use these procedures in different ongoing protocols. We will also use 0.5% proparacaine HCL ophthalmic solution in addition to the inhalant anesthetic, prior to retro-orbital injections, as an additional form of procedural and post-procedural analgesia.

V.4.1.2.2. Pre- and Post-procedural Provisions

Mice will be housed under standard conditions both before and after radiation procedures in accordance with VSD mouse Standard Operating Procedure (see section V.5).

V.4.1.2.3. Paralytics

No paralytic agent will be administered.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched

AGRICOLA
PubMed
IVIS

V.4.1.3.2. Date of Search

AGRICOLA - 6/20/2013
PubMed - 6/20/2013
IVIS - 6/20/2013

V.4.1.3.3. Period of Search

ARGICOLA -1966-2013
PubMed - 1965-2013
IVIS - 1998-2013

V.4.1.3.4. Key Words of Search

"Anti-ceramide" AND "Pain"
"Ceramide AND pain"
"Ionizing radiation" AND "pain"
"Ionizing radiation" AND" mice"
"Ionizing radiation" AND "alternatives"
"Ionizing radiation" AND "toxicity"

V.4.1.3.5. Results of Search

"Anti-ceramide" AND "Pain"
AGRICOLA: 0
PubMed: 0
IVIS: 0

"Ceramide" AND"pain"
AGRICOLA: 0
PubMed: 76
IVIS: 5

"Ionizing radiation" AND "pain"
AGRICOLA: 0
PubMed: 427
IVIS: 21

"Ionizing radiation" AND" mice"
AGRICOLA: 283
PubMed: 427
IVIS: 14

"Ionizing radiation" AND "alternatives"
AGRICOLA: 2
PubMed: 127
IVIS: 2

"Ionizing radiation" AND "toxicity"
AGRICOLA: 329

PubMed: 4628
IVIS: 16

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification

As stated above, irradiated animals finally die due to a compromised immune response and microbial infections. In the event that there is pain and distress for animals, as we have already discussed under above headings, we cannot give anesthetic/analgesic agents to the animals (except at the time of ocular injection or wounds procedures which can't be performed without anesthesia) since these agents interact with the immune system, and in turn will affect experimental results.

There are conflicting reports regarding effects of buprenorphine on the immune system (7, 11, 16, 17, 19, 20, 23, 29). A majority of reports suggest that buprenorphine influences immune response. Since buprenorphine has been shown to influence various arms of cellular and humoral immune response (7, 17, 23, 29), we have decided not to use it in our study. Non-steroidal anti-inflammatory drugs are not suitable for use in such experiments (18).

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure(s): N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures

V.4.3.6.1. Procedures: N/A

V.4.3.6.2 Scientific Justification: N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections

Mice will be injected with anti-ceramide antibody through the retro-orbital sinus (venous plexus behind the eye – for intravenous route) with 27 or 28 G needle with a 1 or 0.5 ml syringe (AFRRI IACUC Operating Policy # 4). This is a commonly used method (2-4, 22, 26, 30, 33). The animals will be temporarily anesthetized by inhalation of isoflurane. Both eyes can be used but PI will select one side and all animals will be injected at the same site for one administration. The eyelid area will be stretched gently to expose the eye and the needle will be inserted slowly into the orbita going beneath the eyeball. The needle is gently inserted bevel downwards until it hits the bone of the orbita. The antibody or bone marrow cell suspension will be injected slowly. If the needle is correctly inserted into the plexus, some resistance will be felt while injecting. After injection of the desired volume, the needle will be removed and the eyelid will be closed. No liquid or blood should leak out. In case, blood leaks out from the eye, sterile cotton gauze will be used to wipe/clean and animal will be carefully observed for 5-10

min. Animals will be returned to the cage and observed until they fully recover from the effects of the anesthesia.

Recently it has been demonstrated that retro-orbital venous sinus injection is less stressful compared to lateral tail vein (27). In mice we have found retro-orbital injection more reproducible than tail vein injection due to difficultly visualizing the tail vein. Since our radiation mitigating agent needs to be injected at a particular time post-irradiation, we have a limited time period to administer the agent to all mice in the study in order to insure post-irradiation time of injection. Because retro-orbital injection is more rapidly performed than tail vein injection, a single technician is able to inject large numbers of mice in a relatively short period of time. Study personnel assigned to this protocol have been trained in this procedure.

We have used such a method for cell, blood, and anti-ceramide antibody injection to mice in several protocols.

Injection volume will be 100 – 150 µl/mouse. In case, any preparation has a low solubility, we may need to inject 200 µl. There will be no need to go above this volume for any preparation. According to the policy from University of California, San Francisco, a maximum of 200 µl can safely be injected via the retro-orbital sinus (web reference: a copy of the UCSF policy can be found here: *www.iacuc.ucsf.edu/Policies/RetroOrbitalInjection.doc*). Under such situation, we will try two options:

1. in a minimum of 5 mice we would try the 100 µl in each eye and
2. in different mice (a minimum of 5), try 200 µl in one eye.

In both cases injection will be administered slowly and/or with rest periods (for the 100 µl) as necessary. Both options will be evaluated with a VSD veterinarian and the best will be used. We have received such approval earlier (Protocol #(b)(6) (b)(6)

Antibody will be also injected im, ip and sc as mentioned above under various experiments.

Antimicrobial therapeutic drugs will be prepared aseptically with sterile diluent including the control vehicles. They will be administered orally. Therapeutic drugs given p.o. will be administered in a 0.2-ml volume with a 1-ml syringe and a 20-gauge feeding canula, which has a ball tip, or a disposable feeding needle, which has a silicone rubber tip. One sterile syringe and needle will be used for mice in each cage. However, the feeding needle will be wiped and disinfected between inoculations with a gauze sponge that is moistened with 70% ethanol to reduce the microorganisms on the needle as an extra precaution even though the mice share the sipper tube in the water bottle and oral microorganisms as well.

The following doses will be used for administration of the therapeutic antimicrobial agents based on earlier studies with mice: amoxicillin (300 - 325 mg/kg PO sid), levofloxacin (90-100 mg/kg PO sid).

V.4.4.1.1. Pharmaceutical Grade Drugs

Vendors (or others as appropriate):

Amerisource-Bergen, Glen Allen, Virginia, Tel. 1-800-262-8470 ext. 4313, Acct. No. 74047 (USUHS)

Henry Schein, Inc., Henry Schein Veterinary Institutional Department, 135 Duryea Road Melville, NY 11747, Phone: 800-872-4346, Fax: 1-800-483-8329

Webster Veterinary Supply, 86 Leominster Road, Sterling, MA 01564, 978-422-8211 (Phone), 800-225-7911 (Toll Free in the U.S.), 978-422-8959 (Fax)

Pharmaceuticals:

0.5% Proparacaine HCL ophthalmic solution (will be procured from USUHS pharmacy).

Amoxicillin (AMX), generic e.g. 150 ml/bottle, Amoxicillin for Oral Suspension, USP (Sandoz, Broomfield, Colorado 80020, 250 mg/5 ml as trihydrate, NDC 0781-6041-55); or 100 ml / bottle, commercial oral suspension (IVAX Pharmaceuticals, Inc., Miami, Florida 33137-3227 by PENN Labs, Inc., Philadelphia, Pennsylvania 19102, amoxicillin for oral suspension USP, 250 mg per 5 ml as the trihydrate, 100 ml total volume when reconstituted with sterile water according to manufacturer's directions, NDC 0172-7418-21). Amoxicillin is a semisynthetic aminopenicillin antibiotic. Stored at 4°C. after reconstitution, durable for up to 14 days.

Levofloxacin (LVX), LEVAQUIN Oral Solution (25 mg/mL, 16 oz. [480 mL], Ortho-McNeil, NDC 0045-1515-01), is a 4-fluoroquinolone antimicrobial agent. It will be diluted to a concentration of 10.4 mg/ml in sterile water to deliver a dose of 90 mg/kg *q.d.* for in 0.2 ml given *p.o.* Oral and i.v. routes are considered interchangeable and achieve the same plasma concentration profile (Ortho-McNeil product information).

The corresponding vehicle is sterile water given to control mice.

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

Anti-ceramide antibody, its fragments and isotype control (non-specific antibody) will be purchased from commercial vendors. Purity is assured because the antibodies are purified by the vendor by Protein G affinity chromatography. These reagents have been tested for endotoxin by the vendor, but have not undergone other tests for infectious agents. Before use in animals, the antibody reagents will be tested for infectious agents using a serological test (MAP test or equivalent) or PCR (MAP-IT test or equivalent). Stability will be ensured by storing the antibodies as lyophilized powder at -20 °C, in a manual defrost freezer, with no repeated thaw/freeze cycles, as recommended by the vendor. Microgram quantity of antibodies will be injected intravenously in pharmaceutical grade sterile phosphate-buffered saline, so osmolarity will not be an issue. Please also see section V.4.4.9. Animal By-Products.

V.4.4.2. Biosamples

Animals will be euthanized by overdose of isoflurane (for blood collection – blood will be stored for future reference), followed by cervical dislocation immediately before tissue (jejunum and femur bone) collection. GI tract and femur bone will be collected post-mortem for histopathology.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAb) Production: N/A

V.4.4.5. Animal Identification

Each cage will have a cage card. In addition to cage card, another experimental card will be displayed before starting the experiment. One cage will have mice of only one treatment/control group. Each card will state the investigator's name, protocol number, experiment number, cage number, start date, end date, species, item number (animal lot), birth date, gender, strain, number of animals in the cage, vendor, arrival date, treatments, and dates of death or euthanasia of moribund mice, including the initials of the staff person who found and removed the dead animals or euthanized the moribund mice.

V.4.4.6. Behavioral Studies: N/A**V.4.4.7. Other Procedures:** None

V.4.4.8. Tissue Sharing: N/A

V.4.4.9. Animal By-Products

Commercially available antibody to ceramide, its fragments, and isotypic control will be used for injecting into mice. As stated above, these products will be tested for adventitious agents prior to use. Such testing will be done for mycoplasma and 18 viral agents by BioReliance, Rockville, MD prior to use (also mentioned under Non-Pharmaceutical Grade Drugs). We have carried out such testing for anti-ceramide antibody earlier for approved protocol # (b)(6) (b)(6) (b)(6) The results of testing will be given to the attending veterinarian or his/her designee for review prior to using these biologics in mice.

V.4.5. Study Endpoint

In all experiments where jejunum or bone marrow cells will be harvested, mice will be anesthetized and euthanized by an overdose of isoflurane followed by cervical dislocation immediately before tissue collection at a preset time as described under experimental design (V.1). All mice that have survived beyond experimental duration (30 day post-irradiation) or for moribund mice, if any, will be euthanized by CO_2 overdose followed by cervical dislocation. Naïve mice, if not used in the experiments, will be transferred to another approved protocol, if possible, or euthanized. Animals in these studies may become morbid and hence it is critical to define criteria for euthanizing mice humanely without compromising the study objective and complying with the IACUC recommendations stipulated in Policy Letter #10. Mice will be considered morbid based on criteria described in policy # 10. Experimental mice that are found morbid, but not moribund, will be allowed to continue in the experimental protocol. Once mice are considered morbid, we will monitor them at least twice daily, early morning and late afternoon.

Mice found moribund will be euthanized by trained and experienced personnel listed on this protocol or by trained/experienced VSD staff. Compressed CO_2 gas in a cylinder with a regulator will be used for euthanasia in accordance with the current American Veterinary Medical Association (AVMA) Guidelines on Euthanasia. Cervical dislocation will be done following CO_2 overdose as a secondary method of euthanasia. Mice will be considered moribund based on criteria described in policy # 10.

The table provided in AFRRI IACUC Operating Policy # 10 (Guidelines for establishment of early end points in experiments with expected mortality) has been included below because this study will involve a large degree of lethality.

VIII. Appendix A: Clinical Observations with Criteria for Euthanasia (Rodent)

DATE:	Time:	Animal ID#	Score
Appearance:			
	Normal (smooth coat, clear eyes/nose)		0
	Hunched and/or fluffed		1
	Ocular discharge, and/or edema		3
	Emaciated, dehydrated (skin tent) **		5
	Soft stools (fecal matter around anus)		5
	Bloody diarrhea		9
Respiratory rate:			
	Normal breathing		0
	Increased breathing (double normal rate, rapid, shallow)		6
	Abdominal breathing (gasping +/- open mouth breathing)*		12
General Behavior:			
	Normal (based on baseline observations)		0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)		1
	Decreased mobility		2
	Ataxia, wobbly, weak**		6
	Inability to stand*		12
Provoked Behavior:			
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))		0
	Subdued; responds to stimulation (moves away briskly)		1
	Subdued even to stimulation (moves away slowly)		3
	Unresponsive to gentle prodding **		6
	Does not right when placed gently on side within 5 seconds*		12
TOTAL			_____

Definitive criteria for morbidity:	**Definitive criteria for moribundity:**
Weight Loss up to 10% baseline (if available)**	Weight Loss > 20% OR <15g (adult mouse)*
Pale, white mucous membranes/ skin * *	Blue mucous membranes/ skin (cyanosis)*
Fever >104F/40C (if available)**	Cool to the touch or <86F/30C (if available)*
**** Regardless of score, notify appropriate person immediately.**	***** Regardless of score, immediately euthanize (death is imminent)**

Score:
< 6 Normal
6 - 9 Morbid: Monitor at least 3 times per day; notify appropriate personnel immediately
> 10 Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*)
Any single criteria of 12* euthanize immediately; consider as 'found dead'

(Note: This form does not have to filled out for every individual at every observation, so long as the criteria are used in informing decisions on increased monitoring and/or euthanasia. This form may be used to make a spreadsheet for greater ease of multiple animal observations, as long as the criteria and numbering system are exactly copied.)

V.4.6. Euthanasia

As already mentioned above under various headings, mice will be euthanized by overdose of isoflurane and cervical dislocation just before tissue collection. Terminal CO_2 euthanasia will be used for mice that have survived beyond experimental duration (30 day post-irradiation) or for moribund mice, if any. Compressed CO_2 gas in a cylinder with a regulator will be used for euthanasia in accordance with the current American Veterinary Medical Association (AVMA) Guidelines on Euthanasia. Cervical dislocation will be done following CO_2 overdose as a secondary method of euthanasia.

V.5. Veterinary Care

V.5.1. Husbandry Considerations

No special husbandry consideration needed.

V.5.1.1. Study Room: As assigned by VSD.

V.5.1.2. Special Husbandry Provisions: None

V.5.1.3. Exceptions

Mice will be socially (group) housed, except where there is a possibility that during survival studies cage mates may be lost through attrition and for a period of time the sole surviving mouse may remain singly housed until the end of the 30-day study.

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care

As per Standard Operating Procedures, VSD staff will monitor all animals at least twice daily during morning and afternoon rounds. Any abnormalities will be reported immediately to the on-call veterinarian and PI. In addition, animals will be observed daily by this protocol's research staff. Any morbid mice will be observed twice a day (early morning and late afternoon) by the research staff. PI staff will be responsible for before and after working hours observations of morbid mice. Any moribund mice will be euthanized as soon as possible. If needed, VSD veterinarian will be contacted for decision.

V.5.2.2. Emergency Veterinary Medical Care

Emergency veterinary medical care will include immediate euthanasia of any moribund mice.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

Mice will be group housed and enrichment will be provided as per the VSD rodent enrichment SOP.

V.5.3.2. Enrichment Restrictions: None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1,3,4,5,7	(b)(6)
				1,3,4,5	
				1,3,4,5	
				1,3,4,5	
				1,3,4,5	
				1,3,4,5,7	
				1,3,4,5,7	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a-ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific

Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital injection)

VII. BIOHAZARDS/SAFETY

There is no plan to use any potential biohazard. Standard laboratory safety precautions will be observed throughout this study

VIII. ENCLOSURES

Form 310

List of references

X. ASSURANCES

Protocol Title: Development of anti-ceramide reagent as mitigators of acute radiation gastrointestinal syndrome in mice (*Mus musculus*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I (**AM**) / **AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / (**WILL NOT**) be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

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DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (October 2012)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	**8/26/2013**	2nd Resubmission Date:	
Approved/Returned for Revision:	**9/30/2013**	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	**10/8/2013**	**EXPIRATION DATE:**	**10/14/2016**
Approved/Returned for Revision:	**10/15/2013**	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. **NAME OF FACILITY: Armed Forces Radiobiology Research Institute**

II. **PROTOCOL NUMBER:** (b)(6)

III. **PROTOCOL TITLE:** Pediatric large animal model of ARS (Gottingen minipigs, *Sus scrofa domestica*)

IV. **PRINCIPAL INVESTIGATOR:** (b)(6)

(b)(6) PhD Date
Principal Investigator, (b)(6) Scientific Research Department
Tel: (b)(6) Fax (b)(6) E-mail: (b)(6)

V. **DEPARTMENT HEAD:** This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date
Head, Scientific Research Department, AFRRI
Telephone: (b)(6) Fax (b)(6)
(b)(6)

VI. **STATISTICAL REVIEW**: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

VII. **ATTENDING VETERINARIAN**: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI

Telephone (b)(6) Fax (b)(6)

(b)(6)

VIII. **SAFETY OFFICER**: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department, USUHS

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

IX. **IACUC APPROVAL**: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.

(Date)

(b)(6) **PhD**

IACUC Chair, (b)(6) Scientific Research Department, AFRRI (b)(6)

Telephone (b)(6) Fax (b)(6)

(b)(6)

PROTOCOL TITLE: Pediatric large animal model of ARS (Gottingen minipigs, *Sus scrofa domestica*)

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6) PhD Date
Principal investigator, (b)(6) Scientific Research Department
Tel. (b)(6) Fax (b)(6) E-mail (b)(6)

CO-INVESTIGATOR(S): (b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS

The relationship between radiation dose and the effect on health has been well established. However, in addition to the dose absorbed other parameters such as genetic background, co-morbidities and age are considered as important risk factors. Very little information is available on the ARS in children, except for the well-known increased risk for cancer development. Because children are still in an active growing phase, the effects of radiation are expected to be more severe than for adults. Furthermore, radiation countermeasures developed for adults cannot be assumed to be effective or safe for children without prior testing, due to the fact that organs of children are immature, and their physiological and metabolic features still developing. Consequences for administration of adult drugs to children include potential differences in drugs' PK/PD and mechanism of action, over- or under-medication and unanticipated adverse events.

Under the FDA Animal Rule, data for radiation countermeasure efficacy testing can be extrapolated from animal models. Development of a pediatric large animal model for characterization of the Acute Radiation Syndrome as well as drug development is critical. The minipig represents an ideal animal model to test the effects of radiation on the pediatric population. Benefits include short reproductive cycle, prolificacy, large litter size, ease of cross-fostering, similarities in organ development, maturation, physiology and anatomy to human pediatric population, multiple administration routes to test intact drug formulation for more immediate translation to humans, regulatory acceptance, large historical database and expertise available. Here, we propose to subject juvenile animals (< 3 mos of age) to irradiation and to determine dose-survival relationship.

II. BACKGROUND

II.1.

There is very limited information available on total body irradiation (TBI)-ARS for demographics of 0-12 years (children) or >75 years (elderly), and no treatment for age-specific radiation injuries are being developed. Limited data suggest accentuated vulnerability in the very young, likely due to the immature immune system (1). At an early age, tissue and organ systems are still developing and are not fully mature. Data from adult animal models cannot be directly translated to the pediatric population because of major differences in the organ anatomy, physiology and metabolism between adults and pediatrics. Examples are represented by the different clearance mechanisms of liver and kidney, the immature immune system, the developing CNS and the rapid growth of bones. Pharmacokinetics and pharmacodynamics are different in children. For the same reason, countermeasures shown to provide benefits to adults must be tested in animal models of age range appropriate to mimic the same developmental characteristics as the target population.

Pediatric animal models, for development and testing of pediatric-friendly drugs, are urgently required. The Gottingen minipig represents a suitable animal model to study the influence of age on susceptibility to radiation insult. Here we proposed to use the Gottingen minipig to study the Acute Radiation Syndrome in the pediatric population. Juvenile minipigs have been used as model for hemorrhagic hypovolemic shock, lung injury, kidney replacement therapy, hemodynamics, cardiovascular pathophysiology, and

asphyxia among others. A table summarizing the age comparison between humans and the G. minipig is reported below.

Table 1. Age comparison: human vs minipig

	Human	Minipig
Neonate	birth to 1 month	birth to 1 month
Infant	1 mo – 2 yr	4 – 8 wk
Child	2 – 12 yr	8 wk – 3 mo
Adolescent	12 – 16 yr	3 – 5 mo

Stages in a minipig life – from: Ellegaard Gottingen Minipigs

In previous years under (b)(4) we have established a basic minipig model for the hematopoietic acute radiation syndrome (H-ARS) (2-4), after total-body irradiation (TBI) and in the absence of supportive care. We characterized dose-response relationships at radiation doses spanning from H-ARS and the gastrointestinal (GI) ARS. Animals used were adults and of an age corresponding to humans >16years. Use of a vascular access port allowed us to obtain serial samples from the same animal. We were thus able to analyze hematological changes over time, hematopoietic progenitor stem cells, kinetics of DNA damage/repair and residual foci (gamma-H2AX), circulating citrulline levels and microbiological status. In addition, we monitored organ function, and isolated peripheral blood mononuclear cells (PBMCs) and platelets for phenotypic and functional studies. Primary cell lines were obtained from the lungs to study mechanisms of DNA repair in vitro.

Our data so far confirm that minipigs provide an ideal system to test drugs and to study the natural history of ARS. One of the many advantages of this large animal model is the opportunity to study different populations (pediatric, juvenile, adults, geriatrics) and both genders. Here, we propose to subject juvenile animals (<3 months of age) to irradiation and to determine dose-survival relationship. Survival studies will be done in the presence of minimum supportive care (antibiotics, antipyretic/anti-inflammatory, and dietary supplementation – no IV fluids or blood supplements), to address the potential issue represented by the proven sensitivity of the juvenile minipig and the expected increase in sensitivity in younger animals. To our knowledge, this is the first attempt to establish a pediatric model of ARS. The adult control group will be represented by animals irradiated at approximately 4-5 months of age, and subjected to identical treatment as the pediatric cohorts. During this project, we will (i) develop and assess basic operating protocols for animal housing, handling, blood collection in a pediatric minipig model, (ii) measure normal growth rate of pediatric minipigs model, and (iii) establish dose-survival relationship in irradiated pediatric minipigs.

II.2. Literature Search for Duplication
II.2.1. Literature Sources Searched BRD, RePORTER, PubMed
II.2.2. Date of Search , July 14, 2013
II.2.3. Period of Search
BRD: 1998 – 2009
RePORTER: 1998-2013
Pubmed: 1998-2013

II.2.4. Key Words of Search
Minipigs OR swine AND Radiation AND pediatrics OR pediatric OR juvenile

II.2.5. Results of Search

BRD database:
Keyword Minipig.....25 results (not relevant)
1) Low-Level Chemical Agent Toxicology: Feasibility of the Minipig as a Non-Rodent Model for Studying Effects of Whole-Body Sarin (GB) Vapor Exposure
2) Inhalation Toxicity of Sarin (GB) and Cyclo-Sarin (GF) Vapor in the Gottingen Minipig: Low-Level Threshold Effects and Lethality
3) Inhalation Toxicity of Sarin (GB) and Cyclo-Sarin (GF) Vapor in the Gottingen Minipig: Low-Level Threshold Effects and Lethality
4) Pilot study: Development of Gottingen minipig (Sus scrofa domestica) as radiation injury model
5) Inhalation Toxicity of Sarin (GB) and Cyclo-Sarin (GF) Vapor in the Gottingen Minipig: Low-Level Threshold Effects and Lethality
6) Determination of Thermal Injury Thresholds in the Yucatan Minipig Using Radio Frequency Radiation
7) Inhalation Toxicity of Sarin (GB) and Cyclo-Sarin (GB) Vapor in the Gottingen Minipig: Low-Level Threshold Effects and Lethality
8) Inhalation Toxicity of Sarin (GB) and Cyclo-Sarin (GB) Vapor in the Gottingen Minipig: Low-Level Threshold Effects and Lethality
9) Miotic and Lethal Effects of VX Vapor Inhalation Exposure in the Gottingen Minipig
10) Determination of Thermal Injury Thresholds in the Yucatan Minipig Using Radio Frequency Radiation
11) Miotic and Lethal Effects of VX Vapor Inhalation Exposure in the Gottingen Minipig
12) Efficacy of Human Butyrylcholinesterase as a Pretreatment Against Nerve Agent Exposure in the Gottingen Minipig
13) Efficacy of Human Butyrylcholinesterase (Hu BChE) as a Pretreatment Against Nerve Agent Exposure
14) Efficacy of Human Butyrylcholinesterase (Hu BChE) as a Pretreatment Against Nerve Agent Exposure
15) Efficacy of Human Butyrylcholinesterase (Hu BChE) as a Pretreatment Against Nerve Agent Exposure
16) Analysis of Depth of Ablation, Thermal Damage, and Wound Healing with a Novel Erbium: YAG Laser
17) Exploratory Assessment of Immediate Behavioral Effects Of TASER-Like Devices On Swine
18) Effects of Vasopressin Infusion on Systemic Organ Perfusion in a Porcine (Sus scrofa) Model of Vasodilatory Shock
19) Exploratory Assessment of Immediate Behavioral Effects Of TASER-Like Devices On Swine
20) Effects of Vasopressin Infusion on Systemic Organ Perfusion in a Porcine (Sus scrofa) Model of Vasodilatory Shock
21) Exploratory Assessment of Immediate Behavioral Effects Of TASER-Like Devices On Swine
22) Characterization of Freeze-Dried Platelet-Rich Plasma for Use in Hemostasis and Wound Healing of Oral and Maxillofacial Trauma
23) Characterization of Freeze-Dried Platelet-Rich Plasma for Use in Hemostasis and Wound Healing of Oral and Maxillofacial Trauma
24) Characterization of Freeze-Dried Platelet-Rich Plasma for Use in Hemostasis and Wound Healing of Oral and Maxillofacial Trauma

25) Comparison of Exposure Duration, Mode of Administration, and Method of Decontamination of VX on LD50 in Male New Zealand White Rabbits

Keyword Minipig + radiation: .....4 results
1) Implantation Techniques and Specific Absorption Rates for Swine (Sus scrofa) in Directed Energy Health and Safety Studies
2) Pilot study: Development of Gottingen minipig (Sus scrofa domestica) as radiation injury model
3) NIRVANA: Non-Ionizing Radiation Vision for a New Army
4) Extended Electro Magnetic Medical Advisor: A Diagnostic Tool for Casualty Triage by Combat Medics

Keyword Minipig + radiation+pediatrics:.....0 results
Keyword Minipig + radiation+pediatric:.....0 results
Keyword Minipig + radiation+juvenile:.....0 results

Keyword Swine:.....223 results
Keyword Swine + radiation: ..... 4 results

1) Implantation Techniques and Specific Absorption Rates for Swine (Sus scrofa) in Directed Energy Health and Safety Studies
2) Pilot study: Development of Gottingen minipig (Sus scrofa domestica) as radiation injury model
3) NIRVANA: Non-Ionizing Radiation Vision for a New Army
4) Extended Electro Magnetic Medical Advisor: A Diagnostic Tool for Casualty Triage by Combat Medics

Keyword Swine+ radiation+pediatrics:
Keyword Swine+ radiation+pediatric:
Keyword Swine+ radiation+juvenile:

RePORTER database:
Keyword Minipig: .....8 results (4 not relevant to this project)

1. DEVELOP RADIATION INJURY MODEL: GOTTINGEN MINIPIG (SUS SCROFA DOMESTICA)
2. DEVELOP RADIATION INJURY MODEL USING THE GOTTINGEN MINIPIG
3. COUNTERMEASURES AGAINST RADIOLOGICAL AND NUCLEAR ATTACK - AFRRI/IAA
4. COUNTERMEASURES AGAINST RADIOLOGICAL AND NUCLEAR ATTACK

Keyword Minipig + radiation: .....4 results (same as above)
Keyword Minipig + radiation+pediatrics:.....0 results
Keyword Minipig + radiation+pediatric:.....0 results
Keyword Minipig + radiation+juvenile:.....0 results

Keyword Swine .....10742 results
Keyword Swine+ radiation: ..... 840 results (not relevant to this project, covering progenitor cells, trabecular response to laser, ssdna viruses, aqueous humor, stents, myoblast therapy and angiomyogenesis, kidney stones, salivary glands, imaging, microfluidics, proton radiation therapy, cancer, ablation, sunlight and vitamins, adrenergic receptors, cardiovascular research, pharmacology, dosimetry in hyperthermia, vaccines, surgical implantations, gene and protein expression, oxidative metabolism, thermobrachytherapy, heat and irradiation, infections, gene/cell therapy, diagnostics, SARS, bowel disease, organ calcification, arterial properties from stimulated acoustical emission, angiogenesis, wound healing, engraftment and others not related to this project)

Keyword Swine+ radiation+pediatrics:.....23 results (not relevant to this project, covering xenografts, extravasation, imaging, circulation, scar expansion, cystic fibrosis)

Keyword Swine+ radiation+pediatric:.....5 results (not relevant to this project)
1) EARLY DETECTION OF NEONATAL & PEDIATRIC EXTRAVASATIONS (1999)
2) EARLY DETECTION OF NEONATAL & PEDIATRIC EXTRAVASATIONS (1998)

3) EARLY DETECTION OF NEONATAL & PEDIATRIC EXTRAVASATIONS (1993)
4) ENDOVASCULAR SURGERY SYSTEM FOR ATRIAL SEPTAL DEFECT REPAIR
5) MINORITY SUMMER RESEARCH TRAINING PROGRAM (MSRTP)

Keyword Swine+ radiation+juvenile:.....8 results (not relevant to this project)
1) SHORT COURSE: INTEGRATIVE AND ORGAN SYSTEMS PHARMACOLOGY
2) MANIPULATING AROMATICITY: CHARACTERIZATION OF AN ULTRA-RAPID INSULIN ANALOG
3) MANIPULATING AROMATICITY: CHARACTERIZATION OF AN ULTRA-RAPID INSULIN ANALOG
4) RAPID MRI MEASURES OF ABSOLUTE FAT MASS IN ADIPOSE TISSUE AND ORGANS
5) RAPID MRI MEASURES OF ABSOLUTE FAT MASS IN ADIPOSE TISSUE AND ORGANS
6) NONINVASIVE ASSESSMENT OF PANCREATIC BETA-CELL MASS
7) NONINVASIVE ASSESSMENT OF PANCREATIC BETA-CELL MASS
8) LONGEVITY AND STRESS RESISTANCE

PUBMED database:
Keyword Minipig .....5959 results
Keyword Minipig + radiation: .....232 results ()
Keyword Minipig + radiation+pediatrics:.....0 results
Keyword Minipig + radiation+pediatric:.....0 results
Keyword Minipig + radiation+juvenile:.....2 results (not relevant to this project)
1) Mortality rates of interventional and surgical procedures performed in domestic juvenile farm pigs and Yucatan mini-pigs.
2) Thymic transplantation in miniature swine. I. Development and function of the "thymokidney".

Keyword Swine .....178021 results
Keyword Swine+ radiation: .....4334 results

Keyword Swine+ radiation+pediatrics: .....10 results (not relevant to this project)
1) Evaluation of ultra-low dose CT in the diagnosis of pediatric-like fractures using an experimental animal study.
2) (EZ)-Cyclobilirubin formation from bilirubin in complex with serum albumin derived from various species.
3) NADPH oxidases and reactive oxygen species at different stages of chronic hypoxia-induced pulmonary hypertension in newborn piglets.
4) Tyrosine phosphorylation of neuronal nitric oxide synthase (nNOS) during hypoxia in the cerebral cortex of newborn piglets: the role of nitric oxide.
5) Reactive oxygen species from NADPH oxidase contribute to altered pulmonary vascular responses in piglets with chronic hypoxia-induced pulmonary hypertension.
6) Dexamethasone prevents alteration of tight junction-associated proteins and barrier function in porcine choroid plexus epithelial cells after infection with Streptococcus suis in vitro.
7) Continuous, noninvasive, and localized microvascular tissue oximetry using visible light spectroscopy.
8) Non-human to human organ transplantation: its biologic basis and a potential role for radiation therapy.
9) Light induces peroxidation in retina by activating prostaglandin G/H synthase.
10) In vivo studies of UV-B-irradiated adult pig islets in diabetic mice.

Keyword Swine+ radiation+pediatric: .....28 results (not relevant to this project)
1) Vesicoureteral reflux in young children: a study of radiometric thermometry as detection modality using an ex vivo porcine model.
2) Pediatric fracture diagnosis--ultra-low-dose CT with an effective dose equal to that of radiographs.
3) Evaluation of ultra-low dose CT in the diagnosis of pediatric-like fractures using an experimental animal study.
4) Enhanced endothelialization on surface modified poly(L-lactic acid) substrates.
5) RF ablation at low frequencies for targeted tumor heating: in vitro and computational modeling results.

6) (EZ)-Cyclobilirubin formation from bilirubin in complex with serum albumin derived from various species.
7) Sequential activation of ground pads reduces skin heating during radiofrequency tumor ablation: in vivo porcine results.
8) NADPH oxidases and reactive oxygen species at different stages of chronic hypoxia-induced pulmonary hypertension in newborn piglets.
9) Diffuse optical monitoring of hemodynamic changes in piglet brain with closed head injury.
10) Tyrosine phosphorylation of neuronal nitric oxide synthase (nNOS) during hypoxia in the cerebral cortex of newborn piglets: the role of nitric oxide.
11) Reactive oxygen species from NADPH oxidase contribute to altered pulmonary vascular responses in piglets with chronic hypoxia-induced pulmonary hypertension.
12) Dexamethasone prevents alteration of tight junction-associated proteins and barrier function in porcine choroid plexus epithelial cells after infection with Streptococcus suis in vitro.
13) Detection of simulated inflicted metaphyseal fractures in a fetal pig model: image optimization and dose reduction with computed radiography.
14) An electrode array that minimizes blood loss for radiofrequency-assisted hepatic resection.
15) Evaluation of a reduced dose protocol for respiratory gated lung computed tomography in an animal model.
16) Radiation exposure reduction during voiding cystourethrography in a pediatric porcine model of vesicoureteral reflux.
17) SLiC technique. A novel approach to percutaneous gastrostomy.
18) 16-MDCT of the posttraumatic pediatric elbow: optimum parameters and associated radiationdose.
19) Continuous, noninvasive, and localized microvascular tissue oximetry using visible light spectroscopy.
20) Porcine kallikrein-4 activation, glycosylation, activity, and expression in prokaryotic and eukaryotic hosts.

Keyword Swine+ radiation+juvenile:.....21 results (not relevant to this project)

1) Reconstructing the sequence of events surrounding body disposition based on color staining of bone.
2) Phenotypic and genetic characterization of a novel phenotype in pigs characterized by juvenilehairlessness and age dependent emphysema.
3) Efficacy and safety of absorbable metallic stents with adjunct intracoronary beta radiation in porcine coronary arteries.
4) Computed tomography dose and variability of airway dimension measurements: how low can we go?
5) Porcine Parkin: molecular cloning of PARK2 cDNA, expression analysis, and identification of a splicing variant.
6) Effect of monopolar radiofrequency treatment over soft-tissue fillers in an animal model: part 2.
7) Effects of monopolar radiofrequency treatment over soft-tissue fillers in an animal model.
8) Prevention of porcine aortic wall calcification by acellularization: necessity for a non-glutaraldehyde-based fixation treatment.
9) Mortality rates of interventional and surgical procedures performed in domestic juvenile farm pigs and Yucatan mini-pigs.
10) Histopathologic alterations after endovascular radiation and antiproliferative stents: similarities and differences.
11) Decreased adventitial neovascularization after intracoronary irradiation in swine: a time course study.
12) Differential remodeling after balloon overstretch injury and either beta- or gamma-intracoronaryradiation of porcine coronary arteries.
13) Radioactive beta-emitting solution-filled balloon treatment prevents porcine coronary restenosis.
14) Thymic transplantation in miniature swine. I. Development and function of the "thymokidney".
15) Effects of intracoronary radiation on thrombosis after balloon overstretch injury in the porcine model.
16) The human intrinsic factor-vitamin B12 receptor, cubilin: molecular characterization and chromosomal mapping of the gene to 10p within the autosomal recessive megaloblastic anemia (MGA1) region.

17) Vascular endothelial growth factor (VEGF) expression during arterial repair in the pig.
18) The role of the adventitia in the arterial response to angioplasty: the effect of intravascularradiation.
19) Intracoronary irradiation: dose response for the prevention of restenosis in swine.
20) Intracoronary irradiation markedly reduces neointimal proliferation after balloon angioplasty in swine: persistent benefit at 6-month follow-up.
21) Intracoronary irradiation markedly reduces restenosis after balloon angioplasty in a porcine model.

III. OBJECTIVE / HYPOTHESIS

Hypothesis: it is possible to establish a pediatric minipig model of ARS (corresponding to humans 1-12 years)

Objectives:
(i) establish protocols and procedures for handling and housing of minipigs <3 months of age at AFRRI

(ii) safely obtain multiple blood samples from pediatric minipigs for CBC counts; and measure animal growth

(iii) irradiate minipigs of pediatric age, establish survival probit curves and monitor signs and symptoms of ARS.

IV. MILITARY RELEVANCE

According to the DoD Chemical and Biological Defense Program 2009 Annual Report to Congress (6): "Although the overall number of nuclear weapons continues to decline because of Russian and U.S. treaty commitments, the United States anticipates an increase in weapon numbers in China, India, and Pakistan. Motivated by economic and strategic interests, Russia and China (or political entities in each) and North Korea continue to supply technologies and components that are dual use and could support weapons of mass destruction and missile programs, especially in the Middle East and South Asia. Iran continues to develop its enrichment program in defiance of United Nations (U.N.) Security Council resolutions and also continues to build a heavy water reactor in Arak, which will be capable of producing plutonium that could be weaponized. While North Korea has halted portions of its nuclear program, it is possible that it has stockpiled several nuclear weapons from plutonium produced at Yongbyon. Non-nuclear radiological dispersal devices (RDD) and radiological exposure devices (RED) pose a significant potential threat especially in the hands of non-state groups ... Contamination and recovery issues associated with operating in a radiological environment remain significant concerns for military operations and underscore the need for robust detection, protection, and decontamination systems."

Although there is no direct military relevance in developing a pediatric animal model of radiation injury, terrorist attacks are renowned for involving also civilian populations, including children. The use of sarin gas on the civilian population in Damascus, Syria, on August 21 2013, left men, women and children injured or dead and it is just one of the testimonies of the type of atrocities perpetrated by extremist groups. Terrorist attacks may extend to military installations housing families with children. Currently, there is no safe and non-toxic radiation countermeasure available for adults or children. Both early and late health effects of radiation exposure are major concerns for the military. Developing countermeasures to radiation exposure is a top priority for the US Department of Defense. Effective countermeasures would expand the options available to field commanders operating Ionizing Radiation (here defined as IR) threat environments, and improve the morale of personnel at risk of exposure to IR. DoD Defense Technology Objective MD.18 states: "Effective mitigation of health consequences [of IR] will (1) reduce casualty load at medical treatment facilities, (2) sustain a more effective operational force after a radiation exposure event, (3) allow commanders to conduct operations in radiation field environments without undue risk of decremented performance of personnel due to acute tissue injury, and (4) reduce the negative psychological impact on personnel tasked to operate in contaminated environments."

An important barrier to advanced development of radiation countermeasures is the paucity of large, long-lived, non-rodent animal models of radiation injury. The only choices at present are NHPs and canines. The advanced evolutionary stage of NHPs, and the expense and danger of working with NHPs, are also disadvantages. Canines are notoriously sensitive to NSAID and vomiting. The addition of miniature swine to available large animal radiation injury models would facilitate development of promising countermeasures.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

The purpose of this study is to establish: 1) Establish protocols and procedures for handling, housing, feeding of pediatric minipigs; 2) Establish protocols and procedures for taking multiple blood samples from pediatric minipig model; and 3) Establish a probit curve for dose-survival relationship.
Health of animals will be monitored at least twice-daily, CBC/differential will be measured on the days indicated below (except in emergency cases, where blood for CBC counts will be taken per VSD veterinarian request). An implantable Micro Identification transponder with programmable Temperature Transponder (Bio Med Data System) will be implanted subcutaneously to monitor body temperature (see section **V.4.4.7. Other Procedures**). We will determine if an externalized catheter will improve ease of blood draws (see section **V.4.4.7. Other Procedures**). In case of catheter malfunction or if the catheter needs to be removed, we will access alternative veins (see list of potential sites under section **V.4.4.2.Biosamples "Collection of blood samples"**). This experiment will utilize irradiation procedures perfected during the experiments performed under IACUC protocol 2008-04-003 and 2011-04-003. Sham-irradiated Gottingen minipigs and minipigs gamma-irradiated at various doses will be compared in studies of radiation/mortality dose response, and invasive and non-invasive biological markers indicating severity of injury.

Number of animals requested for Experiment 1+2 = 6 total (3 animals 5-weeks old + 3 animals 9-weeks old).

Number of animals requested for Experiment 3 = 72 animals = 30 (age 5 weeks) +30 (age 9 weeks) +12 (age 4-5 months).

V.1.1. Experiment 1: handling and housing of pediatric minipigs (5 weeks and older)

Up until now, we have been dealing with adult Gottingen minipigs; protocols and procedures for handling and housing of minipigs <3 months of age at AFRRI are not yet in place and need to be established. Guidelines from the breeder, Marshall Bioresources, will be followed as much as possible (see attached "**Housing and Care Recommendations for the Marshall Gottingen Piglets**" and excerpts below). However, if necessary, modification may be introduced to reflect institutional and project requirements, as described below.

Quarantine and housing (see **V.5.1. Husbandry Considerations**): animals will be quarantined for 7 days, after which an externalized catheter may be implanted in the jugular vein (for animals of age <4 months, use of a VAP for long period of time may not be feasible). During the time of quarantine, animals may be group-housed; however, after implantation of the catheter and/or irradiation, animals will be single housed. Parameters such as food intake need to be monitored on a single animal basis, as anorexia is one of the criteria used for euthanasia. This would not be possible under group-housing conditions. Furthermore, pushing and biting is a normal and expected behavior in social animals while establishing a hierarchy in each new group; because of the immunocompromised and thrombocytopenic status of the irradiated animals, the potential of wounding and infection must be reduced to the minimum.

Feeding, Water (see **V.5.1.2. Special Husbandry Provisions**): normal weaning age is at 4 weeks. When the piglets are weaned their diet is mixed with a milk supplement for the first 2-4 weeks after weaning. This procedure will be continued until 8 weeks of age, when the piglets will be gradually transitioned to a grower maintenance diet (only dry pellets). Milk/formula supplement will be continued after irradiation throughout the duration of the study in addition to the dry pellets. During the weaning time, we will also provide pedialyte as nutritional supplements, in view of the supportive care treatment that the animals will receive after irradiation.

Water is offered ad libitum to piglets from 5 days of birth and older via automatic watering. The proper height for automatic water nipples is about ½ - 1 inch above the top of the pig / piglets shoulder. It is very important to make routine adjustments (weekly) in the height of the water nipples as piglets grow.

V.1.2. Experiment 2: to establish procedures for catheter implantation, blood draw and measurement of animal growth in animals 5 weeks old and older.

Blood counts and the dose that cause 50% of lethality (LD50) are among the most useful terms for ARS comparison across categories (species, age, co-morbidities etc). Indication of blood counts after irradiation together with the determination of the LD50 will be critical in the characterization of a pediatric model of ARS. The minipig is emerging as a valid alternative to NHP and dogs as a large animal model for the ARS. We have optimized the implantation and patency of Vascular Access Port (VAPs) in animals >4 months old, in order to obtain serial blood samples. By this age, skeletal growth rate has slowed down and an internal catheter can be safely left in place for at least 3 months (4). For animals of age <4 months, use of a VAP for long period of time may not be feasible. Growth rate of minipigs is highest between birth and 4 months of age (Figure 1); use of an internal catheter may require frequent adjustments in the catheter length after just a few weeks after implantation. In the case of irradiated minipigs, where blood cytopenia and susceptibility to infection and bleeding are among the leading causes of death, any major surgical intervention is expected to negatively impact the outcome.

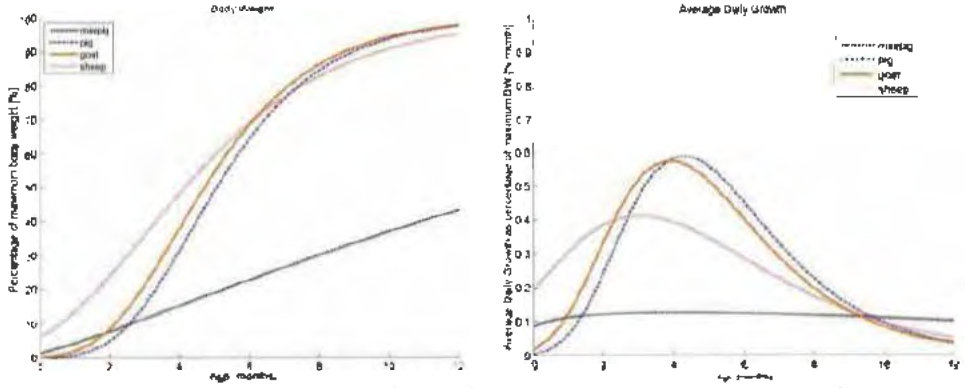


Fig. 1. Body weight (BW) and average daily growth curves expressed as percentage of the maximum BW for Gottingen minipig, Yorkshire pig, Young Hair goat, and Suffolk sheep. Growth curves acquired from Gompertz or Richards growth function as determined in the literature. From: Alex K. Roth, Rob Bogie, Eva Jacobs, , Jacobus J. Arts, Lodewijk W. van Rhijn, Large animal models in fusionless scoliosis correction research: a literature review. The Spine Journal 13 (2013) 675–688.

Our long term goal is to be able to collect sufficient amount of blood for CBC counts and other experiments from irradiated pediatric animals and age-matched sham-controls, within allowed limits, ("*All nonterminal blood collection without replacement of fluids is limited up to 10% of total circulating blood volume in healthy, normal, adult animals on a single occasion and collection may be repeated after 3 to 4 weeks. In case repeated blood samples are required at short intervals, a maximum of 0.6 ml/kg/day or 1.0% of an animal's total blood volume can be removed every 24 hour*"), up to three times a week and up to 60 days after irradiation and to establish basic preliminary, non-invasive assessment for future studies. See below calculated blood volumes per weight of animals. No more than 7% will be drawn in any one 7-day period, followed by a 7-day recovery period, and when performing serial bleeds, 24-hours of rest should be allowed for each 1% of body weight sampled up to a maximum of 21% of total blood volume within a 21-day period. A 7-day rest period must be observed whenever the maximum allowable 21-day volume has been attained.

Table 2: Relationship between age, weight and circulating blood volume.

Age	Weight (kg)	Total circulating blood volume (mL)	1% total circulating blood volume (mL)	10% total circulating blood volume (mL)
4-5 Weeks*	2-4	130-260	1.3 - 2.6	13-26
5-6 Weeks*	2-4	130-260	1.3 - 2.6	13-26
6-7 Weeks*	3-5	198-330	approx 2-3	approx 20-30
7-8 Weeks*	3-5	198-330	approx 2-3	approx 20-30
2 - 3 Months	5-7	330-462	approx 3-4.5	approx 30-45
3 - 4 Months	7-9	462-594	approx 4.5-6	approx 45-60
4 - 5 Months	9-11	594-726	approx 6-7	approx 60-70

On average, the total circulating blood volume is equal to 5.5 -7.0 % (~66 ml/kg) of the animal's body weight.

Blood sampling: blood may be used for CBC counts, and, if time and resource allow it, for in vitro/ex vivo studies (i.e. biodosimetry assays, mechanistic studies, immunophenotypic characterization of immune cell subsets etc).

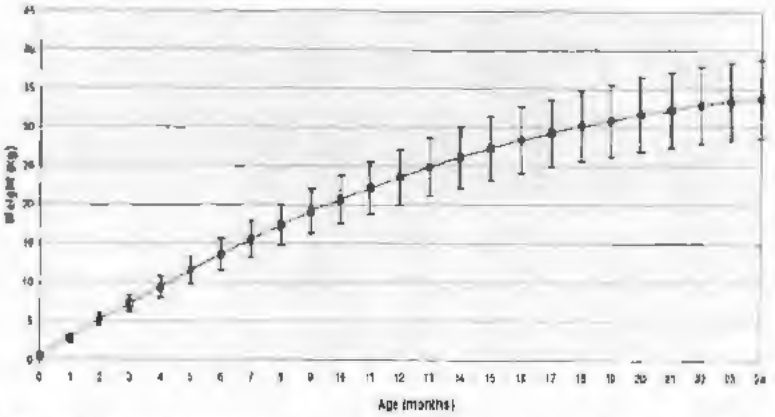
In Experiment 2, we propose to: (i) evaluate alternative ways to obtain serial blood samples, and (ii) assess growth of normal animals.

Experiment 2.1): Evaluate alternative ways to obtain serial blood samples, i.e. from externalized catheters and peripheral veins.

Using animals that will be sham irradiated, VSD personnel will attempt to sonographically guide the placement of an intravenous catheter in the jugular or femoral vein, for serial blood sampling, as described in (5) (see section **V.4.4.7. Other Procedures**). Ultrasound imaging is routinely used to facilitate safe, minimally invasive puncture of deep vessels; the described procedure is fast and results in no physiological complications. Ultrasound is used to visualize the jugular vein and the needle as it enters the vein, enhancing success (otherwise, this would be a blind technique in the pig). VSD has an ultrasound machine.VSD personnel will perform this technique- either a veterinarian or a properly trained technician. We would likely sedate with telazol the same as for irradiation, though it can be performed in the awake animal. Alternatively, if placement of the catheter fails and/or catheter loses patency, we will collect blood from peripheral veins such as auricular, femoral, cephalic, saphenous etc, with the animal under sedation (see section **V.4.4.7. Other Procedures**). Blood will be collected once before irradiation, and, with day 0 being the day of sham-irradiation, on days 0, 1, 3, and once a week after that, up to 60 days. Blood collected will not exceed 1% of total blood volume.

Experiment 2.2): Measurement of normal animal growth

-Body weight and skeletal development. At least once a week, we will measure body weight and take measurements of skeletal development such as: height (cm, from shoulder to floor) and length (cm, from snout to base of tail). As a guide, minipigs should gain roughly 0.5 kg per week, from 2 month of age until they are 12 months of age. We will also record vitals (T, HR and RR), and standard animal health check status (i.e. hydration status, hair coat, skin quality).



- Weight of the main organs. At the time of euthanasia we will take the weight of the main organs (i.e. heart, lungs, spleen, liver, kidney), for comparison with those of irradiated animals (second portion of this study).

Irradiated animals will be followed for at least 45 days after irradiation (45-day survival study); depending on availability of space and funds, animals may be observed up to 60 days from the day of irradiation, to obtain preliminary information about longer 60-day survival. Animals will be euthanized when they reach endpoint criteria or within 60 days from the day of irradiation, whichever comes first.

-Radiographs of the epiphyseal plates may be taken at the time of or prior to euthanasia, on sedated animals. Sedation will be the same as for irradiation (telazol), though dose may be on the lower side as duration of effect does not necessarily need to be so long.

Experiment 3: to determine age group-specific probit curves following irradiation with Co-60 (TBI, 0.6 Gy/min).

There is very limited information available on TBI-ARS for demographics of 0-12 years (children) or >75 years (elderly), and no treatment for age-specific radiation injuries are being developed. We propose to subject juvenile animals (<3 months of age) to irradiation and establish a dose-survival relationship. We will determine 2 probit curves, one for animal in the age range of 4-8 wks (representing 1 mo-2 yr humans) and the other one for animals in the range of 8 wks-3 mo (representing 2-12 yr humans). Animals will be administered minimum supportive care on a pre-fixed, standard regimen (see regimen for supportive care below). Probit curves will be compared to data obtained from young adult animals (5 months of age) administered the identical supportive care as the pediatric animals and irradiated at doses approaching the LD10 and LD90 (1.6 Gy and 2.0 Gy, respectively). This is not a repetition of our past minipig studies employing adult animals, since those studies were done in the absence of supportive care. Inclusion of adult animals is essential to establish preliminary observation regarding age-dependent dose-responses.

Irradiation: Irradiation will be total body, at the dose rate of 0.6 Gy/min (gamma photons, Co-60). Groups will be of 6 animals per dose (n=30 animal total). For each age group, we will begin with doses of 1.6 and 2.0 Gy, and will then choose 3 additional doses based on the results of the initial experiments and in consultation with a statistician. We do not expect to reach doses higher than 3.2 Gy. In order to determine if pediatrics are more sensitive to radiation-induced lethality, we will use a "control" age group, consisting of young adult animals (5 month old). For the control group, we will irradiated at 1.6 and 2.0 Gy, n=6 animals per dose); these doses represent the LD10 and LD100 for 5 month-old animals in the absence of supportive care (our historical data), and will provide a preliminary indication about the effect of supportive care on survival and the age-dependent difference in sensitivity. A complete dose response curve for 5-month old animals in the presence of supportive care is not the focus of this study. Below is reported the study protocol for this experiment.

Table 3: Study protocol

45-day survival study, 1 week quarantine, minimum supportive care	*Age 6 weeks*	*Age 10 weeks*	*Age 5 months*
Group 1 (survival+ blood draws)	6 animals (3x 1.6 Gy, + 3x 2.0)	6 animals (3x 1.6 Gy, + 3x 2.0)	
Group 2 (survival+ blood draws)	6 animals (3x 1.6 Gy, + 3x 2.0)	6 animals (3x 1.6 Gy, + 3x 2.0)	2 animals 1.6 Gy + 2 animals 2.0 Gy
Group 3 (survival+ blood draws)	6 animals (dose t.b.d)	6 animals (dose t.b.d)	2 animals 1.6 Gy + 2 animals 2.0 Gy
Group 4 (survival+ blood draws)	6 animals (dose t.b.d)	6 animals (dose t.b.d)	2 animals 1.6 Gy + 2 animals 2.0 Gy
Group 5 (survival+ blood draws)	6 animals (dose t.b.d)	6 animals (dose t.b.d)	
TOTAL (72)	30	30	12

Endpoints will be 45-day survival, CBC counts (if determined feasible from results of Experiment 2.1 and as per schedule described in **V.4.4.7. Other Procedures**), signs and symptoms, and measurement of animal growth.
Animals will be observed for a minimum of 45 days and up to 60 days after irradiation.
Signs and symptoms: vitals (T, HR and RR), body weight, and standard animal health check status (i.e. hydration status, hair coat, skin quality etc)
Measurement of animal growth will be taken as described in Experiment 1.2)

Supportive care: Animals will be administered minimum supportive care (defined here as antibiotics, antipyretic/anti-inflammatory, and dietary supplementation) on a standardized regimen. No IV fluids will be provided. Treatment schedule is chosen based on our historic dataset with TBI at doses 1.6 -12 Gy and reflects time to recovery of bone marrow after irradiation.

Table 4: Supportive care

Treatment		Regimen	Admin route	Start/end
Antibiotics	Enrofloxacin (Baytril)	For animals 3-10 kg, 3 ml of 0.5% oral solution *SID** For animals >10 kg, 5-7 mg/kg *SID*	*PO* (or *IM* or *IV* if *PO* not possible)	3-30 d
Antipyretic/anti-inflammatory	Carprofen (Rimadyl)	2.2–4.4 mg/kg *BID*	*PO* (or *IM* or *IV* if *PO* not possible)	3-30 d
Dietary Supplementation	Age-appropriate food, milk/formula, Pedialyte, Metamucil, canned fruits and vegetables. NO RAW FOOD			Throughout study, as necessary

* http://www.animalhealth.bayer.com/5293.0.html; if dosage is inadequate to cope with challenges from radiation damage, we will use the "adult" dosage of 5-7 mg/kd *SID*.

V.2. Data Analysis
The dose response relationship (DRR) will be plotted as probit percent mortality versus linear dose. Probit regression analysis will be used to generate the probit curves, using the PASW Statistic 18 software, SPSS Inc, IL, USA and preliminary values for LD30, LD50, and LD70will be obtained. Survival data from

the probit curve generated in Experiment 3 will be compared to our historical probit curve generated in the absence of supportive care (3).

For the generation of the dose-response curve, the LD30, 50 and 70 will be estimated using the probit method. This method allows for construction of confidence intervals to describe the precision of the estimated lethal doses. The width of the confidence interval depends on the number of doses, number of animals per dose, and the slope of the dose-response curve, and a narrow interval indicates a more reliable estimate. We propose to use five doses and six animals per dose. For a dose-response curve ranging from 1.6 to 2.0 Gy with mortality rates of .1, .3, .5, .7 and .9 percent for the five doses, the expected width of the 95% confidence interval for the LD50 will be 0.3 Gy, and the expected width of the 95% confidence interval for the LD30 and LD70 will be .5 Gy. Expected confidence interval widths are based on the average of 1,000 simulated data sets using the study design and mortality parameters indicated.

Survival data from young adult animals, administered identical supportive care as the pediatric population, will serve two purposes: to gain preliminary insight into the effect of age on sensitivity to radiation (group 1 vs group 2), and to gain preliminary insight into the efficacy of supportive care on survival (group 2 vs group 3).

Table 5: Group comparison for data analysis

Group 1	Pediatric animals + supportive care	This study
Group 2	Adult animals + supportive care	This study
Group 3	Adult animals - supportive care	Historical data

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered.

The purpose of this project is to determine the effect of age and supportive care on survival. There is no computer model or tissue culture that can mimic the effect of radiation on the whole organism and predict lethality. Survival depends on an intricate network of organ cross-talks and cellular microenvironment that cannot be recreated *in vitro*. Consequently, administration of supportive care to cellular components grown *in vitro* will not allow any assessment on survival. Development and aging also cannot be reproduced *in vitro*.

We do not know all of the cell types and tissues involved in radiation injury or how radiation injury causes lethality. The concept of multi-organ failure has been suggested, but how the injured organs affect each other's vital functions is unknown. The present state of the art of tissue culture is still incapable of reproducing the *in vivo* relationships. Computer modeling is similarly limited by lack of full characterization of the biological elements and interactions under study.

These considerations are consistent with the FDA requirement for preclinical research in both small and large mammals before granting approval for drug testing in humans.

V.3.2. Animal Model and Species Justification

We have been developing the adult Gottingen minipig as an additional large animal model for the study of the ARS and advanced development of radiation countermeasures. Currently, there is no pediatric animal model to study the ARS. Large, long-lived, and development-matched models are required for drug studies submitted to the FDA for licensure applications. Comparison between adult and pediatric Gottingen minipigs will help characterize the effect of radiation on the pediatric population, for countermeasure development and drug testing. The close similarity to humans in anatomy and physiology of organs such as the liver, pancreas, kidney and heart has made the pig the primary species of interest as organ donors for xenographic procedures. Minipigs are routinely used for toxicity testing. As such, further and rapid characterization at physiological, molecular and genetic level of the minipig is likely to occur, thus

increasing acceptance of this model and facilitating advanced drug development. Our previous studies have employed male Gottingen minipigs to develop a probit curve. Since we are testing the effect of supportive care and age on survival and we are using our own historical data as one of the terms of comparison, we will continue to use male minipigs for this study.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species *Sus scrofa domestica*

V.3.3.2. Strain / Stock Gottingen

V.3.3.3. Source / Vendor Marshall BioResources/Marshall Farms Group Ltd

V.3.3.4. Age 5 weeks to 5 months

V.3.3.5. Weight 2-13 kg

V.3.3.6. Sex Male

V.3.3.7. Special Considerations NA

V.3.4. Number of Animals Required (by species): 78

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement
The study data-points currently mandated by the FDA for approval of radiation countermeasures under the Animal Efficacy Rule is mortality. Hence the primary study data-point in our development of Gottingen minipigs as a model for ARS is survival. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress, using an extensive set of criteria (see animal endpoints, paragraph V.4.5 – Lethargy, weight loss, temperature, respiratory distress, food intake, bleeding etc). Animal care and use procedures will only be performed by trained personnel. We have considerable experience in the care and handling of minipigs. Extensive human interaction every weekday will reduce stress in the animals. Use of slings, topical anesthetics, and the catheters will minimize distress during blood collections and the animals will be gradually acclimated to the slings for these procedures.

V.3.5.2. Reduction
Control animals used to optimize protocols for animal handling, housing and blood draw will be used also to obtain data on growth of normal animals, and to set up dosimetry for future studies and to obtain baseline CBC data in pediatric animals. For irradiated animals, blood samples obtained from minipigs before irradiation will serve as controls, thus reducing the number of animals required for this study. Use of historical controls will also reduce the number of animals needed for this study.

V.3.5.3. Replacement NA

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C ___0__ (# of animals)

V.4.1.1.1.2. Column D ___6__ (# of animals)

V.4.1.1.1.3. Column E __72__ (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1+2	Experiment 1 (Handling and housing) +2 (Procedures for catheter implantation, blood draw and measurement of animal growth)		6		**6**
3	Experiment 3: to determine age group-specific probit curves following irradiation with Co-60 (TBI, 0.6 Gy/min).			**72**	**78**
Totals:					**78**

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization
Irradiation/Catheter placement
Prior to irradiation, animals will be sedated with Telazol® (Telazol, 6-8 mg/kg *IM*; 100 mg/ml, Fort Dodge Laboratories, IA). Alternatively, Telazol® (4.4 mg/kg *IM*), in combination with dexmedtomidine (0.05-0.1 mg/kg *IM*) or ketamine (20 mg/kg *IM*)/xylazine (2 mg/kg *IM*) can also be used for sedation. Atropine (0.05 mg/kg *SC, IM* or *IV*; atropine sulfate, Butler, Columbus, OH) or glycopyrrolate (0.01 mg.kg) may be given to reduce mucosal secretions. At the end of the irradiation procedure, once the animals are back in their quarters, dexmedetomidine can be reversed by atipamezole *IM* (same volume as dexmedotmidine) to facilitate recovery.
For catheter placement, Isoflurane 1-4% may be administered via face mask on as needed basis.

Blood sampling
Animal may be sedated at the time of blood sampling and administration of supportive care with Midazolam (0.1-0.5 mg/kg s.c.or *IM*), or acepromazine (1.1 mg/kg *IM*), 10 minutes prior to venous access. Telazol or Telazol +dexmedetomidine or Ketamine + Xylazine could be used as an option as well.

V.4.1.2.2. Pre- and Post-procedural Provisions
Irradiation
Pre-Irradiation: Animals will be fasted overnight (approximately 16–18 h) and sedated as described above. Once sedated in the VSD animal facility, minipigs will be transported to the AFRRI cobalt facility in an approved, covered transport cage. Heart rate and temperature will be measured prior to irradiation. At least one vet tech or one veterinarian will be present at the time of irradiation. Immediately before irradiation, the vet or vet tech will determine whether or not to administer additional sedative to keep the animal asleep during irradiation.
Irradiation procedure: To enable proper positioning, anesthetized animals will be strapped in place into slings in polyvinylchloride PVC frames for irradiation. Effect of anesthesia will be monitored in real time through video cameras positioned in the Cobalt waiting area. Minipigs will be removed from the slings once irradiation is completed. Temperature and heart rate will be taken before returning the minipigs to their home cages.
Post-Irradiation: After irradiation, animals will be transported back to their cages, where they will be allowed to recover from sedation under the careful surveillance of VSD and/or research staff who will

monitor vital signs and provide thermal support (e.g. Bair Hugger blanket) if necessary. Atipamezole *IM* (same volume as dexmedotmidine) may be used to reverse the anesthetic effect of dexmedetomidine.

V.4.1.2.3. Paralytics N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched Agricola, Pubmed

V.4.1.3.2. Date of Search
V.4.1.3.3. Period of Search All available

V.4.1.3.4. Key Words of Search The aspects of the study relevant to alternatives to painful or distressful procedures are the sequelae to exposure to TBI at doses causing ARS. We used the following key words in the search for alternatives:
swine, radiation, ARS, sequelae, pain, distress, alternatives

V.4.1.3.5. Results of Search

AGRICOLA
Swine AND radiation AND pain:.....0 results found

Swine AND radiation AND distress:..... 0 results found

Swine AND radiation AND alternatives.....5 results (not relevant)
1) Energy for swine facilities II Alternative sources of energy
Fehr, Rl NAL Catalog (AGRICOLA)
2) Evaluation of a porcine lens and fluorescence assay approach for in vitro ocular toxicological investigations
Oriowo, Om NAL Catalog (AGRICOLA)
3) Molecular characterization, chromosomal location, alternative splicing and polymorphism of porcine GFAT1 gene
Liu, K. NAL Catalog (AGRICOLA)
4) Evaluation of alternative techniques to determine pork carcass value
Akridge, Jt NAL Catalog (AGRICOLA)
5) Genomic structure, chromosomal localization and expression profile of a porcine long non-coding RNA isolated from long SAGE libraries
Ren, H. NAL Catalog (AGRICOLA)

Swine AND ARS AND pain:.....0 results found

Swine AND ARS AND distress:..... 0 results found

Swine AND ARS AND alternatives.....2 results (not relevant)

1) Environmental Prevalence and Persistence of Salmonella spp in Outdoor SwineWallows
Callaway, Tr NAL Catalog (AGRICOLA)
2) Factors affecting cellular outgrowth from porcine inner cell masses in vitro
Schilperoort-Haun, Kr NAL Catalog (AGRICOLA)

Swine AND radiation sequelae AND pain:.....0 results found

Swine AND radiation sequelae AND distress:.....0 results found

Swine AND radiation sequelae AND alternatives:.....0 results found

Pubmed

Swine AND radiation AND pain:.....264 results found

Swine AND radiation AND distress:..... 115 results found

Swine AND radiation AND alternatives.....0 results (not relevant)

Swine AND ARS AND pain:.....16 results found (same as those found for

Swine AND acute radiation syndrome AND pain.....16 results (b)(4)

(b)(4),(b)(6)

2) Development of a Fluorogenic 5' Nuclease PCR Assay for Detection of the *ail* Gene of Pathogenic *Yersinia enterocolitica*. Alissa D. Jourdan, Scott C. Johnson, Irene V. Wesley Appl Environ Microbiol. 2000 September; 66(9): 3750–3755.

3) Experimental swine vesicular disease, pathology and immunofluorescence studies. R M Chu, D M Moore, J D Conroy. Can J Comp Med. 1979 January; 43(1): 29–38.

4) Recent advances in small bowel diseases: Part II. Alan BR Thomson, Angeli Chopra, Michael Tom Clandinin, Hugh Freeman. World J Gastroenterol. 2012 July 14; 18(26): 3353–3374.

5) Innate Immune Defenses Induced by CpG Do Not Promote Vaccine-Induced Protection against Foot-and-Mouth Disease Virus in Pigs. M. P. Alves, L. Guzylack-Piriou, V. Juillard, J.-C. Audonnet, T. Doel, H. Dawson, W. T. Golde, H. Gerber, N. Peduto, K. C. McCullough, A. Summerfield. Clin Vaccine Immunol.

6) Ectonucleotidases in Solid Organ and Allogeneic Hematopoietic Cell Transplantation Petya Chernogorova, Robert Zeiser. J Biomed Biotechnol. 2012; 2012

7) Guidelines on Management of Human Infection with the Novel Virus Influenza A (H1N1) – A Report from the Hospital das Clínicas of the University of São Paulo. Ludhmila Abrahao Hajjar, Denise Schout, Filomena Regina Barbosa Gomes Galas, David Everson Uip, Anna Sara Shafferman Levin, Helio Hehl Caiaffa Filho, Pedro Takanori Sakane, Carlos Alberto Suslik, Jose Manoel de Camargo Teixeira, Eloisa Bonfa, Antonio Alci Barone, Milton de Arruda Martins, Marcos Boulos, Jose Otavio Costa Auler, Jr

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Swine AND ARS AND distress:..... 13 results found (not relevant for this study)
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Int J Biol Sci. 2007; 3(3): 179–184.
2) Pacing-Induced Regional Differences in Adenosine Receptors mRNA Expression in a Swine Model of Dilated Cardiomyopathy Silvia Del Ry, Manuela Cabiati, Vincenzo Lionetti, Giovanni D. Aquaro, Alessandro Martino, Letizia Mattii, Maria-Aurora Morales
PLoS One. 2012; 7(10):
3) Experimental Infection of Pigs with the Human 1918 Pandemic Influenza Virus
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Published in final edited form as: J Toxicol Environ Health B Crit Rev. 2007; 10(Suppl 1): 1–269.

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V.4.1.4. Unalleviated Painful or Distressful Procedure Justification
Depending upon the irradiation dose irradiated animals die due to compromised immune responses and opportunistic infections. The percentage of surviving animals is the indicator of the efficacy of a countermeasure. We cannot give systemic anesthetic agents to animals after the irradiation procedures, since they are known to interact with the immune system (see references in Jacobsen, K. O., V. Villa, V. L. Miner, and M. H. Whitnall. 2004. Effects of anesthesia and vehicle injection on circulating blood elements in C3H/HeN male mice. *Contemp Top Lab Anim Sci* 43:8-12.), and would confound the correlation of radiation dose with incidence of moribundity, resulting in a waste of animals. However, we are providing supportive care in terms of antibiotics, analgesics, antipyretics, fluids/ nutritional support to increase survival. Use of supportive care is expected to mitigate the pain associated to the sequelae of irradiation.

V.4.2. Prolonged Restraint
Only short periods of restraint in the sling (<30 minutes) will be necessary for blood collections and the irradiation procedure. To minimize discomfort, we have designed a new type of sling that uses softer, elastic material to support the animal, and utilizes a more efficient restraint design that maintains the animal in place while reducing manipulation by technicians and consequently stress to the animals. After the quarantine period, animals are acclimated to the sling for 5-10 minutes, 2-3 times before initiating blood draws. Acclimation to the sling is very rapid and non-stressful; animals are petted and gently spoken to while restrained. It has been our experience that resting on the sling does not agitate the animal at all.
Animals are anesthetized and transported to the Cobalt Facility holding area before being put into slings for irradiation (<20 minutes). Animals will be kept under continuous observation while in the sling. Slings will be spot-cleaned between animals and will be sanitized at the conclusion of each irradiation day.

V.4.3. Surgery NA

V.4.3.1. Pre-surgical Provisions

V.4.3.2. Procedure(s)

V.4.3.3. Post-surgical Provisions

V.4.3.4. Location

V.4.3.5. Surgeon

V.4.3.6. Multiple Major Survival Operative Procedures NA

V.4.3.6.1. Procedures

V.4.3.6.2 Scientific Justification

V.4.4. Animal Manipulations

V.4.4.1. Injections

All injections listed in section V.4.1.2.1 will be administered *IM*, SC, using sterile needles (23 – 21 gauge) and syringes, or IV through the catheter or *PO*.

V.4.4.1.1. Pharmaceutical Grade Drugs

Lidocain (5%), topical
Telazol®
Ketamine/xylazine
Atropine atropine sulfate
Heparin lock, IV
Saline
Carprofen
Metamucil
Enroflaxacin or baytril
Rimadyl
Midazolam
Acepromazine
All the drugs listed above are ordered from veterinary product distributors by VSD and they all are pharmaceutical grade drugs.
Atipamezole (Antisedan, Pfizer)
Dexmedtomidine (Dexdomitor, Pfizer)

V.4.4.1.2. Non-Pharmaceutical Grade Drugs NA

V.4.4.2. Biosamples
Collection of blood samples. We will obtain blood samples from the externalized catheter or from peripheral veins. If the catheter fails or needs to be removed, the sample will be collected from accessible peripheral vessels (see section **V.4.4.7. Other Procedures**). Mean blood volume for pigs is 65-67 ml/kg. Up to 1% of total blood will be drawn per time point, depending upon the frequency of previous bleeds (Walter Reed Army Institute of Research. 1997. Investigators' Handbook. Section I: Handling Techniques and Noninvasive Procedures).

Tissue and histological samples. Tissue samples, to include organs, blood and hair may be taken at the time of euthanasia for tissue blocks and for frozen tissue repository.

V.4.4.3. Adjuvants NA

V.4.4.4. Monoclonal Antibody (MAb) Production NA

V.4.4.5. Animal Identification Tattoos or ear tags and cage cards/tags

V.4.4.6. Behavioral Studies NA

V.4.4.7. Other Procedures
Catheter implantation
Catheter implantation will be done following the Seldinger technique. Briefly, the procedure is described as follows:
(http://www.dechra.co.uk/Critical-Care-Tips/Central-Venous-Set---Using-the-Seldinger-Technique.aspx)

Placement

1 Clip and aseptically prepare site. Right Jugular vein in the neck area or the right/left femoral vein in the inner thigh may be used for catheter placement.
2. Insert the introducer needle into the vessel (gauge needle: 18-24).
3. Whilst steadying the needle use the adaptor to straighten the j-wire and insert it into the needle.
4. Advance the wire into the vessel.
5. Remove the needle and the wire cover making sure the wire does not advance any further into the vessel.
6 Whilst holding the wire steady advance the dilator over the wire 1 to 1.5 inches into the insertion point and rotate back and forth. A small nick incision (1-2 mm) may be needed in the skin prior to inserting dilator.
7. Remove the dilator.
8. Advance the catheter over the wire into the vessel.
9. Remove the wire.
10 Flush the catheter and secure the catheter to the patient with sutures
11. Attach fluid delivery equipment to the catheter.
12. Cover with sterile dressing and a light bandage.

Care and maintenance
1. Ensure the catheter site is kept clean and treated aseptically throughout use
2. Check suture integrity at regular intervals

Catheter to be used will be single lumen indwelling catheter in Polyurethane material which provides strength during insertion and also softens at body temperature to conform to the body tissues. The complete set of central venous catheter kit for catheter implantation consists of:

- Indwelling catheter
- Catheter holder
- Catheter holder clamp
- Extension line clamp
- Injection cap
- Introducer needle
- J-Tip guide wire
- Luer lock syringe
- Scalpel
- Vessel dilator

The soft Flexible J-Tip Guide wire provides good torque to ensure firm insertion and also prevents vessel perforation. The tip is specialty designed for smooth and easy insertion of catheter. Clear and definite marking facilitates correct placement of catheter tip. The catheter is radio-opaque for proper placement using ultrasound imaging. Animals will be sedated during the procedure. (Sedation procedure has been described in the anesthesia section V 4.1.2.1). After placement, the wound will be protected with aerosol bandage aid and the catheter will be covered with elasticon tape.

Temperature transponder At the time of catheter implantation, a temperature microtransponder (Implantable Electronic ID Transponder IPPT-300, Bio Medic Data Systems, Seaford, DE) will be implanted subcutaneously behind the neck, over the shoulder opposite to the site of VAP surgery, to facilitate body temperature measurements.

Blood draw from externalized catheter. Catheter will be flushed with 5 ml saline and the first 0.5 ml of blood will be discarded. Blood samples will be collected using a new syringe. After blood collection, catheter will be flushed again and locked with 3 ml of heparin lock (100 U).

Blood draw from peripheral veins. Alternatively, if placement of the catheter fails and/or catheter loses patency, we will collect blood from peripheral veins such as auricular, femoral, cephalic, saphenous etc. Animals may be sedated at the time of blood sampling and administration of supportive care (see section V.4.1 2.1) if necessary following the recommendations of an AFRRI veterinarian. All procedures will be

entered into the experimental record. Lidocain (5%) may be applied topically to the site of venipuncture to minimize discomfort to the animals about 15 minutes before starting the procedure; site will be cleaned with betadine and alcohol. Blood will be collected once before irradiation, and, with day 0 being the day of sham-irradiation, on days 0, 1, 3, and once a week after that, up to 60 days. Collection of samples will be performed with the minipig restrained in a sling; at the same time, we will take vitals. The entire procedure takes less than 30 min. After blood draw, animals will be returned to their cages and checked to ensure that the needle puncture wound is closed and the blood has clotted.

Health check status
Body weights and standard animal health check status (hydration status, hair coat, skin quality, vitals) will be determined at least once prior to treatment, on the first day of treatment, and on days of blood collection. Body temperature will be determined during week-days between 8 am and 4 pm, and on weekends if there are animal concerns. The time of each temperature collection will be recorded for each minipig.

V.4.4.8. Tissue Sharing Bio-samples (**V.4.4.2. Biosamples_Tissue and histological samples**) will be collected, to be used for histo-chemistry/protein/molecular assays among others, and to be shared with collaborators upon request and as experiments permit

V.4.4.9. Animal By-Products

V.4.5. Study Endpoint The data-point currently mandated by the FDA for approval of radiation countermeasures under the Animal Efficacy Rule is moribundity or mortality. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress. Euthanasia will be carried out when any one of absolute endpoints or combination of the following non-absolute signs of moribundity are observed and in consultation with AFRRI veterinarians.

Absolute
1. Non responsive, assuming the animal has recovered from anesthesia.
2. Dyspnea
3. Loss of 20% of expected weight (based on Marshall Bio-resources growth chart –weight vs age- and day 0 baseline percentile).
4. Hypothermia (< 36 ºC)

Non-absolute
1. Hyperthermia (>41 ºC)
2. Anorexia (skip 3 consecutive meals)
3. Anemia/pallor, CRT >2 seconds. CRT will be performed behind the ears.
5. Petechiae/ecchymosis
6. Vomiting/diarrhea
7. Lethargy
8. Seizures or vestibular signs (falling, circling or head tilt)
9. Uncontrollable hemorrhage

At the time of euthanasia, the attached observation sheet (Attachment 1) will be filled out, and may be used retrospectively at the end of study to put together a score sheet for euthanasia criteria for minipigs, similarly to that for rodents.

V.4.6. Euthanasia
Animals will be euthanized according to current American Veterinary Medical Association (AVMA) guidelines. Animals will be sedated with Telazol® (6-8 mg/kg *IM*) or ketamine (20 mg/kg *IM*)/xylazine (2 mg/kg *IM*). Animals will then be euthanized with and injected IV Euthasol® (sodium pentobarbital, (1 ml/4.5 kg IV or IC) or another commercial euthanasia solution.
Alternative (only if the peripheral percutaneous venous access is not achieved or the external catheter is not functional in the pediatric model), peripheral or central venous cut down or cardiac stick may be performed, with the animals maintained under general anesthesia using isoflurane gas at a maintenance

rate of 1% to 3% and oxygen flow rate of 1 to 2 l/min. Death will be confirmed by VSD veterinarian or veterinary technician via auscultation of the heart, with cessation of sounds for at least 5 minutes. See item V.4.5 for euthanasia criteria before the end of the study

V.5. Veterinary Care

V.5.1. Husbandry Considerations

Animal observations - Throughout the course of the study, animals will be monitored twice a day by VSD caretakers and technicians. Frequency of observation will increase after animals have been irradiated and appear to get sick; in addition to the VSD check, research staff will monitor animals twice a day during week days, and if necessary, after hours and on weekends.

Housing - During quarantine/acclimation period, minipigs may be group-housed. After surgery and/or after irradiation, they will be singly housed to prevent damage to the catheter and to allow individual assessment of feed consumption and fecal/urine/blood production. Rooms will be maintained on a 12:12 h light (0600 to 1800) dark cycle. For piglets 1 – 2 months in age (weaned) room temperature should be maintained between 75 – 78 degrees F / 24 – 26 degrees C. **All young piglets will be provided with a sleeping mat to keep warm.**

Feed and water provisions – Animals will be fed twice a day according to their weight. Age appropriate food will be provided, after consultation with Harlan nutritionist, together with milk supplement, and additional nutritional support as described in Experiment 1. Each piglet will have 60 mL of milk/formula supplement, per piglet, 2 times daily, mixed with approximately ½ the scheduled pellet diet. Milk, alone and with pellet diet, will be provided either with a bottle or in a dish.The remaining ½ of diet will be offered in feeding trough, shallow dish or floor so the piglets are used to eating a dry diet. This procedure will be continued until animals are 8 weeks of age, when the piglets will be gradually transitioned to a grower maintenance diet (only dry pellets)**. We may continue to offer 60 mL of milk supplement twice a day, and Pedialyte until the animals recover, as a part of supportive care. After feeding, all troughs or dishes must be cleaned and disinfected properly to prevent bacterial infections which may result in piglet diarrhea.** During the weaning time, we will also provide pedialyte as nutritional supplements, to get the animals used to the taste, in view of the supportive care treatment they will receive after irradiation.

Supportive care will be provided after irradiation to all animals, accordingly to Table 1. Age-appropriate liquid food and/or moistened pellet will be offered if observed that the sick animal prefers it to dry food. Water is offered *ad libitum* to piglets from 5 days of birth and older via automatic watering. The proper height for automatic water nipples is about ½ - 1 inch above the top of the pig / piglets shoulder. It is very important to make routine adjustments (weekly) in the height of the water nipples as piglets grow. A bowl of water is also offered to all piglets accompanied with the automatic water for at least 3 days post weaning or until caretakers are sure that all piglets have found the automatic water source. It is a good practice to leave the bowl of water under the automatic water nipple until all piglets adjust to the location of their new water source. Dishes should be sanitized daily and filled with clean fresh water throughout the day as needed. The automatic water source should supply 0.5 – 1.0 quart or liter per minute.

V.5.1.1. Study Room

Minipigs will be located at AFRRI for the duration of the study. They will either be in a VSD surgical suite for catheter placement, in transit to and from the cobalt facility in a covered transport cage, in slings for irradiation in the cobalt facility, or in their housing cage or a VSD prep room for blood collections or euthanasia. They will be housed in stainless-steel cages in an environmentally controlled and continuously monitored animal room as described in Section V.5.1

V.5.1.2. Special Husbandry Provisions

Diet will be controlled in accordance with vendor's recommendations so as to prevent excessive weight gain. No sugar-rich treats, marshmallows, peanut butter, crackers and similar food will be offered to these animals, since they are on an age-appropriate caloric diet to prevent excessive weight. Instead, canned

fruits, vegetables and milk will be offered daily; age-appropriate nutritional supplement will be provided after irradiation. Tap water will be provided ad libitum. All dishes and containers need to be cleaned and sterilized daily.

V.5.1.3. Exceptions NA

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care
Animals will be acclimated for ≥1 week prior to beginning of procedures. Each animal will be observed at least twice daily for evidence of pain or distress. Body weights will be determined at least once a week and on days of blood collection. The time of each daily temperature collection will be recorded for each animal. Animals will be qualitatively assessed at least twice daily (a.m. and p.m.) for general behavioral status and food consumption by VSD techs and/or research techs. The heart rate will be measured at the time of each blood collection. Stool consistency, left-over food, demeanor and overall animal appearance will be recorded. Diarrhea has not been observed in minipigs irradiated at doses <8 Gy. Appropriate standard operating procedures will be followed by staff veterinarians in the event of pain or distress.

V.5.2.2. Emergency Veterinary Medical Care Emergency veterinary medical care is available 24/7 and will be provided based on the restrictions approved in earlier sections of this protocol

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy
Enrichment will be done as per the VSD minipig enrichment SOP, except single housing after catheter implantation and irradiation.

V.5.3.2. Enrichment Restrictions
Minipigs will be single-housed after surgery and after irradiation until completion of the study, to avoid mechanical and infectious complications related to implanted catheters, but they will be in visual, olfactory and auditory contact with other minipigs in the same room.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

Trained personnel are certified by VSD.

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3b, 4, 5, 7 (drug delivery) 1, 3b, 4, 5	(b)(6)

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)

Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY NA

VIII. ENCLOSURES NA

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(b)(4),(b)(6)

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ATTACHMENT 1: Animal observation sheet at euthanasia

PROTOCOL NUMBER:

ANIMAL ID: **AGE AT BEGINNING OF EXPERIMENT:**

Date:

Dose:

Time Point:

Treatment:

1) Observation at euthanasia

Observation time	(i) Activity	(ii) Posture/ Movement	(iii) Stool	(iv) Stool Consistency	(v) Vomit	(vi) Respiratory Activity	(vii) RR	(viii) Food	(ix) Temp

i. Activity score: 0- Normal bright alert and reactive, 1-Periods of inactivity/lying down, 2-Lying down, no effort to get up with stimulus
ii. Posture/Movement Score: 0- Normal gait and posture, 1- hunched appearance or slower movement, 2- dog sitting, shoulders forward
iii. Stools: Y, N
iv. Stool Consistency Score: 0 –Normal Formed Stool, 1- Formed Hard Stool, 2-Loose stool, 3- Diarrhea
v. Vomit: Y, N
vi. Respiratory Activity Score: 0- Normal Respiration, 1- Labored breathing, or intermittent cough 2- gasping, open mouth breathing, or persistent cough
vii. Respiratory rate: beats per minute
viii. Food (left in the cage): Y, N. If Yes, how much (1/4 or 1/2 or 3/4 or all)
ix. Temp: °C

2) Cause of euthanasia

3) Study Endpoints (circle all that applies)

Absolute

1. Non responsive, assuming the animal has recovered from anesthesia
2. Dyspnea
3. Loss of 20% of expected weight (based on Marshall Bio-resources growth chart –weight vs age- and day 0 baseline percentile).
4. Hypothermia (<36 °C)

Non-absolute

5. Hyperthermia (>41° C)
6. Anorexia (skip 3 consecutive meals)
7. Anemia/pallor, CRT >2 seconds. CRT will be performed behind the ears.
8. Petechiae/ecchymosis
9. Vomiting/diarrhea
10. Lethargy

11. Seizures or vestibular signs (falling, circling or head tilt)
12. Uncontrollable hemorrhage

4) Check-up at time of Blood Collection

Body Weight:

Hydration signs:
Skin Tent Time Score: Normal: < 2 sec Mild: 2 sec Moderate: 2-3 sec Severe: >3 sec

Mucous Membranes: Pink Pale Red Cyanotic Dry

Capillary Refill Time: Normal: <2 sec Mild: 2 sec Moderate: > 2sec

Hair coat:
Normal Rough Looses Hair

Skin quality:
Normal (pink) Dry Red Petechia Bruising Hematoma

Eyes:
Normal Discharge (L) (R) Hemorrhage (L) (R) Porfirine (L) (R)

Nose:
Moist Dry Discharge Epistaxis

Heart:
Beats per min:
Rhythm: Regular Irregular
Pulse quality: Weak Strong

Lung:
Sounds: Normal Crackles Wheezes Stridor

Abdominal palpation:
Normal Distended

X. ASSURANCES

Protocol Title: Effect of supportive care on irradiated Gottingen minipigs (*Sus scrofa domestica*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)		
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (March 2013)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	12/12/2013	2nd Resubmission Date:	2/10/2014
Approved/**Returned for Revision**:	1/15/2014	**Approved**/Approval Withheld by IACUC:	2/12/2014
1st Resubmission Date:	1/24/2014	**EXPIRATION DATE:**	2/11/2017
Approved/**Returned for Revision**:	2/6/2014	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: *In vivo* combined therapy for ionizing radiation followed by wound trauma in the laboratory mouse (*Mus musculus*)

IV. PRINCIPAL INVESTIGATOR:

Original signed 12/06/2013

(b)(6) **PhD** **Date**

Principal Investigator
SRD
Tel: (b)(6) Fax: (b)(6)
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date
Head, Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date
Statistician
Telephone (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date
Head, Veterinary Sciences Department, AFRRI
Telephone (b)(6) Fax: (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date
Environmental Health and Safety Department, USUHS
Telephone (b)(6) Fax: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**
IACUC Chair (b)(6) Scientific Research Department, AFRRI
Telephone (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: *In vivo* combined therapy for ionizing radiation followed by wound trauma in the laboratory mouse (*Mus musculus*)

PRINCIPAL INVESTIGATOR: (b)(6)

CO-INVESTIGATOR(S):

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS Terrorist use of radiological weapons is considered an urgent threat to both military and civilian personnel. Radiation exposure combined with other injuries, such as thermal burns, wound, and increased susceptibility to infection characterize short-term casualties in many radiological incident scenarios. Our research has shown that the drugs, granulocyte-colony stimulating factor (G-CSF) and (b)(4) are very effective for increasing survival after irradiation followed by wound trauma (CI) in mice. Since CI results in depletion of neutrophils and platelets, G-CSF stimulates neutrophil generation, and (b)(4) increases platelet formation, we propose here to test combined treatment with G-CSF and (b)(4) for their ability to improve recovery of tissues from the combined effects of radiation and wound. Successful development of such a therapy would increase survival of personnel who are victims of such scenarios.

II. BACKGROUND

II.1. Background Radiation and wound injury would appear to have little in common. Radiation injury is triggered by production of free-radical species within the cell that damage or disable important biomolecules. Wound injury induces tissue trauma and systemic bacterial infection. Despite differences in the initiation events, evidence has accumulated in recent years that many of the subsequent cellular responses have mechanisms in common. Both injuries involve complex biochemical pathways of the stress response in tissues, which are now only beginning to be understood. It is clear that nitric oxide (NO) signaling plays a central role in the extent of damage that occurs after exposure to many stressors. This observation led us to consider the possibility that pharmacological approaches to alter NO metabolism, which we are currently investigating for treating wound trauma, might also be effective for treating radiation stress or a combination of the two injuries combined.

A. Effects of Combined Injury (CI)

Injury from ionizing radiation together with a non-lethal wound can have a wide range of harmful effects on humans, including leucopenia, purpura, hemorrhage, pneumonia, and hair loss between days 0 and 7 after radiation, followed by diarrhea, fever, electrolyte disturbance, convulsions, ataxia, tremor, and death 7-14 days after radiation. High doses of radiation suppress hematopoiesis and the immune responses and increase remarkably the risk of infection, which may become a lethal complication [Brook et al., 2004; Hoppe, 1989; Reigle and Dienger, 2003; (b)(4),(b)(6) Organs such as small intestine, lung, and bone are also relatively sensitive to ionizing

radiation, particularly the proliferative progenitor cells (b)(4) (b)(4) Multiple-organ dysfunction syndrome (MODS) and multiple-organ failure (MOF) can result [Baue, 1998].

The damage that ultimately leads to the generalized, systemic responses described above originates at the cellular and molecular level. Electromagnetic waves or particles constituting the various qualities of ionizing radiation induce damaging ionizations in biological molecules – either directly or indirectly through the production of reactive oxygen species (ROS) intermediates – resulting in altered biochemical activities that disrupt normal cell homeostasis. Studies in a variety of biological systems have shown that these alterations can involve a complex series of events that are often interrelated, affecting gene activities, protein expression, and basic metabolic pathways. For example, ionizing radiation can cause DNA double-strand breaks, which can lead to cell-cycle arrest via ATM-CHK2-NF-kB/p53/CDC25 pathways [review by Houtgraaf *et al.*, 2006]. Wound trauma enhances the DNA double-strand breaks, as indicated by increased γ-H2AX formation (b)(4),(b)(6)

The capacity of ionizing radiation and wound to induce apoptosis and thereby cause damage to organs and systems exemplifies the complexity of a response that might be better understood using the systematic analysis we are proposing. Excessive ROS levels can lead to impaired intracellular ionic homeostasis by damaging cellular macromolecules, including DNA, proteins, and lipids, resulting in caspase-independent apoptosis or cancer induction [Cadet et al., 2004; Ogawa et al., 2003; Olive, 1998]. High concentrations of ROS produced by radiation can attack mitochondria, resulting in the release of cytochrome C into the cytoplasm. Caspase-9 and Apaf-1 conjugate cytochrome C to form apoptosomes that in turn activate caspase-3 and -7. Active caspase-3 then activates caspase-2, -6, -8, and -10, resulting in apoptosis and elimination of the damaged cells [Hill et al., 2003; Kannan and Jain, 2000; Richter, 1993; Simon et al., 2000; Kiang, 2004; Jiang and Wang, 2004].

A possible role for NO metabolism in radiation injury and wound has been recognized only recently (b)(4),(b)(6) A high percentage of mammary tumors in animals receiving a whole-body radiation dose of 1.5 Gy gamma radiation was shown probably to be mediated by increased levels of the inducible form of nitric oxide synthase (iNOS) and NO level [Inano and Onoda, 2005]. Up-regulation of iNOS activity, which leads to increased levels of NO, was observed in cultured rat vascular smooth-muscle cells, which were given 14-25 Gy ^{60}Co gamma photons [Zhong et al., 2004]. Similar results have been reported in skin cells after UVB exposure [Chang et al., 2003]. Increased iNOS and radiation exposure have been shown to induce apoptosis in a caspase-dependent fashion [Chung et al., 2003], and radiation-induced apoptosis can be prevented by an iNOS inhibitor [Hirakawa et al., 2002]. Over-expression of iNOS [Ghosh et al., 2001] and NO induced after irradiation have been shown to be responsible for the subsequent cell death, and we have demonstrated that iNOS regulates caspase-dependent apoptosis (b)(4) atg-8-dependent autophagy (b)(4) and tight junctions between cells [Han et al., 2004].

Preliminary data from our experiments with human T cells indicate that gamma radiation-induced injury is mediated by the iNOS pathway. Inhibition of this pathway reduces radiation lethality and enhances cell survival. Additionally, preliminary data from

our laboratory (b)(4) and others [Antonsson et al., 1997; Hockenberry, 1995; Jacobson and Bergeron 2002; Yang and Korsmeyer, 1996; Di Masi et al., 2006; Li et al., 2007] suggest that interactions among Bcl-2, Bax, and p53 are implicated in radiation-induced cell death. Wound trauma further increases radiation-induced p21, Bax, DNA damage-binding protein 2 (DDB2) and telomerase reverse transcriptase (TERT) (b)(4)

In addition, wound trauma significantly furthers radiation-induced depletion of RBCs, platelets, splenocytes, neutrophils, eosinophils, basophils, and leukocytes (b)(4),(b)(6)

(b)(4),(b)(6) Serum concentrations of IL-1β, -6, -8, and G-CSF after irradiation are magnified by wound as well. Taken together with data on activation of the iNOS signaling, a wound significantly increases radiation-induced lethality (b)(4),(b)(6)

(b)(4),(b)(6)

A number of countermeasure candidates are emerging that display significant efficacy and low toxicity (b)(4) However, no pharmaceuticals are currently approved specifically for acute radiation syndrome (ARS). Preliminary data from our laboratory showed that either G-CSF or (b)(4) independently increased mouse survival significantly after CI (b)(4),(b)(6)

B. Effect of Granulocyte-colony stimulating factor (G-CSF)

Granulocyte-colony stimulating factor is a specific immunomodulator. rhG-CSF (Neupogen®), has a longer half-life in circulation than natural G-CSF and is an approved drug for human use. Consequently, rhG-CSF could be used off-label in radiation casualties to mitigate neutropenia [Waselenko et al., 2004]. Adapting from results of previous studies in irradiated canines and non-human primates (NHP), and following a human accident, Neupogen® will be tested for its ability to enhance survival and reduce neutropenia in irradiated mice. In canines, rhG-CSF was given as a 5-ml bolus, 10 µg/kg/day (100 µg/dog), s.c, once per day. Injections began in the morning one day after exposure, and were given for a total of 14-28 days [MacVittie et al., 2005]. rhG-CSF improved recovery of neutrophils and enhanced survival [MacVittie et al., 2005]. Similarly, in NHP, a dose of 10 µg rhG-CSF/kg/day (25-40 µg/NHP) was given s.c. starting the day after irradiation, continuing for a total of 14 days [Neelis et al., 1997]. In that report, rhG-CSF significantly shortened the period of neutropenia after whole-body irradiation [Neelis et al., 1997]. In 3-month-old unirradiated Göttingen minipigs, rhG-CSF elevated white blood cells [Takai et al., 2008]. In that study, a dose of 5-10 µg rhG-CSF/kg/day (no body mass provided) was injected s.c. once daily in the cervical region for 10 days. No abnormal clinical signs were observed. White blood cells (WBC) averaged about 15 x 10^3/µl in vehicle-injected animals, gradually increasing during rhG-CSF administration, and reaching a mean of 30 x 10^3/µl on day 3, 40-70 x 10^3/µl on day 7, and 40-90 x 10^3/µl on day 10, with no difference observed between rhG-CSF doses or gender. The increase was mostly attributed to segmented neutrophils. No effect of rhG-CSF on blood chemistry parameters was observed. Enlargement and increased weight of spleens, an increased number of granulocytic cells in bone marrow, and increased extramedullary hematopoiesis in liver and spleen were also observed in the rhG-CSF-treated minipigs, which are all expected effects of

G-CSF. However, no adverse changes were observed in any of the tests [Takai et al., 2008]. Pegylated G-CSF combined with stem cell factor and erythropoietin successfully saved a human victim exposed accidentally to 4.5 Gy ionizing radiation [Bertho et al., 2008]. In our B6D2F$_1$/J mouse model, administration of G-CSF s.c. once daily beginning 24 h (day 1) through day 14 after CI in combination with topical application of gentamicin cream once daily to the wound day 1 to day 10 and administration of levofloxacin *p.o.* once daily day 3 to day 21 increased mouse survival. In B6D2F1/J mice, which were given 9.5 Gy ^{60}Co gamma-photon radiation, G-CSF therapy increased survival to 100% compared to 67% in vehicle-treated control animals. In CI mice, G-CSF increased survival to 25% compared to 5% in vehicle-treated mice (b)(4),(b)(6)
(b)(4),(b)(6)

C. Effect of (b)(4)

Hematopoietic tissue is highly susceptible to cytotoxic effects of ionizing radiation, resulting in thrombocytopenia and/or neutropenia, followed by mortality.
(b)(4)
(b)(4) In CD2F$_1$ mice, 2 mg drug given s.c. was effective when given 12 h post-irradiation, increasing survival to 70% after 9-Gy ^{60}Co gamma irradiation at 0.6 Gy/min (b)(4)
(b)(4) In our B6D2F$_1$/J mouse model, s.c. administration of (b)(4) 24 h after CI in combination with topical application of gentamicin cream to the wound day 1 to day 10 and administration of levofloxacin *p.o.* day 3 to day 21 increased mouse survival. The treatment also improved body weight, numbers of platelets, neutrophils, and eosinophils, and spleen weights and the number of splenocytes after CI.

We propose here to evaluate G-CSF given at 4 h and (b)(4) given at 24 h after CI or *vice versa* or given together at the same time in combination with topical gentamicin for 10 days, days 1 through 10, and oral levofloxacin for 14 days, days 3 through 16. From these observations described above, it is **hypothesized** that this combined treatment regimen will demonstrate either additive or synergistic therapeutic effects on survival of CI mice.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched
PubMed 1948-2013; Defense Technical Information Center (DTIC) no time period presented; DoD Biomedical Research Database (BRD) 1998-2009; and NIH Research Portfolio Online Reporting Tools (RePORTer) 1989-2013.

II.2.2. Date of Search December 5, 2013

II.2.3. Period of Search PubMed 1948-2013; Defense Technical Information Center (DTIC) no time period presented; DoD Biomedical Research Database (BRD) 1998-2009; and NIH Research Portfolio Online Reporting Tools (RePORTer) 1989-2013.

II.2.4. Key Words of Search Following key words and their combinations were used to perform the literature search for any duplication: mouse, pain, distress, analgesia, anesthesia, ionizing radiation, wound, combined injury, G-CSF, gentamicin, levofloxacin, and (b)(4)

II.2.5. Results of Search In summary, no duplication effort was found for the proposed study.
Search for key words mouse, pain, distress, analgesia, anesthesia, ionizing radiation, wound, combined injury, G-CSF, gentamicin, levofloxacin, and (b)(4) has reflected in "0" matches on NIH RePORTer, BRD, Pub Med, or DTIC. Therefore, no duplication effort was found.

Terms	**Results**
Mouse/ionizing radiation/wound/G-CSF+(b)(4)	0
Mouse/ionizing radiation/wound/anesthesia/G-CSF+(b)(4)	0
Mouse/ionizing radiation/wound/distress/G-CSF+(b)(4)	0
Mouse/ionizing radiation/wound/analgesia/G-CSF+(b)(4)	0
Mouse/ionizing radiation/wound/pain/G-CSF+(b)(4)	0
Mouse/ionizing radiation/wound/G-CSF+(b)(4) +gentamicin+LVX	0
Mouse/ionizing radiation/wound/anesthesia/G-CSF+(b)(4) +gentamicin+LVX	0
Mouse/ionizing radiation/wound/distress/G-CSF+(b)(4) gentamicin+LVX	0
Mouse/ionizing radiation/wound/analgesia/G-CSF+(b)(4) +gentamicin+LVX	0
Mouse/ionizing radiation/wound/pain/G-CSF+(b)(4) +gentamicin+LVX	0

III. OBJECTIVE / HYPOTHESIS Objectives:

1. Determine mortality after exposure to CI in the presence of G-CSF and (b)(4) It is evident that non-lethal γ-radiation combined with non-lethal wounding in mice significantly increases mortality; whereas mice treated with either drug displayed increased survival after CI. We expect that treatment with G-CSF and (b)(4) shall increase survival additively or synergistically.

2. Determine whether treatment with G-CSF and (b)(4) **ameliorates tissue injury from CI.** We have shown that lethal radiation alone or wound alone, or combination activates the iNOS pathways. We will examine whether treatment with G-CSF and (b)(4) ameliorates tissue injury including bone marrow and small intestine.

3. Elucidate the mechanism underlying the therapy provided by G-CSF and (b)(4) Because either radiation or wound activates the iNOS pathway and induces apoptosis and autophagy, the therapy from combined injury provided by G-CSF and (b)(4) should be mediated by its ability to inhibit the iNOS pathway, hematological depletion, cytokine imbalance and bacterial infection.

Hypothesis:
This combined treatment regimen will demonstrate either additive or synergistic therapeutic effects.

IV. MILITARY RELEVANCE Successful completion of the proposed study holds the prospect of identifying and characterizing highly radiotherapy compounds against acute and chronic forms of ionizing radiation combined injury, thereby helping sustain military operations in "RAD/NUC environments". Our previous and current work clearly demonstrates that either G-CSF or (b)(4) are therapeutically effective to improve survival after acute exposures to γ-rays. Most importantly, once being fully characterized, these compounds could be further developed into novel radiotherappy drugs used both by military and domestic authorities in the event of RAD/NUC incidents.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

General. The overall experimental approach is in evaluating and developing G-CSF and (b)(4) as a safe and effective therapy for persons at risk of CI after RAD/NUC hazards. Our hypothesis is that this combined treatment regimen will demonstrate either additive or synergistic therapeutic effects.

Animals:

B6D2F$_1$/J female mice, approx. 12-22 weeks of age, will be housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Animal rooms will he maintained at 22° ± 4°C with 50% ± 20% relative humidity on a 12-hr light/dark cycle. Animals will be randomized prior to each experiment and housed in small shoebox cages (four animals per cage). Commercial rodent ration (Harlan Teklad Rodent Diet 8604) will be freely available as will acidified (pH=2.5 - 3.0) water to control opportunistic infections. All animal-handling procedures will be performed in compliance with *Guide for the Care and Use of Laboratory Animals*, eighth edition from the National Research Council (2011).

Female mice are used as they are less aggressive to cage mates than the males. Therefore, they will not cause further unnecessary wounding. The animals will be purchased from The Jackson Laboratory.

Irradiation procedures:

Exposure of animals to gamma radiation will be conducted using the AFRRI cobalt-60 source. Thirty to 40 min before irradiation, mice will be placed in perforated acrylic restrainers with partitions (four mice/box with separate compartments for each animal, loaded on a single side) designed for radiation exposure experiments. The calibration factor for the ionization chamber will be calculated by means of a standard obtained from an accredited dosimetry calibration laboratory (M. D. Anderson Cancer Center, Houston, TX) certified by the National Institute of Standards and Technology. Radiation exposures will be bilateral and whole-body as described previously (b)(4),(b)(6) Based on the extensive studies conducted previously at AFRRI, the standard radiation dose rate will be 0.4 Gy/min ^{60}Co at ambient temperature to deliver a dose. A single dose of $LD_{70/30}$ ^{60}Co γ-photon radiation delivered at 0.4 Gy/min will be used. The $LD_{50/30}$ in combined injured B6D2F$_1$/J female mice was determined to be 8.95 Gy in 2009 (b)(4),(b)(6) In the current investigations, a radiation dose of 9.5 Gy, which is approximately $LD_{70/30}$ (i.e., 70% lethal during 30 d following CI), will be used. Sham-treated

control mice will be handled in the same manner as those irradiated but will not be given a dose of gamma photons. The sham-treated mice will be returned to their cages in the vivarium.

Wound Trauma:

One to three days before irradiation and/or wounding, mice will be anesthetized by isoflurane inhalation and then their dorsal hair will be shaved. One h post-irradiation, mice will be anesthetized for approximately 3 to 4 minutes by inhalation of methoxyflurane until respiration rate is approximately once per second in a glass dressing jar on a warming pad at 21-27°C. (The water pump has a rheostat thermistor to control the water temperature.), or, as an alternative, by inhalation of isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane). For induction of trauma, methoxyflurane is preferred because the animals remain predictably unconscious for a sufficient time to perform the procedure as well as to provide temporary pain relief. Isoflurane acts more quickly and animals recover more quickly, less than one minute, after removal from isoflurane-oxygen unit, which would be inappropriate during the wounding procedure, than with methoxyflurane anesthesia.

Similarly, during the cardiac puncture procedure, mice, which are anesthetized by inhalation of methoxyflurane, remain unconscious for a sufficient time to perform the procedure so that animals do not awaken and move; however, isoflurane can be used, if the dispensing machine with tubing and nose cone are readily available for this procedure.

We will collaborate with the veterinary services department to progressively transition to the use of isoflurane, if it can be demonstrated to maintain adequate anesthesia for the wound model (i.e., able to complete the wound procedure using isoflurane and not affect the timing of the wound injury post-irradiation as compared to methoxyflurane).

Non-lethal wounds to a double layer of dorsal surface skin caudal from the neck and between the shoulders in the center of the back will be created with a stainless steel punch (9/16-in) on a Teflon-covered board, both cleansed with 70% alcohol after each use. The panniculus carnosus muscle and overlying skin will be removed. The size of the wound approximates 15% of the total body skin surface (TBSS). Acetaminophen solution, 150 mg/kg, in 0.5 ml sterile saline given as a fluid therapy i.p., will be administered immediately following wounding to reduce pain and distress (b)(4),(b)(6) All wounded animals will be given a second dose of acetaminophen 4 hours after the first dose but not more than 8 hours after the first dose.

Previous studies in this mouse model show that, without treatment, full wound closure after irradiation requires more than 30 days. Closure to 50% of the initial wound size is accomplished in about 14 days. Without irradiation, the complete wound closure time is approximately 14 days (b)(4),(b)(6) . Sham-treated control mice will be handled in the same manner as those wounded but will not be given a wound. The sham-treated mice will be returned to their cages in the vivarium.

Other experimental considerations: The wounding process is labor intensive. It is only possible to wound 20 animals within a limited time frame after radiation exposure. Because the time of wounding has been shown to have an impact on the severity of the interaction, it is important to minimize the timeframe for this process. Consequently, it will be necessary to irradiate treatment groups of mice in pairs in six runs for each experiment.

Test drugs:

(1) Pegylated G-CSF (1000 µg/kg, s.c., 25 µg/mouse) or vehicle [sterile, clear, colorless, preservative-free solution containing acetate (0.35 mg), polysorbate 20 (0.02 mg), sodium (0.02 mg), and sorbitol (30 mg) in 0.6 ml Water for Injection, USP; 0.2 ml/mouse s.c.] and

(2) (b)(4) 1 mg/kg, s.c., 25 µg/mouse) or vehicle (sterile 0.9% sodium chloride solution for injection, USP; 0.2 ml/mouse s.c.) will be administered at specified times after irradiation.

Antimicrobial agents:

Antimicrobial agents, gentamicin sulfate (GEN, 0.1% cream) will be applied topically on the wound site for 10 days, on days 1 through 10; and levofloxacin (LVX, 90-100 mg/kg p.o.) will be given orally once daily for 14 days, on days 3 through 16.

Survival assessment:

After radiation exposure, mice will be returned to the animal facility and housed 4 mice per cage with free access to rations and water. The mice will be observed for 30 days and the percentage of mice surviving that period will be calculated for each treatment group.

Body-weight measurements:

The degree of body weight loss is a good predictor of radiation mortality. We will measure the body weight of mice treated with vehicle or drugs 1, 3, 7, 14, 21, and 28 d after irradiation.

Wound closure:

Wounded animals will be observed for 30 days to monitor wound closure by caliper measurements on days 1, 7, 14, 21, and 28 post skin wound trauma alone or with radiation.

Water consumption:

Daily water consumption for days 1, 2, 3, 4, 5, 6, and 7 after sham, wound, irradiation, and CI will be measured using graduated water bottles.

Blood collection:

At 4-5 hr, 1, 3, 7, 15, and 30 days after irradiation, animals will be anaesthetized appropriately by methoxyflurane inhalation. However, isoflurane can he used, if the dispensing machine with tubing and nose cone are readily available for this procedure. Blood will be collected using cardiac puncture with 23- to 26-G needles.

Tissue collection:

At each time point and after blood collection, the animal under isoflurane inhalation will be euthanized by cervical dislocation before terminal tissue collections are performed (section V.4.4.2-biosamples). Tissues will be frozen at −70°C until evaluated *in vitro*.

V.1.1. Experiment 1. Specific Aim #1: Determine survival, body weights, water consumption, and wound healing after CI and therapy with G-CSF and (b)(4)

Rationale: It is evident that non-lethal γ-radiation combined with wounding in mice significantly increases mortality; whereas mice treated with either drug 24 h after irradiation displayed

increased survival after combined injury. Since Department of Defense expects to treat irradiated personnel with drugs 4 h after irradiation, we will evaluate these drugs at this time point.

Experimental designs:

(i) Experiment 1.1. (240 mice) PEG-G-CSF and (b)(4) will be administered concurrently 4 h after sham, wound, RI, or CI. PEG-G-CSF will also be administered on days 8 and 15.

(ii) Experiment 1.2. (240 mice) PEG-G-CSF and (b)(4) will be administered sequentially 4 h and 24 h after sham, wound, RI, or CI. PEG-G-CSF will also be administered on days 8 and 15.

(iii) Experiment 1.3. (240 mice) (b)(4) and PEG-G-CSF will be administered sequentially 4 h and 24 h after sham, wound, RI, or CI. PEG-G-CSF will also be administered on days 8 and 15.

Beginning on Day 1, 0.1% gentamicin sulfate cream (GEN) will be applied topically daily through Day 10 (i.e., 10 consecutive days) via a sterile swab and gloved finger to specified mice (see table for treatment groups). In addition, levofloxacin (LVX; 90-100 mg/kg in 0.2 ml sterile water solution, p.o.) will be administered to mice once daily for 14 days on Days 3–16 post-irradiation using a 20-gauge oral feeding needle fitted to a 1-ml sterile syringe.

Water intake, bodyweights, wound healing, and survival in these mice will be monitored. On or after day 30, blood collection and terminal tissue collection (no more than 6 animals per group) may take place for analyzing blood cell composition and signal transduction pathways.

Mice will be randomly divided into 12 groups (n=20 per group) for evaluation of each of three drug regimens:

[3 drug regimens x 1 administration route x 1 administration time x 1 radiation dose x 12 experimental groups x 20 animals/group] = (Total: 720 mice)

Experiment 1. Design

Group	Treatment	No. mice
1	Sham	20
2	Sham+Veh	20
3	Sham+G-CSF+(b)(4)	20
4	Wound	20
5	Wound+Veh	20
6	Wound+G-CSF+(b)(4)	20
7	RI (9.5 Gy)	20
8	RI+Veh	20
9	RI+G-CSF+(b)(4)	20
10	RI+Wound (CI)	20
11	CI+Veh	20
12	CI+G-CSF+(b)(4)	20
	Total mice =	240

Sham = no Radiation or Wound;
Wound = skin wound injury (15% TBSA on Day 0);
RI = Radiation Injury (9.5 Gy on Day 0);
CI = RI + Wound;
VEH = vehicles for G-CSF [sterile, clear, colorless, preservative-free solution containing acetate (0.35 mg), polysorbate 20 (0.02 mg), sodium (0.02 mg), and sorbitol (30 mg) in 0.6 ml Water for Injection, USP; 0.2ml/mouse s.c.] and (b)(4) sterile 0.9% sodium chloride solution for injection, USP; 0.2ml/mouse s.c);
LVX = levofloxacin (100 mg/kg, p.o.);
GEN = gentamicin (0.1% gentamicin sulfate cream applied topically to wound site).

Experiment 1. 4×4 statistical design (survival)

Groups	Sham	Wound	RI	CI	Total
Control	N=20	N=20	N=20	N=20	N=80
Veh	N=20	N=20	N=20	N=20	N=80
Drugs	N=20	N=20	N=20	N=20	N=80
Total	N=60	N=60	N=60	N=60	N=240

Expected Results: We expect that, at a minimum, combined treatment with (b)(4) and G-CSF will enhance survival from radiation combined with wound compared to vehicle-treated mice.

V.1.2. Experiment 2. Specific Aim #2. Determine whether treatment with G-CSF and (b)(4) ameliorates tissue injury from radiation combined with wound

Rationale: We have shown that RI alone or CI increase apoptosis and autophagy in ileum and skin 7 days after RI or CI. We will examine whether treatment with G-CSF and (b)(4) ameliorates tissue injury including bone marrow and small intestine.
Experimental designs: Effect of drugs on tissue morphology and hematology.
The optimal treatment regimen of combined PEG-G-CSF and (b)(4) will be determined by results of experiments 1.1, 1.2, and 1.3. A second dose of PEG-G-CSF alone will also be administered on Day 8.

Beginning on Day 1, 0.1% gentamicin sulfate cream (GEN) will be applied topically daily through Day 10 (i.e., 10 consecutive days) via a sterile swab and gloved finger to specified mice (see table for treatment groups). In addition, levofloxacin (LVX; 90-100 mg/kg in 0.2 ml sterile water solution, p.o.) will be administered to mice, which remain in the experiment after sampling intervals, once daily for 12 days on Days 3–14 post-irradiation using a 20-gauge feeding needle fitted to a 1-ml sterile syringe.

(i) Experiment 2.1: Histopathology of bone marrow, ileum, and skin. (360 mice)
At 4-5 h, 1, 3, 7, and 15 days after irradiation and wounding, mice will be removed from cages and euthanized (N=6 mice per group per time point) before administration of GEN and LVX to mice that will remain in the experiment. Their blood, bone marrow, ileum, and skin will be collected and prepared for histopathology with H&E staining and immunofluorescent staining. Apoptosis (TUNEL assay) and autophagy (LC-3 immunofluoresence) will be performed.
(ii) Experiment 2.2: Hematology analysis (0 mice)

Blood collected by cardiac puncture from mice in Experiment 2.1 will be used for blood cell analysis to determine changes in numbers of blood cells, including WBCs, RBCs, hemoglobin, hematocrit, platelets, lymphocyte, neutrophils, monocytes, eosinophils, and basophils.

Mice will be randomly divided into 12 groups (N=6 / group / time point):
[One administration route x 1 drug regimen x 1 administration time x 1 radiation dose x 12 experimental groups x 6 animals/group x 5 collection times = Total 360 mice]

Experiment 2. Design.

Group	Treatment	No. mice
1	Sham	30
2	Sham+Veh	30
3	Sham+G-CSF+(b)(4)	30
4	Wound	30
5	Wound+Veh	30
6	Wound+G-CSF+(b)(4)	30
7	RI (9.5 Gy)	30
8	RI+Veh	30
9	RI+G-CSF+(b)(4)	30
10	RI+Wound (CI)	30
11	CI+Veh	30
12	CI+G-CSF+(b)(4)	30
	Total mice =	360

Experiment 2. Sampling per time point:

Groups	Sham	Wound	RI	CI	Total
Control	N=6	N=6	N=6	N=6	N=24
Veh	N=6	N=6	N=6	N=6	N=24
Drugs	N=6	N=6	N=6	N=6	N=24
Total	N=18	N=18	N=18	N=18	N=72

Expected results: Because G-CSF and (b)(4) stimulate neutrophil generation and platelet production, respectively; we expect to see improvement of bone marrow morphology and hematological profiles. Whether structure of ileum and skin will be improved is not predictable. The results, however, will advance our understanding with two promising drugs as therapies for CI.

V.1.3. Experiment 3. Specific Aim #3. Elucidate the mechanism underlying the therapy provided by G-CSF and (b)(4)

Rationale: Because either radiation or wound activates the iNOS pathway to induce cell apoptosis and autophagy, and drastically increase cytokine concentrations in serum, the therapy for combined injury provided by G-CSF and (b)(4) should be mediated by its ability to inhibit the iNOS pathway and/or other possible signal transduction as well as inhibition of pro-inflammatory cytokines. CI-induced bacterial infection and barrier breakdown shall be blocked by these two drugs as well..

Experimental design: effects of drugs on signal transduction and systemic bacterial infection.
The optimal treatment regimen of combined PEG-G-CSF and (b)(4) will be determined by results of experiments 1.1, 1.2, and 1.3. A second dose of PEG-G-CSF alone will also be administered on Day 8.
Beginning on Day 1, 0.1% gentamicin sulfate cream (GEN) will be applied topically daily through Day 10 (i.e., 10 consecutive days) via a sterile swab and gloved finger to specified mice (see table for treatment groups). In addition, levofloxacin (LVX; 90-100 mg/kg in 0.2 ml sterile water solution, p.o.) will be administered to mice, which remain in the experiment after sampling intervals, once daily for 12 days on Days 3–14 post-irradiation using a 20-gauge feeding needle fitted to a 1-ml sterile syringe.

(i) Experiment 3.1: Measurements of cytokines in serum (Total 360 mice)
At 4-5 h, 1, 3, 7, and 15 days after irradiation and wounding, mice will be removed from cages and euthanized (N=6 mice per group per time point) before administration of GEN and LVX to mice that will remain in the experiment. Their blood will be collected by cardiac puncture and serum will be separated for cytokine measurement using multiplex Luminex assay.
(ii) Experiment 3.2: Detection and identification of systemic bacterial infection (0 mice)
Ventricular heart blood, liver, and spleen from mice in Experiment 3.1 will be collected aseptically and cultured for bacterial detection and identification.
(iii) Experiment 3.3: Determination of signaling transduction in bone marrow, ileum and skin (0 mice). The tissues will be removed from mice in Experiment 3.1 and processed for evaluation. Tissues collected from mice in Experiment 3.1 will be analyzed for iNOS, NO, lipid peroxidation (MDA), MPO, ATP, MMPs, and TLRs.

Mice will be randomly divided into 12 groups (N=6 / group / time point):
[1 administration route x 1 drug regimen x 1 administration time x 1 radiation dose x 12 experimental groups x 6 mice/group x 5 collection times = Total 360 mice]

Experiment 3. Design.

Group	Treatment	No. mice
1	Sham	30
2	Sham+Veh	30
3	Sham+G-CSF+(b)(4)	30
4	Wound	30
5	Wound+Veh	30
6	Wound+G-CSF+(b)(4)	30
7	RI (9.5 Gy)	30
8	RI+Veh	30
9	RI+G-CSF+(b)(4)	30
10	RI+Wound (CI)	30
11	CI+Veh	30
12	CI+G-CSF+(b)(4)	30
	Total mice =	360

Experiment 3. Sampling per time point:

Groups	Sham	Wound	RI	CI	Total

Control	N=6	N=6	N=6	N=6	N=24
Veh	N=6	N=6	N=6	N=6	N=24
Drugs	N=6	N=6	N=6	N=6	N=24
Total	N=18	N=18	N=18	N=18	N=72

Expected results: Because G-CSF and (b)(4) increase 30-day survival after CI, respectively, we expect to see inhibitions of activation of the iNOS pathway, increases in pro-inflammatory cytokine concentrations, and systemic bacterial infection.

V.2. Data Analysis Mean with standard error, or percentage will be reported if applicable. A significance level will be set at 5% for each test. All statistical tests will be two-sided. Kaplan-Meier curve and the log-rank test, ANOVA, one-way ANOVA, post-hoc test, chi-square test, and student's test will be used for statistical analyses.

The sample size N=20 per group for survival analysis and the sample size N=6 per group per time point for biomolecular assays will have 80% power to detect any significant difference between groups if any, given type I error of 5% and delta/sigma has at least 1.8, where the delta is the estimated mean differences between the two groups and sigma is the pooled standard deviation of the two groups.

For experiments addressing Specific Aim #1, data analysis will be performed among 12 groups. Kaplan-Meier plot and log-rank test will be used. For experiments addressing Specific Aims #2 and #3, analysis of variance (ANOVA) will be used to detect if there is a significant difference among the groups. If significant, Student-Newman-Keuls multiple range test will be used to do all the possible pair-wise comparisons to identify which group is significantly different from the other.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

Drug manufacturers use computer modeling – structure function relationship – and screening *in vitro* by cell cultures during the development of candidate drugs. Computer modeling and cell cultures are insufficient to determine whether or not a given regimen (prophylactic or therapeutic) is effective in promoting survival, resisting infection, or enhancing hemopoiesis and biochemical interruption. An integrated, functional, computer model of a mammal has not yet been achieved for the purpose of examining intricate physiological interactions among cells, tissues, and organs that occur after exposure to ionizing radiation. The resiliency of organs, such as the gastrointestinal system or hematopoietic system, to ionizing radiation depends on a complex network of interactive signaling systems to sense the magnitude of tissue damage, and to initiate repair, recovery, and other defense processes. The physiology of these interacting networks is altered by radiation in ways that cannot yet be modeled using non-animal alternatives. In fact, the proposed studies will provide data, which will contribute directly to future models to predict the progress of radiation injury and appropriate medical treatments.

V.3.2. Animal Model and Species Justification
The mouse was chosen as the best species of animal to use for radiation therapy protocol because:

(1) It is one of the least sentient mammalian species that will permit us to achieve our research objectives.
(2) The cellular proliferative characteristics of gastrointestinal tract and hematopoietic tissues in the mouse are qualitatively similar to those in the human.
(3) Studies in mice have had a direct impact on work with larger animals and humans, as well as clinical treatment.
(4) There are extensive published reports available for comparison, review, and analysis of major biological findings.

B6D2F$_1$/J mice have been used extensively by the PI and his co-investigators at AFRRI to study the effects of ionizing radiation on susceptibility to combined injury and bacterial infections. (b)(4),(b)(6)
(b)(4),(b)(6)

Female mice are utilized because they are less aggressive to each other when conspecifics are housed together. B6D2F$_1$/J mice are hybrid animals that offer an excellent comparison for the genomic distribution seen in humans and are not subject to the inbreeding pressures that affect responses to radiation exposure. The genes of the parental strains of this mouse differ markedly simulating the human situation. Unlike inbred mouse strains, F$_1$ hybrid mice are uniformly heterozygous for all loci at which the parental strains differ and are homozygous. They express hybrid vigor, as do all species, in terms of resistance to disease, better survival under stress, and demonstrate greater, longer, natural longevity. Furthermore, the B6D2F$_1$/J hybrid strain of mouse is naturally relatively resistant to radiation and induced infection (b)(4),(b)(6) It is not overly sensitive to microbial infections, the most common reason for early mortality in acutely irradiated mice. We have used this strain for many years and consequently are familiar with its immunological, hematological, microbiological, and pharmacological characteristics and responses. Lastly, both parental strains (C57BL6/J and DBA2/J) of the B6D2F$_1$ mouse have been genetically mapped which may allow focusing future studies on improved therapy strategies for molecular changes included by injuries.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species *Mus musculus*

V.3.3.2. Strain / Stock B6D2F$_1$/J

V.3.3.3. Source / Vendor Jackson Laboratories, Bar Harbor, Maine 04609-1500, 800-422-MICE or 207-288-5845, Fax: 207-288-6150, www.jax.org. USDA registration number 11-R-0001, Customer number 1960. This is the only vendor and the only breeding site that will supply the animals used for our work. JAX is the established premier breeder of healthy B6D2F$_1$/J mice that maintains a highly regarded quality

control breeding facility because only that facility can provide the numbers of mice needed in one shipment and because of the exemplary health record. This mouse strain will be designated for purchase only from the Bar Harbor facility. Our combined injury efforts in this animal use protocol are based upon the uniformity of breeding this mouse that allows comparisons with previously collected data. In our years of working with JAX providing this mouse, not once has an experiment been terminated due to undesirable microbial associations.

V.3.3.4. Age Approx. 10-14 weeks of age at purchase; approx. 12-22 weeks of age at use.

V.3.3.5. Weight Approx. 20-35 grams at the time of experimental manipulation.

V.3.3.6. Sex Female. The B6D2F$_1$/J female gender demonstrates less aggressive behavior than males to conspecifics and under stress conditions such as after wound trauma (b)(4),(b)(6) The estrous cycle of female mice did not interfere with the evaluation of 5-AED (b)(4),(b)(6) It is therefore unlikely that the estrous cycle will interfere with G-CSF and (b)(4) in the proposed study.

V.3.3.7. Special Considerations
Commercially procured animals must be adventitious disease free.
Pathogen-free, including *Pseudomonas aeruginosa* and *Pasteurella* sp. All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus, *Mycoplasma pulmonis*, and *Clostridium piliforme*. Endoparasite- and ectoparasite-free.

V.3.4. Number of Animals Required (by species) B6D2F$_1$/J mice: 1440

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement This protocol will evaluate adjusted early experimental endpoints.
(1) Moribund animals will be considered to have arrived at the endpoint and will be euthanized.
(2) Endpoints that assess morbidity will include histology of ileum and lung, and concentration determinations of biomolecules including iNOS, NO, MDA, 3-N-Tyr protein nitration, ATP, LC3, and defensins at timed intervals for autophagy. The animals will have minimal pain or distress.
(3)
Acetaminophen will be administered after wound injury to help alleviate any discomfort.

(4) Animals will be housed in groups of 4 to encourage social behavior throughout the experiment and nestlets will be provided for comfort and to enhance the cage environment.

V.3.5.2. Reduction A statistician has reviewed this protocol to ensure that the minimum number of animals will be used to gain statistically significant results. We will collect multiple data from individual animals, providing information on histology, apoptosis, autophagy, and several biomolecule assessments.

V.3.5.3. Replacement We attempted to develop an *in vitro* CI model to reduce the number of mice used for CI studies. After a 6-month effort, the synergistic effect observed with our *in vivo* model was not obtained with the *in vitro* model. Therefore, we continue to use our *in vivo* model.

It is not feasible to use non-animal systems in place of animal models to address the research questions in this project. The mouse is considered to be a lower sentient mammalian species than is the rat, rabbit, cavy (guinea pig), canine, minipig, and nonhuman primate, which are other mammals that have been used for radiation research. The mouse is commonly used in laboratory studies to determine basic experimental principles before experimentation in larger mammals or in humans. The immune responses of the mouse are similar to those of the human. Blood cells and serum cytokine components of the mouse, while quantitatively different from other species, are qualitatively similar to other animal species. The intestinal tract of mice contains cellular components similar to those found in higher sentient animals, particularly a major component of the immune system that provides surveillance for and response to microorganisms.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals 1440

V.4.1.1.1.1. Column C 180 (# of animals)

V.4.1.1.1.2. Column D 540 (# of animals)

V.4.1.1.1.3. Column E 720 (# of animals)

V.4.1.1.1.4. Pain Category Assignments Because blood will be collected by cardiac puncture from anesthetized mice, all sham-treated control mice will be included in category D, alleviated pain or distress for experiments 2 and 3.

Exp. #	Experimental/Control Group	C	D	E	Totals

Exp. #	Experimental/Control Group	C	D	E	Totals
1.1	PEG-G-CSF and (b)(4) will be administered concurrently 4 h after sham, wound, RI, or CI (survival).	60	60	120	240
1.2	PEG-G-CSF and (b)(4) will be administered sequentially 4 h and 24 h after sham, wound, RI, or CI (survival).	60	60	120	240
1.3	(b)(4) and PEG-G-CSF will be administered sequentially 4 h and 24 h after sham, wound, RI, or CI (survival).	60	60	120	240
2	Determine whether treatment with optimal combination of PEG-G-CSF and (b)(4) ameliorates tissue injury from radiation combined with wound (early endpoint).	0	180	180	360
3	Elucidate the mechanism underlying the therapy provided by optimal combination of PEG-G-CSF and (b)(4) (early endpoint).	0	180	180	360
Totals:		180	540	720	1440

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

Wound procedure: One to three days before irradiation and/or wounding, mice will be anesthetized by isoflurane inhalation and then their dorsal hair will be shaved. One h postirradiation, mice will be anesthetized for approximately 3 to 4 minutes by inhalation of **methoxyflurane** until respiration rate is approximately once per second in a glass dressing jar on a warming pad at 25°C., or, as an alternative, by inhalation of 1-4% **isoflurane** (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane). For induction of trauma and collection of blood by cardiac puncture, methoxyflurane is preferred because the animals remain predictably unconscious for a sufficient time to perform each of the two procedures. Isoflurane acts more quickly and animals recover more quickly than with methoxyflurane anesthesia. Similarly, during the cardiac puncture procedure, mice, which are anesthetized by inhalation of methoxyflurane, remain unconscious for a sufficient time to perform the procedure so that animals do not awaken and move; however, isoflurane can be used, if the dispensing machine with tubing and nose cone are readily available for this procedure.

Acetaminophen solution, 150 mg/kg , will be given in 0.5 ml sterile saline solution for injection, USP, as a fluid therapy i.p. (b)(4),(b)(6) immediately following wounding to reduce pain and discomfort.

Blood collection: At specified time points, animals will be anaesthetized under isoflurane inhalation. The isoflurane anesthetic system that is available in VSD might be able to overcome movement of mice in many cases by continuous delivery of vapor during this procedure. Blood will be collected using cardiac puncture with 23-26 G needles. Blood will be transferred into EDTA vials (lavender-topped) to for hematology studies and plasma or Capiject vials with gel separator (red-topped) for serum depending on the experimental purpose.

Tissue collection: At the end of blood collection, the animal under isoflurane inhalation will be euthanized with cervical dislocation before terminal tissue collections (section V.4.4.2-biosamples) are implemented. Tissues will be frozen at −80°C until use.

V.4.1.2.2. Pre- and Post-procedural Provisions Animals will be housed and cared for both before and after dosing and irradiation procedures, as described in Section V.5. Veterinary Care. Prior to irradiation, mice will be group-housed 4-8 per cage with conspecifics in Micro-isolator cages (IAW VSDH SOP), and observed at least twice daily by the veterinary staff conducting general health check rounds. Following irradiation and wounding, the investigative staff will monitor animals on experiment at least twice daily, in addition to the routine veterinary staff rounds. Supportive care will be observed more frequently by the investigative staff and moribund animals will be euthanized (See V.4.5. Study Endpoint).

V.4.1.2.3. Paralytics NA

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched
Two sources were searched that contain major compilations of information on painful or distressful procedures. These included PUBMED and DoD Biomedical Research Database (BRD).

V.4.1.3.2. Date of Search December 5, 2013, PUBMED and DoD Biomedical Research Database (BRD)

V.4.1.3.3. Period of Search 1948 to 2013, PUBMED; 1998 to 2009, DoD Biomedical Research Database

V.4.1.3.4. Key Words of Search
Mouse, pain, distress, analgesia, anesthesia, ionizing radiation, wound, combined injury, G-CSF, gentamicin, levofloxacin, (b)(4) and alternative

V.4.1.3.5. Results of Search
There are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. No alternatives for the research effort with mice were found. Radiation itself does not cause pain or distress, particularly at sublethal doses. A sizable number of animals in these experiments will receive either near-lethal or lethal doses. These dose levels will lead to the clinical syndromes following irradiation, which may lead to distress/pain and moribundity. There was no mention of employing tissue culture for combined injury work. Tissue culture testing for radiation combined injury would not be possible as whole animals are required for evaluation. End points such as survival, clinical chemistries, complete blood cell counts, and histopathologies, are, by their nature, not possible in a cell culture system. Whole living animal systems are required for determination of the endpoints identified in this proposal and mice are the species of choice to accomplish those goals and aims.

Terms	Results
Mouse/ionizing radiation/wound/alternative/G-CSF	0
Mouse/ionizing radiation/wound/anesthesia/G-CSF	0
Mouse/ionizing radiation/wound/distress/G-CSF	0
Mouse/ionizing radiation/wound/analgesia/G-CSF	0
Mouse/ionizing radiation/wound/pain/G-CSF	0
Mouse/ionizing radiation/wound/alternative/(b)(4)	0
Mouse/ionizing radiation/wound/anesthesia/(b)(4)	0
Mouse/ionizing radiation/wound/distress/(b)(4)	0
Mouse/ionizing radiation/wound/analgesia/(b)(4)	0
Mouse/ionizing radiation/wound/pain/(b)(4)	0
Mouse/ionizing radiation/wound/alternative/G-CSF+(b)(4)	0
Mouse/ionizing radiation/wound/anesthesia/G-CSF+(b)(4)	0
Mouse/ionizing radiation/wound/distress/G-CSF+(b)(4)	0
Mouse/ionizing radiation/wound/analgesia/G-CSF+(b)(4)	0
Mouse/ionizing radiation/wound/pain/G-CSF+(b)(4)	0

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification Pain will be alleviated by drugs as described in V.4.1.2.1.

V.4.2. Prolonged Restraint Mice will be placed in perforated acrylic plastic boxes for up to two hours, but usually for less time, usually <1 h, during prompt irradiation procedures. Restraint of mice will be performed by the PI, Co-investigators, or qualified trained technical support staff.

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions One to three days before irradiation and/or wounding, mice will be anesthetized by isoflurane inhalation and then their dorsal hair will be shaved with electric clippers.

V.4.3.2. Procedure(s) No surgical procedures are planned. The infliction of wound trauma was described in section V.1., under general procedures.

V.4.3.3. Post-surgical Provisions No post-surgical provisions are planned other than that identified in section V.1., under specific methods following irradiation and wounding.

V.4.3.4. Location The wounding procedure will be performed in an assigned room in the Veterinary Sciences Department of the Armed Forces Radiobiology Research Institute.

V.4.3.5. Surgeon The wounding procedure will be performed by the principal investigator, the co-investigators, and trained technical staff of this research proposal. Technical assistance support such as anesthesia, preparing surgical facilities and obtaining animals for surgery will be provided by trained technical support staff that is identified in section VI Study Personnel and Qualifications.

V.4.3.6. Multiple Major Survival Operative Procedures NA

V.4.3.6.1. Procedures NA

V.4.3.6.2 Scientific Justification NA

V.4.4. Animal Manipulations

V.4.4.1. Injections

V.4.4.1.1. Pharmaceutical Grade Drugs

A. Sterile sodium chloride solution for injection USP
Sterile USP isotonic sodium chloride, 0.9%, will be given i.p., 0.5 ml, as fluid therapy with a 25- to 27-G sterile needle fitted to a sterile syringe after sham-operation, irradiation, wounding, and irradiation followed by wounding, i.e., combined injury. USP grade saline will be obtained from the USUHS pharmacy stock or from the WRNMMC hospital stock (or other suitable alternative sources for scientific supplies).

B. Acetaminophen solution
Acetaminophen for injection (NDC:43825-102-01; OFIRMEV. www.ofirmev.com, or equivalent) will be purchased from AmerisourceBergen (or other suitable alternative sources selected in consultation with a veterinarian) and given i.p., 150 mg/kg, i.e., 3.75 mg/25-g mouse, in 0.5 ml sterile saline for injection, USP (Davis J.A. Mouse and Rat Anesthesia and Analgesia *Curr. Protoc. Neurosci.* 42:A.4B.1-A.4B.21. 2008, John Wiley & Sons, Inc.; Coen M et al.. An Integrated Metabonomic Investigation of Acetaminophen Toxicity in the Mouse Using NMR Spectroscopy, *Chem. Res. Toxicol.* **2003,** *16*, 295-303).

C. Gentamicin
Gentamicin sulfate cream (GEN), 0.1%, pharmaceutical grade, will be purchased from Moore Medical LLC*, 1690 New Britain Ave, Box 4066, Farmington CT 06032-4066 (or other suitable alternative sources selected in consultation with a veterinarian) and will be applied topically daily from day 1 to day 10 for 10 consecutive days via a sterile swab No attempt will be made to quantify or measure the amount of gentamicin cream applied to the wound site. Rather, as in the case of human skin injuries, the site will receive an amount of antibiotic cream necessary to uniformly cover the area. This type of application is that expected to be provided to injuries of personnel involved in nuclear radiation disasters and has been used in burn management situations [Snelling et al., 1978]. In previous work from our laboratory, we never observed manifestations of toxicity to topical applications of gentamicin.

D. Levofloxacin
Levofloxacin (LVX), a sterilized pharmaceutical grade drug, will be purchased from AmerisourceBergen, Glen Allen, Va. (or other suitable alternative sources selected in consultation with a veterinarian). Levofloxacin is provided as Levaquin® oral solution 25mg/ml, Ortho-McNeil, Raritan, N.J., NDC 0045-1515-02, 480 ml, or in 20-ml vials.

LVX will be diluted in sterile water and provided *p.o.* 90-100 mg/kg in a 0.2 ml volume once per day for 14 consecutive days (starting day 3 through day 16 after CI) via a 20-G feeding needle with either silicone tip or stainless steel ball tip and a sterile 1-ml syringe. This therapy schedule is used because once-daily administration of a high dose reduces irritation of the soft tissues in the mouth and pharynx induced by the silicone-tipped feeding needles. The high dose attains a high concentration in blood and tissues, which attains over a 24-hr period similar pharmacokinetics as achieved by a lower dose given q12h.

E. Pegylated G-CSF
Pegylated G-CSF (Peg-G-CSF; Neulasta®), a polyethylene glycol pharmaceutical formulated grade drug, also known as pegfilgrastim, is prepared by Amgen Inc., Thousand oaks, CA and will be purchased from Bioscrip Pharmacy Services, Inc, 2787 Charter St. Columbus, OH 43228 800-378-4786 customercare@drugstore.com. Peg-G-CSF will be diluted to deliver 1000 µg/kg (25 µg for a 25-g average mouse) s.c. injection in a volume of 0.2 ml 4 h or 24 h via 25-28 G needle fitted to a sterile syringe. The PEG formulation has a much longer half-life thus reducing the necessity of daily injections of G-CSF [Scholz et al., 2009].

V.4.4.1.2. Non-Pharmaceutical Grade Drugs
(b)(4)
(b)(4) and will be tested at 1 mg/kg (25 µg for a 25-g average mouse), in 0.2 ml s.c. 4h or 24h as specified in experimental design before, after, or combined with Pegylated G-CSF. (b)(4) The selected vehicle will be the phosphate-buffered saline (Life Technologies, Inc). Needle size for s.c. injection is 25-26G. (b)(4) will be prepared with the phosphate-buffered saline and tested for the levels of endotoxins.

V.4.4.2. Biosamples Following anesthesia and/or euthanasia, tissues including blood, lung, liver, kidney, heart, brain, bone marrow and ileum will be collected. Whole anti-coagulated blood (EDTA) will be used for hematological analysis (complete blood count) or serum will be separated from the clotted blood cells. Ileum, lung, and serum will be evaluated *in vitro* by biochemical assays and immunoblotting analysis. Ileum and lung will also be examined for histopathological changes. Other tissues will be frozen at –80°C for future evaluation.

V.4.4.3. Adjuvants NA

V.4.4.4. Monoclonal Antibody (MAb) Production NA

V.4.4.5. Animal Identification Cage cards will be used in general. Marks on tails with marking pens will be used to identify individual animals in a cage if needed. The investigative staff will perform ear tagging with ear skin disinfected with 70% isopropyl rubbing alcohol, if individual identification is needed for periods greater than 3-4 days. No anesthesia will be used.

V.4.4.6. Behavioral Studies NA

V.4.4.7. Other Procedures Food and water consumption and body weights will be measured as a means of discerning the efficacy of therapy for RI and CI as compared to control-treated animals. Furthermore, we will measure wound closure and compare that endpoint to control-treated animals as another means to determine the efficacy of therapy based upon the fact that therapies may result in improved hematopoietic recovery that in turn enhance wound closure. These four variables will be determined on the same selected cage sets of mice at periodic intervals over the 30-day experiments. No aversive stimuli will be provided to the animals in order to elicit behavioral or physiologic changes other than RI and CI.

V.4.4.8. Tissue Sharing All tissues of the animals used in these investigations are appropriate for tissue sharing. Tissues for histopathologic analyses may also be provided to the AFRRI Veterinary Pathologist to obtain diagnoses to detect reasons for survival improvement (over controls) or lack of survival improvement (over controls).

V.4.4.9. Animal By-Products NA

V.4.5. Study Endpoint Animals in these studies may become morbid and moribund and hence it is critical to define criteria for euthanizing mice humanely without compromising the study objective. Mice will be considered **morbid** when they display mild dyspnea, kyphosis with ruffled hair, dried lacrimal fluid accumulated in the cul-de-sac of their eyes, are cool to the touch, show inappetance or diarrhea (i.e., score of 6-10 on Rodent Intervention Score Sheet, Table 1 in appendix, a modification of IACUC Policy #10). Experimental mice that are found morbid, but **not** moribund, will be allowed to continue in the experimental protocol in order to obtain an optimal number of specimens for evaluation of specimens at each time point, particularly to evaluate wound healing rate, namely to determine whether morbid mice are still likely to respond to the therapy. Once mice are considered morbid, the PI or other members of the investigative team will monitor these morbid mice at least twice daily, early morning and late afternoon. When morbid mice become moribund, the investigative team members will monitor those mice more frequently at two- to four-hour intervals.

Those animals found **moribund** with scores greater than 10 will be euthanized with CO_2. Animals will be considered **moribund** when, in addition to showing some or all of the signs of morbidity, they show severe dyspnea plus either an inability to remain upright or an inability to move when stimulated. VSD veterinarians will be consulted if questions should arise regarding difficult, labored breathing (dyspnea), lethargy, and anorexia or inanition. If any single category is above 12 (e.g., the mouse cannot stand or breathe normally, right itself within 5 seconds when placed gently on its side, or respond when the rear foot pads are pinched gently), the mouse will be euthanized.

For those experiments in which blood and tissues will be removed for assays *in vitro*, the anesthetized mice will be euthanized by cervical dislocation, immediately after drawing blood, without inhalation of CO_2 because use of this procedure is to avoid possible adverse effects of inhaled CO_2, i.e., particularly low pH of tissues following

asphyxiation, on the bacteria (low pH kills bacteria) or on metabolism and function of cells and peptides that are to be assayed. These animals will be scored as dead at the time of removal and will be recorded as euthanized in the medical record. PI, co-investigators, or other trained staff, and/or trained veterinary personnel will perform euthanasia.

V.4.6. Euthanasia For blood collection for analyses of blood cell composition, cytokine concentrations, and other biomolecular assays, mice will be anesthetized with isoflurane at specified time points, blood will be collected while under continuous anesthesia (i.e., isoflurane through a nose cone). Then, the mice under isoflurane inhalation will be euthanized by cervical dislocation and terminal tissue specimens will be collected. When mice are found moribund and euthanized as described above under Study Endpoint, blood and tissues will be collected from the individual mice.

Mice, which are found moribund and are not suitable for collection of specimens to be used for further analysis, will be euthanized by CO_2 inhalation and cervical dislocation, as described by VSD's SOP for euthanasia, by investigators or technicians. Animals, which have successfully survived the experimental procedures, including drug treatment and irradiation, will be euthanized by terminal inhalation of CO_2 and cervical dislocation. Briefly, the method of euthanasia for animals surviving the experimental procedures will be exposure to a regulated flow of CO_2 from a compressed gas cylinder in accordance with the current AVMA Guidelines on Euthanasia (only personnel trained and deemed proficient by the Attending Veterinarian are approved to perform this procedure) and a secondary confirmatory method (e.g., cervical dislocation or induced pneumothorax).

We follow the guide in the IACUC policy, a **Rodent Intervention Score Sheet** (Table 1, Appendix 1), to track morbidity and moribund state in injured groups of mice. Mice that have scores greater than 10 will be considered to be moribund. Mice will be euthanized promptly, If any single category is above 12 (i.e., the mouse cannot stand or breathe normally, right itself within 5 seconds when placed gently on its side, or respond when the rear foot pads are pinched gently). These morbid signs usually appear much earlier than body-weight loss of more than 20% original body weight. If there are no other signs apparent and the body-weight loss is the only indication, then mice with body-weight loss more than 20% will be euthanized immediately. These mice will be monitored very closely for their appearance, general behavior, respiratory rate, and provoked behavior prior to making a determination of euthanasia. Weights, which are measured prior to irradiation or within 6 h after irradiation, will be used as a baseline reference. Mice will be monitored at least twice a day (i.e., early in the morning and then again in the late afternoon) during the actual experiment. When mice appear to be morbid, they will be monitored at least three times a day (i.e., check the animals between 6:00-8:00 a.m., 11:00a.m. -1:00 p.m., and 4:00-6:00 p.m. If, during any of the checks, it is suspected that the animals may succumb before the next observation period, the animals should either be checked again before the next scheduled observation period or be euthanized immediately.

V.5. Veterinary Care

V.5.1. Husbandry Considerations Except as noted below, all mice will be group-housed in Micro-isolator cages in the vivarium in cages of 4 or 8 mice each in accordance with the *Guide for the Care and Use of Laboratory Animals* and the current Veterinary Sciences Department standard operating procedure. The animals will be maintained on a 12-h light-dark cycle and fed rodent chow *ad libitum*. Mice will have free access to acidified water via bottles and sipper tubes. Nestlet pads will be provided in each cage during bedding changes. Following irradiation and skin wounding, cages holding similarly treated mice will be placed on shelves in a vertical arrangement to allow similar exposures to environmental factors to all mice in each treatment within the experimental protocol. Mice will be housed for approximately 30 days during the course of survival experiments or for specified durations during the course of mechanistic experiments.

V.5.1.1. Study Room (b)(6) Room Number(s) as assigned by VSD

V.5.1.2. Special Husbandry Provisions Following irradiation and skin wounding, animals will be housed in cages fitted with Micro-isolator tops at Animal biosafety Level One (ASBL-1) within an assigned room of VSD. Gentle movement of injured animals via the tail (taking great care not to disturb the wound site) for purpose of changing the wood-chip bedding is required to insure that the injury site is not disturbed. Cages will be arranged in a specific vertical order to account for variation in environmental factors within experimental groups in the room as noted under "Husbandry Considerations" above.

V.5.1.3. Exceptions Mice are colonial creatures and thus will be group-housed with regard to housing requirements after experimental manipulation. However, during survival studies, as con-specifics within the cage die or are used for experimental purposes; there will be a period of time when an animal could be singly housed. When this occurs, singly housed animals will be able to view con-specifics in neighboring cages housed on the same rack in the same room.

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care Doses of ionizing radiation delivered in this study are expected to result in some illness or debilitation. Because of this, occasional animals may succumb after irradiation without revealing overt signs of stress. On the other hand, the radiation doses used in conjunction with skin wound trauma may result in significant illness, debilitation and death. To monitor these general events, VSD personnel will observe the animals twice daily during general rounds for general health, husbandry conditions, and humane treatment. In addition, the investigative staff will monitor animals at least two times a day, early morning and late afternoon. Supportive care, other than the use of a one-time i.p. injection of 0.5 ml sterile isotonic saline after irradiation and wounding and the use of topical application of gentamicin, oral administration of levofloxacin, and treatments with peg-G-CSF and (b)(4) will

not be provided. Animals found moribund will be euthanized (See Painful Procedure Justification).

V.5.2.2. Emergency Veterinary Medical Care Emergency veterinary medical care will be available from VSD veterinarians and technicians 24 hours daily to provide emergency veterinary medical care as necessary.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy Sterilized nestlet squares will be placed in the cages of experimental mice. The squares must be changed when cages and bedding are refreshed. Experimental mice will be housed in groups of 2-4 in polycarbonate cages. The cages allow viewing of con-specifics in neighboring cage environments. Husbandry provisions by all VSD staff monitoring activities throughout the day by veterinary personnel, and research staff provide an environment of enrichment.

V.5.3.2. Enrichment Restrictions Only sterilized nestlet squares are requested. The squares do not adhere to the wound site and provide a modicum of heat insulation when mice huddle together in their cage environment. The nestlets must be changed when cages and bedding are refreshed. Sterilized acrylic devices will not be used as enrichment devices because removing animals from under these microenvironments adds temporary stress and interferes with the applications of treatment agents to the mice. Further, previous research has not employed acrylic enrichment devices; use in this protocol could add another variable in the behavior of the animals and affect the outcome of the experiment.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1,2,3(all),4,5(aseptic), 7 irradiation, tail vein, anesthesia, oral feeding	(b)(6)
				1, 3a-c, 4, 5(aseptic), 7 oral feeding, irradiation	
				1,3a-c,5(aseptic), 7anesthesia, irradiation	
				1,3a-b,4a,5(aseptic)	
				1, 3a, 4a, 5, 7oral feeding, tail vein, irradiation	
				1,3b,3c,3d,4a,5,7oral feeding, irradiation	

Procedure and manipulation codes:

Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY An AFRRI Safety and Health Department Protocol Hazard Analysis (AFRRI Form 210b) was submitted and approved by the University Safety Officer. We will meet or exceed ASBL-1 procedures to assure that we keep experimental animals separate from the general colony (Chosewood and Wilson 2009). Gaseous anesthetics, including methoxyflurane and isoflurane, will be used under appropriate recovery devices. All the personnel have been trained either as radiation users or as general radiation users annually. All investigators and technicians while in the vivarium will use appropriate procedures and personal protective equipment as described by Veterinary Sciences Department. This will include wearing of gown, mask, eye glasses, and gloves during manipulations and observations of mice. All investigators and technicians maintain current standards in safety training and medical surveillance.

VIII. ENCLOSURES

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Appendix 1.

Table 1. Rodent Intervention Score Sheet.

Rodent Intervention Score Sheet		
PARAMETER	**DESCRIPTION**	**SCORE**
Appearance	Normal (coats smooth, eyes/nose clear)	**0**
	Reduced grooming OR minor hunching	**1**
	Ocular/nasal discharge AND/OR rough coat and hunching OR facial edema	**3**
	Emaciated, dehydrated, OR soft stools (fecal matter around anus)	**5***
	Presence of bloody diarrhea	**9**
General behavior	Normal	**0**
	Minor changes – writhe or grimace, slightly less active than baseline	**1**
	Moderately less mobile and alert	**2**
	Ataxia, wobbly, appearing weak	**6***
	Unable to stand	**12**
Respiratory Rate	Normal breathing	**0**
	Increased (doubled) breathing rate, rapid or shallow	**6**
	Abdominal breathing (gasping +/- open mouth breathing)	**12**
Provoked behavior	Normal	**0**
	Subdued or weak, but moves away when stimulated	**1**
	Subdued even when stimulated (moves away slowly).	**3**
	Unresponsive when stimulated, weak, pre-comatose	**6***
	Does not right when placed gently on side within 5 seconds, or no response when pinch the paws	**12**
***Notify VSD immediately – may need to euthanize**		
<6 – Normal		
6-9 – Morbid, some pain/distress, monitor at least three times a day.		
>10 - Moribund. Either euthanize or notify VSD. If any single category is at 12, euthanize animal immediately.		

Notes: B6D2F$_1$ mice found to have lost greater than 20% of their body weight (relative to weight at start of experiment) will be brought to the attention of VSD staff. Those mice that lose more than 20% of initial body weight will be euthanized and scored as deceased.

X. ASSURANCES

Protocol Title: *In vivo* combined therapy for ionizing radiation followed by wound trauma in the laboratory mouse (*Mus musculus*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM** / **AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)		mod 01/24/2014
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	12/18/2013	2nd Resubmission Date:	2/3/2014
Approved/**Returned for Revision**:	1/16/2014	1st Tier Approved/Final Approval:	2/6; 3/12/14
1st Resubmission Date:	1/21/2014	**EXPIRATION DATE:**	3/11/2017
Approved/**Returned for Revision**:	2/3/2014	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:	2/11/2014	**Approved**/Returned for Revision:	3/12/2014
Approved/**Returned for Revision**:	3/5/2014	2nd Resubmission Date:	
1st Resubmission Date:	3/11/2014	Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and *subsection*, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER (b)(6)

III. PROTOCOL TITLE: Biomarker and efficacy studies of (b)(4) in Rhesus macaques (*Macaca mulatta*)

IV. PRINCIPAL INVESTIGATOR:

(b)(6) Ph.D. Date
(b)(6)
Radiation Countermeasures Program, AFRRI, (b)(6)
Tel (b)(6) fax (b)(6)
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date

Head, Scientific Research Department, AFRRI

Telephone: (b)(6) Fax (b)(6)

(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department, USUHS

Telephone: (b)(6) Fax (b)(6)

(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.

(Date)

(b)(6) **Ph.D.**

IACUC Chair, (b)(6)

Scientific Research Department, AFRRI

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

PROTOCOL TITLE: Biomarker and efficacy studies of [(b)(4)] in Rhesus macaques (*Macaca mulatta)*

PRINCIPAL INVESTIGATOR:

[(b)(6)] Ph.D. Date
[(b)(6)]
Radiation Countermeasures Program, AFRRI [(b)(6)]
Tel [(b)(6)] fax [(b)(6)]
[(b)(6)]

CO-INVESTIGATOR(S): N/A

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: [(b)(6)]

I. NON-TECHNICAL SYNOPSIS: The threat of a limited radiological attack on American cities (i.e. a "dirty" bomb), or a nuclear accident, requires the development of a radiation countermeasure capable of protecting the gastrointestinal (GI) tract of military personnel, who might have to enter a contaminated area, or capable of mitigating GI lethality in the general population. At present there is no effective treatment for the acute radiation GI syndrome. [(b)(4)] promotes recovery of radiation damaged cells by stimulating DNA repair and cell survival pathways. Studies conducted at AFRRI [(b)(4)] both as a protector and a mitigator of ARS in mouse models. Clinical studies in humans support an excellent safety profile of [(b)(4)] In the current protocol the main focus is to optimize the dose of [(b)(4)] to mitigate the effects of radiation by monitoring the biomarkers, and to conduct efficacy studies in nonhuman primates. For efficacy study, drug will be tested by administering it through subcutaneous and oral routes.

II. BACKGROUND

II.1. Background:

Military and civil defense organizations have an urgent requirement for medically effective radiation countermeasures, including radiation protectors, mitigators and therapeutics. Radiation countermeasures that mitigate the harmful effects and promote recovery from radiation injury will have stringent requirements in terms of lack of toxicity, ease-of-use for administration, storage at environmental temperatures, suited for administration by non-medical personnel, and capability of enhancing survival in the absence of ancillary medical support. Although such efforts were initiated more than half a century ago, no safe and effective radiation countermeasure has been approved by the United States Food and Drug Administration (FDA) for ARS (1, 14, [(b)(4)]

Scientists at [(b)(4)] the Armed Forces Radiobiology Research Institute (AFRRI) [(b)(4)] and [(b)(4)] have conducted extensive studies to demonstrate radiation protecting and mitigating effects of injectable (subcutaneous, sc)

and oral (po) [(b)(4)] Safety and tolerability of [(b)(4)] have been established in GLP-compliant nonclinical studies and in 4 clinical safety trials in healthy adults. These results are published in peer-reviewed journals [(b)(4)] and included in 2 active U.S. FDA IND exemptions [(b)(6)] [(b)(4)] mitigates deleterious radiation effects by enhancing DNA damage repair pathways, rather than intervening in the free-radical cascade. Thus, its window of effectiveness is relatively long, extending from a day before to a day or more after exposure to radiation.

Preliminary studies on biomarker analysis in mouse tissue specimens using Nano-immunoassay: A novel analytical method termed, Nanoscale Immuno Assay

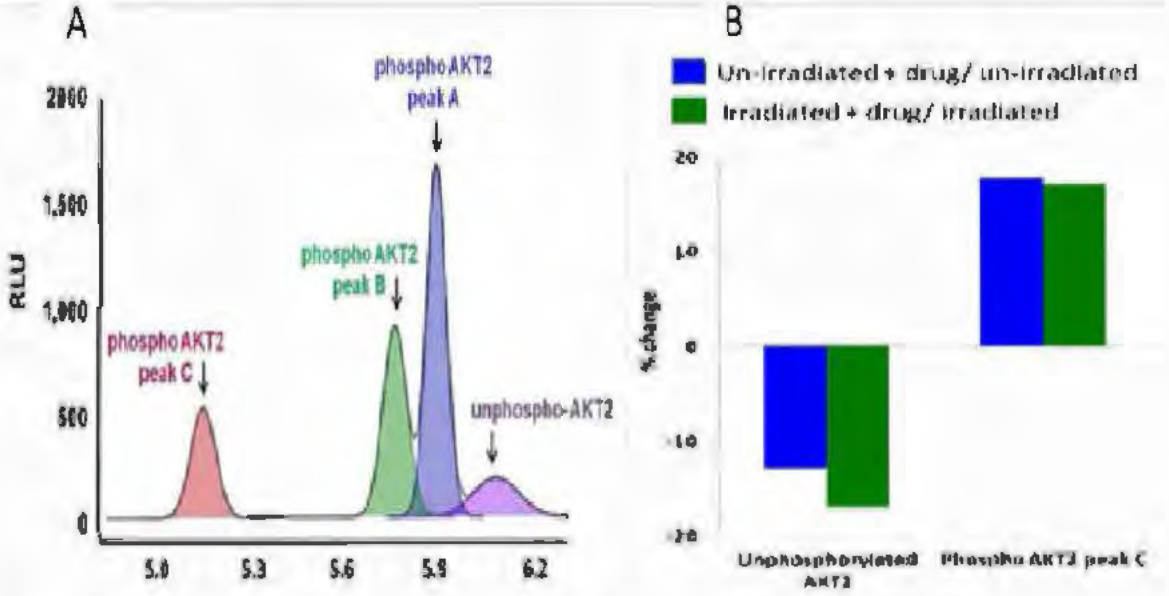

Figure 1. NIA analysis of AKT levels in mouse bone marrow +/- [(b)(4)] administration. A) NIA reveals three phosphorylated peaks of AKT2 in mouse bone marrow (control); B) [(b)(4)] treatment *in vivo* induces similar AKT profiles in bone marrow from un-irradiated and irradiated mice [AKT2 profiles, correlation = 0.94]

(NIA) is capable of rapidly and reproducibly quantifying proteins and their phosphorylation states in very small nonclinical and clinical specimens and has been developed by [(b)(4),(b)(6)] NIA assays have been validated to measure proteins that mediate cell survival, proliferation, cell cycle and apoptotic pathways. Preliminary studies were conducted in mice dosed orally with either vehicle or drug with or without radiation. After 24 h post-irradiation administration of drug, mice were sacrificed, bone marrow (BM) was removed and the cell lysates were analyzed by NIA. The data in **figure 1A** show that the unphosphorylated AKT and the various forms of phosphorylated AKT (A, B and C) were successfully resolved and quantified by NIA. The data in **figure 1B** confirms the up-regulation of Akt2 in BM samples from mice treated with [(b)(4)] with or without radiation. Simultaneously, there is a corresponding down regulation in unphosphorylated AKT. Importantly, correlation in fold change from baseline in AKT2 isoforms between "un-irradiated plus drug" and "irradiated plus drug" was 0.94. This innate effect of drug on the unphosphorylated

levels of AKT and p-AKT, in presence or absence of radiation, could be used to correlate the efficacy of drug in pivotal mouse and NHP studies in therapeutic settings. These preliminary studies demonstrate the potential value of p-AKT2 as a biomarker to follow the drug effect in the absence of radiation during pivotal safety studies in healthy human volunteers.

Understanding changes in signaling and correlating these changes with the mechanism of action provides a path to meeting the challenges of drug approval under the Animal Rule. Results herein provide a promising avenue towards this goal. Additional studies and confirmation under different scenarios, along with validation which is a part of this proposal, may permit correlation of an informative biomarker with expected efficacy parameters.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

Best effort has been made to find any possibility for duplication. The following data bases have been searched:

a. PubMed
b. BRD – DOD Biomedical Research Database
c. FEDRIP

II.2.2. Date of Search

PubMed - 11/7/13
BRD – DOD Biomedical Research Database - 11/6/13
FEDRIP - 11/6/13

II.2.3. Period of Search

PubMed - 1965-2013
BRD - 1998-2013
FEDRIP– 1992-2013

II.2.4. Key Words of Search

1 (b)(4) and radiation,
2 and Primates,
3 and primates and radiation,
4 and biomarker(s),
5 and pancytopenia,
6 and pancytopenia and radiation,
7 and pancytopenia,
8 pancytopenia and radiation and primates,
9 and bone marrow,
10 (b)(4) and radiation and efficacy,
11 Tween-80 and primate,
12. Tween-80 and NHP and radiation**II.2.5. Results of Search**

PubMed

Search string 1: We found 9 references, none of which addressed the questions to be tested in this protocol.
Search string 2: We found 0 references
Search sting 3: We found 0 references
Search string 4: We found 0 references.
Search string 5: We found 1 reference which does not address the questions to be tested in this protocol.
Search string 6: We found 1 reference which does not address the questions to be tested in this protocol.
Search string 7: We found 1 reference which does not address the questions to be tested in this protocol.
Search string 8: We found 0 references.
Search string 9: We found 1 reference which does not address the questions to be tested in this protocol.
Search string 10: We found 2 references none of which addressed the questions to be tested in this protocol.
Search string 11: We found 1,404 references none of which addressed the questions to be tested in this protocol.
Search string 12: We found 0 references.

BRD

Search string 1: We found 11 references, none of which addressed the questions to be tested in this protocol.
Search string 2: We found 5 references none of which addressed the questions to be tested in this protocol.
Search sting 3: We found 5 references none of which addressed the questions to be tested in this protocol.
Search string 4: We found 0 references.
Search string 5: We found 0 references.
Search string 6: We found 0 references.
Search string 7: We found 0 references
Search string 8: We found 0 references.
Search string 9: We found 3 references none of which addressed the questions to be tested in this protocol.
Search string 10: We found 3 references none of which addressed the questions to be tested in this protocol.
Search string 11: We found 0 references.
Search string 12: We found 0 references.

FEDRIP

Search string 1: We found 0 references
Search string 2: We found 0 references
Search sting 3: We found 0 references
Search string 4: We found 0 references.
Search string 5: We found 0 references
Search string 6: We found 0 references

Search string 7: We found 0 references
Search string 8: We found 0 references.
Search string 9: We found 0 references
Search string 10: We found 0 references
Search string 11: We found 0 references
Search string 12: We found 0 references.

There are no references of using (b)(4) for its efficacy against radiation injury in NHPs. Initial studies investigating the efficacy and mechanism of (b)(4) have been conducted in mice and in-vitro models. Efficacy studies with radiation doses ranging from 5 - 15 Gy have been conducted. Results demonstrate that mice administered with drug (sc or po) and exposed to lethal radiation had significant survivors. These studies suggest that (b)(4) is a promising radiation countermeasure and warrants continued development (b)(4) Overall, the search did not reveal any duplication and the proposed study will provide valuable information about the development of (b)(4) as a radiation countermeasure.

III. OBJECTIVE/HYPOTHESIS:

The objective of this protocol is to advance the pre-clinical development of (b)(4) (b)(4) as radioprotector (and radiomitigator) of the acute radiation syndrome. Here, we propose to 1) Optimize the dose of (b)(4) to protect/mitigate the effects of radiation by monitoring the biomarkers in BM and peripheral blood mononuclear cells (PBMC) in NHPs. Preliminary studies in a mouse model have identified p-Akt2 as a potential biomarker for efficacy of (b)(4) as its level is upregulated both in the presence and absence of radiation in a mouse.. We are proposing to validate this marker by analyzing BM and blood samples from NHPs during efficacy studies; 2) Proof of concept study of subcutaneously (sc) administered (b)(4) as a protector/mitigator of hematopoietic ARS in NHPs using survival and hematopoietic rescue as end points; and 3) Confirm the efficacy of orally administered (b)(4) in NHPs using survival and hematopoietic recovery as end points. These studies will form the foundation for future pivotal studies to confirm the efficacy of (b)(4) in two animal models and safety in humans towards licensure from the FDA.

IV. MILITARY RELEVANCE

Currently, there are no FDA approved pharmaceutical agents that can prevent or treat injury from external ionizing radiation. The problem has become more acute in recent times due to the unpredictable nature of terrorist attacks. Recognizing this urgent need, the U.S. Department of Defense has assigned top priority to the "development of medical countermeasures to radiation exposure" against both early and late arising health effects. These concerns imply the urgent need to develop an appropriate countermeasure for radiation injuries potentially sustained by war fighters during combat operations and enable commanders to make judicious decisions in troop deployment. There are several drugs at different stages of the development. This study will perform essential research and development of a promising new agent (b)(4) as a therapy for potential injuries sustained by military personnel who have been accidentally exposed to ionizing radiation.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

We propose to do the following experiments to utilize biomarkers for [redacted] to optimize dose of this drug and to evaluate its efficacy in subsequent experiments. [redacted] will be evaluated as radioprotector/mitigator when administered through sc and po routes. Details of subsequent experiments will be based on results obtained in previous experiment.

V.1.1. Experiment 1. Optimize the dose of [redacted] to mitigate the effects of radiation by monitoring the biomarkers in BM and PBMC in NHP model.

In the present protocol, we plan to optimize the dose of [redacted] administration by analyzing the biomarkers in BM and PBMC. The main objectives of this experiment is to demonstrate drug dose-response by evaluating biomarkers in BM and PBMC. BM and PBMC will be collected from NHPs to analyze the p-Akt2 levels using Western-blot (in-house) and Nanoscale Immuno Assay (NIA) method [redacted] The following specific study will be undertaken:

Two different doses of [redacted] (15 and 30 mg/kg body weight) will be administered to two NHPs in each group. A model based on allometric scaling was built, using the PK parameters in various animal species, to predict the expected concentration-time profile in humans. The results indicate that the predicted clearance of the drug matches very well with the clearance of drug observed in the clinical trial. Allometric data allows prediction of a starting dose in NHP efficacy studies and pivotal human safety trials.

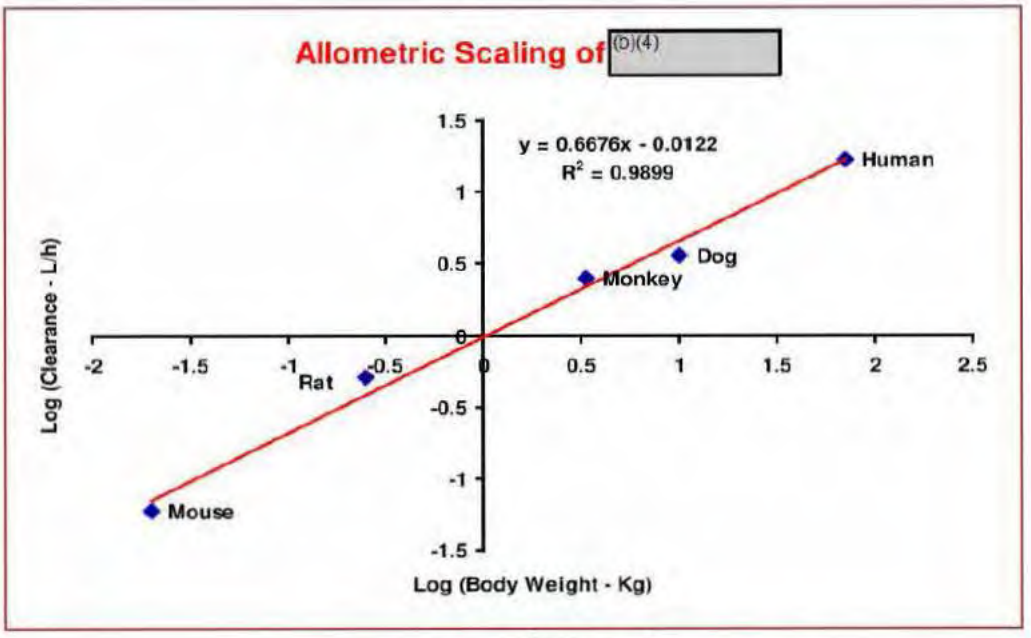

Figure 2. Allometric model and data for [redacted] clearance rates across animal species and humans. *Methodology used to relate pharmacodynamic dose requirement to pharmacokinetic parameter.*

Initially, (b)(4) will be administered (sc) to NHPs followed by collection of BM (2.0 ml) at 24 and 48 h after drug injection and blood (2 ml) (for PBMC) at 12, 24 and 36 h post drug administration. BM (2 ml) and blood (2 ml) collected prior to drug administration (-8 d) will serve as baseline control. If these two doses of drug do not provide dose response in biomarker analysis, same animals will be used after 30 d wash-out period with two additional doses of (b)(4) (above or below the drug dose used in the initial experiment depending on the results of biomarkers). Biomarker identification and validation are important objectives of this protocol. To accomplish this we need frequent BM and blood samplings close to drug administration and radiation exposure. Further, several putative biomarkers will be evaluated by two different techniques (gel electrophoresis/immunoblotting and NIA) for which we will require significant quantity of all samples collected.

Table 1. Sample collection for biomarker studies – sc drug administration

(b)(4) dose mg/kg	Time of blood/BM collection	BM	Blood	Total volume
	-8 d	2 ml	2 ml	4 ml
15 or 30 mg/kg	0 h			
	+12 h		2 ml	2 ml
	+24 h	2 ml	2 ml	4 ml
	+36 h		2 ml	2 ml
	+48 h	2 ml		2 ml

Summary of sample volume:-8 d = 4 ml, one week rest, 0 to 2 d = 10 ml. There will be 3 wk rest before further use of these animals. This will also allow one month wash-out period between drug administrations for two experiments to same animals.

If both sides are needed for a single BM collection attempt, an attempt on that side/site will not be attempted again for 1 week. There will be one week recovery period between pretreatment and post-treatment samplings. This will be applicable to all experiments involving BM collection mentioned below.

Table 2. Sample collection for biomarker studies - sc drug administration 2nd dose set

(b)(4) dose mg/kg	Time of blood/BM collection	BM	Blood	Total volume
	-8 d	2 ml	2 ml	4 ml
Based on results of above experiments	0 d			
	+12 h		2 ml	2 ml

	+24 h	2 ml	2 ml	4 ml
	+36 h		2 ml	2 ml
	+48 h	2 ml		2 ml

Summary of sample volume:-8 d = 4 ml, one week rest, 0 to 2 d = 10 ml. There will be 3 wk rest before further use of these animals. This will also allow one month wash-out period between drug administrations for two experiments to same animals.

Animals from above studies will be reused for biomarker studies with oral drug administration (15 and 30 mg/Kg body weight). Experimental details will be as above except drug administration will be po. BM samples will be collected at -8 d, and 24, and 48 h after drug administration. Blood samples for biomarker analysis will be collected at -8 d, 12, 24 and 36 h post last dose of drug administration.

Table 3. Sample collection for biomarker study - oral drug administration

(b)(4) dose mg/kg	**Time of blood/BM collection**	**BM**	**Blood**	**Total volume**
	-8 d	2 ml	2 ml	4 ml
Based on results of above experiments	0 d			
	+12 h		2 ml	2 ml
	+24 h	2 ml	2 ml	4 ml
	+36 h		2 ml	2 ml
	+48 h	2 ml		2 ml

Summary of sample volume:-8 d = 4 ml, one week rest, 0 to 2 d = 10 ml. There will be 3 wk rest before further use of these animals. This will also allow one month wash-out period between drug administrations for two experiments to same animals.

These animals will be used again after wash-out period of 30 d for study using drug and radiation exposure. Optimal dose of (b)(4) will be administered by sc route at -24 h, -12 h prior to and +4 h to two irradiated animals. Remaining two NHPs will serve as control (receive radiation exposure but no drug). Animals will be exposed to total body radiation (radiation dose $LD_{50/60}$ (6.5 Gy), dose rate 0.6 Gy/min). BM samples (2.0 ml) will be collected at 24 and 48 h after radiation exposure and PBMC will be collected at 12, 24 and 36 h post-irradiation. Again, BM and PBMC collected prior to start of the study (-8 d) before radiation exposure will be used as the baseline control. Drug dose for efficacy studies (expt 2 and 3) will be decided based on the results from above biomarker studies.

Table 4. Sample collection for biomarker study in irradiated animals treated with (b)(4) (b)(4) (sc)

[(b)(4)] dose mg/kg	Time of blood/BM collection	BM	Blood	Total volume
	-8 d	2 ml	2 ml	4 ml
	0 h – 6.5 Gy Radiation			
Based on results of above experiments	-24 h -12 h +4 h			
	+12 h		2 ml	2 ml
	+24 h	2 ml	2 ml	4 ml
	+36 h		2 ml	2 ml
	+48 h	2 ml		2 ml
				Total - 8 d = 4 ml 0 to 2 d = 10 ml

Summary of sample volume:-8 d = 4 ml, one week rest, 0 to 2 d = 10 ml.

The objective of this experiment is to demonstrate that biomarker levels are affected in the same manner by the drug administration both in the presence and absence of radiation. Animals will be euthanized at the end of the experiment (i.e. immediately after last sample collection – 48 h after irradiation). Tissue samples will be collected for histopathology.

V.1.2. Experiment 2. Evaluation of the efficacy of sc administered [(b)(4)] in nonhuman primates using biomarker–guided optimal dosing regimen

This will be a proof-of-concept study in NHPs evaluating the efficacy of optimal dose of [(b)(4)] administered by sc route following a known lethal irradiation dose (i.e. the $LD_{50/60}$ (6.5 Gy). To determine the efficacy of [(b)(4)] by sc route for survival in NHPs, two groups of 4 animals will be used. One will receive optimal dose of [(b)(4)] and other will serve as vehicle control. Optimal dose of [(b)(4)] will be administered by sc route at -24 h, -12 h and +4 h prior to and after radiation exposure. NHPs will be exposed to $LD_{50/60}$ TBI, following sc administration of [(b)(4)] at -24 h and -12 h prior to radiation exposure. One more optimal dose of [(b)(4)] will be administered 4 h after exposure to radiation.

Blood samples collected on -8 d, +12 h, +24 h and +48 h will be used for collecting PBMC for analysis of biomarkers by Western blot and NIA methods. Animals will be monitored for survival for 60 d. The drug efficacy will also be assessed by peripheral blood cell counts (see table 5 for time points). Blood biochemistry and serum citrulline will be also evaluated at various time points after irradiation (table 5). In addition, secondary end points, including 1) significant reduction in the incidence of infection, as manifested by febrile neutropenia, 2) the incidence, severity, and duration

of severe neutropenia (absolute neutrophil count [ANC] < 500/mm^3) following radiation, will be determined.

Blood pressure, pulse and temperature will be recorded before injection, 24 h after irradiation and every time sample is collected. Body weight will be measured before injection and weekly afterwards.

Table 5: Blood and BM collection schedule for experiment 2.

(b)(4) dose mg/kg	Time of blood draw	Blood					Total blood/BM, ml
		CBC 0.5 ml	Biomarker 2.0 ml	Biochemistry 0.5 ml	Bacteremia 0.5 ml	Citrulline 0.5 ml	
	-8 d	+	+	+	+	+	6.0
Optimum dose based on biomarker study	(b)(4) (b)(4) /vehicle oral admn. day –24 h, -12 h, and +4 h						
Radiation exposure 6.5 Gy	Day 0 +12 h		+				2.0
	Day 1 (24 h)	+	+				2.5
	+36 h		+				2.0
	Day 2	+	+				2.5
	Day 4	+				+	1.0
	Day 6	+					0.5
	Day 8	+					0.5
	Day 10	+			+	+	1.5
	Day 12	+					0.5
	Day 14	+					0.5
	Day 16	+					0.5
	Day 18	+					0.5
	Day 20	+				+	1.0
	Day 22	+					0.5
	Day 24	+			+		1.0
	Day 26	+					0.5
	Day 28	+		+		+	1.5
	Day 30	+					0.5
	Day 34	+			+		1.0
	Day 38	+		+		+	1.5
	Day 42	+					1.0
	Day 50	+		+	+	+	1.5
	Day 60	+		+		+	1.5

Summary of sample volume: -8 d = 6 ml. One week rest. 0 to 60 d = 34.5 ml

We will be well within the acceptable published blood collection volumes (4) that are followed at AFRRI. According to these guidelines 1% blood (2.8 ml per day for 4 kg NHP – minimum body weight of experimental NHP) can be withdrawn every 24 h. Up to 10% of circulating blood volume can be drawn in a single day repeated every 3-4 weeks. For the entire span of the study, the total blood drawn will be well below the recommended maximum limits.

V.1.3. Experiment 3. Confirmation of the efficacy of oral (b)(4) in non-human primates and validation of the biomarkers. Survival and hematopoietic recovery will be the primary end points

An oral (b)(4) would be attractive, convenient and able to deliver effective dose levels particularly to children, elderly and chronically-ill patients. Preliminary studies have shown that uptake of orally-administered (b)(4) was rapid, with higher plasma C_{max} and AUC values achieved than those observed with corresponding sc doses.

Similar to experiment proposed under experiment 2, two groups of 4 NHPs each will be used. During distribution of NHPs into two groups, various health parameters will be considered and distributed in a manner so that both groups should receive NHPs of similar health status. One will receive the optimal dose of (b)(4) and the other will get vehicle control. NHPs will be exposed to $LD_{50/60}$ or $LD_{70/60}$ TBI, following oral administration of (b)(4) at -24 h and -12 h prior to radiation exposure. One more optimal dose of (b)(4) will be administered 4 h after exposure to radiation. Animals will be sedated (ketamine 10-20 mg/kg, im) for oral gavage and gastric tube (3-7 fr or as VSD veterinarian recommendation) attached with syringe (needle 25 G) will be used for this purpose.

Blood samples collected on -8 d, +12 h, +24 h and +48 h will be used for collecting PBMC for analysis of biomarkers by Western blot and NIA methods as described above. Animals will be monitored for survival for 60 d. The drug efficacy will also be assessed by peripheral blood cell counts (see table 6 for time points). Blood biochemistry and serum citrulline will be also evaluated at various time points after irradiation (table 6). In addition, secondary end points, including 1) significant reduction in the incidence of infection, as manifested by febrile neutropenia, 2) the incidence, severity, and duration of severe neutropenia (absolute neutrophil count [ANC] < $500/mm^3$) following radiation, will be determined.

Blood pressure, pulse and temperature will be recorded before injection, 24 h after irradiation and every time sample is collected. Body weight will be measured before injection and weekly afterwards.

Table 6: Blood and BM collection schedule for experiment 3

(b)(4) dose mg/kg	Time of blood draw	Blood					Total blood/BM, ml
		CBC 0.5 ml	Biomarker 2.0 ml	Biochemistry 0.5 ml	Bacteremia 0.5 ml	Citrulline 0.5 ml	
	-8 d	+	+	+	+	+	6.0
Optimum dose based on biomarker study	(b)(4) (b)(4) vehicle oral admn. day –24 h, -12 h, and +4 h						
Radiation exposure 6.5 Gy	Day 0 +12 h		+				2.0
	Day 1 (24 h)	+	+				2.5
	+36 h		+				2.0
	Day 2	+	+				2.5
	Day 4	+				+	1.0
	Day 6	+					0.5
	Day 8	+					0.5
	Day 10	+			+	+	1.5
	Day 12	+					0.5
	Day 14	+					0.5
	Day 16	+					0.5
	Day 18	+					0.5
	Day 20	+				+	1.0
	Day 22	+					0.5
	Day 24	+			+		1.0
	Day 26	+					0.5
	Day 28	+		+		+	1.5
	Day 30	+					0.5
	Day 34	+			+		1.0
	Day 38	+		+		+	1.5
	Day 42	+					1.0
	Day 50	+		+	+	+	1.5
	Day 60	+		+		+	1.5

Summary of sample volume: -8 d = 6 ml, One week rest, 0 to 60 d = 34.5 ml

We will be well within the acceptable published blood collection volume (4) that is followed at AFRRI. For the entire span of the study, the total blood drawn will be well below the recommended maximum limits.

Total NHP needed for this Protocol:

Experiments	Total

Experiment 1	4
Experiment 2	8
Experiment 3	8
Total NHP required for this protocol	20

V.2. Data Analysis

Mean with standard errors, or percentages, will be reported if applicable. The significance level will be set at 5% for each test.

For experiment 1 to analyze the significant difference in the biomarker expression between the 2 groups of animals, the data will be tested by one-way ANOVA with Bonferonni's post-test correction to determine the statistically significant difference in biomarker profile. When needed we will also validate using a two-sided Student's *t* test, when comparing two groups to determine significant difference among sampling time. Values of $p < 0.05$ will be considered statistically significant. Values will be expressed as means ± standard error (SE).

Comparison of survival curves will be made using the log-rank test. Fisher's exact test will be used to detect if there is a significant difference across the groups in survival rates by performing pair-wise comparisons. Four NHPs (n = 4) per group would have 80% power to detect a significant difference between two groups if any, given type I error of 5% and delta/sigma of the differences between two group at least 2.4, where sigma is the common standard deviation, and delta is the mean difference (26).

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

Both computer models and tissue culture have been considered as alternatives to animals. These alternatives lack the knowledge base and complexity needed to evaluate the effects of radiation and radiation countermeasures on the immune system. The phenomena under study involve complex information-processing networks comprising large number of cell types and biological signal transduction molecules. Responses to irradiation and radioprotective agents involve interactions between the central nervous system, the peripheral nervous system including the enteric nervous system, endocrine glands, the immune and inflammatory systems, hematopoietic system, gastrointestinal system etc. We do not know all of the cell types and tissues involved and many of the signal transduction molecules have not been discovered yet. Even if all of the tissues, cell types and signaling molecules were known, the present state of tissue culture would be incapable of reproducing their *in vivo* relationships. One would have to reproduce much of an entire mammalian organism in culture to study these phenomena *in vitro*. As for computer models, the most powerful supercomputers available in the near future would be incapable of analyzing interactions between so many elements in the network. Thus these drugs necessitate investigation in whole animal models to see overall outcome.

V.3.2. Animal Model and Species Justification:

As described above, preliminary studies have shown that (b)(4) is effective in mice when administered either sc or po as a protector or as a mitigator. Demonstrating

efficacy in a large animal species (LAS) is a critical requirement of the FDA Animal Rule. *Rhesus macaque* has 95+% DNA sequence identity with humans. NHPs are necessary for the pre-clinical development of a drug candidate intended for use in humans, because drug metabolism and physiology are so similar between NHPs and humans. Rhesus macaques are the model of choice for investigations of toxicity, pharmacokinetics, biomarkers, radiation injury, and countermeasures, because of the large database available from the existing literature. The FDA has accepted Rhesus macaques as the appropriate animal model for pivotal efficacy testing of radiation countermeasures under the Animal Efficacy Rule, where efficacy testing cannot be performed in humans. The biomarker and efficacy of the drug to be tested in this protocol have already been demonstrated in mice. Previous experience with other radiation countermeasures (e.g. Neummune or HE 2100 or 5-AED) has shown that testing in dogs is not always relevant to pharmacokinetics and efficacy in primates. Metabolism of 5-AED in dog hepatocytes was much different than in NHPs and human hepatocytes. Animal model of pig (specifically Gottingen minipig) is currently under initial stage of the development for studying radiation injury. This model is not well defined and mature enough to evaluate radiation countermeasures. Our ultimate goal is to generate data which will be submitted to US FDA and our data should be acceptable to them for approval of this radiation countermeasure (b)(4) Minipig model has not reached to that stage of acceptance yet. Therefore, this study in Rhesus macaque is being undertaken to guide future development of this class of radiation countermeasures. There are several reports for use of NHPs in radiation research and countermeasure development (6-8, 19).

V.3.3. Laboratory Animals

V.3.3.1. Genus/Species

*Macaca mulatta (*Rhesus macaques*)*

V.3.3.2. Strain/Stock

Chinese or Indian origin

V.3.3.3. Source/Vendor

AFRRI VSD will procure animals from vendors registered with the USDA or DoD.

V.3.3.4. Age

2.5 to 7 years

V.3.3.5. Weight

4 to 8 kg

V.3.3.6. Sex

Male and female

V.3.3.7. Special Considerations

Animals should test antibody negative for Herpes B virus (aka Macacine herpesvirus), Simian T-cell leukemia virus type 1 (STLV-1), Simian Immunodeficiency virus (SIV) and Simian Retrovirus (SRV) Types 1, 2, 3, and 5. Animals shall also test

negative by means of virus isolation or polymerase chain reaction (PCR) for SRV Type 2. NHPs will either be vaccinated for measles or, in the case of previously measles-vaccinated NHPs, tested for the presence of measles antibodies. NHPs shall come from the vendor colony negative for Salmonella, Shigella and Campylobacter. Additionally, the animals shall also test negative for Klebsiella pneumoniae. Animals will be pole and collar trained before use in experiment.

V.3.4. Number of Animals Required (by species)

Macaca mulatta: 20 (10 males and 10 females)

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement

For biomarker study end point will be measurement of various parameters till predetermined days. For this purpose blood and BM samples will be collected at predetermined time points and analysis of relevant parameters will be carried out.

For efficacy study, the endpoint currently mandated by the FDA for approval of radiation countermeasures is mortality. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress, using an extensive set of criteria (listed under section V.4.5). The actual irradiation procedure does not cause pain or distress. Animal care and use procedures will only be performed by trained and certified personnel.

V.3.5.2. Reduction

Two different doses of (b)(4) will be tested in two animals each for biomarkers. In case we don't get desired results, same animals will be used again after 30 d wash period with additional doses of the drug. We also plan to use same animals in biomarker study with radiation exposure. After 30 d wash period, these animals will be irradiated and dosed with (b)(4) In efficacy study, drug administration through two different routes (sc and po) will be tested. In each study, there will be only 4 NHPs in vehicle and another 4 in drug-treated group. Tissues will be shared with other investigators, wherever possible without compromising the objectives of the project.

As we move forward with experiments, plans will be reevaluated at every stage of the experiments, in light of incoming data in an attempt to reduce the numbers of experiments and groups, if possible. If deemed not necessary based on incoming data, some investigations may not be carried out to optimize the use of experimental animals.

V.3.5.3. Replacement

There are no *in vitro* techniques available to demonstrate that this drug will counter the effects of whole-body irradiation in humans. Efficacy and safety have already been demonstrated in rodents. The preferred large animal model for preclinical evaluation of radiation countermeasures intended for use in humans is non-human primates. This study represents the next step necessary to develop this drug for FDA approval. According to FDA scientists and the radiobiology community, the large database of radiation studies in *Macaca mulatta* makes this the most useful large animal model to evaluate interactions of radiation injury and drugs intended for use in humans.

V.4. Technical Methods

V.4.1. Pain/Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C.....0

V.4.1.1.1.2. Column D.....0

V.4.1.1.1.3. Column E.....20

V.4.1.1.1.4. Pain Category Assignments

Experiments	Total	C	D	E
Experiment 1	4	0	0	4
Experiment 2	8	0	0	8
Experiment 3	8	0	0	8
Total mice required for this protocol	20	0	0	20

V.4.1.2. Pain Relief/Prevention

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization

Microchips will be implanted to animals before initiating the other experimental procedures. BM will be collected under anesthesia. This procedure has been described in detail under section V.4.3.2 (not elaborated here to avoid duplication). Buprenorphine (0.005 – 0.03 mg/kg, im or sc or iv, every 12 h) will be administered for 3 days after each BM collection (will be given for longer if animals show limping or any other sign of pain is noticed) to NHPs of experiment 1. Since anesthesia, analgesia, and tranquilizers are known to affect functions of the immune system of animals, we plan to minimize their use during course of the experiment after irradiation (2, 9, 10).

Pain is not expected from actual irradiation process. The sequelae of high dose of radiation may result in pain but the animals treated with (b)(4) will experience reduced infections observed in immunosuppressed animals. As observed in mice, (b)(4) (b)(4) may enhance hematopoietic recovery after radiation and if it does, this should lead to improved health. Use of other bactericidal agents to combat unresolved infections during (b)(4) management is not warranted in this study as the scientific end points identified above will be abridged. It is important to note that the principle purpose of this study is to validate the biomarkers (Experiment 1) and evaluate efficacy of (b)(4) (Experiment 2 & 3). The use of antibacterial agents will interfere with principle purpose (efficacy study under experiment 2 & 3). However, if pain and/or distress are observed, the PI and attending veterinarian staff will be notified to evaluate and determine the appropriate treatment. If pain and/or distress cannot be alleviated, the animal will be evaluated for euthanasia.

Some animals may feel temporary discomfort such as nausea, emesis, and diarrhea that may occur after irradiation. In such situation, administration of fluid using bottle will be considered. No treatments for radiation-induced behavioral manifestations

will be provided as they may interfere with the evaluation of (b)(4) in post-irradiation recovery.

Use of ketamine for BM biopsy has been elaborated under section V.4.3.1. Pre-Surgical Provisions.

V.4.1.2.2. Pre- and Post-procedural Provisions

Animals will be observed for signs of pain and distress by either the veterinary or research staff at least twice daily. Before procedures that require general anesthesia, NHPs will be fasted for 12 h prior to administration of systemic anesthesia. After surgical procedure (such as BM aspiration in experiment 1), NHPs will be monitored at least twice daily for signs of surgical site complication such as infection or trauma. Buprenorphine (0.005 – 0.03 mg/kg, im or sc or iv) will be administered every 12 h for 3 days or longer after each BM collection (if animals show limping or any other sign of pain is noticed) to NHPs of experiment 1.

V.4.1.2.3. Paralytics

No paralytic agent will be used in this study.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched

AGRICOLA
PubMed
IVIS

V.4.1.3.2. Date of Search

AGRICOLA – 12/11/2013
PubMed - 12/11/2013
IVIS - 12/11/2013

V.4.1.3.3. Period of Search

ARGICOLA -1966-2013
PubMed - 1965-2013
IVIS - 1998-2013

V.4.1.3.4. Key Words of Search

(b)(4) AND "Pain"
"Ionizing radiation" AND "pain"
"Ionizing radiation" AND " bone marrow"
"Ionizing radiation" AND " primate"
"Ionizing radiation" AND "alternatives"
"Ionizing radiation" AND "toxicity"

V.4.1.3.5. Results of Search

(b)(4) AND "Pain"
AGRICOLA: 0
Pubmed: 0
IVIS: 0

"Ionizing radiation AND pain"
AGRICOLA: 0
PubMed: 445
IVIS: 21

"Ionizing radiation AND bone marrow"
AGRICOLA: 674
Pubmed: 13
IVIS: 0

"Ionizing radiation AND primate"
AGRICOLA: 0
Pubmed: 24
IVIS: 0

"Ionizing radiation" AND "alternatives"
AGRICOLA: 2
Pubmed: 133
IVIS: 2

"Ionizing radiation" AND "toxicity"
AGRICOLA: 351
Pubmed: 4703
IVIS: 16

No reports were found indicating administration of (b)(4) causes pain. Similarly, there were no reports indicating that irradiation itself causes pain. It has been reported that whole-body irradiation can cause sedative effect, as indicated in the tail-flick test in rats.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification

(a): BM aspiration: Animals will get proper analgesics for this procedure and they will not be in painful or distressful condition.

(b) Irradiation: As stated above, irradiated animals finally die due to a compromised immune response and microbial infections. In the event that there is pain and distress for animals, as we have already discussed under above headings, we cannot give anesthetic/analgesic agents to the animals (except at the time of BM aspiration which can't be performed without anesthesia) since these agents interact with the immune system, and in turn will affect experimental results. Buprenorphine (0.005 – 0.03 mg/kg, im or sc or iv) will be administered for 3 d or longer after BM collection if animals show limping or any other sign of pain is noticed.

There are conflicting reports regarding effects of buprenorphine on the immune system (3, 13, 15, 16, 18, 20, 21, 25). A majority of reports suggest that buprenorphine influences immune response. Since buprenorphine has been shown to influence various arms of cellular and humoral immune response (3, 16, 21, 25), we have decided not to use it in our efficacy study (experiment 2 and 3). This gent will be administered to NHPs

of experiment 1 for biomarker study as stated above. Non-steroidal anti-inflammatory drugs are not suitable for use in such experiments (17).

V.4.2. Prolonged Restraint:

Animals to be irradiated will be placed in restraint devices for irradiation in the cobalt facility. Animals will be sedated for this purpose (ketamine 10-20 mg/kg, im).

For blood collection, NHPs will be placed in a primate restraint chair. NHPs will not be left in the restraint chair for more than 30 minutes.

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions:

BM collection proposed in experiment 1 is considered a surgical procedure. Animals will be assessed for signs of pain and distress by either the veterinary or research staff at least twice per day or as often as needed. Antibacterial prophylaxis will be administered: Cefazolin iv or sc (20 - 25 mg/kg, one time before BM collection) or ampicillin (15 - 20 mg/kg, iv or im one time before BM collection). Food will be withheld from the animals 12 h prior to administration of the anesthetic. The NHPs will be chemically sedated with ketamine (5-15 mg/kg im) or Telazol (5-10 mg/kg im) with Atropiune (o.05 mg/kg im) or glycopyrrolate (0.005 – 0.1 mg/kg im) (needle 25 G). For induction, 3-5% isoflurane in 100% oxygen by mask will be used. For maintenance 1-3% isoflurane will be administered in 100% oxygen via endotracheal tube or mask. While the animal is under the anesthesia, vital signs will be monitored including SpO2, pulse, respiration rate and body temperature. Bair Hugger heated surgical table will be used for keeping animal warm. Animal body temperature will be recorded frequently using a rectal thermometer during the procedure.

V.4.3.2. Procedure(s):

The site of BM collection may be the iliac crest, scapula, ischial tuberosity, trochanteric fossa of the femur, tibial crest, or the proximal humerous. The site will be prepared as a surgical site: hair will be clipped using # 40 surgical blade and the site scrubbed at least 3 times using either betadine or chlorhexidine and 70% alcohol. Animal will be positioned appropriately (dorsal or ventral or lateral recumbency depending on the site being used for the BM collection. A sterile drape will be used to cover the site. A small stab incision (just enough for a 16 G needle to go through) will be made in the skin at the site of BM collection. Then, BM aspiration needle will be advanced through the incision into the cortex of the bone. The stylet is removed from the BM needle. A 2 - 5 ml syringe will be used to aspirate the BM. The needle will be removed and incision will be closed with tissue glue or a suture after BM collection. Either non absorbable nylon or absorbable monocryl suture will be used to close the skin. Only autoclaved or disposable sterile instruments will be used. Only one attempt will be made per site and only two sites will be attempted at any one sampling point. No more than 3 ml of BM will be collected per animal.

Food will be withheld for about 12 h prior to sedation.

V.4.3.3. Post-surgical Provisions:

Animals will be monitored at least twice per day for signs of surgical complications such as infection or trauma. If treatment for such conditions is needed, a VSD veterinarian will provide the appropriate medical intervention: Baytril 5-10 mg/kg im or po, sid for at least 5 days or longer (may be given to avoid infection). Buprenorphine (0.005 – 0.03 mg/kg, im or sc or iv) will be administered for 3 d or longer after BM collection if animals show limping or any other sign of pain is noticed. Cefazolin (20-22 mg/kg, iv) will be used as needed based on opinion of veterinarian.

V.4.3.4. Location:

The procedure will occur in the VSD surgical suite at the veterinarian's discretion.

V.4.3.5. Surgeon:

VSD personnel trained in the procedure and aspetic technique.

V.4.3.6. Multiple Major Survival Operative Procedures

V.4.3.6.1. Procedures: N/A

V.4.3.6.2 Scientific Justification:

Though BM collection is not major procedure per se, we are conducting repeated collections which may make it fall into this section. Biomarker identification and validation are important objectives of this protocol. To accomplish these objectives, we need frequent BM and blood samplings close to drug administration and radiation exposure (stated above in various tables). Further, several protein biomarkers will be evaluated by two different techniques (gel electrophoresis/immunoblotting and NIA) for which we will require significant quantity of all samples collected.

As stated above, one site will be used only once for the BM collection in an experiment over a period of one month. Same site will not be used for BM collection again in specific study. Same animal will be used to repeat study after one month rest period (applicable for experiment 1 only). We have identified several sites for BM collection in NHPs of experiment 1. There will be no bM collection in experiment 2 and 3.

V.4.4. Animal Manipulations

V.4.4.1. Injections

NHPs will be dosed with (b)(4) subcutaneously in the dorsal scapular region (midline). Drug will be injected with a sterile 21 – 23 gauge needle. Each injection volume will not exceed 1 ml. Injections will be performed by appropriately trained staff listed in this protocol under section VI.

V.4.4.1.1. Pharmaceutical Grade Drugs:

Currently (b)(4) is in Phase 1 clinical trials for safety and toxicity. (b)(4) (b)(4) for administration in healthy volunteers. We will be using the same formulation in this study.

(b)(4)

(b)(4) All aspects of the solution preparation involve GLP grade formulation.

V.4.4.1.2. Non-Pharmaceutical Grade Drugs: None

V.4.4.2. Biosamples

Blood withdrawals will be carried out according to the schedule presented above. Blood will be collected from a peripheral vessel rather than a femoral vein with a 21 - 23 gauge sterile needle attached to a needle holder, to reduce the chances of bleeding after blood collection in irradiated animals.

The area for the blood draw (samples for bacterial culture) will be swabbed with providone-iodine twice before inserting the needle. Providone-iodine will be swabbed off to reduce irritation to the tissue. For samples collected for other tests, only alcohol swab will be used. The blood sample will be drawn as described above. While withdrawing needle, pressure will be applied at the same time with a sterile bandage to reduce bleeding from the blood draw site. While making regular routine observation of animals, the blood-draw site will be checked for bleeding. In the event the site becomes infected, topical triple antibiotics (bacitracin, neomycin, and polymyxin) or similar agent will be applied.

BM samples will be collected as stated above under section V.4.3.2

V.4.4.3. Adjuvants:

No adjuvant will be used.

V.4.4.4. Monoclonal Antibody (MAb) Production: N/A

V.4.4.5. Animal Identification

Animals arrive tattooed and such tattoo will be used for identification. Additionally cage tags are used for identification.

V.4.4.6. Behavioral Studies:

No behavioral studies will be performed in this protocol.

V.4.4.7. Other Procedures:

Oral administration of (b)(4)

Animals will be sedated with 5 - 15 mg/kg ketamine im (needle 25 G). We will use either the stomach tube or nasogastric tube to administer (b)(4) Before drug administration, the length of the tube will be measured from mouth or tip of the nose up to the last rib. The tube will be lubricated before passing through the nose or the oral cavity. Appropriate size (diameter) tube will be used based on the animal's nostril diameter or trachea diameter. After drug administration, the tube will be flushed with normal saline to make sure the drug reaches to the stomach. Then the tube will be pulled out and animals will be held in vertical position until fully recovered from sedation. Then the animal will be transferred to home cage and any sign of vomiting will be monitored. If there is any discomfort to the animal, veterinarian will be called. This procedure will be conducted under close supervision/guidance of the veterinarian.

Drug volume will be well within the limit stated in Diehl's *et. al.* guidelines (max 15 ml/kg) (4). Currently drug is available at 50 mg/ml concentration (pharmaceutical grade). For 4 kg animal, at a dose of 30 mg/kg, volume will be 2.4 ml

Transport plan

The sedated animals in positioning aide devices will be transported inside a cart to the High Level Gamma Radiation (HLGR) facility. At no time will the animals be removed from the restraint boxes while outside of the VSD vivarium. Two personnel will accompany the animals with additional drugs as needed for chemical restraint (animals are not awake during irradiation), and provide animal handling support.

After irradiation, the NHPs in their restraint boxes will be removed from the platform, returned to the transport cart and returned to the vivarium.

(b)(4) will be administered orally to NHPs in experiment 3.

V.4.4.8. Tissue Sharing:

BM/PBMC samples will be sent to Dr (b)(6) (b)(6) for Nanoscale Immuno Assay (NIA) for validation of biomarkers. Tissue sharing form will be completed before sending sample to above collaborators.

Unused biological samples will be retained for additional studies. Studies planned will depend upon additional funding for supplies and staff in future.

V.4.4.9. Animal By-Products

No animal by-products will be used in the proposed study.

V.4.5. Study Endpoint

In biomarker study without radiation exposure (first phase of experiment 1), blood and BM samples will be collected at predetermined time points as stated above. No mortality or morbidity is expected in this study. BM collection should not be a painful procedure since we are using pain medication. There may be mortality or morbidity in biomarker study with radiation exposure (second phase of experiment 1) depending on dose of radiation used. These animals will be taken care as described below for efficacy study (experiment 1 and 2).

The endpoint used in the efficacy studies (experiment 2 and 3) is mortality. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress. Euthanasia will be carried out when one or more of the following signs and symptoms of moribundity are observed as determined by or in consultation with AFRRI veterinarians. Once platelet count is <30x10^3/µl, PM checks ((~5-6 PM) will begin and continue until solid evidence of recovery in consultation with AFRRI VSD veterinarian.Necropsy and histopathological analysis will be performed on all euthanized animals by the board certified veterinary pathologist. Following parameters will provide the guidelines for moribundity (all signs will be taken together along with the recommendation of a VSD veterinarian for euthanasia decision):

1. Weight loss: loss of more than 20% body weight over a 3-day period.

2. Inappetance: complete anorexia for 2 days with deteriorating conditions based on clinical examination

3. Weakness/inability to obtain feed or water: Inability or extreme reluctance to stand which persists for 1 h.

4. Minimal or absence of response to stimuli (animal does not move when prodded).

5. Core body temperature: Below 96.6ºF following a period of febrile neutropenia (such as >103 ^{0}F and <500 neutrophils/μl).

6. Severe acute anemia: <13% hematocrit or <40 g/L hemoglobin (decision in consultation with AFRRI veterinarians).

7. Severe thrombocytopenia (<10,000 platelets/μl) (decision in consultation with AFRRI veterinarians)

8. Other signs of severe organ system dysfunction with a poor prognosis as determined by a veterinarian:

8.1 Respiratory: any dyspnea or severe cyanosis.
8.2 Gastrointestinal: sustained vomiting or diarrhea, obstruction, intussusceptions; peritonitis. NOTE: Transient vomiting and diarrhea are expected results of whole-body gamma irradiation. Therefore, as criteria for euthanasia, these symptoms should be severe and life-threatening per se, i.e., hemorrhagic diarrhea to the point of causing anemia as listed above or severe uncompensated dehydration.
8.4. Urogenital: renal failure
8.3 Nervous: sustained CNS depression, seizures, paralysis of one or more extremities.
8.4 Integumentary: Non-healing wounds, repeated self-trauma, severe skin infections, indicating severe organ system dysfunction with a poor prognosis.

The actual irradiation procedure does not cause pain or distress. Animal care and use procedures will only be performed by trained and certified personnel. Secondary endpoints will be incidence of bacteremia, standard hematology measurements in peripheral blood (numbers of neutrophils, platelets, erythrocytes, lymphocytes, mean corpuscular hemoglobin, etc.), body temperature, body weight, and occurrence of bloody stools. Body weights will be determined at least once prior to treatment, on the first day of treatment, and at the time of blood collection. Body temperature (implanted chip) will be determined daily between 10 am and 12 noon beginning approximately one week prior to the first treatment. The time of each temperature collection will be recorded for each NHP.

Surviving animals at the end of the study (60 d post-irradiation) will be euthanized for gross necropsy and tissue collection as described under section V.4.6. Histopathology of various organs will be conducted to study the effects of radiation and drug.

V.4.6. Euthanasia

Animals will be humanely euthanized using American Veterinary Medical Association (AVMA) guidelines. These animals will be given sodium pentobarbital

intravenously (100 mg/kg). Prior to pentobarbital administration, animal will be sedated using Ketamine (5-15 mg/kg intramuscularly).The animals will be euthanized only under the guidance and supervision of a staff veterinarian. After sodium pentobarbital administration, the animals will be examined (heart auscultation and pulse) to confirm death. A full body necropsy and histopathology analysis will be performed by a Board Certified Veterinary pathologist, after confirmation of the death.

V.5. Veterinary Care

V.5.1. Husbandry Considerations

Special care will be given to irradiated animals as their natural immunity will be suppressed (more prone to infections). Irradiation boxes will be sanitized after every use or more often as needed.

V.5.1.1. Study Room:

As assigned by VSD.

V.5.1.2. Special Husbandry Provisions: None

V.5.1.3. Exceptions: None

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care

All animals will be quarantined for 45 days prior to any experiment. As per Standard Operating Procedures, VSD staff will monitor all animals at least twice daily during morning and afternoon rounds. Any abnormalities will be reported immediately to the on-call veterinarian and PI. In addition, animals will be observed daily by this protocol's research staff. Any sick animal will be observed twice a day (early morning and late afternoon/night) by the research staff. When animals become ill or debilitated, a veterinarian will be contacted immediately to assess the animal and provide adequate/emergency care. If the animal becomes moribund, it will be euthanized via intravenous administration of pentobarbital sodium at a dose of 100 mg/kg body weight under the guidance of the veterinarian. Prior to pentobarbital administration, animal will be sedated using Ketamine (5-15 mg/kg intramuscularly) (needle 25 G).

V.5.2.2. Emergency Veterinary Medical Care

The on-call veterinary office will be available via 24 h a day.

Euthanasia will be carried out according to pre-defined criteria as determined by AFRRI veterinarians (see criteria in section V.4.5, and also section V.4.6 for details).

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

Food will be withheld for about 12 h prior to sedation. Citrus fruits will be restricted to animals under experiment # 2 and 3 as citrulline will be measured in experiment 2 and 3.

V.5.3.2. Enrichment Restrictions:

None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

Following employees are trained for NHP work. They have handled NHP work of earlier protocols at AFRRI.

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1,3,4,5,7 for mice 7, NHP (off-h observation)	(b)(6)
				1,3,4,5 mice 1,3,4,5, mice 1,3,4,5, mice 1,3,4,5, mice 1,7, NHP (off-h observation)	
				1,3,4,5,7, mice 1,3b,3d, 4,5,7, NHP (off-h observation)	
				1,3,4,5,7, mice 1,3b,3d, 4,5,7, NHP (off-h observation)	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY

There is no plan to use any potential biohazard. Standard laboratory safety precautions will be observed throughout this study

VIII. ENCLOSURES

Form 310

List of references

X. ASSURANCES

Protocol Title: Biomarker and efficacy studies of (b)(4) in Rhesus macaques (*Macaca mulatta)*

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard/Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures/manipulations/observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM** / **AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)	_____	_____
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

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DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (March 2013)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

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* ***Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.***

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This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	1/8/2014	2nd Resubmission Date:	2/14/2014
Approved/**Returned for Revision**:	2/4/2014	**Approved**/Approval Withheld by IACUC:	2/19/2014
1st Resubmission Date:	2/5/2014	**EXPIRATION DATE:**	2/18/2017
Approved/**Returned for Revision**:	2/12/2014	Previous Protocol Number *(if related)*	2010-12-019
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6) **(revised 2-5-2014)**

III. PROTOCOL TITLE: Radiation Countermeasure Screening for Protection and Mitigation in the lab Mouse, *Mus musculus*.

IV. PRINCIPAL INVESTIGATOR:

(b)(6) PhD,
Research Biologist, SRD, AFRRI.
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date

Head, Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACVPM, DACLAM** Date

Head, Veterinary Sciences Department
Armed Forces Radiobiology Research Institute
(b)(6)
Office: (b)(6)
Fax: (b)(6)
Email: (b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department, USUHS
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**

IACUC Chair (b)(6) Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: Radiation Countermeasure Screening for Protection and Mitigation in the lab Mouse, *Mus musculus*.

PRINCIPAL INVESTIGATOR:
(b)(6) PhD,
Research Biologist,
AFRRI
Phone (b)(6) Fax (b)(6)

CO-INVESTIGATOR(S): (b)(6) Ph.D.
Research Scientist, AFRRI, Bethesda, MD 20889
Phone: (b)(6) Fax: (b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBERS: (b)(6)
(b)(6)

I. NON-TECHNICAL SYNOPSIS

The first objective of the protocol is to provide, in the event of radiological/nuclear accident, an extra level of biological protection to first responders including military personnel, fire fighters, and security personnel. The second objective is to identify therapeutic agents to treat civilian population who may be accidently exposed to ionizing radiation. Acute total body irradiation (TBI) at a moderate dose induces extensive apoptotic/mitotic death of actively dividing progenitors wherein the hematopoietic system is identified as among the most highly susceptible tissues to radiation injury. Profound and persistent hematological syndromes develop as a result of death and ablation of the blood forming elements in bone marrow resulting in severe neutropenia (loss of circulating neutrophils) and thrombocytopenia (loss of platelets), thereby increasing the risk of death due to opportunistic infections and/or hemorrhage.

The (b)(4) AFRRI interagency agreement (IAA) has been established to evaluate several agents @ AFRRI as radiation countermeasure in a mouse model for potential post exposure countermeasure treatment against nuclear threats to the civilian population. The Department of Defense (DoD) is interested in providing additional protection to the military and first responders in harm's way to a nuclear incident and pre-exposure countermeasure (the prophylaxis) study arm addresses this issue and is supported by the AFRRI Intramural funds.

A standard protocol developed at AFRRI will be used to evaluate 10 potential countermeasures per year for efficacy in reducing radiation mortality. Depending upon these screening tests, some drugs will be short listed for further detailed investigations to include a. optimization studies (drug route, concentration, multiple administrations and time of drug administration), b. Dose reduction factors (DRF) studies (testing efficacy of promising candidates using various radiation doses), and c. hematological and mechanistic studies (to elucidate drug response).

Due to the inherent variations in radiation sensitivities arising from genetic differences, we will test promising radiation medical countermeasures (MCM) in rodent strains

representative of most sensitive (C3H/HeN) to radioresistant (B6C3F1) strains. The C57BL/6 strain represents the wild type, moderately radioresistant model most commonly used for screening radiation MCMs. The current protocol will continue our efforts to study efficacy of promising MCM in different mouse strains.

II. BACKGROUND

II.1. Background Acute Radiation Syndrome (ARS) represents the clinical response of key radiation-sensitive tissues following whole body or extended partial body exposure to high doses of ionizing radiation. New drugs and new therapeutic strategies are still required to cope with the high morbidity/mortality of ARS. The Acute Radiation Syndrome represents a dose-dependent graduate response of key tissues of decreasing radiosensitivity and the clinical continuum of hematopoietic (>2 Gy TBI), gastrointestinal (>10Gy) then neurovascular (>30 Gy) syndromes (Stone *et al* 2004). The (b)(4) is charged with implementing research to identify, characterize and develop medical countermeasures (MCM) against injuries caused by radiological/nuclear attack. A robust research and development program, initiated in 2005, supports the development of many MCM to treat radiation injury as well as development of diagnostic/radiation dose triage tools. (b)(4) partnered with AFRRI via an interagency agreement (IAA) to screen agents in a mouse model with potential for radiation MCM efficacy dating back to 2006. This protocol represents the continuation of the (b)(4) AFRRI radiation countermeasure screening program under protocol (b)(6) due to expire in February, 2014.

We have established a standard system for evaluating MCM for radiation countermeasure efficacy at AFRRI using the CD2F1 mouse model. This screening program was instrumental in identifying several promising MCM, some which have advanced to studies in higher mammals (b)(4) The screening program is a conduit for low through-put screening of drugs. Other AFRRI investigators select promising drugs from this pool for product development in specific areas such as mixed field irradiation, mechanistic studies, and in combined injury models.

Funding sources: PI requests the inclusion of 3 site numbers (b)(6) (b)(6) in the protocol since they are all related to successful evaluation of potential radiation countermeasures.

The major thrust of the protocol will be supported by the (b)(4) AFRRI interagency agreement (IAA). The emphasis will be the therapeutic/mitigation (drug administered after irradiation) modality. Each of the agents being screened were selected based on technical input from biotechnology companies, and scientific interactions between (b)(4) and AFRRI scientists. Several criteria, such as cytoprotective effects, radical scavenging activity, hematopoietic growth factor activity, and colony stimulating activity that rescue or replenish hematopoietic cells and other radiation sensitive organs including GI, are considered in selection. (Herodin *et al* 2007). The funding for this will be under site number (b)(6)

In addition, promising candidates from the previous years that need additional studies (for e.g. optimization) are funded by both (b)(6) (b)(4) and AFRRI (b)(6) Further, prophylaxis arm of the drugs being screened as well as some of the mechanistic studies will be supported by (b)(6) and PI requests that these funding lines be conveyed in a single protocol to maximize animal use. Strain comparison studies to be conducted in the present proposal will either be supported by (b)(6) depending on the experimental conditions (prophylaxis vs. mitigation).

Table 7 in section V.3.4. "Number of animals required (by Species)" provides a breakdown of animal numbers per site number.

Classification of drugs (for the study):
The new drugs included in the current protocol are subject to legal requirements of confidentiality and material transfer agreement (moderated (b)(4) (b)(4). As of this submission, several compounds are undergoing legal due process. Once approved, they will be added to the current protocol by an amendment. Further, since the (b)(4) AFRRI contract is valid up to 2018, the process of testing new drugs will continue in the next Fiscal year (2015) under this protocol.

New drugs: (b)(4) (b)(4) (b)(4) (Table 3 in section V.1.2 details route and schedule of administration and Table 9 in section V.4.4.1.summarizes physical characteristics and proposed drug doses).

Optimization studies: (b)(4) a drug that showed mitigation efficacy in earlier screening requires additional optimization. Other candidates from the current list may also require optimization depending on the preliminary screening study.

Strain comparison studies: (b)(4) and **Filgrastim** are two drugs to be evaluated in different mouse strains (Table 4 in section V.1.2)

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched BRD, NIHRePORTER, PUBMED , Embase

II.2.2. Date of Search 11/25/2013 to 12/10/2013

II.2.3. Period of Search The search period extended from 1998 to 2005 for BRD, 2009-present for NIHReporter, 1970 to present for PubMed, 2006 to present for Embase.

II.2.4. Key Words of Search Each individual term (b)(4) (b)(4)

(b)(4)

II.2.5. Results of Search

Literature search in PubMed and Embase resulted in >**5000** hits; the relevant references have been used for background information and cited under bibliography. There were **no hits** under **BRD**. These were the results using NIHRePORTER:

(b)(4),(b)(6)

The studies proposed in the current amendment are not a duplication of any of the above-mentioned studies.

Radiation: 64730 hits
Radiation+countermeasures: 290 hits
Radiation+mitigation: 267 hits

Table 1 provides a summary of these searches.

Table 1: Summary of relevant studies on radiation countermeasures (NIHRePORTER)

Project Title	Project Number	Contact PI / Project Leader
RADIATION INJURY TO THE HEART PARATHYROID HORMONE IN PREVENTION AND MITIGATION OF THROMBOCYTOPENIA	(b)(6)	

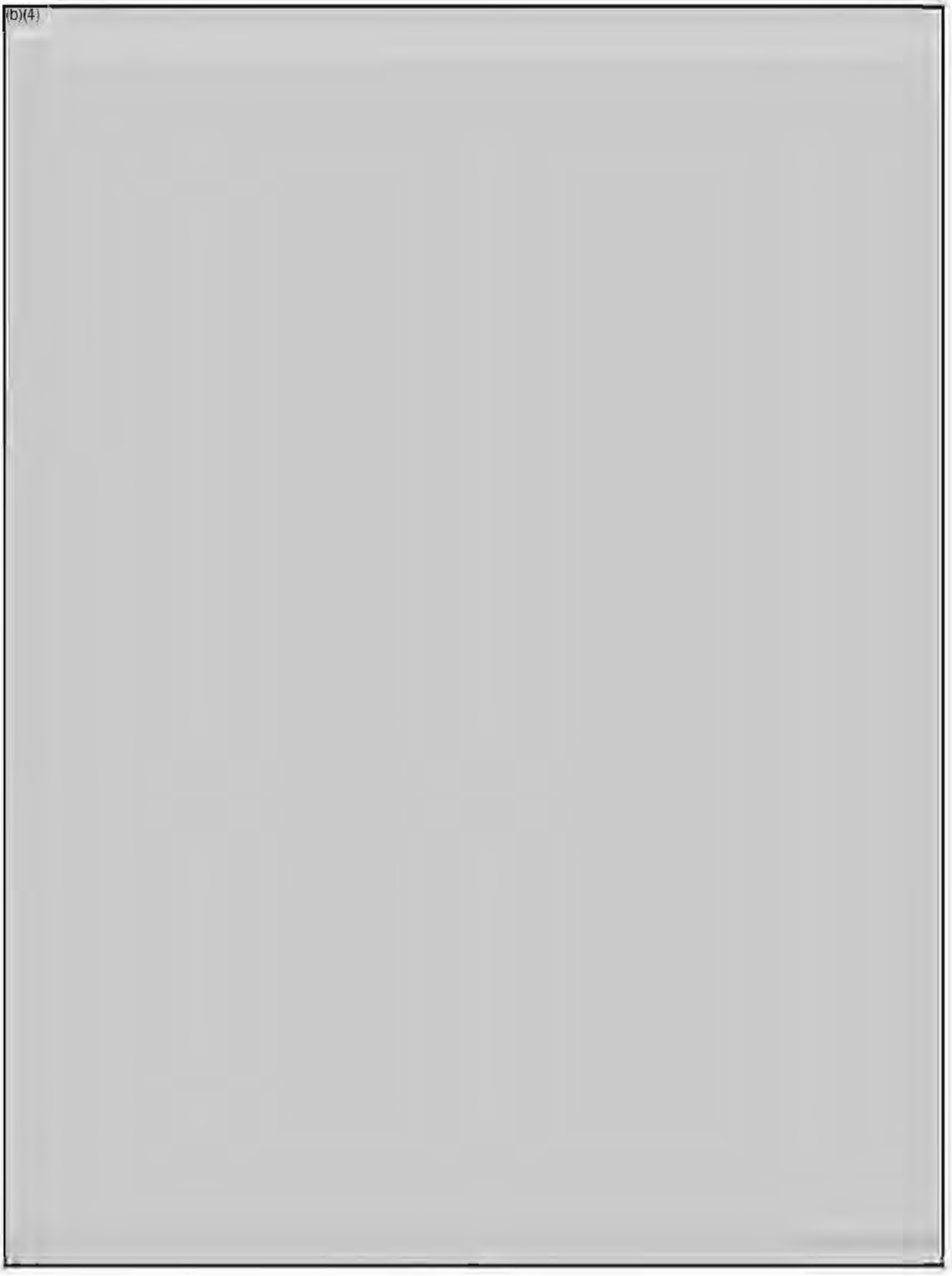
CENTERS FOR MEDICAL COUNTERMEASURES AGAINST RADIATION MOLECULAR-TARGETED RADIATION THERAPY LONG-ACTING G-CSF ANALOGS FOR TREATING ACUTE RADIATION SYNDROME GM-CSF ANALOGS FOR TREATING ACUTE RADIATION SYNDROME LONG-ACTING IL-11 ANALOG FOR TREATING ACUTE RADIATION SYNDROME PILOT PROJECTS NOVEL CUTANEOUS RADIATION INJURY COUNTERMEASURES NOVEL CUTANEOUS RADIATION INJURY COUNTERMEASURES COMBINED RADIATION AND TRAUMATIC INJURY AFFECT HIPPOCAMPAL STRUCTURE AND FUNCTION MITIGATION AND MODELING OF RADIATION EFFECTS IN THE CONTEXT OF MULTI-ORGAN/MODEL IMPROVING GASTROINTESTINAL RECOVERY AFTER RADIATION SOMATOSTATIN ANALOGS AS COUNTERMEASURES AGAINST INTESTINAL RADIATION TOXICITY PHARMACOLOGY AND DRUG DEVELOPMENT CORE NOVEL AGENTS FOR MITIGATION OF RADIATION-INDUCED PULMONARY INJURY ORAL RADIATION NUCLEAR DECORPORATION AGENTS MITIGATING CUTANEOUS RADIATION INJURY WITH CXCR4 ANTAGONIST MECHANISMS OF LATE EFFECTS OF EXPOSURE TO RADIATION SKIN & IMMUNOLOGY - MITIGATION OF RADIATION AND COMBINED INJURY TO THE SKIN RADIATION NUCLEAR COUNTERMEASURES PRODUCT DEVELOPMENT SUPPORT SERVICES CONTRACT DEVELOPMENT OF ACE INHIBITOR AS COUNTERMEASURE FOR RADIATION-INDUCED LUNG INJURY DEVELOPMENT OF ACE INHIBITOR AS COUNTERMEASURE FOR RADIATION-INDUCED LUNG INJURY EVALUATION OF NEUROPROTECTIVE EFFECTS OF AEOL10150 AGAINST CHEMICAL THREAT AGENTS STUDIES OF POPULATIONS EXPOSED TO ENVIRONMENTAL SOURCES OF RADIATION SELECTION OF AN ANTI-CERAMIDE MITIGATOR OF ACUTE RADIATION TOXICITY OF THE GI TRA IMMUNOSENESCENCE STUDIES OF ACUTE RADIATION EXPOSURE IN ATOMIC BOMB SURVIVORS EVALUATING RADIATION MEDICAL COUNTERMEASURES IL-10 COMBINED THERAPY FOR RADIATION COMBINED INJURY SELECTION OF AN ANTI-CERAMIDE MITIGATOR OF ACUTE RADIATION TOXICITY OF THE GI TRA IMMUNOSENESCENCE STUDIES OF ACUTE RADIATION EXPOSURE IN ATOMIC BOMB SURVIVORS EVALUATING RADIATION MEDICAL COUNTERMEASURES	(b)(6)

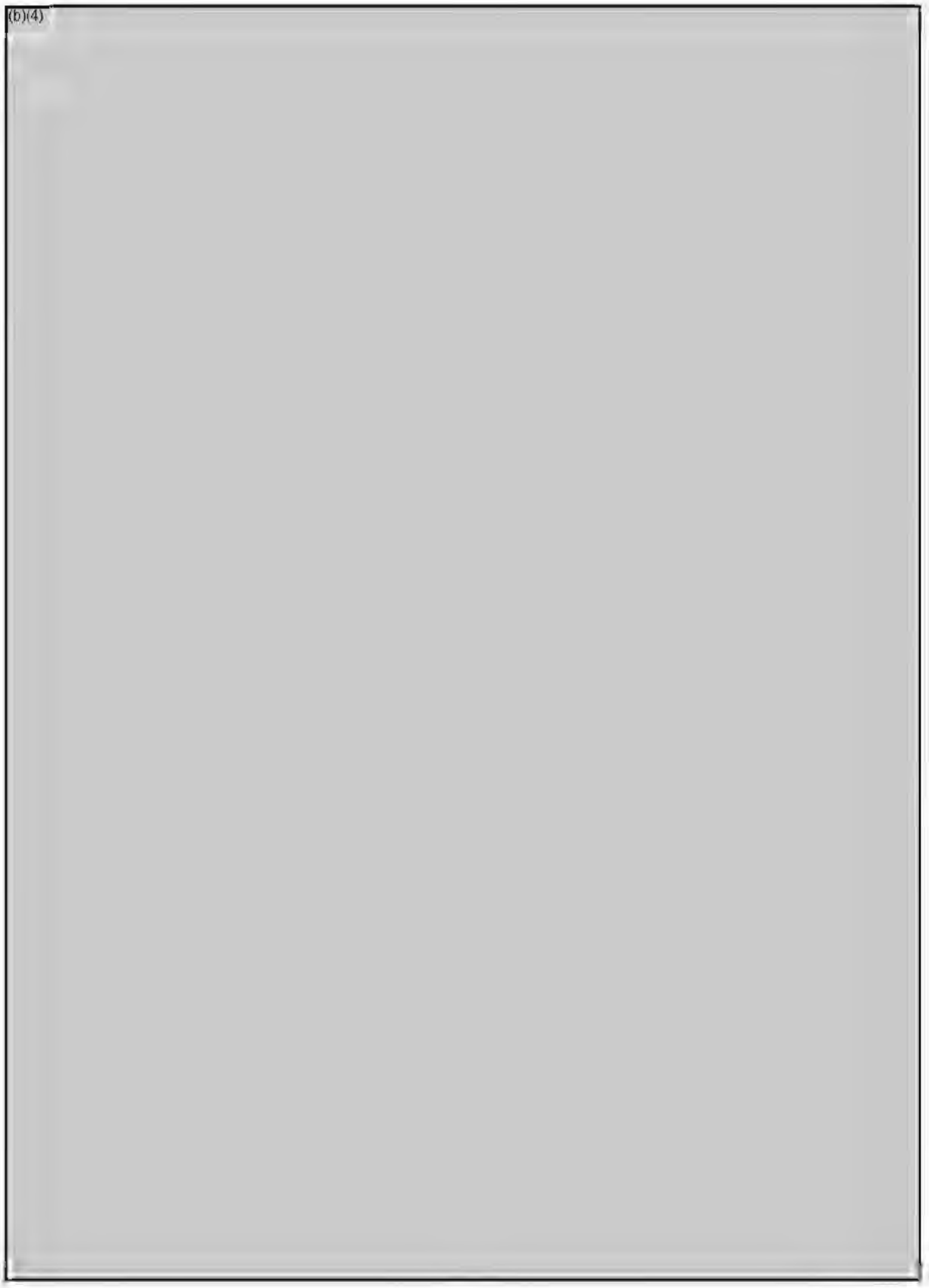
IL-10 COMBINED THERAPY FOR RADIATION COMBINED INJURY RADIATION PROTECTION WITH SOD MIMETICS UNIVERSITY OF ROCHESTER CENTER FOR MEDICAL COUNTERMEASURES AGAINST RADIATION BIOACTIVE PEPTIDES FOR MITIGATION OF AGS AND ACUTE RADIATION INJURY ROLE OF P38 MAPK IN HSC SELF-RENEWAL AND RADIATION-INDUCED BONE MARROW INJURY RADIATION-INDUCED LYMPHOID AND HEMATOPOIETIC TOXICITY	(b)(6)

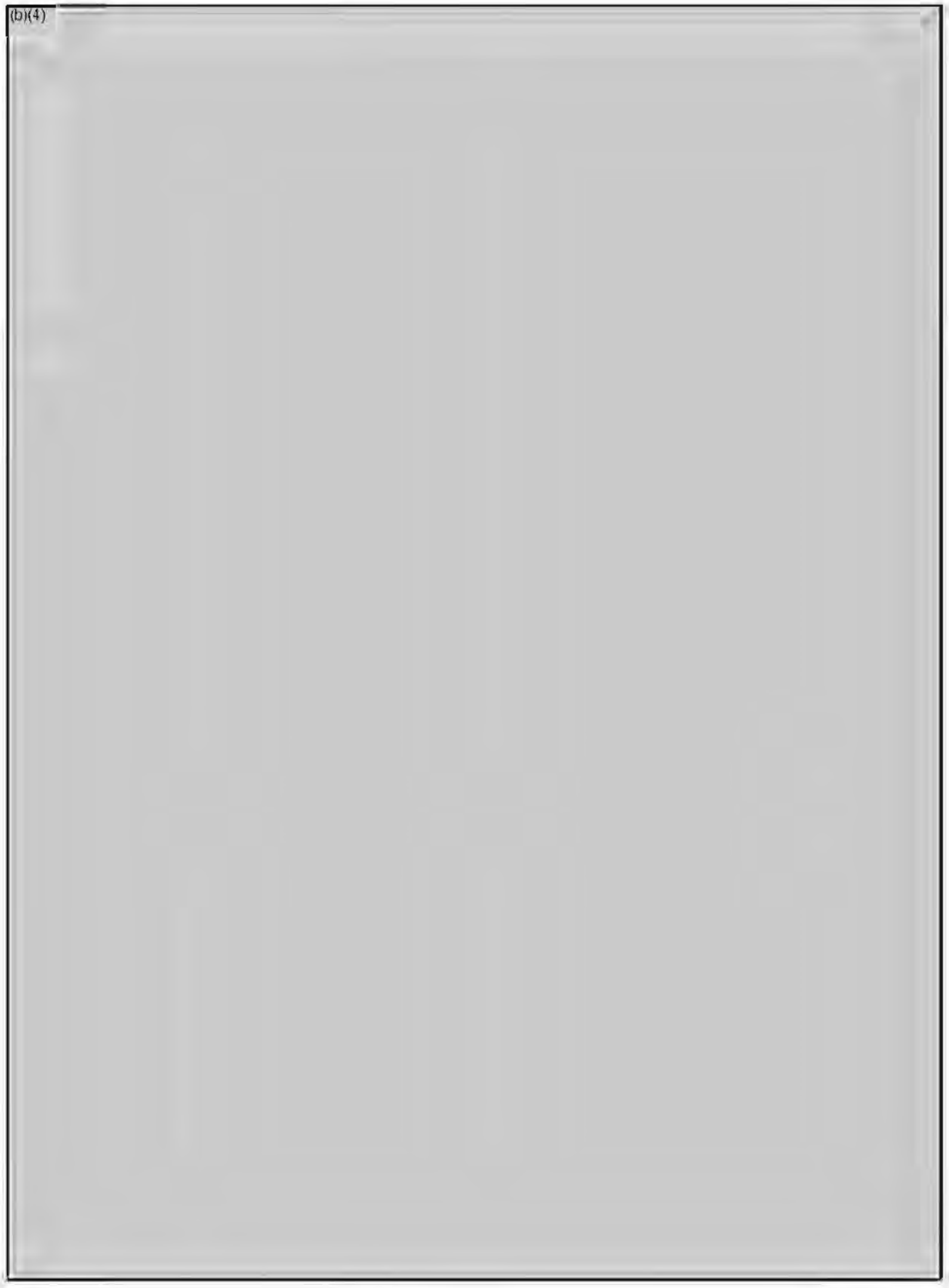
However, none of the studies are duplicated here.

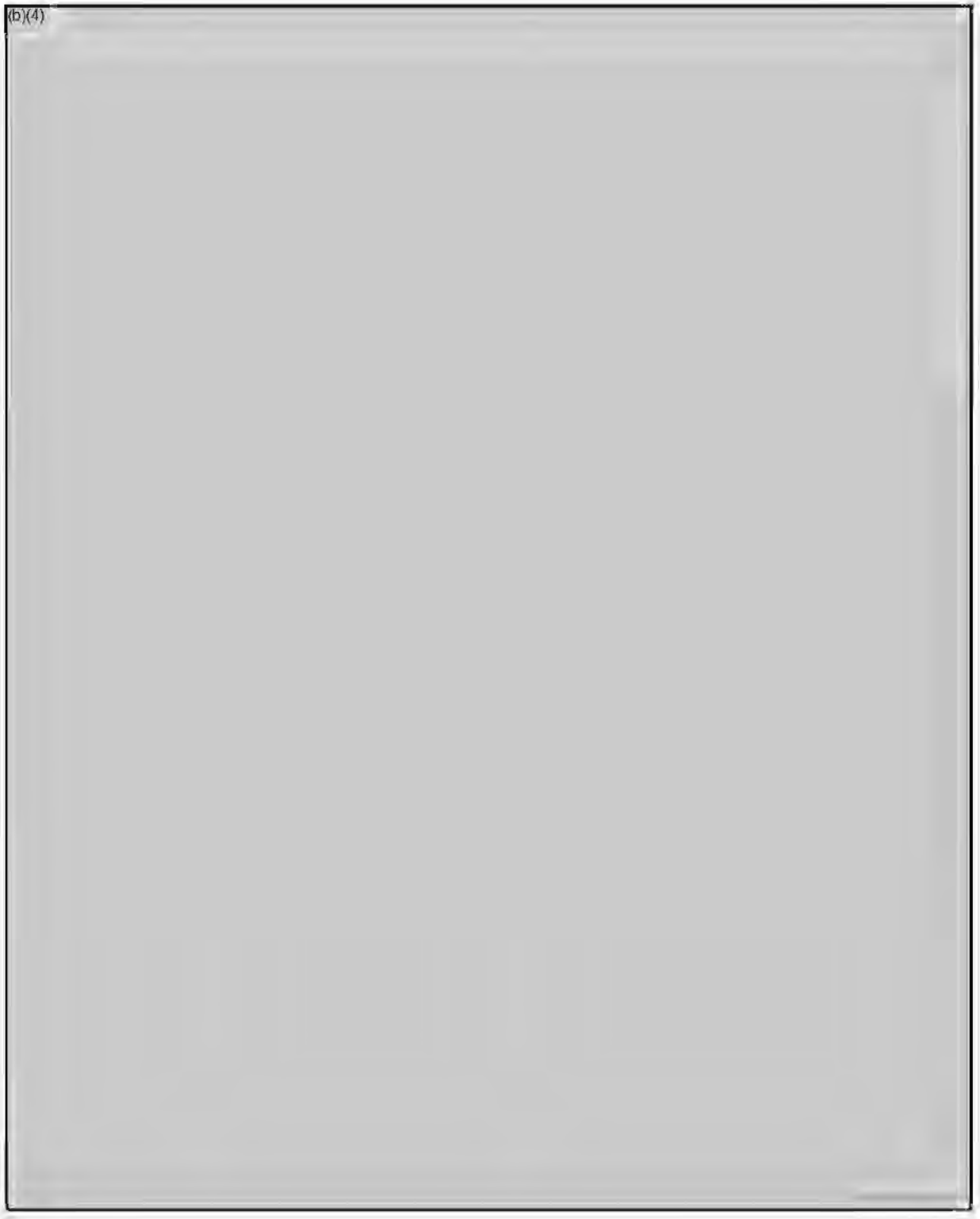
Background information on drugs included in the protocol

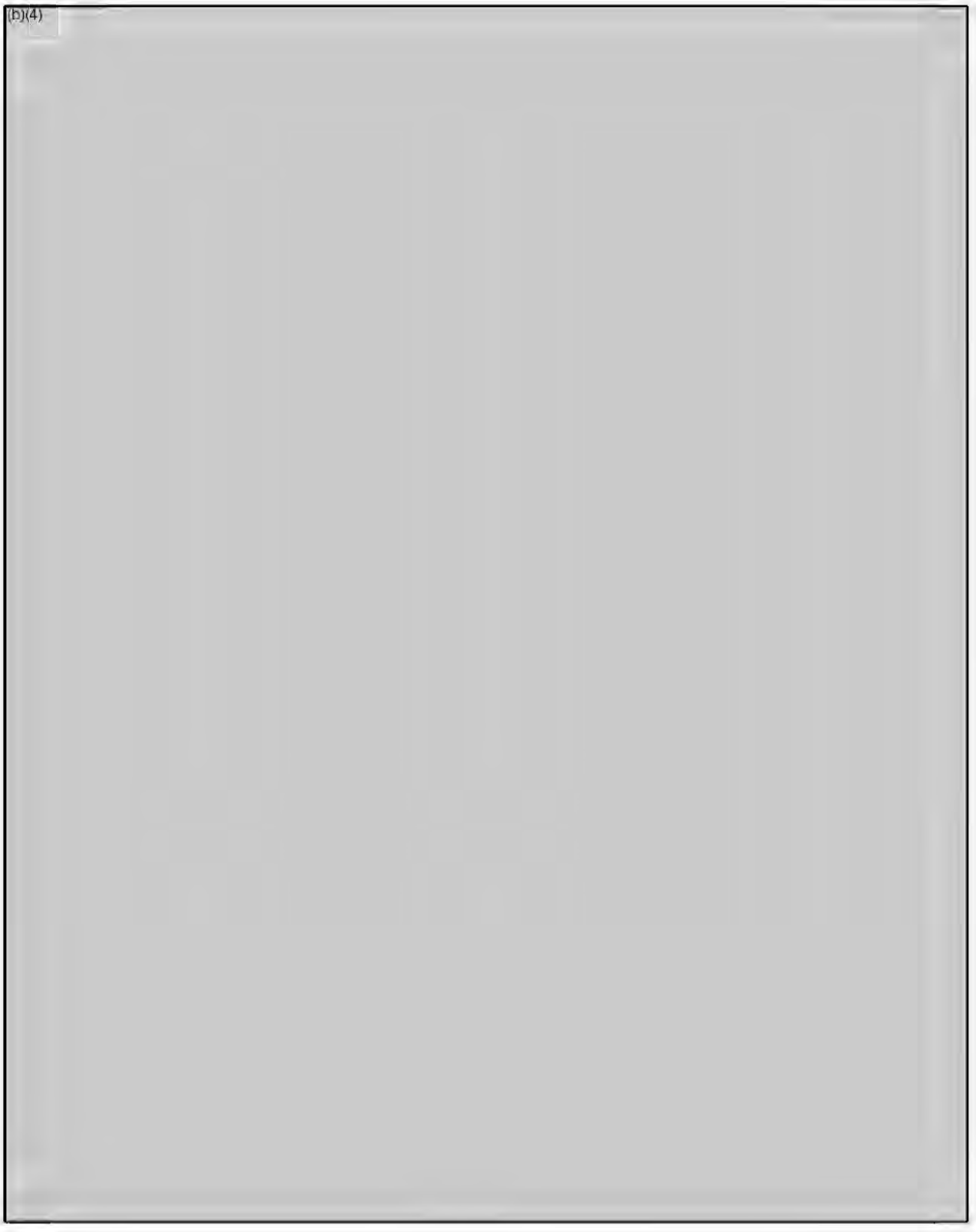
(b)(4)


(b)(4)


(b)(4)


(b)(4)


(b)(4)


(b)(4)

(b)(4)

Filgrastim: G-CSF is a hematopoietic cytokine that increases granulocyte regeneration in radiation-induced myelosuppressed animals (McVittie *et al* 1990, Patchen *et al* 1990). The most relevant study in literature is by Patchen *et al* (1991), where 2.5 µg/mouse G-CSF in saline administered from 1-16 d following TBI increased the survival benefit (DRF=1.06) (Figure 6; from Patchen *et al* 1991).

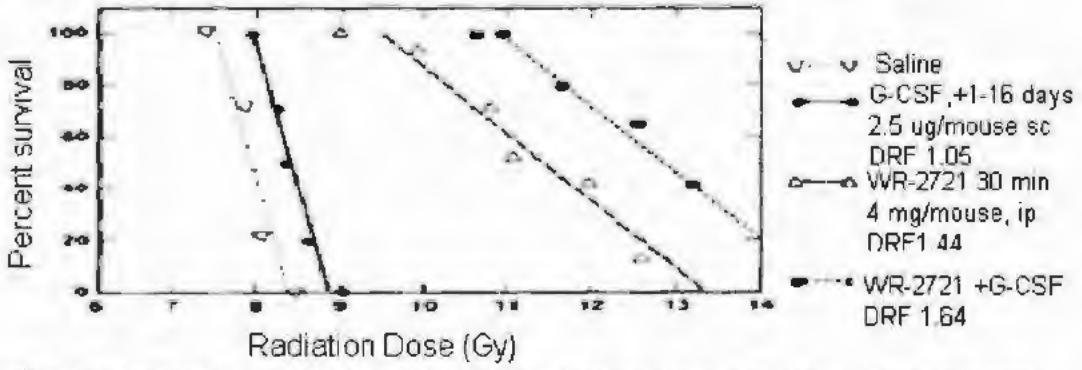


Figure 6 Effect of saline, G-CSF, WR-2721, and WR-2721+G-CSF treatments on survival of mice. C3H/HeN mice were administered WR-2721 4 mg/mouse, ip) 30 min before Cobalt 60 gamma irradiation and G-CSF (2.5 ug/mouse sc) on days 1-16 after irradiation. each data point represents results obtained from 30 mice. Patchen et al 1991, IJROBP, 22: 773-779

The proposed study differs from the reported one in that we will use the pharmaceutical grade Filgrastim in the recommended vehicle, i.e. dextrose. We will use the optimal drug dose schedule of 0.17 mg/kg Filgrastim administered 4 h, 1 and 2 d or 1-3 d post-TBI in the strains.

III. OBJECTIVE / HYPOTHESIS We hypothesize that drugs screened in the current protocol will lead to identifying promising radiation countermeasures in mice that can be advanced to efficacy studies in large animal model, and ultimately for human use in the event of a large scale nuclear casualty.

Due to genetic variations in individuals, the radiation response to the drugs will also differ. We will test any promising radiation MCM in three other strains (C3H/HeN, C57BL/6 and B6C3F1) for differences in survival.

IV. MILITARY RELEVANCE The current lack of radiation protectants (pre-TBI) and mitigators (post-TBI) that are safe, effective and approved for use in victims of an accidental or deliberate radiation exposure constitutes a major problem for emergency preparedness. In the event of a nuclear related accident or terrorist activity, first responders will be comprised of the HAZMAT team, military, fire and police departments and experts from the Radiological Assistance Program; potentially they will all be at risk for exposure. Death is certain at doses of 10 Gy and above while exposure to 2-8 Gy results in severe hemaptopoietic ablation, GI injury and ensuing infectious diseases. All triage procedure instructs first responders and other health care providers to provide treatment, including antibiotic administration and blood product support and supportive care. In a mass casualty scenario, there will be significant delays in transport, disruption of communication and overfilling of limited hospital resources. At these times, the availability of safe and effective countermeasures is a valid alternative for medical management of patients. The agents proposed for testing in the current protocol are derived from various backgrounds, with abilities such as scavenging of deleterious radicals, increase in survival, proliferation and differentiation of hematopoietic progenitors, antiapoptotic, enhancement of microbicidal activity, management of radiation-induced bone marrow aplasia and immunmodulators. The current AFRRI protocol meets the goals of the Department of Defense (DoD) mission in protecting the armed personnel and the Human Health Services (HHS) interest of treating civilian population.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

Section V.1.1.

The ODDS method for determining safe dose (modified up and down procedure [UDP]): Previously, we have used the modified up and down procedure at AFRRI for selection of a safe dose for radiation studies. This was derived from the original UDP for establishing LD50 toxicity by oral route. The UDP is an in vivo acute systemic toxicity test adopted by the US regulatory agencies to **reduce and refine** animal use (OEDC Test Guidelines 425) which have replaced the conventional acute toxicity test method. According to these guidelines, testing can be carried out using **6 animals**, the animals can be of the **same sex** (usually females, but males can be used when justified) and dosing is escalated in **fixed-time interval** (48 h) survival outcomes of all the animals up to that time. This is especially applicable when there is very little information available to make a preliminary estimate of the LD50 dose of the agent and the slope of the dose-response curve. In such a case, starting doses near 175 mg/kg and using half-log units (dose progression of factor 3.2) will increase efficiency of animal use and increases accuracy in prediction of the LD50 dose.

The AFRRI approach is similar to the UDP test, with a few variations. We use 6 animals/group/dose and stagger the doses over 48 h intervals. Only males are used, since all our radiation studies are carried out in male mice. After dosing, the animals

are observed continuously for 1 h, and 4 h and then daily for 14 days using the observation codes listed (Table 2). After the initial 48 h observations, if no changes in body weight, behavior, activity, temperature and irritation at injection site, or mortality is observed, the drug dose is escalated 3 times (a half log unit, note: a change of 3.2 times theoretically equals half log unit). If any adverse effect is noted (weight loss, decreased or increased activity, death), the dose is reduced to 3 times the tested dose. Drug dose for radiation countermeasure studies is 1/4th the non-toxic dose. However, when there is sufficient background information about the drug or indication of effective dose, a single dose four times higher than the dose to be used in the radiation screening study will be tested as a reduction and refinement strategy. Given that 1 or 2 drugs out of 10 may pass the initial screening for radiation, this is a vastly preferable procedure to the conventional toxicity study using 2 genders of mice and a much larger number.

Table 2:	**OBSERVATION CODES**		
Normal	**N**	Head	**LH1**
		Ears	**LH2**
Dyspnea	**A1**	Ear-Right	**LH3**
Tachypnea	**A2**	Ear-Left	**LH4**
		Eyes	**LH5**
Hyperthermia	**B1**	Eye-Right	**LH6**
Hypothermia	**B2**	Eye-Left	**LH7**
		Mouth	**LH8**
Edema (L)	**C1**	Nose	**LH9**
Irritation (L)	**C2**		
Erythema (L)	**C3**	Dorsal	**LD1**
Alopecia (L)	**C4**	Dorsal-Cervical	**LD2**
Piloerection (L)	**C5**	Dorsal-Lumbar	**LD3**
Necrosis (L)	**C6**	Dorsal-Sacral	**LD4**
Cyst (L)	**C7**		
Abscess (L)	**C8**	Lateral-Right	**LS1**
		Lateral-Left	**LS2**
Ataxia	**D1**		
Convulsions	**D2**	Ventral	**LV1**
Tremors	**D3**	Ventral-Thoracic	**LV2**
Prostrate	**D4**	Ventral-Abdominal	**LV3**
Catalepsy	**D5**	Ventral-Inguinal	**LV4**
		Ventral-Inguinal-Right	**LV5**
Lachrymation (L)	**E1**	Ventral-Inguinal-Left	**LV6**
Exophthalmos (L)	**E2**	Ventral-Axillary-Right	**LV7**
Ptosis (L)	**E3**	Ventral-Axillary-Left	**LV8**
Diarrhea	**E4**		
Diuresis	**E5**	Limbs	**LL1**
Rhinorhea	**E6**	Limb-Fore-Right	**LL2**
		Limb-Fore-Left	**LL3**
Paralysis (L)	**F1**	Limb-Hind-Right	**LL4**
Circling	**F2**	Limb-Hind-Left	**LL5**
Languid	**F3**	Paws	**LL6**
Hunched	**F4**	Paw-Fore-Right	**LL7**

Thin	**F5**	Paw-Fore-Left	**LL8**
Head Tilt	**F6**	Paw-Hind-Right	**LL9**
Lame (L)	**F7**	Paw-Hind-Left	**LL10**
Rough Coat	**F8**	Tail	**LL11**

Procedure: There will be six animals per group housed three per box. Each mouse will be individually monitored for weight changes by standard ear punch/notch system. The punches or holes correspond to a predetermined numbering code for individual animals. Here, in a group of three, we will have the right ear punch, left ear punch and no punch to distinguish between each animal. In case of 5 animals, we will have right and left punch, right and left notch, and a no punch/notch to identify individual animals. The procedure is described under section G (surgical methods or procedures).

(b)(4)

different routes (b)(4) *was previously tested in CD2F1 mice* (b)(4),(b)(6) (b)(4) *and no mice are requested in this protocol to evaluate the* (b)(4) Similarly, (b)(4) and filgrastim will not be tested in this section.

(b)(4)

6 groups (1 naïve, 1 vehicle, 4 doses) x 1 route (*sc*) x 6 mice per group x 6 drugs
Subtotal = **216 CD2F1 mice**

(b)(4) will be tested via the oral route, using oral gavage.

6 groups (1 naïve, 1 vehicle, 4 doses) x 1 route (*po*) x 6 mice per group x 4 drug
Subtotal = **144 CD2F1 mice**

Intravenous route: (b)(4) will be tested via this route.

6 groups (1 naïve, 1 vehicle, 4 doses) x 1 route (*iv*) x 6 mice per group x 1 drug
Subtotal = **36 CD2F1 mice**

Intraperitoneal route: (b)(4) will be tested via this route.

6 groups (1 naïve, 1 vehicle, 4 doses) x 1 route (*ip*) x 6 mice per group x 1 drug
Subtotal = **36 CD2F1 mice**

Section V.1.2.
Radiation survival study:

1) We will use the LD-70 radiation dose, a dose that results in 70% (±20%) mortality within 30 days following exposure.

2) There are three major arms to the survival study: one prophylaxis (24 h pre-TBI), and two mitigation (post-irradiation) arms, viz. 4 h and 24 h post-TBI.

3) Survival of ≥30% over vehicle is considered the 'pass criteria'.

4) Only those countermeasures that meet the AFRRI pass criteria in CD2F1 mice will be evaluated in C3H/HeN, C57BL/6 and B6C3F1 strains.

The efficacy of each drug in enhancing survival of lethally irradiated CD2F1 mice will be tested in both the prophylactic and therapeutic regimen (-24 h, +4 h or +24 h after exposure [0 h]) to LD-70 radiation) as described below. Numbers of mice include two iterations.

For each drug, the animal requirement was calculated as follows:

Drug + vehicle (2 groups) x 3 regimen (one pre- and two post-TBI) x 1 rad dose (LD-70/30) x 1 route (*sc*, *po*, or *iv*) x 24 CD2F1 mice per group = **144 mice/drug**. (Table 3)

Positive controls

Vehicle + 5-AED control (2 groups) x 1 regimen (-24 h) x 1 radiation dose x 1 route (*sc*) x 10 mice per group x 7 iterations Subtotal = **140 CD2F1 mice**

Iterations

Assuming five of the listed agents achieve ~30% increase in survival over vehicle, the study will be repeated for concordance.

Drug+vehicle (2 groups) x 3 regimen (-24 h, +4 or +24 h) x 1 radiation dose x 1 route (*sc*, *po*, *iv*, or *ip*) x 24 mice per group x 5 drugs Subtotal = **720 CD2F1 mice**

Table 3: Preliminary Radiation Survival Studies in CD2F1 (LD-70dose)

List of agents selected FY13 countermeasure program						
Drug	**Vehicle**	**Route**	**Administration time point(s)**			**# of animals**
			Prophylactic	**Therapeutic**		
(b)(4)	PBS	*po*	-4, 3, 2, 1 d	+4 h, 1- 3 d	+1- 4 d	144
		sc	-1 d	+4 h, 2 d	+1, 2 d	
	PBS	*po*	-4, 3, 2, 1 d	+4 h, 1- 3 d	+1- 4 d	144
	PBS	*sc*	-1 d	+4 h	+1 d	144
	PBS	*sc*	-1 d	+4 h	+1 d	144
	PBS	*sc*	-1 d	+4 h	+1 d	144
	Saline	*sc*	-3, 2, 1 d	+4 h, 1, 2 d	+1, 2, 3 d	144
	Saline	*sc*	-1 d	+4 h	+1 d	144
		po	-4, 3, 2, 1 d	+4 h, 1- 3 d	+1- 4 d	

(b)(4)		*po*	-4, 3, 2, 1 d	+4 h, 1- 3 d	+1- 4 d	144
	PBS	*iv*	-1 d	+4 h	+1 d	
	PBS	*ip*	-1 d	+4 h	+1 d	144

Sub-Total CD2F1 mice=1296

MCM efficacy of lead countermeasures in different strains

There are inherent differences in response to total/partial body ionizing radiation arising from genetic differences in the same species. Certain drugs that improve survival following TBI in one mouse strain will not necessarily show similar efficacy in another strain. An ideal countermeasure should be able effective across strain variations. To this end, (b)(4) directive is to test the gold standard, Filgrastim, in several strains. Similarly, AFRRI intramural funding supports development of (b)(4)

Table 4: Countermeasure efficacy in mouse strains: C3H/HeN, C57BL/6 and B6C3F1

Drug	Vehicle	Route	Pre-TBI	Post-TBI	Post-TBI	#animals
(b)(4)	PBS	*sc*	-1 d	+4 h	+1 d	144
Filgrastim*	5% dextrose	*sc*	-1 d	+4 h, 1, 2 d	+1, 2, 3 d	144
*These two agents showed significant mitigation in CD2F1 mice previously- hence no CD2F1 mice will be required.						

Number of mice required for V.1.2. (Table 5)

Strain	C3H/HeN	C57BL/6	B6C3F1	CD2F1
# of mice needed	288	288	288	0

Section V.1.3. Optimization studies: Optimization studies will be initiated in CD2F1 mice only if any of the drugs tested meets the AFRRI criteria of >30% survival than vehicle in the preliminary radiation survival screen. Assuming that three of the eleven drugs meet the AFRRI criteria, we request animals to carry out optimization studies and DRF for three drugs.

Drug dose optimization study: 6 groups (Five drug doses + 1 vehicle) x 1 radiation dose (LD-70) x 2 regimen (pre- and post-exposure) x 1 route (*sc*, *iv*, or *po*) x 3 drugs x 24 animals per group Subtotal = **864 CD2F1 mice**

Time optimization study: 4 different time points x 1 optimum dose + 1 vehicle (2 groups) x 1 radiation dose (LD-70) x 1 route (*sc*, *iv*, or *po*) x 3 drugs x 24 animals per group Subtotal = **576 CD2F1 mice**

Section V.1.4. Dose reduction factor study (DRF):

The dose reduction factor (DRF) studies will use the optimum drug dose and times to evaluate the effectiveness of the drug against a range of radiation dose in CD2F1 mice.

6 irradiated vehicle groups (8.5, 9, 9.5, 9.75, 10 and 10.5 Gy, 0.6 Gy/min)* + 6 irradiated drug groups (8.5, 9, 9.5, 10, 10.5, and 11 Gy)* x 24 mice per group x 3 drugs. Subtotal = **864 CD2F1 mice**

*These radiation doses are projected based on an earlier probit study and are considered a guidance. The appropriate radiation doses will be determined using the new dosimetry map (02/28/2013)

Section V.1.5. Hematology, clinical chemistry, and bone marrow studies .These studies are anticipated only for those candidates that demonstrate 30% increase in survival. Data from these studies will form the basis of understanding the mode of action of the drug,
Naïve + vehicle + drug alone (3 groups) x 7 time-points x 10 mice per group x 1 route of administration x 2 drugs **Subtotal = 420 CD2F1 mice**

2 experimental groups (vehicle-irradiated, Drug-irradiated) X 7 time-points X 10 mice per group x 1 route of administration X 2 radiation doses x 2 drugs **= 560 CD2F1 mice**

V.2. Data Analysis All data will be reported as means with standards errors or percentages where applicable. Experiment V.1.1 (ODDS) –probit analysis will be used to generate LD_{50} of the drugs under study using PASW Statistics (version 18). V.1.2 (preliminary survival study) and V.1.3 (Optimization study)- Using calculations based on log rank test with 80% power and 5% two-sided significance level, we have determined that this protocol design has adequate power for screening purposes. The standard for declaring a test promising is a 30 percent increase in survival over vehicle from a radiation dose producing approximately 70 percent mortality (±10%) in the vehicle group. With N of 24 for the vehicle and treated groups this has a one-tailed p value of approximately 0.001 and with N of 20 this has a one-tailed p value of 0.005. This low p value is required, rather than the usual 0.05 for significance, which would use fewer mice, due to sources of intrinsic variability in the biological system that cause departure from the underlying assumption of independence, e.g. occasional cage effects where most or all the mice co-housed in one cage (4 mice per cage) die due to infection. Therefore, further reduction of N below 24 is not feasible. Experiment V.1.4. (Dose Reduction Factor study)- In past experience, 20 animals per dose with six radiation doses has been sufficient to determine a probit line fit to mortality data for DRF calculations. At least three out of the six doses should sustain survival other than 100 or 0 percent. Finney's methods will be adhered to in making probit fits and estimating DRF.
Experiment V.1.5: For testing the overall difference between groups (averaging over time points), a repeated measures ANOVA with 6 animals per group measured at 7 time points will have 80% power to detect a difference of 0.91 standard deviations if the within-subject correlation is 0.2. For comparisons between groups at a single time point, the proposed sample size will have 80% power to detect a difference of 1.8 standard deviations between groups, based on a t test for independent samples with a 5%, two-sided significance level. Previous studies have yielded differences of this magnitude.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered Although high throughput screening and computer modeling have advanced significantly in recent years in identification of promising drugs, it would be simplistic to assume that cell systems or artificial intelligence modeling can replace the data that can be generated by using an animal model. Irradiation of animals will result in a cascade of changes including death of stem and progenitor cells in bone marrow, alterations in signal transduction pathways, multiple organ dysfunctions etc. due to differing radiosensitivities of organs. Currently, there are no alternatives in existence that can predict or compute the complexity of the response to total body irradiation. The US FDA recognizes the ethical considerations involved in testing the efficacy of radioprotective drugs in humans. Therefore FDA requires preclinical drug assessments (safety and efficacy) to be performed using both small and large animal models (FDA 2002) prior to granting approval for safety testing in humans.

V.3.2. Animal Model and Species Justification Recent advances in molecular techniques have allowed significant improvements in understanding the cellular and biochemical processes. These advances are possible only due to whole animal modeling of human ailments. The mouse, in particular, has high sequence homology to humans at a genomic level as well as similar hematological and immunological response to radiation. Mouse models not only confirm primary pathological processes but have also provided a venue for studying basic molecular, cellular, biochemical, and cytological processes. Further, there are extensive data on the mouse for comparison, review and analysis. The practicalities of breeding and housing these small mammals have made the mouse model invaluable. The Principal Investigator's group at AFRRI has significant training and experience in handling mice in radiation countermeasure studies.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species: Mouse, *Mus musculus*

V.3.3.2. Strain / Stock: CD2F1, C3H/HeN, B6C3F1 and C57BL/6

V.3.3.3. Source / Vendor: CD2F1, C3H/HeN, B6C3F1from Harlan Inc., VA, and C57BL/6 from Charles River Laboratories or Jackson labs.

V.3.3.4. Age: (procurement) 7-8 weeks

V.3.3.5. Weight (delivery) 25-30 grams

V.3.3.6. Sex Males have been used historically and will continue to be used in this study.

V.3.3.7. Special Considerations Commercially procured animals must be adventitious disease free. Specific pathogen-free, including *Pseudomonas*

aeruginosa, and *Pasteurella*: All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus, *Mycoplasma pulmonis*, and *Clostridium piliforme*. Endoparasite- and ectoparasite-free.

V.3.4. Number of Animals Required (by species) (Table 6)

Animal (genus, species and common names)	**Original**
CD2F1	5872
C57BL/6	288
C3H/HeN	288
B6C3F1	288

Total number of animals in different fund sites (Table 7)

Site number	**C57BL/6**	**C3H/HeN**	**B6C3F1**	**CD2F1**
G1B2DB14	144	144	144	2780
G1B2HF13	0	0	0	1440
RAB2GY14	144	144	144	1652

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement: PI will take necessary steps to all personnel in the project to be rigorously trained in handling techniques (ear-punch, injections, oral gavage, intraperitoneal injections, and routine cage-side observations) to minimize distress and pain to the animals. Specifically, during capture and restraint of the rodents, care will be taken to ensure that handling does not contribute to distress. Topical ointment/lubrication gel will be used, if necessary, to prevent drying of the cornea as well as at the site of drug injection in consultation with attending veterinarian and veterinary staff.

V.3.5.2. Reduction The protocol will use a tiered approach to reduce the number of animals for testing 10 drugs/year. Although basic toxicity and survival will be carried out on all 10 drugs, additional studies will depend on the initial results. Positive controls and negative controls are used to confirm robustness of experimental conditions and drugs that don't meet our minimal criteria of 30% increased survival over the vehicle control will be eliminated. Based on previous experience, we have proposed that only 3 drugs out 10 may be advanced for optimization and dose reduction factor studies (see section V.1.3 and V.1.4). Further, control groups (such as vehicle) will be shared whenever possible between drugs that are dissolved in similar vehicles (for example Phosphate buffered saline-PBS). In classical toxicology testing, groups of 10-20 mice and both sexes were routinely used; in using the ODDS method (current protocol), we reduce the number of animals to 6 per group, and use only males, thereby reducing animal numbers significantly. Five drug doses have been

proposed for toxicity screening; however, in some cases a smaller number (i.e. 3) might be selected based on literature search and recommendation by the manufacturers. Similarly, several routes of drug administration have been contemplated in the experimental design; but depending on the existing data and company suggestions, a single and optimal route may be chosen. Wherever possible we have reduced the number of repeats planned for the experiments shown above.

V.3.5.3. Replacement It is not feasible to use non-animal systems in place of actual animal models to address the questions in this project. Rodents are considered sentient and hence rodents will be used for screening large number of potential radiation countermeasures. Only those drugs that pass the AFRRI criteria for significant survival benefit will advance to studies in higher mammals.

V.4. Technical Methods
V.4.1. Pain / Distress Assessment
V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals 6736

V.4.1.1.1.1. Column C 0 (# of animals)

V.4.1.1.1.2. Column D 852 (# of animals) These mice have the potential to be sickand die due to drug administration (toxicity study, #432) or will have blood collected via cardiac stick or the inferior vena cava.

V.4.1.1.1.3. Column E 5884 (# of animals). These mice will be irradiated and will not receive any intervention for pain relief. However, they will be euthanized if found moribund.

V.4.1.1.1.4. Pain Category Assignments: (Table 8)

Exp. #	Experimental/Control Group	C	D	E	Totals
V.1.1.	ODDS safety/toxicity study		432		432
V.1.2.	Radiation survival studies			3020	3020
V.1.3.	Optimization studies			1440	1440
V.1.4.	Dose reduction factor studies			864	864
V.1.5.	Hematology, clinical chemistry and bone marrow studies		420	560	980
Totals:			852	5884	6736

V.4.1.2. Pain Relief / Prevention Minimizing and alleviating pain in laboratory animals without compromising the methodological integrity of a research project is important both ethically and legally. Pain in animals is difficult to access, mostly due to lack of methods that can validate and objectively measure it.

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization The protocol will not use anesthetics or analgesics during radiation since they will adversely affect the outcome of the experiments. The proposed study is to determine the effects of radiation and

countermeasures on the function of the immune and blood forming systems; use of any analgesics and anesthetics would interfere with the function of the immune system and therefore compromise the interpretation of the results. It is anticipated that at high radiation doses, animals will demonstrate varying degrees of morbidity and moribund, which makes it critical to define the criteria for euthanizing the animals without undermining the study objective. Euthanasia will be performed by trained personnel in accordance to the current AVMA guidelines (AMVA Guidelines on Euthanasia, 2013). In toxicity studies, if animals show discomfort (hunching, decreased activity, inappetence, separation from cage-mates), that animal will be under careful observation on consultation with the attending VSD veterinarian. If the animal becomes moribund (defined in V.4.5.), it will be euthanized. Antibiotics will be applied if wounding occurs via in-cage fighting, under the guidance of the clinical veterinarian in accordance with standard treatment measures.
Anesthesia using standard Isoflurane apparatus under the guidance of the VSD will be carried out in mice receiving intravenous injections and for blood collection. All personnel will be extensively trained for efficient administration of the agents as well as to minimize handling stress.
Animals will be placed in the Isoflurane chamber and delivered a metered amount of 5% Isoflurane mixed with 100% oxygen until all voluntary motor movement ceases and the animal is recumbent for 10 seconds. Animal's respiration rate will be closely monitored through the Plexiglas chamber during induction of anesthesia. When animals are recumbent, they will be removed from the chamber and their nose/mouth placed in the anesthesia apparatus breathing/gas administration cone for maintenance of anesthesia at 3% Isoflurane and 100% O_2.

V.4.1.2.2. Pre- and Post-procedural Provisions Mice will be housed under standard conditions both before and after radiation procedures in accordance with current VSD rodent husbandry Standard Operating Procedures (see section V.5).

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched AGRICOLA, PubMed, EMBASE

V.4.1.3.2. Date of Search 12/10/2013

V.4.1.3.3. Period of Search AGRICOLA: 1975-2004, EMBASE 1974-present PubMed~1950 to present.

V.4.1.3.4. Key Words of Search Radiation and pain, [Filgrastim, (b)(4) (b)(4) and pain, and [drug name]+toxicity, anesthesia, analgesia, distress, humane.
In order to increase specificity of literature review, several combinations such as radiation plus specific drug, mice plus specific drug, radiation plus specific drug plus mice were evaluated.

V.4.1.3.5. Results of Search There are no alternatives to irradiation. While radiation itself does not cause pain or distress, it induces a number of changes in the body that alters immune response and destroys hematological tissues, resulting in opportunistic infections, and multiorgan dysfunction leading to death. It is anticipated that animals exposed to high lethal radiation doses will become moribund. Unfortunately, literature clearly demonstrates that use of analgesics alters radiation response and therefore leads to increase in animal number for better statistical results or for iteration of entire experiments. The nonsteriodal analgesic (b)(4) is reported to decrease the effect of radiation therapy in head and neck cancer cells (Czemirek *et al* 2009). Ginseng, with known analgesic properties, is a potent radioprotector (Lee *et al* 2005).
No references were found suggesting distress due to the drugs, though some drugs are well documented as having side effects associated with the pathophysiology of biological response to the drug. For instance, (b)(4) increases white blood cells and platelets, as does (b)(4) Filgrastim is documented as causing bone pain, but is well tolerated in human volunteers. (b)(4) is reported to cause feelings of discomfort, lower back pain, decreased appetite and diarrhea.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: Because exposure to irradiation is potentially lethal or cause debilitating effects in humans, it is ethically impermissible to test the effectiveness of radiation countermeasures on human volunteers. Irradiation compromises the immune and blood forming systems leading to mortality. The radiation-induced mortality and potential percentage increase of survivors over 30 days using potential radiation countermeasures are study endpoints for this protocol. Administration of drugs and analgesics is not an option since it is well documented that they interact with the immune system which will confuse the interpretation (Galley *et al*, 1997, 1998). Pain relieving measures are not used because such measures may compromise the experimental integrity of the study.

V.4.2. Prolonged Restraint : The experimental animals will receive radiation at a dose rate 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (dosimetry 02/28/2013;or the latest dosimetry map) using plastic racks. Mice will be inserted into the standard radiation boxes just prior to irradiation (~15 min of exposure time) and taken to the cobalt facility for irradiation. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period. They will be monitored daily (twice a day when necessary) for 30 days and euthanized at the completion of the observational period.

V.4.3. Surgery: N/A

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure(s) : N/A

V.4.3.3. Post-surgical Provisions : N/A

V.4.3.4. Location : N/A

V.4.3.5. Surgeon : N/A

V.4.3.6. Multiple Major Survival Operative Procedures : N/A

V.4.4. **Animal Manipulations:**
V.4.4.1 Ear punch: Three animals/group will be housed per shoe box during toxicity study (section V.1.1.). They will be identified by ear punch (one animal will have a right punch, the second will have a left punch and the third will have no punch).

Procedure for ear-punch

a. Soak the ear punch (Fine Science Tools, Cat# 24212) in a disinfectant (100% ethanol) before use and between animals.
b. Place the device on the pinna of the ear (external ear) where the skin is thin avoiding the cartilage.

Press firmly to punch a circular hole through the ear

For the oral route, drug(s) will be administered using a 20 G gavage needle (Fisherbrand-Animal feeding needles, disposable-sterile, non-toxic and nonpyrogenic-20 G, 1 ½" length). Non-disposable, metal ball ended, oral gavage needles are included as an alternative to the disposable needles. Drug volume will be 0.2 ml (with a maximum of 0.5 ml). The animal will be restrained in a vertical position to align the spine straight. The bulb will be introduced into the mouth horizontally. Using the needle as lever, the syringe will be moved into a vertical position and the needle dropped down the esophagus into the stomach. The needle contents will be administered when the needle hub touches the animal's mouth. All precautions will be taken to ensure that the placement of the needle is properly positioned and that the animal is not struggling severely. No forceful feeding will be done to prevent perforation of the esophagus.

Intravenous injections: Mice will be anesthetized as described earlier, in the Isoflurane units. Once deeply unconscious (tail or toe pinch), the animals will be moved to a station with nose cone for continued anesthesia. The tail will be cleaned with alcohol and the test agent injected to the tail vein using a 25 or 27 G needle. Mice will be returned to their cages and monitored until they recover before being placed in their assigned rooms. Heat packs, warm platform, or heat lamps may be employed during anesthesia to prevent hypothermia.

Irradiation: These will follow the standard AFRRI protocol with mice placed in restrainers shortly prior to exposure, irradiation at the cobalt facility and return to VSD for further monitoring. Briefly, the experimental animals will receive radiation at a dose rate 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (dosimetry 02/28/2013) using plastic racks. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period. They will be monitored daily

(twice a day when necessary) for 30 days and euthanized at the completion of the observational period. In case of non-lethal exposures, mice will be harvested at specific time-points after irradiation, and tissues collected after terminal blood draw.

Blood collection: Mice will be anesthetized as described under section V.4.1.2.1 (anesthesia). Blood draw for hematology is collected either by cardiac stick or from the inferior vena cava (IVC). The animals will be deeply anesthetized in the rodent anesthesia machine; the tail will be pinched for reflexive movement, indicative of insufficient anesthesia. If there is no response, the animal will be moved to the station with individual nose cone for continued anesthesia. An incision will be made on the right side below the abdomen of the animal, closest to the IVC, the vein exposed and blood drawn with a 23 G needle. Similarly, cardiac stick will be performed on the animal positioned at the platform under anesthesia. In both cases, the animals will be immediately euthanized on completion of blood draw by cervical dislocation.

V.4.4.2. Injections: The vehicles for the different compounds depends upon solubility and include sterile water for injection, phosphate buffered saline, polyethylene glycol-400 (PEG-400). Based on literature data, PEG-400 is associated with limited radioprotective property and hence will be administered as a control in a given study. Because there is a drift in $LD_{70/30}$ over time each experiment requires an internal vehicle group from the same batch of mice. Subcutaneous injections will be given with either 23 or 25 G needle to the nape of the neck of 0.1 ml (maximum volume 1 ml for 25 to 38 g animals). Intravenous injections to tail vein will use a 25 or 27 G needle in a volume of 0.1 ml per mouse (maximum volume-0.2 ml for 25 to 38 g animals).Intraperitoneal injections will be given with either a 23 or 25 G needle in a volume of 0.25 ml (maximum volume 1 ml for 25 to 38 g animals). Disposable sterile needle will be used for all *sc* and *iv* injections. One needle will be used per cage containing generally 4 animals. The purpose of the table shown below is to provide available information for the drugs to be tested. The information in the table should be considered only as guidance. It should be noted that the information on several drugs is incomplete since material transfer agreements (MTA) are not in place and the companies have not shared information with us. Once MTAs are in place, the PI will request additional information on the drug including details of preferred vehicle, pH, osmolarity, endotoxin level, schedules, and the route of injection, from the collaborator/manufacturer (if purchased in the open market or through a pharmaceutical company [pharmaceutical grade]). It is expected that the osmolarity will not be more than AFRRI IACUC's recommended levels (Policy#9). Further, ODDS method clearly states that the PI will decide on the drug dose based on limited toxicity studies unless otherwise provided by the collaborator/drug manufacturer. The PI will initiate ODDS method using a safe dose (using the data provided in the Table shown below) or a dose recommended by the collaborator in order to obtain the best drug dose for survival studies.

Table 9: Physical characteristics and vehicles for the countermeasure agents

Drug	Vehicle	Projected Dosage*	pH range	Endotoxin levels	Grade
(b)(4)	PEG-400	30 mg/kg	6-8	Not done**	N-PG
	PBS	2000 µg/kg	7	0.014 EU/ml	N-PG

(b)(4)	PBS	50 mg/kg	¥	<0.0025 EU/mg	N-PG
	Saline	150 mg/kg	¥	Not done**	N-PG
	PBS	100-250 µg/kg	7-7.4	Not done**	N-PG
	PBS	350-750 µg/Kg	¥	Not done**	N-PG
	PBS	100 µg/kg	¥	Not done**	N-PG
	PBS	180 mg/kg	6	<0.05 EN/ml	N-PG
	PBS	TBD	¥	Not done**	PG
	Saline	250 mg/kg	¥	Not done**	N-PG
	PBS	2.5 mg/kg	¥	Not done**	N-PG
	PBS	1-8 mg/kg	7.4	<0.001 EU/mg	N-PG
Filgrastim	5% dextrose	0.17 mg/kg	5.3	N/A	PG

*These doses should be considered only as guidance. Actual drug doses will be determined by PI based on additional information made available from collaborators/manufacturers and/or by ODDS test. PG=pharmaceutical grade; N-PG=non pharmaceutical grade drug

¥ To be evaluated.

**Endotoxin testing for all drugs will be completed prior to any animal testing at AFRRI and a report submitted for IACUC/VSD review and approval. The endotoxin tests are conducted by Charles River Laboratories. At the same time, pH will be tested to ensure that it falls in the range (6-8 for *sc* administration) stipulated by IACUC.

However, if there are significant changes in the protocol including drug dose or route of administration, the PI will initiate an IACUC amendment prior to evaluating that specific drug.

V.4.4.1.1. Pharmaceutical Grade Drugs We will use pharmaceutical grade Filgrastim, 5% Dextrose, and (b)(4) All attempts will be made to obtain pharmaceutical or veterinarian grade drug(s) for the protocol. However, most drugs listed here are of research grade.

V.4.4.1.2. Non-Pharmaceutical Grade Drugs Some of the drugs (eg. (b)(4) (b)(4) under study will require a carrier such as poly ethylene glycol (PEG 400). All drugs will be tested for endotoxin levels if this information is not supplied by the manufacturers. Physical characterization, including osmolality and pH of final solutions will be in the range stipulated by IACUC by its policy letter on testing non-pharmaceutical grade drugs (policy #9). We do not anticipate filtering the test agents due to the limited availability of drugs, and uncertainty as to how much of the drug will be filtered out. However, we will ensure that drug preparation is carried out under hygienic conditions (use of PPE, clean surface area for drug preparation, use of clinical grade solvents) to preclude contamination of test samples.

(b)(4) an inhibitor of microtubule polymerization will be injected intraperitoneally (*ip*) into mice (0.5% in saline) 2 h before euthanasia and collection of bone marrow cells for processing chromosomal aberrations in metaphase spreads (b)(4), (b)(6)

V.4.4.2. Biosamples: N/A

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAb) Production : N/A

V.4.4.5. Animal Identification Generally, cage cards will be used to distinguish drug-treated and control groups. Some experiments might necessitate ear punch or notch by personnel to identify individual animals in a cage. Ear punch is performed as described in V.4.4.1).

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures : N/A

V.4.4.8. Tissue Sharing All tissues from the animals used in toxicity study will be preserved for histopathological analysis by the VSD pathologist. The pluck method will be used to harvest all the organs from tongue to rectum, brain, sternum, femur and blood may be collected at euthanasia. Tissue from irradiated mice (brain, GI, spleen, lung, liver, kidney) will be collected for histopathology at intervals.

V.4.4.9. Animal By-Products: N/A

V.4.5. Study Endpoint The time frame for experiment V.1.1 (basic toxicity) will be 14 days after final drug dose administration (for instance, oral gavages are administered once a day for four days and the day of the last dose is considered day 0).
The radiation survival studies will span 30 days after radiation exposure (experiments V.1.2, V.1.3. and V.1.4.). Serial sampling by terminal procedures is projected on 7 different time points after drug or irradiation for experiment V.1.5.

It is anticipated that the animals involved in these studies may succumb to death either due to a drug administration or radiation or a combination of the two. To maintain a suitable baseline for humane treatment of the animals while adhering to the study objective, the IACUC recommendations stipulated in Policy Letter #10 will be implemented for judging morbidity and moribundity. Signs of morbidity in the mouse include difficulty in breathing, ruffling of hair, drying of lachrymal fluid, fall in body temperature, loss of appetite with/ without diarrhea. Morbid animals will proceed in the experimental observation to sustain survival accuracy and to monitor the efficacy of drug intervention. No supportive care is proposed, but the morbid mice will be examined at least twice daily, early morning and late afternoon by the research staff (including weekends and holidays) in addition to the regular VSD health checks.

The moribund condition is defined as a clinically irreversible condition leading inevitably to death. Animals involved in experiments that may lead to moribundity or death will be monitored daily by personnel experienced in recognizing signs of moribundity (illness, injury, or abnormal behavior) for at least the following: abnormal posture, rough hair coat, head tucked into abdomen, exudates around eyes and/ or nose, skin lesions, or abnormal breathing, difficulty with ambulation, cyanosis, decreased food or water intake, or self-mutilation (Toth 1997, AFRRI policy #10).

Animals will be will be immediately euthanized when they display abnormal breathing, are recumbent, or unable to respond to mild external stimulus by the

research staff and scored as dead on removal, while recorded as euthanized in the medical records. VSD veterinarians will be consulted for all matters regarding the animal welfare.

V.4.6. Euthanasia Mice found moribund will be euthanized by CO_2 inhalation plus confirmatory cervical dislocation by investigator/ technician according to the directives of the VSD-SOP and in accordance with current AVMA Guidelines on Euthanasia (AMVA 2013). At the conclusion of the experimental time frame, all animals that survived the procedures of irradiation, drug intervention and controls, will be sacrificed by CO_2 inhalation plus confirmatory cervical dislocation and their carcasses disposed of according to VSD regulations.

V.5. Veterinary Care

V.5.1. Husbandry Considerations The AFRRI vivarium follows stringent protocols for the housing and care of all animals. The PI and designated team members will adhere to all the policy and guidelines set forth by standard operating protocols of the VSD. The specific requirements for mice housing should meet the following criteria of opportunity for social interaction, opportunity to carry out normal behavior and the opportunity to rest and withdraw from each other (AWIC website). Mice are social animals and hence will be caged together in groups of 4 or 8. For ODDS testing, mice will be housed in groups of 3 or 5 mice per cage and identified individually as described. Nestling pads will be provided in each cage during bedding changes. In cage shelters will also be provided as an enrichment strategy at the discretion of the PI depending on experimental conditions. Animals will receive Harlan Teklad Rodent Diet (w) 8604 and water *ad libitum.* Acidified water (pH 2.5 – 3.0) is routinely provided to AFRRI mice used in radiological experiments. Cages will be regularly changed and cleaned by VSD staff in accordance with Standard Operating Procedure. Animals will have 12 h light/dark cycle. Animals will be habituated to their surroundings and routine procedures prior to experimentation. In the event of procedures that last into the night cycle, care will be taken to minimize exposure to light and the quality of light used will be of the diffused, dim type.

V.5.1.1. Study Room As assigned by VSD

V.5.1.2. Special Husbandry Provisions N/A

V.5.1.3. Exceptions Mice will be socially (group) housed as described above, except when there is a possibility that during survival studies cage mates may be lost through attrition and for a period of time the sole surviving mouse may remain singly housed until the end of the 30-day study.

V.5.2. Veterinary Medical Care We do not project routine veterinary medical care. However, in some cases of drug testing, if the mice are anesthetized, we will use a topical lube to prevent the eyes from drying out, on consultation with the VSD. Further, in case of minor injuries arising from fighting, topical application of antibiotics is acceptable with consensus from the PI and VSD.

V.5.2.1. Routine Veterinary Medical Care Experimental animals will be observed daily by either the investigator or technician or both. VSD personnel will monitor all animals during their routine rounds as per VSD SOPs. In the course of the study, as animals approach morbidity, the research staff observations will be increased to a minimum of twice a day, early morning and late afternoon (includes weekends and holidays). There is no supportive therapy contemplated since this would compromise the experimental results (described earlier in the end point and pain category sections).

V.5.2.2. Emergency Veterinary Medical Care Moribund animals (gasping, recumbent, non-responsive to mild stimulus) will be euthanized by trained personnel.

V.5.3. Environmental Enrichment: As provided by the husbandry staff to animals not on active experiments.

V.5.3.1. Enrichment Strategy Nestling pads will be provided in each cage during bedding changes as per AFRRI-VSD SOPs.

V.5.3.2. Enrichment Restrictions

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING (Table 10)

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3, 4b, 5	(b)(6)
				1, 3, 3d, 4a, 4b, 5, 7*	
				1, 3, 4a, 5	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a-*ip*, 3b-*sc*, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-*iv*
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)
*Trained in retro-orbital injection, tail vein injection and tumor implantation.

VII. BIOHAZARDS/SAFETY All personnel working in the protocol have undergone necessary training in handling experimental animals. The amount of drug used will be small and standard safety precautions will be taken while handling them in the laboratory and in the vivarium. All personnel listed in the present protocol have been trained as general radiation users on a regular basis. All investigators and personnel will use appropriate protective gear while in the vivarium as described by the VSD. All listed personnel are current in safety training and medical surveillance.

VIII. ENCLOSURES none
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(b)(4),(b)(6)
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X. ASSURANCES

Protocol Title: Radiation Countermeasure Screening for Protection and Mitigation in the lab Mouse, *Mus musculus*.

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): **I AM** / **AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator (Printed Name) **Principal Investigator (Signature)** **2-5-2014** (Date)

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
8901 WISCONSIN AVENUE, BUILDING 42
BETHESDA, MARYLAND 20889-5603

July 28, 2015

MEMORANDUM FOR: IACUC Administration

SUBJECT: Unexpected outcome of the study (b)(6) amendment# (b)(6)

As part of the (b)(4) countermeasure screening program under IACUC protocol (b)(6) was begun on Tuesday, July 14, 2015 to test synthetic pre-implantation factor (b)(4) as a potential radiation countermeasure. Drug (b)(4) or vehicle was injected twice a day for 14 days as described below (Table 1). 160 animals were included in this study as follows:

Table 1. (b)(6) Design for radiation countermeasure screening with sPIF, sc

Group #	Cage #	Drug	Dose	Volume	Route	Admin (time)
1 a-b	1-2	PEG	-	0.1 ml	sc	-1 d
2 a-b	3-4	5-AED	30 mg/kg	0.1 ml	sc	-1 d
3 a-f	5-10	PBS	-	0.1 ml	sc	-24 h, -12 h, (+0 – 13 d bid)
4 a-f	11-16	(b)(4)	0.75 mg/kg	0.1 ml	sc	-24 h, -12 h, (+0 – 13 d bid)
5 a-f	17-22	PBS	-	0.1 ml	sc	+4 h, (+1-14 d bid)
6 a-f	23-28	(b)(4)	0.75 mg/kg	0.1 ml	sc	+4 h, (+1-14 d bid)
7 a-f	29-34	PBS	-	0.1 ml	sc	+24 h, (+1-14 d bid)
8 a-f	35-40	(b)(4)	0.75 mg/kg	0.1 ml	sc	+24 h, (+1-14 d bid)

Research staff began the dosing schedule on Tuesday, July 14, 2015, according to the above table. Mice were injected with 0.75 mg/kg (b)(4) *sc* at a volume of 0.1 mL per dose every 10-12 hours. Staff performed injections using a one-handed restraint technique, and one needle per cage of four animals was used, as is stated in the (b)(6) protocol. All mice were irradiated on Wednesday, July 15, at AFRRI's cobalt-60 facility at an $LD_{70/30}$ dose of 9.25 Gy at 0.6 Gy/min.

Critical period began on Wednesday, July 22. The first death was observed on Thursday, July 23. On Saturday, July 25, research staff noticed a large, clotted wound on the right side of an animal's abdomen. On the following day, research staff euthanized five mice with clotted wounds on the right side of the body, either on the abdomen or nape of the neck. Three of these mice did not meet criteria for euthanasia as stated in IACUC Policy 10, but research staff decided to euthanize to prevent possible infection in the rodent colony. Research staff also changed any cages where dried blood was observed. A total of six mice with wounds were euthanized at this time; three mice from the PBS vehicle groups and three mice from the (b)(4) groups were euthanized. Please note that the unexpected clinical signs were observed only in groups with multiple injections (in random animals either PBS or (b)(4) treated), but not in the 5-AED or PEG-400 treated groups (injected only once).

An emergency meeting was called with veterinary staff on Monday, July 27. Dr (b)(6) instructed research staff to continue with dosing with increased monitoring of injection technique by a vet tech for

Monday evening's dosing. Prior to this meeting, research staff placed green concern cards on three cages where animals exhibited open wounds. Directly after this meeting, research staff showed these animals to Drs. (b)(6) and photographs were taken for documentation. Two animals that were found dead at this time, one from the PEG-400 vehicle group and one from a (b)(4) group, were taken for histopathology analysis.

Research staff is continuing to monitor surviving animals closely in coordination with veterinary staff. The study will end on Aug 14, 2015.

Sincerely,

(b)(6) PhD
Principal Investigator, SRD

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (October 2012)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	**2/26/2014**	2nd Resubmission Date:	
Approved/**Returned for Revision**:	**3/19/2014**	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	**3/24/2014**	**EXPIRATION DATE:**	**3/30/2017**
Approved/Returned for Revision:	**3/31/2014**	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. **NAME OF FACILITY: Armed Forces Radiobiology Research Institute**

II. **PROTOCOL NUMBER:** (b)(6)

III. **PROTOCOL TITLE:** Minipig model of H-ARS for drug screening protocols relevant to mass casualty-scale treatment (Gottingen minipigs, *Sus scrofa domestica*)

IV. **PRINCIPAL INVESTIGATOR:** (b)(6)

(b)(6) PhD Date
Principal investigator, (b)(6) Scientific Research Department
Tel (b)(6) Fax (b)(6) E-mail: (b)(6)

V. **DEPARTMENT HEAD**: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date
Head, Scientific Research Department, AFRRI
Telephone (b)(6) Fax (b)(6)

(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician

Telephone (b)(6) Fax (b)(6)

(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI

Telephone (b)(6) Fax: (b)(6)

(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department, USUHS

Telephone (b)(6) Fax: (b)(6)

(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____
(Date)

(b)(6) **PhD**

IACUC Chair (b)(6) Scientific Research Department (b)(6)

Telephone (b)(6) Fax: (b)(6)

(b)(6)

PROTOCOL TITLE: Minipig model of H-ARS for drug screening protocols relevant to mass casualty-scale treatment

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6) PhD Date
Principal investigator (b)(6) Scientific Research Department
Tel (b)(6) Fax (b)(6) E-mail: (b)(6)

CO-INVESTIGATOR(S):

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER:

I. NON-TECHNICAL SYNOPSIS

Development of large animal models for drug testing under the U.S. Food and Drug Administration (FDA) Two Animal Rule is still representing the main bottleneck for drug licensure and commercialization. In spite of the many promising drug candidates developed for the Acute Radiation Syndrome (ARS) and proven successful in rodents, only one drug (G-CSF, Neupogen®) has been approved by the FDA for use in nuclear/radiological emergency under an Emergency Use Authorization (EUA). Only three large animal models have been characterized for the ARS: the Non-Human Primate (NHP), the dog, and the minipig (M-P). However, both NHP and the dog have several practical and ethical limitations. High regulatory oversight, low availability, and public opinion sensitivity issues are among the major factors limiting the applicability of these animal models for drug testing. The minipig provides an ideal system for drug testing, of scientific rigor and efficacy equal to other non-rodent models.

We propose to continue to characterize the minipig (M-P) as a less controversial alternative non-rodent model of hematopoietic ARS (H-ARS) for the evaluation, development and licensure of radiation mitigators and therapeutics for use in a mass casualty scenario. To address FDA requirements that test drugs need to be assessed under the same conditions they will most likely be used (i.e. in combination with antibiotics, cytokines, etc), we will establish a probit curve at doses bracketing the hematopoietic syndrome in the presence of minimal supportive care, and we will follow survival for 60 days. In light of the relationship between poor nutritional status, weight loss and poor outcome after radiation treatments, and to align our model with other large animal models, we will provide nutritional supplementation together with minimal supportive care. Our hypothesis is that minimal supportive care and nutritional support will increase the hematopoietic LD50 and reduce the steepness of the slope.

Our approach significantly differs from what had been done by us in the past and from what currently is being done by other centers working with the M-P model. Our (b)(4) project focuses on nutritional support in pediatric animals, and our (b)(4) project evaluates the effect on 30-day survival of gentamicin+amoxicillin, rimadyl, oral hydration (through a watered down preparation of pumpkin or applesauce) and blood products in adult animals. The current protocol uses adult animals and addresses not only minimal medical management but also nutritional supplementation and extended survival time (60-day period), to be in alignment with other large animal models.

II. BACKGROUND

II.1. In case of the Acute Radiation Syndrome (ARS), damage to a specific organ appears to affect proper function of many other organs, and multiple organ failure (MOF) is being recognized as one of the main causes of radiation-induced lethality. Swine display close similarity to humans in terms of overall organ anatomy and physiology and have been extensively used in biomedical research in a variety of areas (cardiovascular, gastrointestinal, metabolic, liver, reproductive and infectious disease). We therefore selected the minipig because of its physiological properties similar to humans across several organs (14), with the expectation that it will more faithfully reproduce the physiopathology of MOF observed in ARS, and will provide an ideal tool for drug efficacy testing for total body irradiation (TBI).

(b)(4) We have taken a step-by-step approach to the development of the minipig as an animal model for drug testing [redacted] and we established a basic model for the hematopoietic Acute Radiation Syndrome (H-ARS) [redacted] in the absence of supportive care and under TBI conditions, and characterized dose-response relationship for the Gottingen minipig at doses spanning from H-ARS to GI-ARS. During our past studies, we have optimized procedure for housing, handling and sampling in the M-P. Animals were kept on a calorie-restricted diet, as per provider recommendation, to maintain the small size characteristic of this animal. Use of a vascular access port [redacted] allowed us to obtain serial samples and to analyze hematological changes over time, hematopoietic progenitor stem cells, kinetics of DNA damage/repair and residual foci (gamma-H2AX), circulating citrulline levels and microbiological status. From those studies, we demonstrated that the bone marrow syndrome (H-ARS) in the minipig closely resembles that in humans and other large animal models. The sequence of sign development, dynamics of hematological cell loss, susceptibility to infection and occurrence of multi-organ dysfunction leading to death, all confirmed the potential validity of this model to study ARS [redacted] Kinetics of DNA double strand break formation and repair were nearly identical between M-P, humans and NHP [redacted] Administration of G-CSF to irradiated minipigs stimulated recovery of bone marrow, induced mobilization of progenitor stem cells, hastened neutrophil recovery and increased survival, similarly to what was described for NHP, canine and mouse models of ARS (6), confirming the high potential of the model for radiation countermeasure screening. The only major difference between M-P and other animal models or human was represented by extensive hemorrhages and a significant lower LD50 (no supportive care): M-P 1.73 Gy (3) and 1.96 Gy [redacted] -personal communication); dog 2.6 Gy (6); human 3.2-4.5 Gy (7-9); NHP 4.9 – 6.71 Gy) [redacted]

(b)(4) (b)(4) (b)(4) (b)(6) (b)(4)

During our previous proof-of-concept study, survival was followed for 30 days, and the animals were kept on a calorie-restricted diet as per provider recommendation. Here we propose to address the sensitivity of the minipig to radiation using minimal supportive care and nutritional supplementation. Our ultimate goal is to characterize the model for testing of medical drugs to be used in a mass casualty scenario, under the FDA Animal Rule. In a mass casualty scenario, the standard of care used for small-scale accidents (administration of antibiotics, iv fluids, parenteral nutrition, and blood products) may be problematic. We will use minimal supportive care (defined here as antibiotics and dietary supplementation –no iv fluids, blood products or parenteral nutrition), as a potential tool for testing drugs relevant to Warfighter population as well as civilians. Nutritional intake will be increased with a diet containing 20% protein instead of 16% (historical diet, Tekland 8753), to be comparable to that in dogs and NHP. The amount of food provided will be equivalent to 3-4% of body weight, instead of 2.3-3.3% as recommended by Marshall Bioresources (Gottingen minipig provider) (see Appendix 1: Housing and Feeding of Gottingen Minipigs). Because of the possibility of potential delayed mortality, dissociated hematologic recovery, and secondary relapse, we will extend the observation period to 60 days, as routinely done for NHPs.

II.2. Literature Search for Duplication
II.2.1. Literature Sources Searched BRD, RePORTER, PubMed
II.2.2. Date of Search January 6, 2014
II.2.3. Period of Search
BRD: 1998 – 2009
RePORTER: 1998-2013
Pubmed: 1998-2013

II.2.4. Key Words of Search
Minipigs OR pigs OR swine AND Radiation AND supportive care OR antibiotic

II.2.5. Results of Search
Search of the combination minipig OR pig OR swine AND radiation AND supportive care did not result in any document.

Search of the combination minipig OR pig OR swine AND radiation AND antibiotic resulted in articles about dermal phototoxicity, vascular access port implantation (our publication), dentine formation, bone marrow transplantation, restenosis, ex vivo ocular toxicity, and effect of antibiotic on small intestine mucosa. These studies are irrelevant to our project. Other articles met the key words search criteria but were not relevant to this study as they pertained to Guinea pigs or ex vivo/in vitro observations.

III. OBJECTIVE / HYPOTHESIS

Rationale: in order to develop a new countermeasure under the FDA Animal Rule, it is required that the mechanism of action are understood and the interaction between test drug and supportive care (additive, synergistic, antagonistic or none) is investigated. Recommendations for medical management of radiation victims under mass casualty scenario requiring immediate and delayed treatment include administration of antibiotics and routine care. It is therefore imperative to develop an animal model that will mimic conditions under which mitigators will be administered. It is reasonable to assume that antibiotics, oral fluids and nutritional support will be available in a mass casualty scenario.

Hypothesis: it is possible to estimate the dose response relationship (DRR) for total-body gamma-irradiation and moribundity in a M-P model of H-ARS, in the presence of minimal supportive care (antibiotics, and nutritional support). The resulting probit curve will provide estimates of lethal doses ranging from the LD10/60 to LD90/60, to be used for testing radiation countermeasures.

IV. MILITARY RELEVANCE

According to the DoD Chemical and Biological Defense Program 2009 Annual Report to Congress: "Although the overall number of nuclear weapons continues to decline because of Russian and U.S. treaty commitments, the United States anticipates an increase in weapon numbers in China, India, and Pakistan. Motivated by economic and strategic interests, Russia and China (or political entities in each) and North Korea continue to supply technologies and components that are dual use and could support weapons of mass destruction and missile programs, especially in the Middle East and South Asia. Iran continues to develop its enrichment program in defiance of United Nations (U.N.) Security Council resolutions and also continues to build a heavy water reactor in Arak, which will be capable of producing plutonium that could be weaponized. While North Korea has halted portions of its nuclear program, it is possible that it has stockpiled several nuclear weapons from plutonium produced at Yongbyon. Non-nuclear radiological dispersal devices (RDD) and radiological exposure devices (RED) pose a significant potential threat especially in the hands of non-state groups. Contamination and recovery issues associated with operating in a radiological environment remain significant concerns for military operations and underscore the need for robust detection, protection, and decontamination systems."

An important barrier to advanced development of radiation countermeasures is the paucity of large, long-lived, non-rodent animal models of radiation injury. The only choices at present are NHPs and canines. The advanced evolutionary stage of NHPs, and the danger of working with NHPs, are also disadvantages. Canines are notoriously sensitive to NSAID and vomiting. The addition of miniature swine to available large animal radiation injury models will facilitate development of promising countermeasures.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

The purpose of this study is (i) to establish the effect on the survival of irradiated minipigs of a prophylactic empirical antibiotic regimen, similar to that likely to be administered to patients in case of a radiological accident, in combination with dietary supplementation; and (ii) to build a novel probit curve under these conditions. The expected results from empirical antibiotic treatment and dietary supplementation are a higher LD50 and a less steep dose-survival curve.

Primary endopoints will be 60 day survival. Secondary endpoints will be CBC-counts, clinical signs, gross- and histo-pathology, and C-reactive protein. Measurements of blood elements and circulating analytes will be useful to establish the utility of the minipig as a biodosimetry model and will provide a useful term of comparison for intra- and inter-laboratory validation efforts.

Prophylactic empirical antibiotic regimen.
Guidelines for treatment of neutropenic patients from chemotherapy and from ablative radiation treatment

for hematopoietic stem cell transplantation (13; 14; 15), as well as prototype medical orders during a radiation incident (17) suggest administration of prophylactic empirical antibiotic treatment to reduce the risk of infections. Antibiotic prophylaxis with fluoroquinolones is recommended for afebrile neutropenic patients (15), as it significantly reduces all-cause mortality for cancer patients following chemotherapy (16). Broad spectrum oral fluoroquinolone plus amoxicillin/clavulanate are among the recommended antibiotics for initial empiric therapy in neutropenic patients; length of treatment is based on bone marrow recovery and attainment of stable neutrophil levels above neutropenia. In case of resistance to fluoroquinolone (fever for >3-5 days during fluoroquinolone treatment), it is recommended to switch to gentamicin+amoxicillin/clavulanate (NCCN), and to start on oral triazole (antifungal) if fever still persists for >3-5 days.

A number of micro-organisms have been identified in the blood and tissues of the irradiated minipigs (b)(4),(b)(6) Gram-positive (*Staphylococcus aureus, S. chromogenes, S. saprophyticus, S. warneri, Streptococcus agalactiae, S. alactolyticus, S. dysgalactiae* ssp. *equisimilis, S. infantarius* ssp. *coli, S. mitis/S. oralis, Streptococcus anginosus, Streptococcus bovis, Streptococcus hyointestinalis, Enterococcus cecorum, E. columbae, E. faecalis, Enterococcus faecium, Enterococcus hirae,* Enterococcus sp., *Gemella morbillorum,* Leuconostoc mesenteroides ssp. cremoris, *Actinomyces naeslundii,* Bifidobacterium sp., *Lactobacillus gasseri, Actinomyces naeslundii,* Corynebacterium sp.), Gram-negative (*Acinetobacter baumannii, Aeromonas hydrophila, Escherichia coli, Klebsiella pneumonia,* ssp. *pneumoniae, Pasteurella penumotropica, Pseudomonas aeruginosa*), Yeast (*Candida tropicalis*). The variety of opportunistic pathogens that is capable of producing infections in the minipig justifies the use of broad spectrum antibiotics and anti-fungal that we propose to use for prophylactic empirical antibiotic regimen.

We will follow the regimen for neutropenic, afebrile patients suggested by the American Society of Cancer Oncology, ASCO (15), and the guidelines from the Department of Health and Human Services, Radiation emergency medical management and the Radiation Injury Treatment Network, RITN (17). Furthermore, we will define neutropenia as neutrophil counts <500/μL (18) to be in alignment with the NHP model, and we will use this parameter as trigger point to start/end treatment.

We will start treatment at ANC<500/μL or expected to decrease to <500/μL within the next 48 hours, with or without concurrence of fever (Table 2). We will administer levofloxacin plus amoxicillin/clavulanate (po) for 3 days or until bone marrow recovers and ANC remain stable above >500/μL. In case of resistance to levofloxacin (temperature higher than 39° Celsius for >3-5 days during fluoroquinolone treatment), we will switch to a different class of antibiotic and we will use gentamicin in combination with amoxicillin/clavulanate (13). If fever still persists for >3-5 days, we will switch to fluconazole. Oral administration of drugs is preferred over iv administration because of negative impact of venipuncture on animal survival. Oral administration is also likely to be preferred in case of mass casualties due to limited availability of infrastructure for iv administration of drugs. The table below summarizes drug regimen. If the animals show an adverse reaction to levofloxacin, we will switch to ciprofloxacin.

Table 1: Prophylactic empirical antibiotic regimen: trigger points to start/end treatment	
ANC<500/μL or expected to decrease to <500/μL within the next 48 hours	**Start** levofloxacin plus amoxicillin/clavulanate
ANC stable above >500/μL (2 consecutive CBC reading at 3-4 days distance) and T<39° C	**Stop** treatment
If T > 39° C for >3-5 days while taking levofloxacin	**Stop** levofloxacin plus amoxicillin/clavulanate. **Start** gentamicin plus amoxicillin/clavulanate. Administer until ANC stable above >500/μL (2 consecutive CBC reading at 3-4 days distance) and T<39° C
T > 39° C for >3-5 days while taking gentamicin	**Stop** gentamicin plus amoxicillin/clavulanate. **Start** fluconazole plus amoxicillin/clavulanate. Administer until ANC stable above >500/μL (2 consecutive CBC reading at 3-4 days distance) and T<39° C

Levofloxacin (LEVAQUIN®)
Levofloxacin is a fluoroquinolone agent for oral and intravenous administration with antibiotic activity against a broad spectrum of Gram-negative and Gram-positive bacteria responsible for infections to the respiratory, urinary and gastrointestinal tract. Spectrum of activity includes the Gram- negative *Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Legionella pneumophila, Moraxella catarrhalis, Proteus mirabilis,* and *Pseudomonas aeruginosa,* and the Gram positive *Staphylococcus Aureus, Streptococcus pneumoniae, Staphylococcus epidermidis, Enterococcus faecalis, and Streptococcus pyrogenes*), as well as atypical bacterial pathogens (*Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*). Fluoroquinolones (ciprofloxacin and levofloxacin) are widely used in the treatment of human disease and are likely to be used in the event of radiation exposure to prevent infections. Because levofloxacin exhibits enhanced activity against *Streptococcus pneumoniae* with respect to ciprofloxacin, we selected to characterize the model in the presence of levofloxacin although we maintain ciprofloxacin as a possible alternative in case oral administration of levofloxacin proves difficult in the minipigs. Table 2 below summarizes drug administration routes and doses.

Amoxicillin/clavulanate (Augmentin)
Amoxicillin/clavulanate is an oral antibacterial combination consisting of amoxicillin and the beta-lactamase inhibitor, clavulanate potassium. Amoxicillin is a semisynthetic, third generation penicillin (beta-lactam antibiotic); clavulanate is a beta-lactam that inhibits beta lactamase, the major bacterial enzyme responsible for penicillin resistance. Amoxicillin/clavulanate is used to treat mild-to-moderate bacterial infections including sinusitis, bronchitis, and otitis media. Amoxicillin/clavulanate is the recommended antibiotic to be used in combination with fluoroquinoloes for empiric therapy regimen (Flower, 2013). Table 2 below summarizes drug administration routes and doses.

Fluconazole (DIFLUCAN®)
Fluconazole, an oral triazole, is a drug used in the treatment and prophylaxis of superficial and systemic fungal infections. Fluconazole is effective against a number of fungi including Candida. Table 2 below summarizes drug administration routes and doses.

Table 2: Prophylactic empirical antibiotic regimen: drug dosage and modality of administration	
Drug	**Regimen/route**
Levofloxacin (Levaquin)	500 mg SID, po *
Ciprofloxacin	2.5 - 7.5 mg/kg, BID, po. *
Gentamicin	2-6 mg/kg SID, po. *
Amoxicillin/clavulanate	11-13 mg/kg every 12-24 h, po. *
Fluconazole (Diflucan)	10-12 mg/kg, SID, po. *

*or iv or im if PO not possible, at dosage prescribed by and in consultation with VSO veterinarian or assigned stufy veterinarian

Dietary supplementation

In our initial, historical studies we have been using a 16% protein diet for minipigs and we have kept the animals on a calorie-restricted diet in order to avoid excessive weight gain, as per provider recommendation (Marshall Bioresources, see Appendix 1). No supportive care was given to the animals after irradiation. Indeed, it is well known that nutritional support for cancer patients undergoing radiation therapy is vital, and other large animal models (dogs and NHPs) receive supportive care after irradiation, to include nutritional support rich in proteins, anti-oxidants and fats.

For the current study, we will increase the food amount to 3-4% (grams/animal kg) instead of 2-3% as recommended by the vendor. We will use a "starter" diet for minipigs which has a higher content of proteins (20% instead of the 16% protein content, as done in our previous studies); the diet will be supplemented with fatty acids and probiotics to counteract the effects of antibiotics on the intestinal flora (Table 1). The proposed nutritional support is what is currently been used for piglets (ongoing study, approved protocol (b)(6) has shown no adverse effects and has provided some advantage in terms of survival at 2 Gy (preliminary data, n=3 per radiation dose). Traditional enrichment will be maintained as part of the diet.

Nutritional support	
Starter diet (i.e. 5080 from Quality Lab Products)	20% protein, 4% grams/kg animal (average for group of animals and updated on a weekly basis)
Probiotics	approximately 2 grams, daily
omega-3 fatty acids	DHA, 1 capsule every 3 kg body weight
electrolytes, hydrating gels	as needed
milk formula	up to 20ml/kg, twice a day as necessary
yogurt	daily
nutritional support if not eating solid food	as needed, includes additional milk formula
only canned/dried fruits and vegetables	as needed
metamucil	as needed

Probit curve

Briefly, a vascular access port (VAP) will be implanted approximately one-to-two weeks after the arrival of animals at AFRRI, to facilitate collection of blood samples. Three weeks after VAP implantation, we will expose minipigs to a range of doses of whole-body gamma irradiation to bracket the hematopoietic

syndrome. Animals will be administered the minimal supportive care listed in Table 1, and survival will be observed for 60 days.

V.1.1. Experiment 1: LD50/60 range finding pilot study

To limit the number of animals used, we will adopt the "range finding approach" for the initial assessment of a rough estimate of the LD50/60.

In toxicology, the purpose of conducting a dose range finding study is to establish a dose response and to provide preliminary data to enable appropriate dose selection for full-scale studies (15). A range-finding test ordinarily consists of a down-scaled test in which small groups of animals are exposed to several widely-spaced doses, to estimate the LD50/60. The study design usually includes an assessment of basic toxicological parameters including clinical observations, hematology, chemistry and pathology. This study design is in alignment with 'three R' concepts for animal welfare (refinement, reduction, and replacement) and is intended to obtain optimal, standardized study conditions for protocol transferability and data reproducibility.

In Experiment 1, we will use 2 animals per dose and we will test 4 separate doses for each of three reiterations (24 animals, total). Since administration of supportive care in other species (i.e. dogs, NHP) and in humans increased survival so that the LD50 was shifted of up to 0.5-2 Gy, we will test doses ranging between 2.0 and 3.5 Gy, given that the LD100 in the M-P in the absence of supportive care is 2.0 Gy. Initially we will test doses equivalent to 2.0, 2.5, 3.0 and 3.5 Gy (2 animals per dose; group A). Based on the results of the group A, we will then refine the dose interval in consultation with the statistician, and perform 2 additional experiments, each consisting of 4 doses and 8 animals (2 animals per dose; group B and C). This pilot study will provide a rough estimate of the LD50, as well as the doses bracketing the hematopoietic syndrome (LD10 and LD90).

Animal requested for experiment 1: 2 animals/dose, 12 doses = 24 animals

V.1.2. Experiment 2: **Construct a full lethality probit curve to be used for establishing radiation doses during countermeasures drug testing**

For the generation of the dose-response curve, the LD30, 50 and 70 will be estimated using the probit method, and including results from experiment 1 (pilot study). This method allows for construction of confidence intervals to describe the precision of the estimated lethal doses. The width of the confidence interval depends on the number of doses, number of animals per dose, and the slope of the dose-response curve, and a narrow interval indicates a more reliable estimate. We propose to use five doses and six animals per dose (0.6 Gy/min, TBI, Cobalt-60). Doses will be evenly spaced. Minipigs already irradiated at those doses in the pilot study will be incorporated into the data from this experiment, to increase statistical power.

Animal requested for experiment 2: 4 animals X 5 doses = 20

V.2. Data Analysis

The dose response relationship (DRR) will be plotted as probit percent mortality versus linear dose. Probit regression analysis will be used to generate the probit curves, using the PASW Statistic 18 software (SPSS Inc, IL, USA), and preliminary values for LD30, LD50, and LD70 will be obtained. For a sample size of 6 per dose (including 2 per dose from experiment 1), a dose-response curve ranging from 1.5 to 3.5 Gy with mortality rates of .1, .3, .5, .7 and .9 percent for the five doses, the expected width of the 95% confidence interval for the LD50 will be 1.5 Gy, and the expected width of the 95% confidence interval for the LD30 and LD70 will be 2.5 Gy. Expected confidence interval widths are based on the average of 1,000 simulated data sets using the study design and mortality parameters indicated. Actual confidence interval widths will vary depending on the specific doses selected and the slope of the dose-response curve.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered.

The purpose of this project is to determine the effect of minimal supportive care on survival. There is no computer model or tissue culture that can mimic the effect of radiation on the whole organism and predict lethality. Survival depends on an intricate network of organ cross-talks and cellular microenvironment that cannot be recreated *in vitro*. Consequently, administration of supportive care to cellular components grown *in vitro* will not allow any assessment on survival. Effect of supportive care also cannot be reproduced *in vitro*.

We do not know all of the cell types and tissues involved in radiation injury or how radiation injury causes lethality. The concept of multi-organ failure has been suggested, but how the injured organs affect each other's vital functions is unknown. The present state of the art of tissue culture is still incapable of reproducing the *in vivo* relationships. Computer modeling is similarly limited by lack of full characterization of the biological elements and interactions under study.

These considerations are consistent with the FDA requirement for preclinical research in both small and large mammals before granting approval for drug testing in humans.

V.3.2. Animal Model and Species Justification
We have been developing the adult Gottingen minipig as an additional large animal model for the study of the ARS and advanced development of radiation countermeasures. Large, long-lived, and development-matched models are required for drug studies submitted to the FDA for licensure applications. The close similarity to humans in anatomy and physiology of organs such as the liver, pancreas, kidney and heart has made the pig the primary species of interest as organ donors for xenographic procedures. Minipigs are routinely used for toxicity testing. As such, further and rapid characterization at physiological, molecular and genetic level of the minipig is likely to occur, thus increasing acceptance of this model and facilitating advanced drug development. Our previous studies have employed male Gottingen minipigs to develop a probit curve. Since we are testing the effect of minimal supportive care on survival and we are using our own historical data as one of the terms of comparison, we will continue to use male minipigs for this study.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species *Sus scrofa domestica*

V.3.3.2. Strain / Stock Gottingen

V.3.3.3. Source / Vendor Marshall BioResources/Marshall Farms Group Ltd

V.3.3.4. Age Approximately 4 months at arrival

V.3.3.5. Weight Age appropriate (9-11 kg)

V.3.3.6. Sex Male

V.3.3.7. Special Considerations NA

V.3.4. Number of Animals Required (by species): 46

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement
The study data-points currently mandated by the FDA for approval of radiation countermeasures under the Animal Efficacy Rule is mortality. Hence the primary study data-point in our development of Gottingen minipigs as a model for ARS is survival. Moribundity will be used as a surrogate for mortality, and

euthanasia will be used in order to minimize pain and distress, using an extensive set of criteria (see animal endpoints, paragraph V.4.5 – Lethargy, weight loss, temperature, respiratory distress, food intake, bleeding etc). Animal care and use procedures will only be performed by trained personnel. We have considerable experience in the care and handling of minipigs. Extensive human interaction every weekday will reduce stress in the animals. Use of slings, topical anesthetics, and the catheters will minimize distress during blood collections and the animals will be gradually acclimated to the slings for these procedures.

V.3.5.2. Reduction
Control animals used to optimize protocols for animal handling, housing and blood draw will be used also to obtain data on growth of normal animals, and to set up dosimetry for future studies and to obtain baseline CBC data in pediatric animals. For irradiated animals, blood samples obtained from minipigs before irradiation will serve as controls, thus reducing the number of animals required for this study. Use of historical controls will also reduce the number of animals needed for this study.

V.3.5.3. Replacement NA

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C _____ (# of animals)

V.4.1.1.1.2. Column D _____ (# of animals)

V.4.1.1.1.3. Column E __46__ (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1	**LD50/60 range finding pilot study**			**24**	**24**
2	**Construct a full lethality probit curve to be used for establishing radiation doses during countermeasures drug testing**			**22**	**22**
Totals:				**46**	**46**

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization
Irradiation
Prior to irradiation, animals will be sedated with Telazol® (Telazol, 6-8 mg/kg *IM*, Fort Dodge Laboratories, IA). Alternatively, Telazol® (4.4 mg/kg *IM*), in combination with dexmedtomidine (0.05-0.1 mg/kg *IM*) or ketamine (20 mg/kg *IM*)/xylazine (2 mg/kg *IM*) can also be used for sedation. Atropine (0.5 ml *IM* or *IV*; atropine sulfate, Butler, Columbus, OH) or glycopyrrolate (0.01 mg.kg) may be given to reduce mucosal secretions. At the end of the irradiation procedure, once the animals are back in their quarters, dexmedetomidine can be reversed by atipamezole *IM* (same volume as dexmedotmidine) to facilitate recovery.

Blood sampling
Animal may be sedated at the time of blood sampling and administration of minimal supportive care with Midazolam (0.1-0.5 mg/kg s.c.or *IM*), or acepromazine (1.1 mg/kg *IM*), 10 minutes prior to venous access. Telazol or Telazol +dexmedetomidine or Ketamine + Xylazine or isoflurane could be used as an option as well.

V.4.1.2.2. Pre- and Post-procedural Provisions
Irradiation
Pre-Irradiation: Animals will be fasted overnight (approximately 16–18 h) and sedated as described above. Once sedated in the VSD animal facility, minipigs will be transported to the AFRRI cobalt facility in an approved, covered transport cage. Heart rate and temperature will be measured prior to irradiation. At least one vet tech or one veterinarian will be present at the time of irradiation. Immediately before irradiation, the vet or vet tech will determine whether or not to administer additional sedative to keep the animal asleep during irradiation.

Irradiation procedure: To enable proper positioning, anesthetized animals will be strapped in place into slings in polyvinylchloride PVC frames for irradiation. Effect of anesthesia will be monitored in real time through video cameras positioned in the Cobalt waiting area; if, during irradiation, signs are observed that the animal is attempting to get loose, we will immediately stop the irradiation and we will promptly recover the minipig. Minipigs will be removed from the slings once irradiation is completed. Once in the procedure room adjacent to Cobalt and before returning the minipigs to their home cages, temperature will be taken with the transponder or rectally; heart rate will be measured using a stethoscope. .

Post-Irradiation: After irradiation, animals will be transported back to their cages, where they will be allowed to recover from sedation under the careful surveillance of VSD and/or research staff who will monitor vital signs and provide thermal support (e.g. Bair Hugger blanket) if necessary. Atipamezole *IM* (same volume as dexmedetomidine) may be used to reverse the anesthetic effect of dexmedetomidine.

V.4.1.2.3. Paralytics N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched Altweb, Pubmed

V.4.1.3.2. Date of Search

V.4.1.3.3. Period of Search All available

V.4.1.3.4. Key Words of Search The aspects of the study relevant to alternatives to painful or distressful procedures are the sequelae to exposure to TBI at doses causing ARS. We used the following key words in the search for alternatives: swine, radiation, ARS, sequelae, pain, distress, alternatives

V.4.1.3.5. Results of Search
Cell culture based alternative were found to study the intestinal barrier function of piglets and for organ specific drug toxicity testing. However techniques are not fully developed yet and are not representative the entire organism.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification
Depending upon the irradiation dose irradiated animals die due to compromised immune responses and opportunistic infections. The percentage of surviving animals is the indicator of the efficacy of a countermeasure. We are not planning to administer analgesics post-irradiation, as NSAID may aggravate bleeding disorders in combination with the radiation-induced thrombocytopenia, and opiates can induce sedation and drowsiness and may confound proposed euthanasia criteria. However, we are providing

minimal supportive care in terms of antibiotics, and fluids/ nutritional support to increase survival. Use of supportive care is expected to mitigate the pain associated to the sequelae of irradiation.

V.4.2. Prolonged Restraint
Only short periods of restraint in the sling (<30 minutes) will be necessary for blood collections and the irradiation procedure. To minimize discomfort, we have designed a new type of sling that uses softer, elastic material to support the animal, and utilizes a more efficient restraint design that maintains the animal in place while reducing manipulation by technicians and consequently stress to the animals. After the quarantine period, animals are acclimated to the sling for 5-10 minutes, 2-3 times before initiating blood draws. Acclimation to the sling is very rapid and non-stressful; animals are petted and gently spoken to while restrained. It has been our experience that resting on the sling does not agitate the animal at all. Animals are anesthetized and transported to the Cobalt Facility holding area before being put into slings for irradiation (<20 minutes). Animals will be kept under continuous observation while in the sling. Slings will be spot-cleaned between animals and sent to wash at the end of each collection day; slings will be sanitized at the conclusion of each irradiation day.

V.4.3. Surgery: implantation of vascular access port (VAP).

V.4.3.1. Pre-surgical Provisions Animals will be cared for according to section V.5.1. They will be fasted overnight (approximately 16 – 18 h) prior to VAP implantation surgery.
For antibiotic, Cefazolin sodium 20 mg/kg *IV* or ampicillin sodium 10-20 mg/kg *IV* will be given just prior to the surgery, or intra-operatively, for prophylaxis. At the time of surgery, a temperature microtransponder (Implantable Electronic ID Transponder IPPT-300, Bio Medic Data Systems, Seaford, DE) will be implanted subcutaneously behind the neck, over the shoulder opposite to the site of VAP surgery, to facilitate body temperature measurements.

V.4.3.2. Procedure(s) VAP catheters (SoloPort subcutaneous access port, either PMID or MID model; Instech Solomon, PA) will be surgically implanted in the right external jugular veins under general anesthesia according to the principles of aseptic surgery. Animals will be lying on their back; vital signs will be taken at regular intervals by vet techs. The catheter tip will be introduced through a 2-3 mm incision in the right jugular vein and advanced to the junction of the external jugular vein and vena cava. Prior to securing the VAP catheter within the vein, sutures will be used to ligate the vessel just cranial to the insertion point of the catheter tip. Additional ligature will be placed around the caudal portion of the vessel containing the VAP catheter and retention bead, with 1 ligature on either side of the bead. The port will be secured within the incision site on the neck to the underlying musculature by use of sutures, at a minimum of 2 of the 5 anchor holes on the port. Sutures will be 3-0 PDS or others (with the exception of silk) as deemed appropriate by surgeon. Catheter function will be confirmed intra-operatively through aspiration of locking solution and withdrawal of a blood sample via the septum. The port reservoir and catheter will be flushed again with 6 ml saline and locked with 3 ml of a heparin lock flush solution (100 IU/ml) all subsequent lock solutions will be heparin (100 IU/ml).

During closure of the surgical sites, special attention will be given to eliminating dead space around the port and around the catheter loop on the neck. The muscle and subcutaneous layers will be closed with sutures. Skin incisions will be closed in a continuous intradermal pattern. Tissue glue (VetBond, 3M, St Paul, MN) may be used to help seal surgical sites as necessary to help prevent contamination.

V.4.3.3. Post-surgical Provisions Pre-, intra- or postoperative intramuscular buprenorphine (0.01 to 0.02 mg/kg; Buprenex, Reckitt Benckiser, Richmond, VA) or Carprofen (2.2 mg/kg) will be provided for analgesia before full recovery from anesthesia. Additional post-operative analgesia will be given as Carprofen (2.2 mg/kg *po* BID) for three days starting the day after surgery, or as recommended by an AFRRI veterinarian in consultation with the PI. Alternatively, fentanyl transdermal patch at 5mcg/kg/hr or buprenorphine sustained release 0.12 – 0.27 mg/kg sc for analgesia can be used as determined by the veterinarian. The fentanyl patch, if used, will be applied to a shaved portion of skin and secured. It takes approximately 12 hrs for the fentanyl patch to take effect. Animals will continue to be monitored until fully

recovered from anesthesia; they will be returned to their home cage when ambulatory. Supplemental heat may be provided in the home cage post-operatively (e.g. Bair Hugger warm air blanket)

After surgery, 25-50 mg/kg trimethoprim-sulfa *po* (500 mg *po* for 10 kg pig or 5mg/kg im), *SID*, will be given for 5 days or more as determined by veterinarian to lessen the risk of post-op infection. Alternatively, enrofloxacin 5-10 mg/kg iv/po can be given every 24 hrs for 3-5 days or more if needed as determined by the veterinarian. Convenia 5-10 mg/kg sc may be used as an antibiotic as determined by the veterinarian. The advantage of this antibiotic is that the single injection provides protection against the bacterial infection for several days.

Postsurgical recovery and pain will be monitored twice daily by visual examination of the incisions and overall conditions of the animals. Parameters monitored will be food and water intake, activity, alertness, vocalization, guarding, and response to human contact.

V.4.3.4. Location VSD surgical suite

V.4.3.5. Surgeon Surgeons will be AFRRI veterinarians

V.4.3.6. Multiple Major Survival Operative Procedures NA

V.4.3.6.1. Procedures

V.4.3.6.2 Scientific Justification

V.4.4. Animal Manipulations

V.4.4.1. Injections

All injections listed in section **V**.4.1.2.1 will be administered *im, sc*, using sterile needles (23 – 21 gauge) and syringes, or iv through the catheter or *po*.

V.4.4.1.1. Pharmaceutical Grade Drugs

Bactrim (sulfamethoxazole and trimethoprim)
Lidocain (5%), topical
Telazol®
Ketamine/xylazine
Atropine atropine sulfate
Heparin lock, IV
Saline
Metamucil
Levofloxacin
Ciprofloxacin
Gentamicin
Amoxicilline/clavulanate
Fluconazole
Midazolam
Acepromazine
All the drugs listed above are ordered from veterinary product distributors by VSD and they all are pharmaceutical grade drugs.
Atipamezole (Antisedan, Pfizer)
Dexmedtomidine (Dexdomitor, Pfizer)

V.4.4.1.2. Non-Pharmaceutical Grade Drugs NA

V.4.4.2. Biosamples

Collection of blood samples. We will obtain blood samples from the VAP or from peripheral veins. If the VAP fails, the sample will be collected from accessible peripheral vessels (see section **V.4.4.7. Other Procedures**). Mean blood volume for pigs is 55-65 ml/kg. at arrival, animals weigh approximately 9 kg and gain 0.5 kg per week. Up to 1% of total blood will be drawn per time point (corresponding to 4.9 ml - 5.8 ml for a 9 kg animal), and no more than 7.5 % per week.

Depending on the volume of blood taken, appropriate recovery time will be allowed between sampling intervals (21, 22):
0.75% circulatory blood removed = 1 day recovery period
7.5% circulatory blood removed = 1-2 weeks recovery period

Collection of samples will be performed with the minipig restrained in a sling; at the same time, we will take vitals. The entire procedure takes less than 30 min. After blood draw, animals will be returned to their cages and checked to ensure that the needle puncture wound is closed and the blood has clotted. Blood samples will be obtained at these time points: with day 0 being the day of irradiation, we will collect blood at -2 weeks, -1 week, -1 day, day 0 and on days +1, +2, +3, +7, +10, +14, +17, +20, +23, +27, +35, +42, +49, +56 and at euthanasia.

Time point (day)	volume	% total blood volume*	Total % blood volume (Cumulative volumes from the beginning of the study)
-14	1 ml	0.17	0.17
-7	3 ml	0.5	0.67
-1	3 ml	0.5	1.17
0	1 ml	0.17	1.34
1	1 ml	0.17	1.51
2	1 ml	0.17	1.68
3	1 ml	0.17	1.85
7	1 ml	0.17	2.02
10	1 ml	0.17	2.19
14	1 ml	0.17	2.36
17	1 ml	0.17	2.53
20	1 ml	0.17	2.7
23	1 ml	0.17	2.87
27	1 ml	0.17	3.04
35	1 ml	0.17	3.21
42	1 ml	0.17	3.38
49	1 ml	0.17	3.55
56	1 ml	0.17	3.72

*Assuming a body weight of 9kg and total blood volume of 585 ml

Dates of collection may be changed by +/- 48 hours or collection may be cancelled if contingencies arise. VAPs will still be flushed at least once a week.

Tissue and histological samples. Tissue samples, to include organs, blood and hair may be taken at the time of euthanasia for tissue blocks and for frozen tissue repository.

V.4.4.3. Adjuvants NA

V.4.4.4. Monoclonal Antibody (MAb) Production NA

V.4.4.5. Animal Identification Tattoos or ear tags and cage cards/tags

V.4.4.6. Behavioral Studies NA

V.4.4.7. Other Procedures
Catheter implantation

Temperature transponder At the time of catheter implantation, a temperature microtransponder (Implantable Electronic ID Transponder IPPT-300, Bio Medic Data Systems, Seaford, DE) will be implanted subcutaneously behind the neck, over the shoulder opposite to the site of VAP surgery, to facilitate body temperature measurements.

Blood draw from peripheral veins. Alternatively, if VAP fails we will collect blood from peripheral veins such as auricular, femoral, cephalic, saphenous, cranial vena cava.. Animals may be sedated at the time of blood sampling and administration of minimal supportive care (see section V.4.1.2.1) if necessary, following the recommendations of an AFRRI veterinarian. All procedures will be entered into the experimental record. Lidocaine (5% ointment) may be applied topically to the site of venipuncture to minimize discomfort to the animals about 15 minutes before starting the procedure; site will be cleaned with alcohol.

Health check status
Body weights and standard animal health check status (hydration status, hair coat, skin quality, vitals) will be determined at least once prior to treatment, on the first day of treatment, and on days of blood collection. Body temperature will be determined during week-days between 7 am and 4 pm, and on weekends if there are animal concerns. The time of each temperature collection will be recorded for each minipig. Monitoring will be increased during critical period.

V.4.4.8. Tissue Sharing Bio-samples (**V.4.4.2. Biosamples_Tissue and histological samples**) will be collected, to be used for histo-chemistry/protein/molecular assays among others, and to be shared with collaborators upon request and as experiments permit

V.4.4.9. Animal By-Products

V.4.5. Study Endpoint The data-point currently mandated by the FDA for approval of radiation countermeasures under the Animal Efficacy Rule is moribundity or mortality. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress. Euthanasia will be carried out when any one of absolute endpoints or combination of the following non-absolute signs of moribundity are observed and in consultation with AFRRI veterinarians.

Absolute
1. Non responsive, assuming the animal has recovered from anesthesia.
2. Dyspnea
3. Loss of 20% of expected weight (based on Marshall Bio-resources growth chart –weight vs age- and day 0 baseline percentile).
4. Hypothermia (< 36ºC)

Non-absolute
1. Hyperthermia (>41ºC)
2. Anorexia (skip 2-3 consecutive meals)
3. Anemia/pallor, CRT >2 seconds (CRT will be performed behind the ears), sudden severe drop in HCT (>10% over 3 consecutive days).
5. Petechiae/ecchymosis
6. Vomiting/diarrhea

7. Lethargy
8. Seizures or vestibular signs (falling, circling or head tilt)
9. Uncontrollable hemorrhage

When non-absolute endpoints are being evaluated, the frequency of monitoring of the animals will be increased to four times per day. At the time of euthanasia, the attached observation sheet (Attachment 1) will be filled out, and may be used retrospectively at the end of study to put together a score sheet for euthanasia criteria for minipigs, similarly to that for rodents.

V.4.6. Euthanasia
Animals will be euthanized according to current American Veterinary Medical Association (AVMA 2013) guidelines. Animals will be sedated with Telazol® (6-8 mg/kg im) or ketamine (20 mg/kg im)/xylazine (2 mg/kg im). Animals will then be euthanized through the VAP with and injected iv Euthasol® (sodium pentobarbital, (1 ml/4.5 kg iv or intracardiac) or another commercial euthanasia solution.
Alternative, in case of VAP malfunction or loss, peripheral or central venous cut down or cardiac stick or catheter without cutdown may be performed, with the animals maintained under general anesthesia using isoflurane gas at a maintenance rate of 1% to 3% and oxygen flow rate of 1 to 2 l/min, to prevent the possibility that the telazol might wear off during this time. Death will be confirmed by VSD veterinarian, assigned study veterinarian, or veterinary technician via auscultation of the heart, with cessation of sounds for at least 5 minutes. See item V.4.5 for euthanasia criteria before the end of the study

V.5. Veterinary Care

V.5.1. Husbandry Considerations

Minipigs will be housed in stainless steel cages/runs, in tactile, visual and olfactory contact with adjacent animals. During quarantine/acclimation period, up to day of surgery, minipigs may be group-housed. After surgery, they will be singly housed to prevent damage to the VAPs and to allow individual assessment of feed consumption and fecal/urine/blood production. Rooms will be maintained on a 12:12 h light (0600 to 1800) dark cycle with the temperature set to 27 to 29°C and 30% to 70% relative humidity. Commercial diet with 20% protein content will be provided (i.e. Labdiet Minipig starter diet 5080). Water will be provided ad libitum. Minimal supportive care will be provided after irradiation to all animals, accordingly to Tables 1-3.

V.5.1.1. Study Room
Minipigs will be located at AFRRI for the duration of the study. They will either be in a VSD surgical suite for catheter placement, in transit to and from the cobalt facility in a covered transport cage, in slings for irradiation in the cobalt facility, or in their housing cage or a VSD prep room for blood collections or euthanasia. They will be housed in stainless-steel cages in an environmentally controlled and continuously monitored animal room as described in Section V.5.1

V.5.1.2. Special Husbandry Provisions
No sugar-rich treats, marshmallows, peanut butter, cereal, crackers and similar food will be offered to these animals. Instead, any one of canned fruits, vegetables, yogurt and milk will be offered daily; age-appropriate nutritional supplement will be provided after irradiation. Tap water will be provided ad libitum. Pedialyte or other oral rehydration fluid will be offered when the animals show signs of dehydration.

V.5.1.3. Exceptions NA

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care
Animals will be acclimated for ≥1 week prior to beginning of procedures. Each animal will be observed at least twice daily for evidence of pain or distress. Body weights will be determined at least once a week and on days of blood collection. The time of each daily temperature collection will be recorded for each animal. Animals will be qualitatively assessed at least twice daily (a.m. and p.m.) for general behavioral

status and food consumption by VSD techs and/or research techs. The heart rate will be measured at the time of each blood collection. Stool consistency, left-over food, demeanor and overall animal appearance will be recorded. Diarrhea has not been observed in minipigs irradiated at doses <8 Gy. Appropriate standard operating procedures will be followed by staff veterinarians in the event of pain or distress.

V.5.2.2. Emergency Veterinary Medical Care Emergency veterinary medical care is available 24/7 and will be provided based on the restrictions approved in earlier sections of this protocol

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy
Enrichment will be done as per the VSD minipig enrichment SOP, except single housing after catheter implantation and irradiation.

V.5.3.2. Enrichment Restrictions
Minipigs will be single-housed after surgery and after irradiation until completion of the study, to avoid mechanical and infectious complications related to implanted catheters, but they will be in visual, olfactory and auditory contact with other minipigs in the same room.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				**1, 3b, 4, 5, 7 (drug delivery)**	(b)(6)
				1,2,3,4,7	
				1, 3	
				1,2,3b, 4, 5	
				1,2,3b, 5	

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)					(b)(6)
				1, 2, 3, 4, 5	
				3, 4	

Trained personnel are certified by VSD.
Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY NA

VIII. ENCLOSURES NA

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(b)(4),(b)(5)

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X. ASSURANCES

Protocol Title: Minipig model of H-ARS for drug screening protocols relevant to mass casualty-scale treatment

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of enesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment

(b)(6)

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (March 2013)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	3/4/2014	2nd Resubmission Date:	
Approved/**Returned for Revision**:	3/19/2014	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	3/21/2014	**EXPIRATION DATE:**	3/27/2017
Approved/Returned for Revision:	3/28/2014	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

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- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Characterization and validation of biomarkers of (b)(4) a promising radiation countermeasure, in mice (*Mus musculus*)

IV. PRINCIPAL INVESTIGATOR:

(b)(6) Ph.D. Date
(b)(6)
Radiation Countermeasures Program, AFRRI (b)(6)
Tel (b)(6) fax (b)(6)
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, Lt Col, USAF** Date
Head, Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date
Statistician
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date
Head, Veterinary Sciences Department, AFRRI
Telephone (b)(6) Fax: (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date
Environmental Health and Safety Department, USUHS
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**
IACUC Chair (b)(6)
Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: Characterization and validation of biomarkers of (b)(4) a promising radiation countermeasure, in mice (*Mus musculus)*

PRINCIPAL INVESTIGATOR:

(b)(6) Ph.D. Date
(b)(6)
Radiation Countermeasures Program, AFRRI, (b)(6)
Tel (b)(6) fax (b)(6)
(b)(6)

CO-INVESTIGATOR(S): N/A**AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER:** (b)(6)

I. NON-TECHNICAL SYNOPSIS: The threat of a limited radiological attack on American cities (i.e. a "dirty" bomb), or a nuclear accident, requires the development of a radiation countermeasure capable of protecting the hematopoietic system and gastrointestinal (GI) tract of military personnel, who might have to enter a contaminated area, or capable of mitigating radiation lethality in the general population. At present there is no effective treatment for the acute radiation syndrome. (b)(4) (b)(4) promotes recovery of radiation damaged cells by stimulating DNA repair and cell survival pathways. Studies conducted at AFRRI (b)(4) have shown promising efficacy of (b)(4) both as a protector and a mitigator of acute radiation syndrome (ARS) in mouse models. Clinical studies in humans support an excellent safety profile of (b)(4) In the current protocol the main focus is to optimize the dose of (b)(4) to mitigate the effects of radiation by monitoring the biomarkers, and to conduct efficacy study in mice. For efficacy study, drug will be tested by administering it through subcutaneous and oral routes. Drug will be evaluated in two different strains of mice.

II. BACKGROUND

II.1. Background:

Military and civil defense organizations have an urgent requirement for medically effective radiation countermeasures, including radiation protectors, mitigators and therapeutics. Radiation countermeasures that mitigate the harmful effects and promote recovery from radiation injury will have stringent requirements in terms of lack of toxicity, ease-of-use for administration, storage at environmental temperatures, suited for administration by non-medical personnel, and capability of enhancing survival in the absence of ancillary medical support. Although such efforts were initiated more than half a century ago, no safe and effective radiation countermeasure has been approved by the United States Food and Drug Administration (FDA) for ARS (1, 10, 19).

Scientists at (b)(4) the Armed Forces Radiobiology Research Institute (AFRRI) (b)(4) (b)(4) have conducted extensive studies to demonstrate radiation protecting and mitigating effects of injectable (subcutaneous, sc)

and oral (po) [(b)(4)] Safety and tolerability of [(b)(4)] have been established in GLP-compliant nonclinical studies and in 4 clinical safety trials in healthy adults. These results are published in peer-reviewed journals [(b)(4)] and included in 2 active U.S. FDA IND exemptions [(b)(6)] [(b)(4)] mitigates deleterious radiation effects by enhancing DNA damage repair pathways, rather than intervening in the free-radical cascade. Thus, its window of effectiveness is relatively long, extending from a day before to a day or more after exposure to radiation.

Preliminary studies on biomarker analysis in mouse tissue specimens using Nano-immunoassay: A novel analytical method termed, Nanoscale Immuno Assay (NIA) is capable of rapidly and reproducibly quantifying proteins and their phosphorylation states in very small nonclinical and clinical specimens and has been developed by [(b)(4),(b)(6)] NIA assays have been validated to measure proteins that mediate cell survival, proliferation, cell cycle and apoptotic pathways. Preliminary studies were conducted in mice dosed orally with either

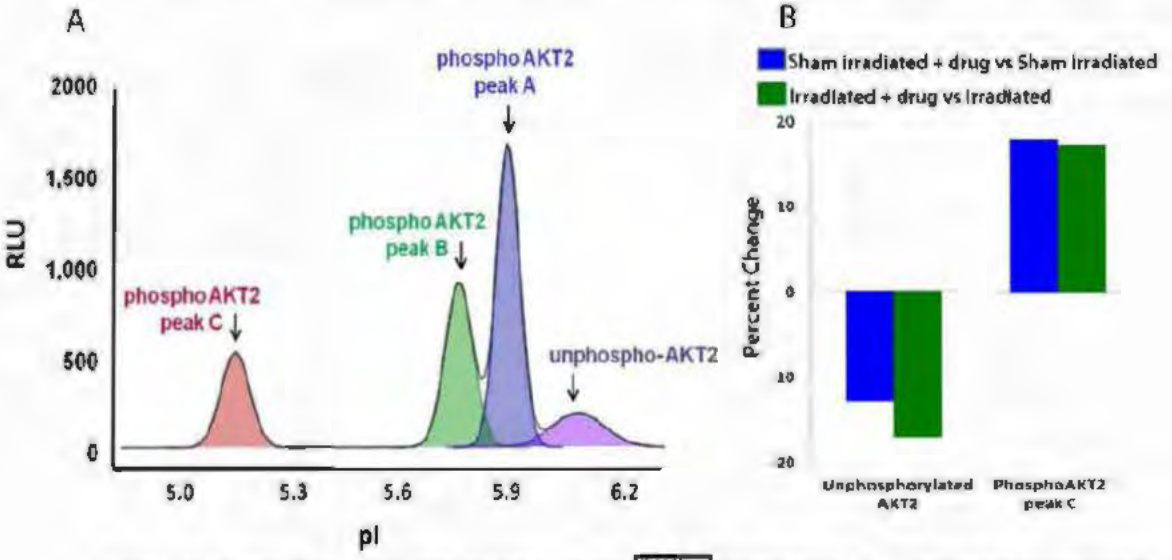

Figure 1. NIA analysis of AKT levels in mouse bone marrow +/- [(b)(4)] administration. A) NIA reveals three phosphorylated peaks of AKT2 in mouse bone marrow (control); **B)** [(b)(4)] treatment *in vivo* induces similar AKT profiles in bone marrow from sham-irradiated and irradiated mice (AKT2 profiles, correlation = 0.94)

vehicle or drug with or without radiation. After 24 h post-irradiation administration of drug, mice were sacrificed, bone marrow was removed and the cell lysates were analyzed by NIA. The data in **figure 1A** show that the unphosphorylated AKT and the various forms of phosphorylated AKT (A, B and C) were successfully resolved and quantified by NIA. The data in **figure 1B** confirms the up-regulation of Akt2 in bone marrow samples from mice treated with [(b)(4)] with or without radiation. Simultaneously, there is a corresponding down regulation in unphosphorylated AKT. Importantly, correlation in percent change from baseline in AKT2 isoforms between "sham irradiated plus drug" and "irradiated plus drug" was 0.94. This innate effect of drug on the unphosphorylated levels of AKT and p-AKT, in presence or absence of radiation, could be used to correlate the efficacy of drug in pivotal mouse and NHP studies in therapeutic settings. These preliminary studies demonstrate the potential

value of p-AKT2 as a biomarker to follow the drug effect in the absence of radiation during pivotal safety studies in healthy human volunteers.

Understanding changes in signaling and correlating these changes with the mechanism of action provides a path to meeting the challenges of drug approval under the Animal Rule. Results herein provide a promising avenue towards this goal. Additional studies and confirmation under different scenarios, along with validation which is a part of this proposal, may permit correlation of an informative biomarker with expected efficacy parameters.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

Best effort has been made to find any possibility for duplication. The following data bases have been searched:

a. PubMed
b. BRD – DOD Biomedical Research Database
c. RePORTER

II.2.2. Date of Search

Pubmed- 12-20-2013
BRD- 12-20-2013
RePORTER- 12-20-2013

II.2.3. Period of Search

PubMed - 1965-2013
BRD - 1998-2013
FEDRIP– 1992-2013

II.2.4. Key Words of Search

1. (b)(4) AND radiation
2. [redacted] AND mice
3. [redacted] AND mice AND radiation
4. [redacted] AND radiation AND efficacy
5. [redacted] AND pancytopenia
6. [redacted] AND bone marrow
7. Tween-80 AND mice
8. Tween 80 AND mice AND radiation

II.2.5. Results of Search
PubMed

Search string 1: We found 9 references, which did not address the questions to be tested in this protocol.
Search string 2: We found 6 references, none of which addressed the questions to be tested in this protocol.
Search sting 3: We found 6 references, none of which addressed the questions to be tested in this protocol.

Search string 4: We found 2 references which does not address the questions to be tested in this protocol.
Search string 5: We found 0 references.
Search String 6: We found 3 references, none of which addressed the questions to be tested in this protocol.
Search String 7: We found 3 references, none of which addressed the questions to be tested in this protocol.
Search String 8: We found 22 references, none of which addressed the questions to be tested in this protocol.

BRD

Search string 1: We found 11 references, none of which addressed the questions to be tested in this protocol.
Search string 2: We found 3 references, none of which addressed the questions to be tested in this protocol.
Search sting 3: We found 3 references, none of which addressed the questions to be tested in this protocol.
Search string 4: We found 3 references, none of which addressed the questions to be tested in this protocol.
Search string 5: We found 1 reference, which does not address the questions to be tested in this protocol.
Search String 6: We found 1 reference, which does not address the questions to be tested in this protocol.
Search String 7: We found 569 references, none of which addressed the questions to be tested in this protocol.
Search String 8: We found 1 reference, which does not address the questions to be tested in this protocol.

RePORTER

Search string 1: We found 0 references.
Search string 2: We found 0 references.
Search sting 3: We found 0 references.
Search string 4: We found 0 references.
Search string 5: We found 0 references.
Search String 6: We found 0 references.
Search String 7: We found 70 references, none of which addressed the questions to be tested in this protocol.
Search String 8: We found 23 references, none of which addressed the questions to be tested in this protocol.

Initial studies investigating the efficacy and mechanism of (b)(4) have been conducted in mice and in-vitro models. Efficacy studies with radiation doses ranging from 5 - 15 Gy have been conducted. Previous results demonstrated that mice injected with the drug subcutaneously at one dose at -24h and -15 min prior to TBI resulted in significant protection of mice. These preliminary studies suggested that (b)(4) is a promising radiation countermeasure and warrants continued development. Previous studies have not systematically determined the optimal dose and schedule of

administration using biomarkers and oral route of administration. In this study we will be using biomarkers, identified from the *in vitro* cell-based studies, to optimize the dose and schedule of administration of (b)(4) and to correlate the biomarker levels with its efficacy. In addition we are evaluating the oral route of administration to determine the efficacy of (b)(4) We believe that this is a systematic approach to optimize the dosing regimen, evaluate the efficacy of (b)(4) and validate the biomarkers at the same time. Overall, the search did not reveal any duplication and the proposed study would provide valuable information about the development of (b)(4) as a radiation countermeasure.

III. OBJECTIVE/HYPOTHESIS:

In the current protocol we are proposing to utilize the biomarkers to optimize dose and schedule of administration and validate these markers in mouse models of radiation protection. Validation of biomarkers is a critical step in the development of radiation countermeasures since human efficacy studies are neither ethical nor feasible. FDA guidance on "Animal Rule" (21 CFR 314.600 for drugs) states, "The data or information on the pharmacokinetics and pharmacodynamics of the product or other relevant data or information, in animals and humans, allows selection of an effective dose in humans." (21 CFR 314.610(a) (1)-(4); 21 CFR 601.91(a)(1)-(4)). Hence, a validated biomarker will be a critical milestone for optimizing the effective dose in humans (1, 23).

We plan to optimize the dose and schedule of oral administration of (b)(4) in mouse models using biomarker expression in bone marrow (BM) and peripheral blood mononuclear cells (PBMCs). Using these optimal conditions, we will evaluate the efficacy of (b)(4) in mouse models of radiation protection and also validate the biomarkers. The main objectives are (a) optimize the dose and schedule of administration based on biomarker response; (b) demonstrate drug dose-response in enhancing survival and hematopoietic recovery; (c) validate the biomarkers identified from cell line studies; d) determine dose reduction factor (DRF) at optimum (b)(4) dose and schedule; and e) evaluate the effectiveness of (b)(4) in two additional mouse strains (C57BL/6 and CD2F1), to address the genetic heterogeneity such as encountered in human populations.

IV. MILITARY RELEVANCE

Currently, there are no FDA approved pharmaceutical agents that can prevent or treat injury from external ionizing radiation. The problem has become more acute in recent times due to the unpredictable nature of terrorist attacks. Recognizing this urgent need, the U.S. Department of Defense has assigned top priority to the "development of medical countermeasures to radiation exposure" against both early and late arising health effects. These concerns imply the urgent need to develop an appropriate countermeasure for radiation injuries potentially sustained by war fighters during combat operations and enable commanders to make judicious decisions in troop deployment. There are several drugs at different stages of the development. This study will perform essential research and development of a promising new agent (b)(4) as a therapy for potential injuries sustained by military personnel who have been accidentally exposed to ionizing radiation.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

We propose to do the following experiments to utilize biomarkers of (b)(4) treatment to optimize dose and schedule of administration. Optimal dose and schedule of (b)(4) administration will be used to evaluate its in protecting mice from total body irradiation and thus validate the biomarkers. Details of subsequent experiments will be based on results obtained in previous experiment.

V.1.1. Experiment 1. Dose optimization of (b)(4) using biomarker levels in C3H/HeN mice: In this experiment our main objective is to analyze biomarker (p53, mdm2, p21, p73, c-abl, chk1, pAKT, pGsk-3, pS6, pMAPK, pRAF, PTEN etc) levels, identified from the cell-based assays, in bone marrow (BM) and peripheral blood mononuclear cells (PBMCs) of mice as a function of (b)(4) dose response. C3H/HeN mice will be dosed orally with (b)(4) (Low = 250 mg/kg, medium = 500 mg/kg; high = 750 mg/kg body weight) along with appropriate vehicle controls at -24 h and -15 minutes prior to TBI (LD50/30). Following groups will be included in the study: 1) Radiation alone, 2) Vehicle control (oral) + Radiation, 3) Radiation +250 mg/kg of drug (oral), 4) Radiation+ 500 mg/kg of drug (oral), 5) Radiation + 750 mg/kg of drug (oral), 6) Drug alone at 250 mg/kg (oral), 7) Drug alone at 500 mg/kg (oral), 8) Drug alone at 750 mg/kg (oral), 9) Vehicle alone. Mice will be sacrificed 24 and 48 h after final drug administration to collect BM and PBMCs. Biomarker levels will be analyzed by Western-blot. Radiation dose for this study will be 7.5 Gy (TBI). We expect the biomarker level to be responsive to (b)(4) administration in a dose dependent manner both in the presence and absence of radiation. Earlier studies indicate optimal dose is 500 mg/kg. (b)(4)

C3H/HeN mice needed: 9 treatment groups x 6 mice per group x two time points (24 and 48 h) = 108

V.1.2. Experiment 2. Biomarker-guided optimization of schedule of (b)(4) administration in C3H/HeN mice: The objective here is to optimize the schedule of administration of (b)(4) using biomarker levels in BM and PBMCs and the optimal dose determined from the previous experiment. Previous studies have used -24 h and -15 min prior to TBI to administer (b)(4) by sc route. We will explore administering multiple doses of (b)(4) prior to TBI to determine the effect on biomarker levels in C3H/HeN mice and determine the optimal schedule of administration. Optimal dose of (b)(4) as determined from the above experiment will be administered orally to C3H/HeN mice (6 mice per group + appropriate vehicle controls for each time point) at 1) -24 h and -15 min; 2) -24 h, -12 h and -15 min; 3) -24 h, -18 h, -12 h and -15 min; 4) -24 h, -18 h, -12 h, -6 h and -15 min and 5) vehicle control for each schedule administered before TBI (7.5 Gy). Mice will be sacrificed to collect BM and PBMCs at 24 and 48 h after final drug administration. BM and PBMC cell pellets will be lysed for biomarker analysis by Western-blot.

C3H/HeN mice needed: 2 treatments groups (drug and vehicle) x 5 treatment regimens x 6 mice per group x 2 time points = 120 mice

V.1.3. Experiment 3. Experiment 3. Dose response survival study and validation of biomarkers in C3H/HeN mice: To determine survival efficacy as a function of oral drug dose post-TBI and to correlate with biomarker levels from previous experiments, C3H/HeN mice will be dosed orally with optimal dose of (b)(4) and one dose below and one dose above the optimal dose, along with appropriate vehicle controls, at the optimal schedule as determined from above experiment. Following groups will be included in the study: 1) Radiation alone, 2) Vehicle control for lower dose of (b)(4) (oral) + Radiation, 3) Radiation + (b)(4) dose lower than optimal dose (oral), 4) vehicle control for optimal drug dose + radiation, 5) Radiation+ optimal dose of (b)(4) (oral), 6) vehicle control for higher drug dose + radiation, 7) Radiation + (b)(4) dose higher than optimal dose (oral), and 8) subcutaneous (sc) administration of drug at 500 mg/kg body weight and radiation as positive control. Mice will be monitored for survival for 30 d post-irradiation. Radiation dose for this study will be 7.5 Gy (TBI).

C3H/HeN mice needed: 8 treatment groups x 16 mice per group x 2 experiments = 256

V.1.4. Experiment 4. Dose reduction factor (DRF) survival study at optimum dose and schedule in C3H/HeN mice: The objective of this experiment is to determine the dose reduction factor (DRF) at the optimal oral dose and schedule determined from above studies along with appropriate vehicle controls. C3H/HeN mice (16 mice per group) will be administered drug prior to TBI at 6 doses (6 different doses of radiation between 6 and 11 Gy, sub-lethal to 100% lethal for both sets of mice) will be selected for vehicle and drug treated animals. We have published such studies earlier with several agents (b)(4) Mice will be monitored for survival for 30 d.

C3H/HeN mice needed: 2 treatments groups (drug and vehicle) x 6 radiation doses x 16 mice per group = 192)

V.1.5. Experiment 5. Hematopoietic rescue study in C3H/HeN mice

To evaluate hematopoietic rescue by oral (b)(4) administration in C3H/HeN mice, mice (6 mice per group per time point) will be exposed to sublethal TBI dose (4 Gy), followed by optimal oral dose regimen of (b)(4) (+ appropriate vehicle controls). Hematopoietic response will be assessed by peripheral blood cell counts and by the number of surviving BM progenitors committed to the myeloid lineage of differentiation. Peripheral blood will be collected from posterior vena cava using 1 ml syringe and 23 G needle (please also see section V.4.4.2. Biosamples) and counted at 3, 5, 7, 14, 21 and 28 d after TBI. We have published such results with other radiation countermeasures (17). Values for vehicle and drug alone (no radiation) at 3 d will serve as baseline controls.

C3H/HeN mice needed: (2 treatments groups (drug and vehicle) x 6 time points x 6 mice per group + 12 animals for baseline control without radiation – with and without drug – 6 each) x 2 experiments = 168

V.1.6. Experiment 6. Radiation dose survival study in C57BL/6 and CD2F1 mice strains: To determine radiation dose-response on oral (b)(4) efficacy in two other mouse strains to address genetic heterogeneity, C57BL/6 mice (16 mice per group) and CD2F1 mice (16 mice per group) will be exposed to varying doses of radiation including $LD_{30/30}$, $LD_{50/30}$, and $LD_{70/30}$ (will be determined based on results above experiment # 4

for DRF) after oral administration of (b)(4) or vehicle at optimal dose and schedule. Mice will be scored for survival for 30 d.

C57BL/6 and CD2F1 mice needed: 2 treatments groups (drug and vehicle) x 3 radiation doses x 16 mice per group x 2 experiments = 192 of each strain

V.1.7. Experiment 7. Hematopoietic rescue study in C57BL/6 mice and CD2F1 mice strains: To confirm the hematopoietic rescue by oral (b)(4) in two other mouse strains to address genetic heterogeneity, C57BL/6 mice (6 mice per time point) and CD2F1 strain (6 mice per time point) will be exposed to sub-lethal dose of radiation after oral administration of (b)(4) or vehicle at optimal dose and schedule. Peripheral blood cell counts and the number of surviving BM progenitors committed to the myeloid lineage of differentiation will be determined at 3, 5, 7, 14, 21 and 30 d after TBI as described above under experiment 5. Values for vehicle and drug alone (no radiation) at 3 d will serve as baseline controls.

C57BL/6 and CD2F1 mice needed: (2 treatments groups (drug and vehicle) x 6 time points x 6 mice per group + 12 animals for baseline control without radiation – with and without drug – 6 each) x 2 experiments = 168 of each strain

Total C3H/HeN mice needed for this Protocol:

Experiments	Total	C	D	E
Experiment 1	108		48	60
Experiment 2	120	0	0	120
Experiment 3	256	0	0	256
Experiment 4	192	0	0	192
Experiment 5	168	0	24	144
Sub total	844	0	72	772
For training and technique establishment, odd weight (5%)	42	0	42	
Total C3H/HeN mice required for this protocol	886	0	114	772

Total C57BL/6 mice needed for this Protocol:

Experiments	Total	C	D	E
Experiment 6	192	0	0	192
Experiment 7	168	0	24	144
Sub total	360	0	24	336
For training and technique establishment, odd weight (5%)	18	0	18	0
Total C57BL/6 mice required for this protocol	378	0	42	336

Total CD2F1 mice needed for this Protocol:

Experiments	Total	C	D	E
Experiment 6	192	0	0	192

Experiment 7	168	0	24	144
Sub total	360	0	24	336
For training and technique establishment, odd weight (5%)	18	0	18	0
Total CD2F1 mice required for this protocol	378	0	42	336

Unirradiated animals used for blood collection under anesthesia have been placed under category D.

V.2. Data Analysis

Mean with standard errors, or percentages, will be reported if applicable. The significance level will be set at 5% for each test. Under the similar situations for a given experiment, two independent replicates of data will be pooled. Poolability will be assessed. Statistical software, PC SAS will be used for statistical analyses.

Experiment 1: Analysis of variance (ANOVA) will be used to detect if there is a significant difference among the groups for a biomarker. If significant, Student-Newman-Keuls multiple range test will be used to do all the possible pair-wise comparisons to identify which group is significantly different from the other. A two-way ANOVA will be used to detect if there is any significant difference between the two replicated experiments across the groups. If not significant, then we will pool the two replicated experiments for each group for our statistical analysis. If we can pool them (as expected), then the sample size per group (N=16) would have 80% power to detect a significant difference between two groups if any, given type I error of 5% and delta/sigma has at least 1.03, where the delta is the estimated mean differences between the two groups and sigma is the standard deviation of the ANOVA model (14).

Experiment 2, 3 and 6: Comparison of survival curves will be made using the log-rank test. Fisher's exact test will be used to detect if there is a significant difference across the groups in survival rates. N = 16 mice per group would have 80% power to detect a significant difference between two groups, given type I error of 5%, a treatment group survival rate of at least 79%, and vehicle group survival of 25%. A similar statement would apply, provided the treatment group survival was at least 84%, 88%, or 91%, if the vehicle group survival was 30%, 35% or 40%, respectively (*52*).

Experiment 4: Probit analysis will be used to estimate the DRF. The DRF will be estimated as the ratio of the radiation LD-50/30 for mMPc versus vehicle, and 95% confidence interval will be derived.

Experiment 5 and 7: Analysis of variance (ANOVA) will be used to find overall significant differences among treatment groups. If a difference is significant, pair-wise Tukey-Kramer comparisons will be used to identify specific differences between groups. Similarly additional data will be analyzed.
The eight mice per group for each experiment, N=8 per group will have 80% power to detect any significant difference between groups if any, given type I error of 5% and delta/sigma has at least 1.6, where the delta is the estimated mean differences between the two groups and sigma is the pooled standard deviation of the two groups [26].

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

Both computer models and tissue culture have been considered as alternatives to animals. These alternatives lack the knowledge base and complexity needed to evaluate the effects of radiation and radiation countermeasures on the immune system. The phenomena under study involve complex information-processing networks comprising large number of cell types and biological signal transduction molecules. Responses to irradiation and radioprotective agents involve interactions between the central nervous system, the peripheral nervous system including the enteric nervous system, endocrine glands, the immune and inflammatory systems, hematopoietic system, gastrointestinal system etc. We do not know all of the cell types and tissues involved and many of the signal transduction molecules have not been discovered yet. Even if all of the tissues, cell types and signaling molecules were known, the present state of tissue culture would be incapable of reproducing their *in vivo* relationships. One would have to reproduce much of an entire mammalian organism in culture to study these phenomena *in vitro*. As for computer models, the most powerful supercomputers available in the near future would be incapable of analyzing interactions between so many elements in the network. Thus, these drugs necessitate investigation in whole animal models to see overall outcome.

V.3.2. Animal Model and Species Justification: Evaluation of radiation countermeasures in *in vivo* model is the only option available for identifying clinically safe and non-toxic radiation countermeasures. The murine model is immunologically the most well defined animal model for research. The murine model is also the most studied animal model, and has significant immunological similarity to higher animals and humans. This model is also preferred over any other *in vivo* model due to readily available immunological and molecular biology research reagents. AFRRI staff has extensive experience with several strains of mice in radiation injury and radioprotection research. PI has extensive experience with C57BL/6, CD2F1, and C3H/HeN mice. We will be using C3H/HeN, C57BL/6, and CD2F1 mice for selected experiments in this protocol. (b)(4) is effective against moderately low dose of radiation and has been evaluated earlier only in C3H/HeN strain of mice (mostly sc route, few expt with oral route) as stated above. C3H/HeN is comparatively radiosensitive strain. Its LD_{50} value is lower than C57BL/6 and CD2F1. We are interested to conduct limited evaluation in two other strains (C57BL/6 and CD2F1 – comparatively radioresistant) of mice. Such study will demonstrate that this drug efficacy is not strain specific or efficacy is limited to radiosensitive sensitive strains.**V.3.3. Laboratory Animals**

V.3.3.1. Genus/Species

Mus musculus, Laboratory mouse

V.3.3.2. Strain/Stock

C3H/HeN, C57BL/6, and CD2F1

V.3.3.3. Source/Vendor

C3H/HeN, C57BL/6, and CD2F1 mice will be purchased from a VSD approved vendor (the preferred vendor for C57BL/6 is Jackson Laboratory, 600 Main St., Bar

Harbor, Maine 04609-1500; Tel: 207-288-5845; State of Maine animal welfare license is R012, the preferred vendor for C3H/HeN and CD2F1 mice is Harlan Labs Indianapolis, Indiana 46229 – 0176; USDA License Number 32-A-0083).

V.3.3.4. Age

6-12 weeks old

V.3.3.5. Weight

20 – 25 g

V.3.3.6. Sex

Male

Justification for using only male mice: Previous studies have been performed using male mice, so males are needed for data comparison. Also there is possibility that female hormone variation may interfere with our results. To see the effect of this drug in both sexes, we need to repeat such experiments in females. To minimize the number of animals, and to keep variables minimized, it was decided that the study will be performed only in one sex (male).

V.3.3.7. Special Considerations

Commercially procured animals must be adventitious disease free, including: *Pseudomonas aeruginosa,* and *Pasteurella.* All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus, *Mycoplasma pulmonis*, *Clostridium piliforme, and C. difficile.* Mice should also be endoparasite- and ectoparasite-free.

V.3.4. Number of Animals Required (by species)

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement

Moribund animals will be euthanized immediately to minimize the magnitude and duration of suffering those animals might experience when they are subjected to toxic effects of ionizing radiation. Under such situation moribundity will be used as a surrogate for death in animals and will be considered to have arrived at the endpoint. Pain experienced by injection will be minimized by the use of higher gauge needles.

V.3.5.2. Reduction

As we move forward with experiments, plans will be reevaluated at every stage of the experiments, in light of incoming data in an attempt to reduce the numbers of experiments and groups, if possible. If deemed not necessary based on incoming data, some investigations may not be carried out to optimize the use of experimental animals.

V.3.5.3. Replacement

It is not feasible to use non-animal systems in place of *in vivo* animal models to address the research questions proposed in this IACUC protocol.

V.4. Technical Methods

V.4.1. Pain/Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C.....0

V.4.1.1.1.2. Column D.....198

V.4.1.1.1.3. Column E.....1,444

V.4.1.1.1.4. Pain Category Assignments

Experiments	Total	C	D	E
Experiment 1	108	0	48	60
Experiment 2	120	0	0	120
Experiment 3	256	0	0	256
Experiment 4	192	0	0	192
Experiment 5	168	0	24	144
Experiment 6	384	0	0	384
Experiment 7	336	0	48	288
Sub total	1,564	0	120	
For training & technique establishment, odd weight (5%)	78	0	78	0
Total mice required for this protocol	1,642	0	198	1,444

Unirradiated animals used for blood collection under anesthesia have been placed under category D

V.4.1.2. Pain Relief/Prevention

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization

Since anesthesia, analgesia, and tranquilizers are known to affect functions of the immune system of animals, we do not plan to use analgesic/anesthetic agents during course of the experiment after irradiation (2, 5, 6).

Animals in experiments involving blood/tissue collection (different organs) will be deeply anesthetized using isofluorane before blood collection. Next, animals will be euthanized by exsanguination with confirmation by cervical dislocation before tissue collection.

V.4.1.2.2. Pre- and Post-procedural Provisions

Mice will be housed under standard conditions both before and after radiation procedures in accordance with VSD mouse Standard Operating Procedure (see section V.5).

V.4.1.2.3. Paralytics

No paralytic agent will be used in this study.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched

AGRICOLA
PubMed
IVIS

V.4.1.3.2. Date of Search

Agricola-12/19/2013
PubMed-12/19/2013
IVIS-12/19/2013

V.4.1.3.3. Period of Search

ARGICOLA -1966-2013
PubMed - 1965-2013
IVIS - 1998-2013

V.4.1.3.4. Key Words of Search

(b)(4) AND "Pain"
"Ionizing radiation" AND "pain"
"Ionizing radiation" AND "mice"
"Ionizing radiation" AND "alternatives"
"Ionizing radiation" AND "toxicity"

V.4.1.3.5. Results of Search

(b)(4) AND "Pain"

AGRICOLA: 0
Pubmed: 0
IVIS: 0

"Ionizing radiation" AND "pain"

AGRICOLA: 0
Pubmed: 451
IVIS: 21

"Ionizing radiation" AND "mice"

AGRICOLA: 23
Pubmed: 3543
IVIS: 15

"Ionizing radiation" AND "alternatives"

AGRICOLA: 2
Pubmed: 135
IVIS: 2

"Ionizing radiation" AND "toxicity"

AGRICOLA: 20
Pubmed: 4729
IVIS: 16

No reports were found indicating administration of (b)(4) causes pain. Similarly, there were no reports indicating that irradiation itself causes pain. It has been reported that whole-body irradiation can cause sedative effect, as indicated in the tail-flick test in rats.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification

As stated above, irradiated mice succumb to a compromised immune response. In the event that there is pain and distress for animals, as we have already discussed under above headings, we cannot give anesthetic/analgesic agents to the animals since these agents interact with the immune system, and in turn will affect experimental results.

There are conflicting reports regarding effects of buprenorphine on the immune system (3, 9, 11, 12, 14-16, 24). A majority of reports suggest that buprenorphine influences immune response. Since buprenorphine has been shown to influence various arms of cellular and humoral immune response (3, 12, 16, 24), we have decided not to use it in our study. Non-steroidal anti-inflammatory drugs are not suitable for use in such experiments (13).

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure(s): N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures

V.4.3.6.1. Procedures:

Irradiation: Mice will be placed in a perforated and well ventilated acrylic plastic restrainer (maximum 8 mice in one restrainer). These mice will be exposed to desired dose of gamma radiation from bilaterally positioned cobalt-60 sources.

V.4.3.6.2 Scientific Justification: N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections

(b)(4) will be administered sc with 23 G needle in the nape of the neck. The maximum volume for sc administrations will be 0.20 ml per 20 - 25 g animal.

(b)(4) given *p.o.* will be administered in a 0.2-ml volume with a 1-ml syringe and a 20-gauge feeding canula, which has a ball tip, or a disposable feeding needle, which has a silicone rubber tip. One sterile syringe and needle will be used for mice in each cage. The feeding needle will be wiped and disinfected between inoculations on a gauze sponge that is moistened with 70% ethanol (absolute alcohol diluted to 70%) to reduce the microorganisms and saliva on the needle as an extra precaution even though the mice share the sipper tube in the water bottle and oral microorganisms as well. First p.p. experiment will be conducted in the presence of a veterinarian/vet tech.

V.4.4.1.1. Pharmaceutical Grade Drugs:

Currently (b)(4) is in Phase 1 clinical trials for safety and toxicity. (b)(4) (b)(4) for administration in healthy volunteers. We will be using the same formulation in this study.

(b)(4) (b)(4) All aspects of the solution preparation involve GLP grade formulation.

V.4.4.1.2. Non-Pharmaceutical Grade Drugs: N/A

V.4.4.2. Biosamples

Animals in experiments involving blood/tissue collection (different organs) will be deeply anesthetized using isofluorane before blood collection (posterior vena cava). Next, animals will be euthanized by exsanguination with confirmation by cervical dislocation before tissue collection tissue (jejunum, sternum and femur bones). GI tract, sternum and femur bones will be collected post-mortem for histopathology and bone marrow analysis for biomarkers. Blood will be used for CBC, blood chemistry, citrulline, and biomarker analysis.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAb) Production: N/A

V.4.4.5. Animal Identification

Each cage will have a cage card. In addition to cage card, another experimental card will be displayed before starting the experiment. One cage will have mice of only one treatment/control group. Each card will state the investigator's name, protocol number, experiment number, cage number, start date, end date, species, item number (animal lot), birthdate, gender, strain, number of animals in the cage, vendor, arrival date, treatments, and dates of death, including the initials of the staff person who found and removed the dead animals.

V.4.4.6. Behavioral Studies: N/A**V.4.4.7. Other Procedures: None**

V.4.4.8. Tissue Sharing:

Bone marrow/PBMC samples will be sent to Dr. (b)(6) (b)(6) for Nanoscale Immuno Assay (NIA) for validation of biomarkers. Once protocol is approved, tissue sharing document will be initiated.

Unused biological samples will be retained for additional studies. Studies planned will depend upon additional funding for supplies and staff in future.

V.4.4.9. Animal By-Products: N/A

V.4.5. Study Endpoint

In all experiments involving blood/tissue collection, mice will be deeply anesthetized using isofluorane before blood collection (posterior vena cava). Next, animals will be euthanized by exsanguination with confirmation by cervical dislocation before tissue (jejunum, sternum and femur bones) collection at a preset time as described under experimental design section. All mice that have survived beyond experimental duration (30 day post-irradiation) or for moribund mice, if any, will be euthanized by CO_2 followed by cervical dislocation. Naïve mice, if not used in the experiments, will be transferred to another approved protocol, if possible, or euthanized. Animals in these studies may become morbid and hence it is critical to define criteria for euthanizing mice humanely without compromising the study objective and complying with the IACUC recommendations stipulated in Policy Letter #10. Mice will be considered morbid based on criteria described in policy # 10. Experimental mice that are found morbid, but not moribund, will be allowed to continue in the experimental protocol. Once mice are considered morbid, we will monitor them more than twice daily, early morning and late afternoon, and evening.

Mice found moribund will be euthanized by trained and experienced personnel listed on this protocol or by trained/experienced VSD staff. Compressed CO_2 gas in a cylinder with a regulator will be used for euthanasia in accordance with the current American Veterinary Medical Association (AVMA) Guidelines on Euthanasia - 2013. Cervical dislocation will be done following CO_2 overdose as a secondary method of euthanasia. Mice will be considered moribund based on criteria described in policy # 10.

V.4.6. Euthanasia

As already mentioned above under various headings, in all experiments involving blood/tissue collection, mice will be deeply anesthetized using isofluorane before blood collection (posterior vena cava). Next, animals will be euthanized by exsanguination with confirmation by cervical dislocation before tissue collection. Terminal CO_2 euthanasia will be used for mice that have survived beyond experimental duration (30 day post-irradiation) or for moribund mice, if any. Compressed CO_2 gas in a cylinder with a regulator will be used for euthanasia in accordance with the current American Veterinary Medical Association (AVMA) Guidelines on Euthanasia - 2013.

VIII. Appendix A: Clinical Observations with Criteria for Euthanasia (Rodent)

DATE:	Time:	Animal ID#	Score
Appearance:			
	Normal (smooth coat, clear eyes/nose)		0
	Hunched and/or fluffed		1
	Ocular discharge, and/or edema		3
	Emaciated, dehydrated (skin tent) **		5
	Soft stools (fecal matter around anus)		5
	Bloody diarrhea		9
Respiratory rate:			
	Normal breathing		0
	Increased breathing (double normal rate, rapid, shallow)		6
	Abdominal breathing (gasping +/- open mouth breathing)*		12
General Behavior:			
	Normal (based on baseline observations)		0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)		1
	Decreased mobility		2
	Ataxia, wobbly, weak**		6
	Inability to stand*		12
Provoked Behavior:			
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))		0
	Subdued; responds to stimulation (moves away briskly)		1
	Subdued even to stimulation (moves away slowly)		3
	Unresponsive to gentle prodding **		6
	Does not right when placed gently on side within 5 seconds*		12
TOTAL			_____

Definitive criteria for morbidity:
Weight Loss up to 10% baseline (if available)**
Pale, white mucous membranes/ skin * *
Fever >104F/40C (if available)**

Definitive criteria for moribundity:
Weight Loss > 20% OR <15g (adult mouse)*
Blue mucous membranes/ skin (cyanosis)*
Cool to the touch or <86F/30C (if available)*

**** Regardless of score, notify appropriate person immediately.**

*** Regardless of score, immediately euthanize (death is imminent)**

Score:
< 6 Normal
6 - 9 Morbid: Monitor at least 3 times per day; notify appropriate personnel immediately
> 10 Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*)
Any single criteria of 12* euthanize immediately; consider as 'found dead'

(Note: This form does not have to filled out for every individual at every observation, so long as the criteria are used in informing decisions on increased monitoring and/or euthanasia. This form may be used to make a spreadsheet for greater ease of multiple animal observations, as long as the criteria and numbering system are exactly copied.)

V.5. Veterinary Care

V.5.1. Husbandry Considerations

No special husbandry consideration needed.

V.5.1.1. Study Room:

As assigned by VSD.

V.5.1.2. Special Husbandry Provisions: None

V.5.1.3. Exceptions:

Mice will be socially (group) housed, except where there is a possibility that during survival studies cage mates may be lost through attrition and for a period of time the sole surviving mouse may remain singly housed until the end of the 30-day study.

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care

As per Standard Operating Procedures, VSD staff will monitor all animals at least twice daily during morning and afternoon rounds. Any abnormalities will be reported immediately to the on-call veterinarian and PI. In addition, animals will be observed daily by this protocol's research staff. Any morbid mice will be observed twice a day (early morning and late afternoon) by the research staff. PI staff will be responsible for before and after working h observations of morbid mice. Any moribund mice will be euthanized as soon as possible. If needed, VSD veterinarian will be contacted for decision.

V.5.2.2. Emergency Veterinary Medical Care

Emergency veterinary medical care will include immediate euthanasia of any moribund mice.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

Mice will be group housed and enrichment will be provided as per the VSD rodent enrichment SOP.

V.5.3.2. Enrichment Restrictions: None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

Following employees are trained for mice work. They have handled mice work of earlier protocols at AFRRI.

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1,3,4,5,7	(b)(6)
				1,3,4,5	
				1,3,4,5	
				1,3,4,5	
				1,3,4,5	
				1,3,4,5,7	
				1,3,4,5,7	
				1,3,4	
				1,3,4	
				1, 2	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a-ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY

There is no plan to use any potential biohazard. Standard laboratory safety precautions will be observed throughout this study

VIII. ENCLOSURES

Form 310

List of references

X. ASSURANCES

Protocol Title: Characterization and validation of biomarkers of (b)(4) a promising radiation countermeasure, in mice (*Mus musculus)*

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard/Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures/manipulations/observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I (**AM**) / **AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / (**WILL NOT**) be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) _____ _____

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

References

1. **Aebersold, P.** 2012. FDA Experience with Medical Countermeasures under the Animal Rule. Adv. Prev. Med. **2012**:507571.
2. **Beilin, B., Y. Shavit, J. Hart, B. Mordashov, S. Cohn, I. Notti, and H. Bessler.** 1996. Effects of anesthesia based on large versus small doses of fentanyl on natural killer cell cytotoxicity in the perioperative period. Anesth. Analg. **82:**492-497.
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(b)(4)

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(b)(4)

9. **Gomez-Flores, R., and R. J. Weber.** 2000. Differential effects of buprenorphine and morphine on immune and neuroendocrine functions following acute administration in the rat mesencephalon periaqueductal gray. Immunopharmacology **48:**145-156.
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15. **Martucci, C., A. E. Panerai, and P. Sacerdote.** 2004. Chronic fentanyl or buprenorphine infusion in the mouse: similar analgesic profile but different effects on immune responses. Pain **110**:385-392.
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(b)(4),(b)(6)

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rDOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (December 2013)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	3/24/2014	2nd Resubmission Date:	5/7/2014
Approved/**Returned for Revision**:	4/17/2014	**Approved**/Approval Withheld by IACUC:	5/12/2014
1st Resubmission Date:	4/23/2014	**EXPIRATION DATE:**	5/11/2017
Full Committee Review:	4/24/2014	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: (b)(4) Protection from Radiation-Induced (b)(4) Injuries in a Murine Model (*Mus musculus*)

IV. PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6) **Ph.D.** Date
Associate Professor (b)(6)
(b)(6) office (b)(6) fax: (b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **Ph.D.** Date
Chair (b)(6)
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician
Telephone: (b)(6) Fax (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI
Telephone (b)(6) Fax: (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department, USUHS
Telephone: (b)(6) Fax (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**

IACUC Chair (b)(6) Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: (b)(4) Protection from Radiation-Induced (b)(4) Injuries in a Murine Model (*Mus musculus*)

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

CO-INVESTIGATOR(S): none

USUHS SCIENTIFIC RESEARCH PROTOCOL NUMBER: to be assigned

I. NON-TECHNICAL SYNOPSIS

Despite advances in precision of targeting tumors with radiation therapy (RT), regrowth of tumors does occur. Higher doses of radiation that would be needed to completely destroy a tumor cannot be used because of damage to the underlying normal tissues of the body. Tumors in which regional control is difficult include locally advanced breast, lung, head and neck, brain, cervical cancer, and others. Two other situations where radiation protection may be useful are: 1) in children, where RT can impair normal growth and development; and 2) in patients receiving combined RT and chemotherapy, where the acute effects of treatment may be severe, particularly when RT and chemotherapy are given concurrently. One method to improve the therapeutic index is by use of agents that differentially protect normal tissues relative to tumors.

The only FDA-approved radioprotector is amifostine. Amifostine is approved to reduce side effects in patients receiving head and neck irradiation, but its use is limited because it must be given intravenously shortly before RT and it has systemic toxicity (b)(4)

(b)(4)

(b)(4) Our proposed studies here will examine the effects of (b)(4) radiation injuries in murine models for these injuries. We will utilize C57L mice exposed to (b)(4) X-ray irradiation at (b)(4) to determine the efficacy and mechanisms of (b)(4) for the protection of the (b)(4) against radiation injury. Our aim is to develop an effective, non-toxic radioprotectant/ radiotherapeutic treatment strategy that can be used for protection against radiation injury to normal tissues during clinical radiation for cancer treatment.

II. BACKGROUND

II.1. Background The acute toxic effects of exposure to high doses of ionizing radiation in humans have been shown to occur due to two sequential effects: first the ablation of immune cells, resulting in loss of immune function and increasing likelihood of pathogenic infection (b)(4) (b)(4) and subsequently the loss of epithelial progenitor cells of the gastrointestinal (GI) crypt (b)(4) Both of these events have been shown to be due to the loss of fast-growing pluripotent and/or progenitor cell types. The time course of the acute injuries correlates with the life span of the mature cell types of the tissue, which are not replaced because of radiation damage to the adult pluripotent cells and adult stem cells. There is currently no FDA approved prophylactic or treatment for prevention of acute radiation injuries following high-dose (b)(4) radiation exposure.

(b)(4)

(b)(4)

In humans, severe radiation (b)(4) can result in death (b)(4) (b)(4) Cumulative evidence from a number of laboratories, including our own, shows that there is a threshold dose of radiation of (b)(4) to induce sufficient (b)(4) for the (b)(4) to occur in mice; below these doses, no permanent injury manifests, although some short-term effects are observed (b)(4) (b)(4) However, in humans, there is currently no accepted protocol for preventative treatment of radiation-induced (b)(4) and morbidity as high as 50% is associated with each phase (b)(4) (b)(4) injuries from radiation is considered to be a serious problem in the field for the development of agents to prevent these radiation injuries.

(b) as a radioprotectant:

Preliminary Studies:

(b)(4),(b)(6)

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

Biomedical Research Database (BRD), NIH RePORTER (replacement database for CRISP) *http://projectreporter.nih.gov/reporter.cfm*, and PubMed

II.2.2. Date of Search Data base searches conducted on 1/30/14.

II.2.3. Period of Search Searches conducted for the entire time periods available in each data base. For BRD, 1998-2014; for RePorter, 1972-2014. For PubMed, 1970-2014.

II.2.4. Key Words of Search Searches were performed using the following key word combinations: (b)(4)

(b)(4)

II.2.5. Results of Search

1) BRD: Search resulted in 0 projects with these key words
2) A RePORTER search: Search resulted in 0 projects with these key words
3) PUBMED Search *8/3/09*

(b)(4),(b)(6)

Summary of other publications:

(b)(4)

(b)(4)

III. OBJECTIVE / HYPOTHESIS

(b)(4)

IV. MILITARY RELEVANCE

Military personnel and their families receive medical treatment for cancer, including clinical radiation. The development of agents for the protection of normal tissues during cancer radiotherapy would allow the use of higher doses of radiation which has been shown to improve the level of tumor control and cancer irradiation. The goals of this research are consistent with improved cancer treatment in military personnel and their families.

Additionally, the Department of Defense is prepared to execute military missions within radiation environments, to manage radiation crises associated with terrorist activities, and to manage consequences in the event of nuclear weapons detonation. Recent studies indicate that there is an increasing risk of exposure to ionizing radiation to military personnel due to increasing terrorist activities, including potential use of stolen nuclear weapons or dispersal of radioactive material in a "dirty bomb" (b)(4) Because of potential exposure of military personnel to radiation, the DoD is committed to protect members of the Armed Services from the adverse health effects of ionizing radiation. High dose ionizing radiation causes short term, acute injuries including hematopoietic and GI syndromes. However, both high and low dose ionizing radiation (as may result from a radiation dispersal device) can cause (b)(4) as demonstrated by Japanese atomic bomb survivor data as well as accidental exposures to Russian populations (b)(4) . While early effects of radiation injury now have treatment/ prophylactic strategies, the delayed effects currently have no treatment. Lack of treatment for late effects of radiation, including (b)(4) will likely lead to delayed morbidity and mortality for personnel exposed to ionizing radiation.

The Department of Defense (DoD) Chemical and Biological Defense Program 2007 Annual Report to Congress, Chapter 2.7.6 Medical Radiological Defense, addresses the requirements for the development of radioprotectants. This priority is outlined for the development of

protectants for both acute and delayed radiation injuries, with an emphasis on (b)(4) (b)(4) According to this report, the mitigation of (b)(4) is a major research thrust area. This project is also designed to investigate mechanism(s) of protection to aid in the development of such agents.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

The goal of this project is to investigate the protective effects and mechanisms of (b)(4) for radiation-induced (b)(4) injuries.

(b) Rationale for Doses and Times of Administration:

(b)(4),(b)(6)

(b)(4) For animals receiving i.p. injections on consecutive days, inspections will be performed by the AFRRI veterinary staff to ensure that peritonitis does not occur. If any indications occur of abdominal swelling, sensitivity or other potential indicators of peritonitis, animals will be switched to s.c. injection.

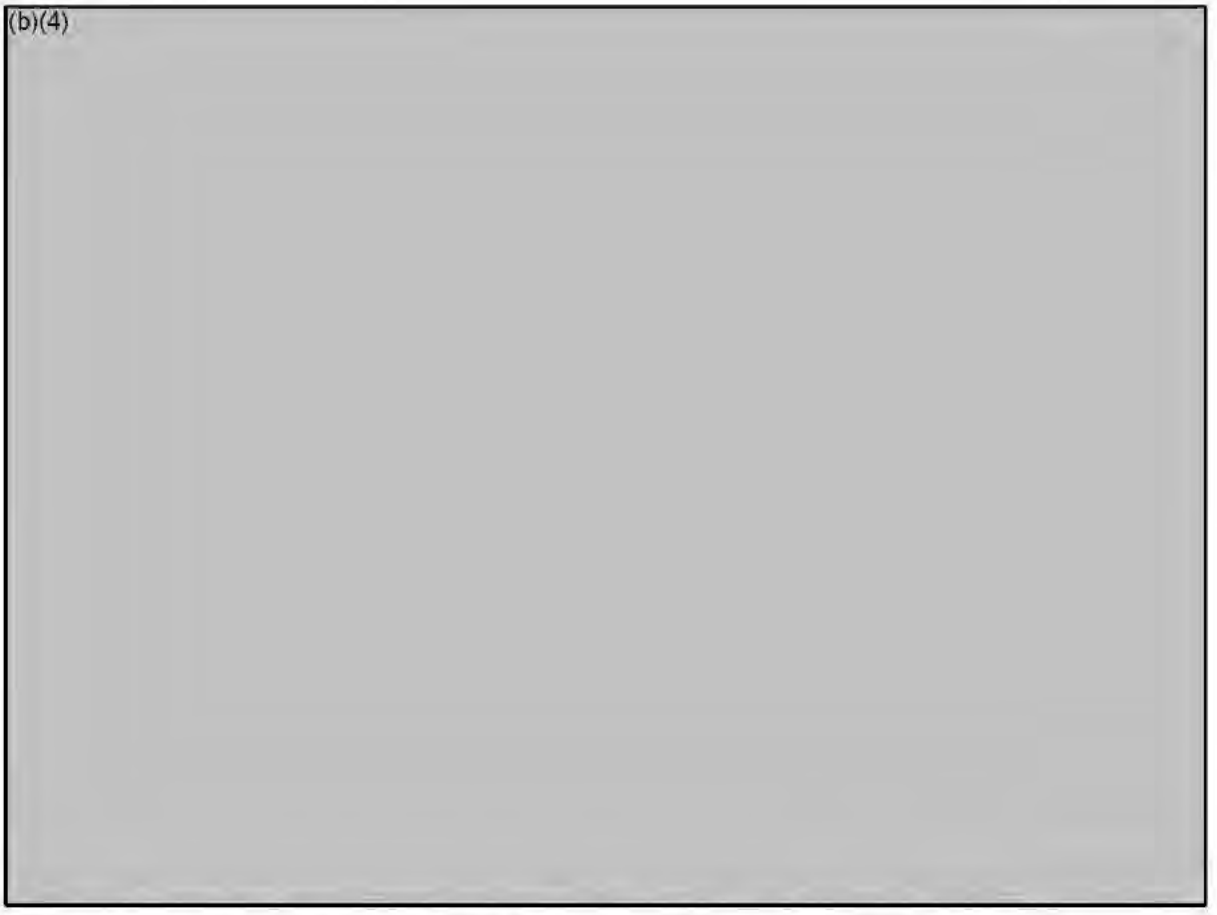
(b)(4) will also be administered by gavage, for the same time course as for injected routes of administration. The survival outcomes will be observed for injectable or gavage administration in Experiment 1 (see below), and the optimal route of administration will be selected for subsequent experiments. The optimal route of administration will be utilized throughout an entire study. If injection is selected as the optimal route of administration, the site of injection will be altered in consecutive injections to avoid injuries from multiple injections in the same site.

Radiation Rationale for Doses and Times of Administration

(b)(4)

5 Criteria for Determining Radiation-induced (b)(4)

(b)(4)



V.1.1. Experiment 1. Pilot study for protection C57L mice: category E)

This pilot experiment will be performed to assess survival from

Four groups will be studied:

Survival will be determined at days postirradiation. For these studies mice are required per radiation dose

Animals will be examined by AFRRI veterinarians following initiation of injections to ensure that peritonitis is not an issue for these injections. If it is determined that animals exhibit any potential indications of peritonitis (for example, abdominal swelling or sensitivity), then the route of administration will be switched to injection.

Because the time course for radiation-induced is shorter than for radiation-induced animals will be euthanized from this study prior to the completion of experiments in this AFRRI protocol.

This information can also be used to determine the effects of administration of

Future experiments will be performed following the completion of experiment 1 and are dependent upon the results of experiment 1. If improved survival is not observed with any of the routes of

administration for the proposed times of administration, further discussions will be held with the AFRRI IACUC prior to the initiation of further experiments.

(b)(4) [redacted] mice per group) x (4 treatment groups) x (1 time point) = [redacted] mice (total) (b)(4)

Endpoint: The final endpoint for the experiment is [redacted] days. Mice displaying obvious dyspnea, lethargy, or other markers of moribundity and which appear to be in distress will be euthanized. (b)(4) [redacted] Mice scoring a total of >10 under any measurement on the revised pain score sheet (based on Appendix A: Clinical Observations with Criteria for Euthanasia [rodent]) will be euthanized.

V.1.2. Experiment 2 Dose-modifying effect of (b)(4) protection ([redacted] mice: [redacted] in category C, [redacted] in category E) (b)(4)

(b)(4) Once we have established the efficacy (b)(4) for protecting mice from (b)(4) irradiation at the (b)(4) level, we will determine the dose modifying effect using three doses of (b)(4) irradiation (b)(4) (b)(4) (b)(4) Two groups of mice will be studied: 1) ionizing radiation +vehicle (control) and 2) ionizing radiation (b)(4) (experimental). For these studies, we will require (b)(mice per group in the radiation groups. We will include controls with (b) animals per group: 1) Sham irradiation + vehicle and 2) Sham irradiation (no treatment). The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

Radiation groups

(b) (4) mice per group) x (2 treatment groups) x (1 time point) x (3 (b)(4) radiation levels) = (b)(4) mice/experiment) x (2 experiments) = (b)(4 mice

Control groups

(b)(4) [redacted] mice per group) x (2 treatment groups) x (1 time point) = [redacted] mice (b)(4)

(b)(4) Total mice required for this experiment = [redacted] mice (total)

Endpoint: The final endpoint for the experiment is (b) days. Mice displaying obvious dyspnea, lethargy, or other markers of moribundity and which appear to be in distress will be euthanized. (b)(4) [redacted] Mice scoring a total of >10 under any measurement on the revised pain score sheet (based on Appendix A: Clinical Observations with Criteria for Euthanasia [rodent]) will be euthanized.

V.1.3. Experiment 3 Survival from fractionated-dose irradiation [redacted] mice: [redacted] in category E) (b)(4)

Humans receive radiation for cancer treatment in fractionated doses. Our preliminary studies will utilize single doses of radiation for the induction of (b)(4) injuries. Once we have established the efficacy (b)(4) for the protection of (b) tissue against radiation injury from single high doses of radiation, we will investigate the use of (b)(4 for protection against fractionated radiation. A schedule of fractionated radiation (b)(4) given equally spaced (b)(4)

(b)(4) over [redacted] days, has been demonstrated in a murine model to be effective for the induction of radiation (b)(4 injuries (b)(4) We will utilize two treatment groups: 1) ionizing radiation + vehicle; 2) ionizing radiation + (b)(4) The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) [redacted] mice per group) x (2 treatment groups) x (1 time point) = (b)(4)

(b)(4) mice/experiment) x (2 experiments) = mice (total)

Endpoint: The final endpoint for the experiment is days. Mice displaying obvious dyspnea, (b)(4) lethargy, or other markers of moribundity and which appear to be in distress will be euthanized. (b)(4) Mice scoring a total of >10 under any measurement on the revised pain score sheet (based on Appendix A: Clinical Observations with Criteria for Euthanasia [rodent]) will be euthanized.

V.1.4. Experiment 4 (b)(4) protection against (b)(4) mice: in category C; in category E) (b)(4)

(b)(4) We will investigate the degree of protection against radiation-induced (b)(4) We will determine levels of (b)(4) C57L mice will develop significant (b)(4) and we want to have as much survival as possible for the endpoint of this experiment. The selection of (b)(4) will allow increased survival while still provide a significant (b)(4) For this experiment we will require only (b)(4) mice/group, and we will examine 4 groups: 1) sham irradiation + vehicle; 2) sham irradiation + (b)(4) 3) ionizing radiation + vehicle; 4) ionizing radiation + (b)(4) The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) mice per group) x (4 treatment groups) x (1 time point) = (b)(4)

(b)(4) mice/experiment) x (2 experiments) = mice (total)

Endpoint: The final endpoint for the experiment is days. Mice displaying obvious dyspnea, (b)(4) lethargy, or other markers of moribundity and which appear to be in distress will be euthanized. (b)(4) Mice scoring a total of >10 under any measurement on the revised pain score sheet (based on Appendix A: Clinical Observations with Criteria for Euthanasia [rodent]) will be euthanized.

(b)(4) **V.1.5. Experiment 5 protection against (b)(4) (b)(4) mice: in category C; in (b)(4) category E)**

(b)(4)

(b)(4) For this

(b)(4) experiment we will require only mice/group, and we will examine 4 groups: 1) sham irradiation + vehicle; 2) sham irradiation+(b)(4) 3) ionizing radiation + vehicle; 4) ionizing radiation +(b)(4) The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) mice per group) x (4 treatment groups) x (4 time points) = (b)(4)

(b)(4) mice/experiment) x (2 experiments) = mice (total)

Endpoints: Endpoints for the experiments will be (b)(4) days. Early time points will be taken if any animal exhibits signs or symptoms of morbidity or moribundity. Such animals will be euthanized at the time of observation. Mice scoring a total of >10 under any measurement on the revised pain score sheet (based on Appendix A: Clinical Observations with Criteria for Euthanasia [rodent]) will be euthanized.

V.2. Data Analysis

Mean with standard error, or percentage will be reported if applicable. A significance level is set at 5% for each test. All statistical tests are two-sided. A statistical software, SAS will be used for statistical analyses.

Experiments 1-3: Analysis of variance (ANOVA) will be used to detect significant difference among the groups. If significant, then the pair-wise comparison by Tukey-Kramer will be used to identify which group is different from the other. For experiment 2, N = [redacted] mice per group could have 80% power to detect any significant difference between groups if any, given type I error of 5% and delta/sigma has at least 1.4, where the delta is the estimated mean differences between the two groups and sigma is the pooled standard deviation of the two groups (b)(4) (b)(4) [redacted]

Experiments 4 and 5: Previous data from this laboratory indicate that the coefficient of variation (CV) of the data is about 0.5, and that differences of between 2- and 30- fold are likely to be observed. A sample size of [redacted] per group will have 80% power to detect 3-fold increases in biomarkers based on a lognormal t test with a 5% two-sided significance level and a CV of 0.5. (b)(4)

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

For the past 9 years, we have utilized primary cell culture systems to examine anti-apoptotic mechanisms; however, we must determine whether our culture findings are relevant in whole animals. We will continue to use primary cell culture whenever possible to avoid unnecessary use of animals. We have also considered computer models as alternatives to animals. However, this alternative also lacks the knowledge base and complexity needed to evaluate radioprotective and radiotherapeutic agents.

V.3.2. Animal Model and Species Justification

Tissue culture conditions cannot fully substitute for the complexities of whole organisms in response to radiation injury. Such injury involves the interactions between the (b)(4) [redacted] system and other organ systems. In order to develop viable treatments for humans, we find that our research must be conducted in whole animals.

Work by (b)(6) [redacted] also advocates the mouse as the best species to use for radiation protection and radiotherapy protocols: 1) mice provide the least sentient species available whose response to radiation has been clearly identified, with physiological responses to radiation and radioprotectants that are similar to human physiology, 2) extensive literature is available on responses of mice to radiation and countermeasures, 3) more reagents are available to study the relevant cell types and molecules in mice than in any other species except humans, and the availability of transgenic and knockout mice make this model extremely attractive for further research into molecular mechanisms, and 4) (b)(6) [redacted] (b)(6) have extensive experience with the mouse model in radiation (b)(4) injury and radioprotection research.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species *Mus musculus*

V.3.3.2. Strain / Stock C57L (NOT C57BL/6)

V.3.3.3. Source / Vendor (b)(4) [redacted]

V.3.3.4. Age animals will be purchased at 6 weeks of age and held (b)(6) until they are irradiated at 10-14 weeks

V.3.3.5. Weight Normal adult weight range for this strain: 17-21 g.

V.3.3.6. Sex
Female. Only females will be used in this study for the following reasons: 1) we have so far utilized female mice to lay the foundation of our studies for radiation-induced (b)(4) (b)(4) injuries; 2) because of long housing periods, it is advantageous to use female mice that are less aggressive toward each other.

V.3.3.7. Special Considerations Commercially procured animals must be adventitious disease free. Mice- Pathogen-free, including *Pseudomonas aeruginosa*, and Pasteurella: All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus; *Helicobacter* spp. *Mycoplasma pulmonis*, and *Clostridium piliforme*. Endoparasite- and ectoparasite-free.

V.3.4. Number of Animals Required (by species) C57L (b)(4)

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement
Animals demonstrating moribundity due to radiation exposure (b)(4) will be considered to have arrived at the study endpoint and will be immediately euthanized (See sections V.4.5 Study Endpoint and V.4.6 Euthanasia).

Ketamine/xylazine and/or inhaled isoflurane anesthesia will be used during X-ray irradiation to prevent stress from the time the mice are placed in restraint. Isoflurane will be the preferred method. Mice will be restrained using either an approved jig or using surgical tape to secure the animal to the SARRP platform. Under the training protocol at AFRRI inconjunction with the AFRRI veterinary staff, we found that in female CBA or C57BL/6J mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in ~30-60 min anesthesia with no morbidity or mortality (b)(4) (b)(4) We have also observed that the animals require at least 10-20 additional minutes on heating pads to become fully ambulatory. The injectable anesthesia may also be used in combination with isoflurane if deemed necessary by the AFRRI veterinary staff.

As stated above, we use a heating pad to keep mice warm during the period of recovery from anesthesia. The temperature in room where the mice will be irradiated will be measured, and if necessary an alternate heating divice approved by VSD (such as Beir hugger to blow warm air into the room) will be used to increase the temperature. Standard intraperitoneal injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which all laboratory personnel have received training at AFRRI.

V.3.5.2. Reduction
Whenever possible, control groups will be shared between experimental groups for our experiments. Tissues will be collected for multiple assays from the same animals further

reducing the total number of animals needed. At every stage of the research, planned experiments will be reevaluated, in an attempt to reduce the numbers of experiments and eliminate repeats of experiments where statistical significance has already been obtained.

V.3.5.3. Replacement

At this time, it is not feasible to use non-animal systems in place of animal models to address the research questions in this protocol.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals C57L

V.4.1.1.1.1. Column C (b)(4) (# of animals)

V.4.1.1.1.2. Column D (# of animals)

V.4.1.1.1.3. Column E (# of animals)

V.4.1.1.1.4. Pain Category Assignments

(b)(4)

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

A portion of our study is aimed at determining the effect of the radiation countermeasure on all effects of radiation, including those effects on the (b)(4) especially radiation-induced (b)(4) Some analgesics may suppress (b)(4) function that may interfere with the interpretation of our results (b)(4) Additionally, some nonsteroidal anti-inflammatory drugs (NSAIDs) have been demonstrated to exacerbate (b)(4) inflammation in some disease states (b)(4) The response to murine (b)(4) to analgesics following radiation has not been thoroughly investigated, but the induction of additional levels of inflammation would severely compromise our experimental results.

Several studies indicate that exposure to γ-radiation induces altered reactions of opioid receptors to nociception and causes the direct release of endogenous beta-endophins, thus altering the effectiveness of opioid administration (b)(4) (b)(4) Some sedatives have been reported to contribute to postoperative (b)(4) which likely reduce some functions of radiation.

Published IACUC Guidelines define prolonged restraint as the physical restraint of an animal for more than 30 min. Restraint of mice for more than 30 min will be required for the time of irradiation plus time required for positioning the animal in the SARRP. For prolonged restraint required for (b)(4) irradiation, mice will be anesthetized with inhaled and/or injectable anesthesia.

For inhaled isoflorane anesthesia, a protocol will be developed in conjunction with the AFRRI veterinary staff. We will use 3-5% for the induction of anesthesia and 1-2% for maintenance during irradiation. Isoflorane will be delivered using 100% oxygen.

An injectable anesthesia protocol was developed for use during the X-ray irradiation under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff. In female C57BL/6 and CBA mice, ~12 weeks old, i.p. injection of 100-150 mg/kg ketamine plus 10-18 mg/kg xylazine (25 G needle) results in ~30-60 min anesthesia with no morbidity or mortality (b)(4) (b)(4) We have also observed that the animals require at least 10-20 additional minutes on heating pads to become fully ambulatory. Injectable anesthesia may include ketamine 50-100 mg/kg + xylazine 5-10 mg/kg + acepromazine 1-2 mg/kg. Total volumes of injection will not exceed 0.5 ml/mouse. Standard i.p. injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which laboratory personnel have received training at AFRRI.

V.4.1.2.2. Pre- and Post-procedural Provisions
Animals will be housed and cared for as described in Section V.5. Veterinary Care both before and after dose administrations and irradiation procedures.

V.4.1.2.3. Paralytics none

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched DoD Biomedical Research Data Base (BRD), CRISP, PubMED

V.4.1.3.2. Date of Search 1/30/14

V.4.1.3.3. Period of Search Search covered published manuscripts from ~1965-present. (BRD search included all available years 1998 -2014).

V.4.1.3.4. Key Words of Search Pain, analgesia, anesthesia, mice, radiation, ionizing radiation, humane, (b)(4)

V.4.1.3.5. Results of Search
The literature search revealed that, at present, there are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. In some cases radiation has been demonstrated to reduce pain in laboratory mice (b)(4) and has been in humans reported to alleviate cancer related bone pain (b)(4) (b)(4) A recent report showed that administration of botulinum toxin A was effective for reduction of pain and muscle spasms after clinical radiation for treatment of tumors (b)(4) (b)(4) Application of natural honey 20 min prior to radiation exposure was also shown to reduce mucositis with associated pain in humans following head/neck radiotherapy (b)(4) it has not been determined whether honey acts as a local radioprotectant. Spinal cord stimulation has also been reported in humans to alleviate pain

resulting from radiotherapy (b)(4) however, this method would not be practical for use in mice because of the extensive spinal cord manipulation required. Human administration of morphine also relieves pain (b)(4) but again this would not be practical for mice in our experiments because of side effects of the drug. In humans, exposure to total body irradiation may induce vomiting and diarrhea, causing pain and distress. However, mice are not susceptible to vomiting.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification

Anesthesia will be used before (b)(4) irradiation to avoid stress from restraint. (b)(4) (b)(4) (b)(4) we will be unable to use analgesics after irradiation since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4) (b)(4) (b)(4). Pain will be evaluated using several standard indications including: grimacing, abnormal breathing, hunched or fluffed fur and inactivity, or ataxia. In experiments where tissue collection is needed, animals will be euthanized with pentobarbitol prior to sample collection as described elsewhere. We will utilize the attached score sheets to provide a scale for measurement of criteria for euthanasia. Any animal scoring Mice scoring a total of >10 under any measurement on the revised pain score sheet (based on Appendix A: Clinical Observations with Criteria for Euthanasia [rodent]) will be euthanized.

V.4.2. Prolonged Restraint

(b)(4) irradiation will require restraint in designed jigs or using surgical tape for ~20-40 min for SARRP positioning to receive X-ray exposure (b)(4) animals will be anesthetized during this period and tails and limbs will be taped with surgical tape. The maximal time for actual irradiation is 30 minutes; the maximal estimated time for the SARRP setup for the irradiation is 20 minutes. The estimated maximal total time for restraint is therefore 50 minutes.

The jigs for use during (b)(4) irradiation were examined and approved by the AFRRI Veterinary staff. An image of the jigs is attached, and jigs are available for inspection. The restraint is needed to ensure that animals do not move during irradiation. Positioning of the mice must be done carefully to ensure that all animals receive the same exposures. AFFRI Veterinary staff provided training and supervision for the placement of anesthetized animals in the approved jigs for irradiation.

V.4.3. Surgery Not applicable

V.4.3.1. Pre-surgical Provisions Not applicable

V.4.3.2. Procedure(s)

V.4.3.3. Post-surgical Provisions Not applicable

V.4.3.4. Location Not applicable

V.4.3.5. Surgeon Not applicable

V.4.3.6. Multiple Major Survival Operative Procedures Not applicable

V.4.3.6.1. Procedures Not applicable

V.4.3.6.2 Scientific Justification Not applicable

V.4.4. Animal Manipulations

V.4.4.1. Injections

The methodology of intraperitoneal (i.p.) injections by our laboratory technical staff will be monitored and approved by the AFFRI veterinary staff prior to the initiation of i.p. injections of (b)(4) for this study.

Intraperitoneal injections of pentobarbital for euthanasia 0.01-0.02 ml/mouse Fatal Plus (39-78 mg pentobarbital/mouse) will be performed with a 23-25 G needle. Injectable anesthesia administered i.p. may include ketamine 50-100 mg/kg + xylazine 5-10 mg/kg + acepromazine 1-2 mg/kg. Total volumes of injection will not exceed 0.5 ml/mouse, and will be done using a 23-25 G needle. This level of anesthesia was first developed for our use in collaboration with the AFRRI Veterinary staff. This drug combination provides ~30-60 min anesthesia with no morbidity or mortality (b)(4) (b)(4) We have also observed that the animals require at least 10-20 additional minutes on heating pads to become fully ambulatory.

(b)(4)

(b)(4)
(b)(4) Because the time course for radiation-induced (b)(4) is shorter than for radiation-induced (b)(4) animals will be euthanized from this study prior to the completion of experiments in this AFRRI protocol. (b)(4)
(b)(4)
(b)(4) If this information is available prior to the initiation of Experiment 1 of this protocol, this information will be used to determine whether the route of (b)(4) administration in the AFFRI studies.

When possible, same route of administration will be utilized throughout an entire study, but the site of injection will be changed to avoid injuries from multiple injections in the same site. (b)(4) Animals receiving injections will be examined by the AFRRI veterinary staff daily to ensure that there are no signs of (b)(4) If it is determined that peritonitis is a risk from injection (b)(4) (b)(4) or if it is determined that any individual animal is exhibiting signs of (b)(4) the route of administration will be switched to (b)(4)

V.4.4.1.1. Pharmaceutical Grade Drugs

(b)(4)

V.4.4.1.2. Non-Pharmaceutical Grade Drugs None

V.4.4.2. Biosamples

All biosamples will be taken after euthanasia. (b)(4) will be removed *en bloc*. (b)(4) will be removed from (b)(4) as control tissue. All other tissues will be available to other researchers for tissue sharing.

V.4.4.3. Adjuvants None

V.4.4.4. Monoclonal Antibody (MAb) Production Not applicable

V.4.4.5. Animal Identification
All cages will have cage cards identifying the treatment groups for each experiment.

V.4.4.6. Behavioral Studies Not applicable

V.4.4.7. Other Procedures

(b)(4) **irradiation**
(b)(4) irradiation will be performed using (b)(4),(b)(6) The methodology for (b)(4) irradiation of mice (b)(4) will be developed with the AFRRI IACUC and (b)(4) Once the procedure is approved by VSD, an amendment to this protocol with a standard operating procedure (SOP) will be submitted prior to the initiating the studies.

Mice will be anesthetized by injection and/or using inhaled anesthesia. Anesthetized mice will be placed in ventilated Lucite jigs, or animals will be restrained using surgical tape to prevent lateral or vertical movement. Tails and forelimbs will be taped to the jig to prevent any forward motion of animals and to keep the limbs out of the radiation field. Mice will be irradiated at (b)(4) (b)(4) irradiation. The temperature in room where the mice will be irradiated will be measured, and if necessary a space heater or other method approved by VSD will be used to increase the temperature. Following radiation exposure, mice will be recovered in a VSD and IACUC approved location or immediately returned to the AFRRI vivarium and allowed to recover from anesthesia under supervision with heating pads. Immediately upon recovery, animals will be returned to standard cages that house 4 animals per cage. Lucite jigs will be sanitized after each use by washing with soap and water.

V.4.4.8. Tissue Sharing
For the proposed studies, (b)(4) will be obtained. All other tissues will be available for tissue sharing with other investigators.

V.4.4.9. Animal By-Products None

V.4.5. Study Endpoint
(b)(4) The endpoint for Experiment 1-3 is days. The endpoint for Experiment 4 is days, and (b)(4) the endpoints for Experiment 5 are (b)(4) days postirradiation. Morbid animals will be monitored at least three times daily. Monitoring will be performed twice by the PI or laboratory technical staff. The experimental design does not contemplate supportive therapy. Criteria to be used for health evaluation while the animals are on study include: severe weight loss, ruffled fur, dehydration, decreased activity, and hunched body position, dyspnea, or panting. Animals exhibiting these signs may be euthanized, and considered to be at the study endpoint. From our previous experience, (b)(4) (b)(4)

V.4.6. Euthanasia

Animals will be euthanized at the stated early endpoints of the experiments by i.p. injection of 0.01-0.02 ml/mouse Fatal Plus. PHS policy is intraperitoneal injection of at least 200 mg/kg sodium pentobarbital euthanasia solution; Fatal Plus: 390 mg pentobarbitol/1 ml

1 ml/390 ml x 0.02 kg/mouse x 200 mg/1 kg = 0.01-0.02 ml

Injection i.p. will be performed using a 25 G needle. (Please note that Euthasol cannot be used as additives in this preparation have effects on (b)(4) IP using a 25 G needle. Pentobarbital has been utilized as a euthanasia methodology by our laboratory to induce as little injury as possible to (b)(4) CO_2 would be unacceptable because of potential damage (b)(4) and cervical dislocation could potentially tear the trachea (b)(4) (b)(4)

Animals which appear to be experiencing pain or distress as a result of radiation exposure or are found to be moribund will also be euthanized by pentobarbitol and necropsied. If a moribund animal is found that must be euthanized by VSD techs working on the weekend or holidays, 100% CO_2 euthanasia may be used instead of pentobarbitol. At the end of experiments, unused animals will be transferred to other protocols or euthanized by exposure to 100% CO_2 from a compressed gas cylinder in accordance with the current American Veterinary Medical Association (AVMA) Guidelines for Euthanasia. Cervical dislocation will be used to ensure euthanasia.

V.5. Veterinary Care

V.5.1. Husbandry Considerations

Routine animal husbandry will be provided in accordance with AFRRI VSD Husbandry SOPs for each species in this protocol. Food and acidified water and Rodent Diet will be available ad libitum. The 12:12 hr (lights off at 6 pm) room light cycle is fine for all the studies.

V.5.1.1. Study Room

(b)(6)

V.5.1.2. Special Husbandry Provisions None

V.5.1.3. Exceptions None

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care

Experimental animals will be observed at least twice a day, including weekends and holidays, by one of the study investigators or technicians during the period (b)(4) days postirradiation (b)(4) when effects of radiation are expected to be incurred. VSD personnel will also monitor the animals twice a day during their rounds. As described in the study endpoint section animals will be monitored at least three times daily, morning, early afternoon and late afternoon during the critical period of the experiment following (b)(4) irradiation, from (b) days postirradiation through the study endpoint. The experimental design does not contemplate supportive therapy. The justification for not providing supportive care has been described in the study end point section as well as in the pain category section. Criteria to be used for health evaluation while the animals are on study include: severe weight loss, ruffled fur, dehydration, decreased activity, and hunched body position, dyspnea, or panting. Animals exhibiting these signs may be euthanized, and considered to be at the study endpoint. Mice scoring a total of >10 under any

measurement on the revised pain score sheet (based on Appendix A: Clinical Observations with Criteria for Euthanasia [rodent]) will be euthanized.

V.5.2.2. Emergency Veterinary Medical Care Emergency veterinary care is provided 24 hrs/7 days a week by on call VSD veterinary technician and veterinarian as per the AFRRI-VSD SOPs. Essential husbandry procedures and health rounds are conducted by VSD personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

All animals on this protocol will be provided with routine environmental enrichment in accordance with AFRRI VSD SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions None.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

The methodology of intraperitoneal (i.p.) injections by our laboratory technical staff will be monitored and approved by the AFFRI veterinary staff prior to the initiation of i.p. injections of DIM for this study.

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(4)	Ph.D.	AFRRI	(b)(4)	1,3,5	10
	M.S.	AFRRI/AALAS	Multiple dates	1,3,5	9+

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a-ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Ear tagging

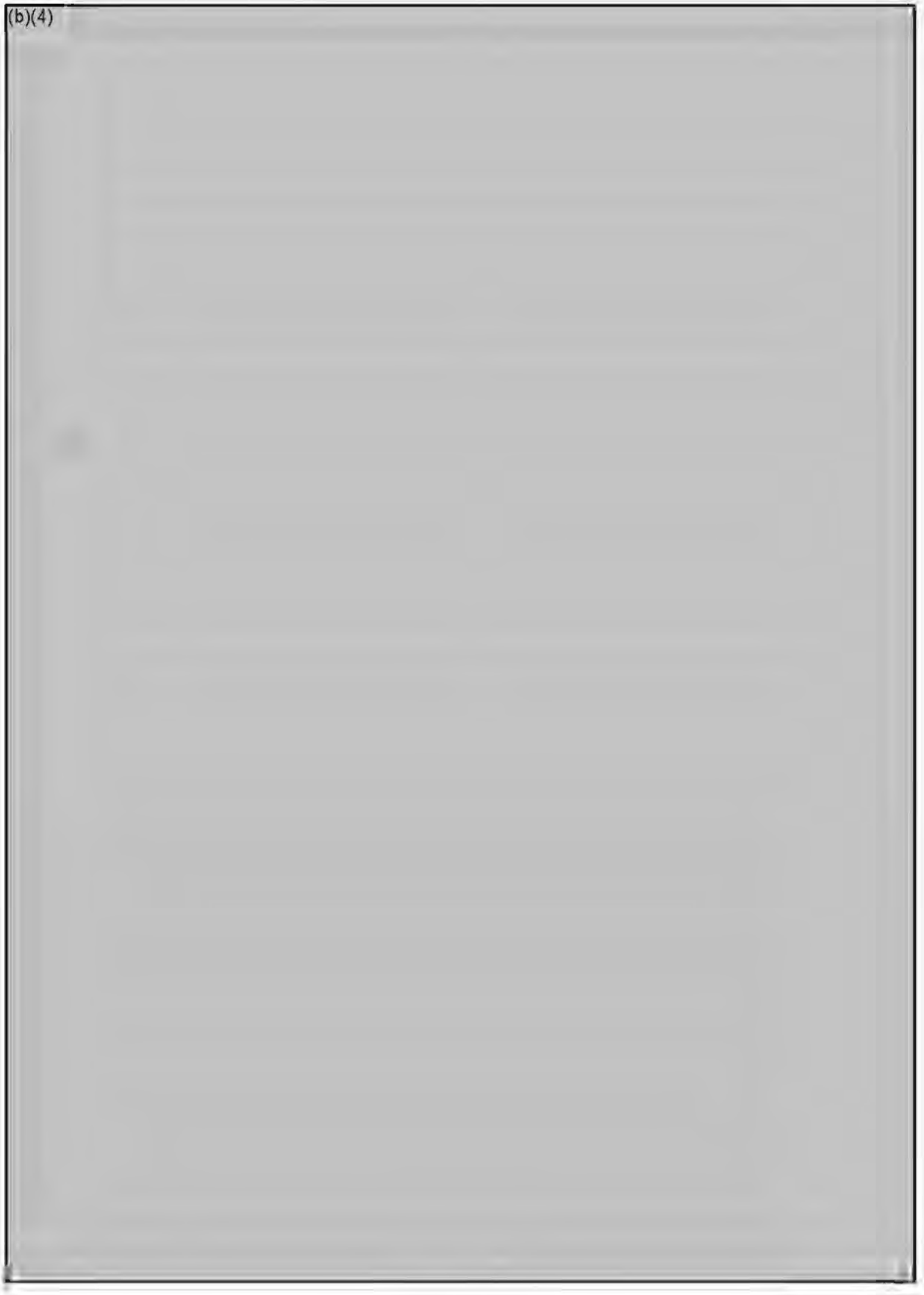
VII. BIOHAZARDS/SAFETY None

VIII. ENCLOSURES

Pain score sheet, criteria for early euthanasia, image of Lucite jigs, references are included for (b)(4)

References:

(b)(4)


(b)(4)


(b)(4)

X. ASSURANCES

Protocol Title: (b)(4) Protection from Radiation-Induced (b)(4) Injuries in a Murine Model (*Mus musculus*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET (Revised protocol 7-15-2014)

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	6-10-2014	2nd Resubmission Date:	8/5/2014
Approved/Returned for Revision:	6/30/2014	1st Tier Approved:	8/5/2014
1st Resubmission Date:	7/15/2014	EXPIRATION DATE:	8/11/2017
Approved/Returned for Revision:	8/4/2014	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:	8/5/2014	Approved/Returned for Revision:	
2nd Tier Approved:	8/11/2014	2nd Resubmission Date:	
1st Resubmission Date:		Final Approval:	8/12/2014

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Radiation countermeasure studies using (b)(4) and G-CSF in Nonhuman Primates (*Macaca mulatta*)

IV. PRINCIPAL INVESTIGATOR:

(b)(6) **PhD** 7-15-2014 Date

Research Biologist, Scientific Research Department, AFRRI

(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, LTC, USA** Date

Head, Scientific Research Department, AFRRI

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

- Or –

(b)(6) **PhD, CDR, USN** Date

Head, Scientific Research Department, AFRRI

Telephone (b)(6)

(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician

Telephone (b)(6) Fax: (b)(6)

(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI

Telephone (b)(6) Fax (b)(6)

(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department, USUHS

Telephone (b)(6) Fax: (b)(6)

(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**

IACUC Chair, (b)(6) Scientific Research Department, AFRRI

Telephone (b)(6) Fax: (b)(6)

(b)(6)

PROTOCOL TITLE: Radiation countermeasure studies using (b)(4) and G-CSF in Nonhuman Primates (*Macaca mulatta*)

PRINCIPAL INVESTIGATOR: (b)(6) PhD
Research Biologist, (b)(6)

CO-INVESTIGATOR(S): (b)(6) PhD
Staff Scientist, (b)(6)
(b)(6) PhD
Research Associate II, (b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS Exposure to ionizing radiation can lead to death due to infection and failure to recovery from infection by loss of white blood cell precursors in the body, especially within bone marrow. This loss of blood cell precursors results in a low number of circulating white blood cells and platelets which protect the body from opportunistic infections and uncontrolled bleeding, respectively. We have shown that (b)(4) specifically increases platelets by 5-fold compared to normal untreated mice. Other studies at AFRRI have established that (b)(4) a good radiation countermeasure in mice. Advancement of this drug (alternatively, the class of compounds that enhance platelets in circulation) alone or combined with other countermeasures such as Granulocyte Colony Stimulating Factor (G-CSF) to human use requires studies in a large animal model such as nonhuman primates (NHPs). We evaluated (b)(4) in NHPs for safety and efficacy. Our preliminary studies show that the drug administered as a single subcutaneous injection or multiple injections does not cause any adverse effects. It also significantly enhanced platelets in both non-irradiated and irradiated NHPs. G-CSF on the other hand has been widely studied as a radiation countermeasure in several animal models. In the present protocol, we will evaluate various combinations of these two potential radiation countermeasures in NHPs subjected to cobalt 60 gamma radiation.

General note to IACUC members: Technically this new protocol represents continuation of an ongoing NHP project. The current active protocol will be expiring on July 31, 2014. The experiments described in this (new) protocol were previously approved as an amendment to the existing protocol. Further, PI has not proposed any new experiments. However, a new arm (G-CSF alone) has been added based on full committee review and recommendation of IACUC members. The reasons for the new protocol and continuation of the project are as follows:

a. Animal procurement: AFRRI has an established vendor selected through proper USUHS contracting procedures. This vendor has not met the required orders and animals for this project have not arrived. (b)(4)
(b)(4)
b. Program manager notification: The funding agency has been informed of the delay.
c. Completion: PI hopes to complete the project within one year of the approval.
d. This protocol should be considered as a pilot study with the intent of demonstrating potential efficacy of radiation mitigators.

II. BACKGROUND

II.1. Background

Because of increasing terrorist activity and dissemination of nuclear materials, the chances of military or civilian personnel being exposed to dirty bombs or improvised nuclear devices have risen dramatically in recent years. The prevailing sentiment of the US government is that it is a matter of when, not if, an attack with radiological or nuclear weapons will take place. The nation needs to be prepared for this eventuality. Acute radiation syndrome (ARS) results when ionizing irradiation causes defective hematopoiesis as a function of the radiation dose, dose-rate, and radiation quality. Defective hematopoiesis results in a decline in all blood cell counts for several weeks after initial exposure. The primary cause of death during the early phase of the radiation-induced hematopoietic syndrome is sepsis resulting from opportunistic infection due to a decline in the numbers of neutrophils (neutropenia) and increased translocation of bacteria across the gastrointestinal mucosa. Sepsis and shock result in eventual multiple organ failure and death. Acute radiation syndrome is often complicated by hemorrhage as a consequence of thrombocytopenia (reduced platelet counts Stickney *et al,* 2007).

The current treatment practice for radiation victims is the off-label administration of bone marrow growth factors, which are not yet approved for this purpose by the Food and Drug Administration (FDA), and must be used under the constant supervision of physicians because of possible adverse effects. Other agents that may be used for radiation victims, like potassium iodide (KI), Prussian blue, Calcium diethylene-triamine-penta-acetate (Ca-DTPA), and Zinc diethylene-triamine-penta-acetate (Zn-DTPA), address the internalization of specific radioactive isotopes, not the general injury caused by external exposure to ionizing radiation. For example, KI is used to prevent thyroid cancers by competing with internalized radioactive iodine for binding sites. Other potential radiation countermeasures are still in the pre-clinical research stage. There is a pressing need to develop countermeasures to ionizing radiation that will have a general beneficial effect on blood-forming tissues and immune competence. In order to expedite such studies which cannot be undertaken in humans due to ethical reasons, the FDA established "the animal efficacy rule" (FDA 2002). Under this rule, pivotal efficacy studies in Rhesus macaques exposed to lethal doses of ionizing radiation are appropriate to support approval of novel radiation countermeasure agents for human use.

The effectiveness of drugs to reduce or eliminate deleterious biological effects of ionizing radiation depends mostly on their effectiveness in controlling neutropenia, thrombocytopenia, and possibly anemia. Off-label drugs such as growth factors (GFs), G-CSF, and GM-CSF are available to treat neutropenia, but no such treatment exists for thrombocytopenia (although thrombopoietic GFs interleukin 11 [IL-11] and thrombopoietin [TPO] have been studied). However, first generation TPOs caused adverse effects in clinical trials (Kuter 2002, 2007, 2008) (b)(4)

(b)(4)

Brief background information on (b)(4) as a radiation countermeasure agent:

The drug for the present study will be synthesized by incorporation of a 14 amino acid peptide into a fully human antibody (PA83) scaffold at both the end region of the light chain

and the hinge region of the heavy chain (shown below). Control antibody without the 14 amino acid drug ligand will be used as control.

(b)(4)

We evaluated this (b)(4) (b)(4) as a radiation countermeasure in mice. We showed that the drug administered subcutaneously pre- or post-irradiation enhanced 30-day survival (b)(4),(b)(6) Briefly, thrombopoietin (TPO) receptor agonists lacking sequence homology to TPO were designed by grafting a known peptide sequence into the hinge and/or kappa constant regions of a human anti-anthrax antibody. Some of these proteins were equipotent to TPO in stimulating cMpl-r (the product of the c-mpl proto-oncogene, mpl= Myeloproliferative leukemia virus oncogene) activity *in vitro*, and in increasing platelet levels *in vivo*. (b)(4) the best agonist in this series with a Kd of 30 nM for cMpl-r, exhibited potent activity as a radiation countermeasure in CD2F1 mice exposed to lethal total body irradiation (TBI of 9.25 Gy) from a cobalt-60 gamma source. (b)(4) once either 24 h pre- or 6 h post-TBI showed superior protection to five daily doses given pre- or post-TBI. Prophylactic administration (aver. 82% 30-day survival) was superior to therapeutic schedules (60% 30-day survival). (b)(4) conferred significant survival benefit ($p<0.01$) when administered 4 days before or even 12 h after exposure, and across a dose range of 0.1 to 8 mg/Kg. The dose reduction factors (DRFs) with a single dose of (b)(4) 24 h pre- or 12 h post-TBI were 1.32 and 1.11, respectively ($p<0.0001$). Furthermore, (b)(4) increased bone marrow cellularity, megakaryocytic development, and accelerated multi-lineage hematopoietic recovery in irradiated mice demonstrating the potential of (b)(4) as both protectant and mitigator in the event of a radiological incident.

Nonhuman primate studies using (b)(4) (b)(4) rhesus macaque. Our results show that the drug (b)(4) is effective in enhancing platelets although to a lesser degree than seen in mice (Figure 1A). The research strategy for the present study is to evaluate a combination study involving (b)(4) and G-CSF or multiple (b)(4) administration on post-irradiation survival in NHPs. We showed recently (b)(4) (b)(4) (b)(4) that the proposed drug (b)(4) is safe for use with NHPs. Figure 1 shows preliminary data on enhancement of platelets (1A) in unirradiated animals administered a single *sc* injection of (b)(4) as well as changes in neutrophil counts (1B).

Figure 2 shows that a single *sc* injection of 2 mg/kg of (b)(4) administered either pre-irradiation (-24 h) or post-irradiation (+24 h) to NHPs subjected to 6.5 Gy resulted in survival and recovery of circulating platelets (platelet levels > 20,000/µl) by day 18. Although the non-drug treated control animal survived, it had a prolonged platelet recovery (day 29). The data represents animals that survived the 60 study.

Figures 3A (platelet levels) and 3B (absolute neutrophil counts) summarizes the hematological data for NHPs that were administered a single higher dose of the drug (b)(4) (b)(4) either pre-irradiation (-24 h) or post-irradiation (+24 h) and subjected to 7 Gy radiation. The results of drug treatment were not as effective as shown with the lower dose of radiation (Figure 2). We therefore proposed a combination of pre irradiation and multiple post irradiation drug administrations (the current protocol).

(b)(4)

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched PubMed, BRD, NIH Reporter, Embase

II.2.2. Date of Search: 5-20-2014

II.2.3. Period of Search: Pubmed 1946-present, NIH Reporter Fy1989 to present, BRD 1987 to active, and Embase 1966-present.

II.2.4. Key Words of Search

Keywords	PubMed 1946-present	NIH Reporter FY 1989-present	BRD 1987-active	Embase 1966-present
TPO	3896	652	1	29
(b)(4)	79	5	0	0
+ radiation	4	1	0	0
+ radiation +primates	2	0	0	0
+ radiation + primates + hematopoiesis+pharmacodynamics + bone marrow	0	0	0	0
(b)(4)	0	0	0	3
+ TPO	0	0	0	0
+ TPO + platelets	0	0	0	0
+ TPO + primates	0	0	0	0
+ TPO + radiation + cobalt 60	1*	0	0	0
G-CSF + primates	27949	35	0	0
(b)(4) + G-CSF + primates	0	0	0	0
+ G-CSF + primates + cobalt 60	0	0	0	0
(b)(4) + G-CSF + primates + cobalt 60 + mitigator	0	0	0	0
(b)(4),(b)(6)				

II.2.5. Results of Search There is no duplication based on the literature search.

III. OBJECTIVE / HYPOTHESIS Exposure to ionizing radiation can result in significant biological effects leading to mortality. The mission requirements of our military and homeland security personnel operating in a radiological environment are to provide rescue operations, security, radiological surveying, intelligence gathering, and decontamination. In a mass exposure scenario, where personnel are cut off from clinical support and treatment time is of the essence and administration of countermeasure agents could ameliorate the deleterious effects of ionizing radiation. The (b)(4) (b)(4) is a promising candidate based on mouse data. It is being evaluated in NHPs under the existing active but expiring protocol. The present protocol (new) will continue the NHP studies and extend ongoing observations with (b)(4) We intend to study (b)(4) either alone (pre and post irradiation) as a multiple regimen or combined with G-CSF.

Note: Please refer to the note to IACUC members on page 4 (below non-technical summary). This proposal represents continuation of an approved project- no new experiments are proposed although an extra arm (G-CSF alone) has been added to experiment 1 and 2 based on recommendation of IACUC members during full committee review.

Scientific relevance and hypothesis: Exposure to ionizing radiation increases susceptibility to infection and mortality due to significant loss of hematological regeneration capabilities. This is relevant to the context of military personnel, first responders, and other personnel who may be involved in rescue, security, and decontamination associated with accidental nuclear exposures. Over the years, several promising candidates have evolved that enhanced hematological recovery. Although none of these have been approved for treating radiation injuries, the FDA has recommended the use of cytokines, such as G-CSF (already in the U.S. stockpile) to control neutropenia in combating opportunistic infections. The relevance of this proposal is one that addresses an area (platelet recovery) that has not been extensively studied. Radiation-induced thrombocytopenia associated with loss of platelets has been implicated in the deleterious effects of ionizing radiation, especially mortality in NHP under acute exposure (Stickney *et al* 2007). Since other investigators have shown that enhancement of platelet response is associated with improved quality of life in patients with thrombocytopenia, it is imperative to develop drugs that can work by reducing radiation-induced thrombocytopenia. The present proposal will evaluate (b)(4) in nonhuman primates (NHPs) exposed to cobalt-60 gamma radiation. We hypothesize, based on our radiation countermeasure studies in CD2F1 male mice that the drug will be as effective in NHP as it has been in mice. Further, we expect (b)(4) will produce robust hematological recovery that will not be restricted to a single lineage. For instance, administration of this drug will not only increase circulating platelets but will also increase neutrophils and monocytes.

IV. MILITARY RELEVANCE Nuclear proliferation, terrorist activity, and the distribution of nuclear and radioactive materials through underground networks make incidents involving radiation injuries increasingly likely. As explained in a Department of Defense (DoD) Report to Congress (http://www.acq.osd.mil/cp), Al-Qaeda is known to have ambitious plans to acquire or develop nuclear weapons and is receptive to outside nuclear assistance. That group has also attempted to purchase enriched uranium. In view of the increasing likelihood of accidental radiation exposure and bioterrorism, the need for field able radiation countermeasures has been recognized as a high priority by the DoD.

Advances in radiation countermeasure strategies may enable military forces to operate, when required, in nuclear or radioactive combat environments while minimizing both short- and long-term consequences of exposure to ionizing radiation. Accurate casualty prediction models promote effective command decisions and force structure planning. Advanced biological dosimetry methodology is used in triage, treatment decisions, and risk assessment. Together, the results of the three research thrusts improve therapeutic strategies for the treatment and prevention of early and long-term health effects and mitigate the risks to our personnel and their offspring (AFRRI mission statement).

Most deaths due to radiation occur as a result of bone marrow damage and secondary infection. Any treatment that stimulates hematopoietic recovery could be advantageous.

We have recently shown that (b)(4) enhanced platelet levels in circulation 3-5 fold in normal and sublethally irradiated mice. This protocol involving evaluation of the drug in NHPs will allow advancement of the drug to stockpiling for emergency use against ionizing radiation.

The scientific basis for the combination of pre- and post- administration of a drug or a combination of drugs is to enhance the beneficiary effects of radiation countermeasures. Using two drugs with different modes of action (b)(4) promoting platelet regeneration; G-CSF stimulating neutrophils) is hypothesized to be beneficial in irradiated subjects. We have previously shown that (b)(4) is a good radioprotectant (administered -24h) and a mitigator (effective +12 h and in some instances up to +24 h) in mice. Farese *et al.*, (2012) recently demonstrated that G-CSF (10 µg/kg) administered daily (until absolute neutrophil counts (ANC) ≥ 1000 cells/µl) and in conjunction with full supportive care increased survival of lethally irradiated Nonhuman primates (NHPs). Growing evidence in large animal models such as NHPs, have demonstrated that G-CSF plays a significant role in the treatment of the acute radiation syndrome (Bertho *et al.*, 2005; Drouet *et al.*, 2008). More recently (May, 2013) FDA has recommended that G-CSF could be used in radiation patients without the President's directive; however, the FDA panel also indicated the need for additional studies to optimize the drug response in NHPs or other large animal models.

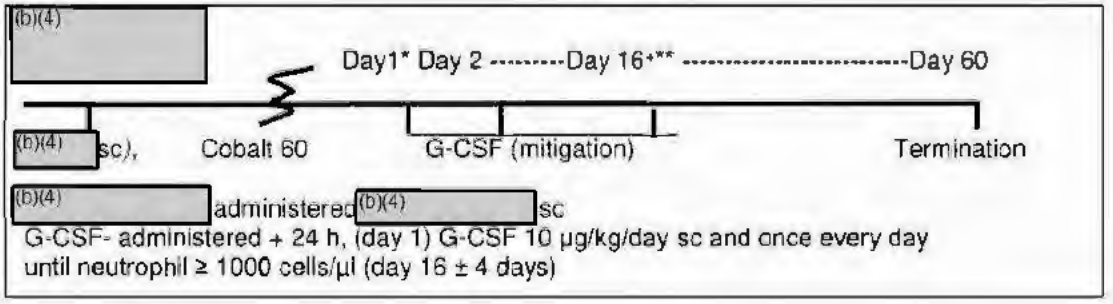
We will be conducting studies in animals with minimal supportive care (no antibiotic or blood transfusions). As described below in Experiment 1, we intend to pretreat animals with a single dose of (b)(4) and subject them to whole body Cobalt 60 gamma radiation. These animals will receive G-CSF following radiation exposure. The end point will be survival and hematological changes over 60-days post-irradiation. We hypothesize that pretreatment with (b)(4) and followed by G-CSF treatment post irradiation will synergistically increase radiation survival of NHPs.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

V.1.1. Experiment 1: Effect of 1 pre-irradiation administration of (b)(4) + multiple postirradiation administrations of G-CSF (once daily) until neutrophil recovery: The first experiment will evaluate hematological indices at periodic intervals and monitor animals for survival until 60 days postirradiation. In this experiment based on experimental design (b)(4) will be administered (b)(4) prior to irradiation (Schematic 1). Filgrastim (G-CSF) administration will be initiated +24 h postirradiation and will be continued on a daily basis until neutrophils return back to ≥ 1000 cells/µl. This is anticipated to be around 16 days (± 4 days).

Schematic 1



Experimental design

The following groups will be included in the study:

Group 1-Control – will receive PBS sc pre- and postirradiation, n = 3

Group 2- G-CSF alone- will receive G-CSF daily (starting +24 h, and until neutrophil recovery (≥ 1000 cells/µl) n = 3

Group 3-(b)(4) -G-CSF- Will receive (b)(4) and G-CSF (+24 h, and until neutrophil recovery (≥ 1000 cells/µl) n = 3

Radiation dose: The dose in the present study will be 7.2 Gy @ 0.6 Gy/min representing approximately the LD70/60 dose with minimal supportive care (unpublished preliminary data).

(b)(4) regimen: (b)(4) will be administered sc 24 h before irradiation

G-CSF regimen: The first G-CSF administration (10 µg/kg) will be administered sc 24 h postirradiation. Additional G-CSF will be administered sc daily (10 µg/kg) until absolute neutrophil count (ANC) increases to ≥ 1000 cells/µL.

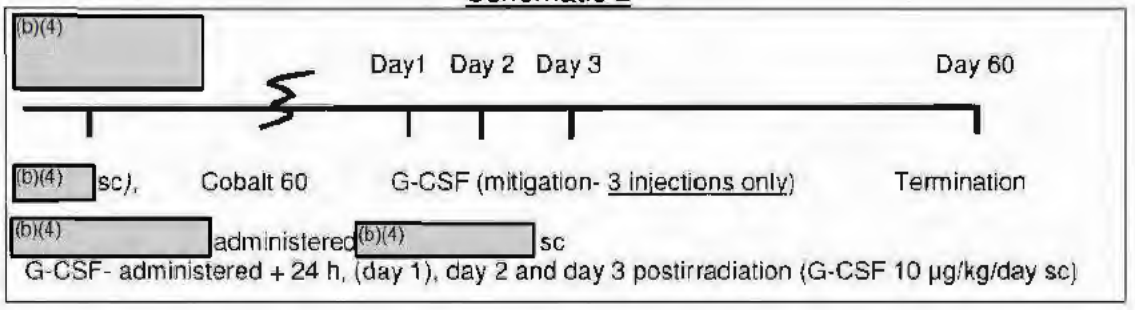
Postirradiation monitoring: Animals will be monitored for 60 days postirradiation.

Euthanasia: Experimental animals will be euthanized at the end of 60 day observation period and all relevant tissues including bone marrow will be collected for histopathological and biochemical analysis.

V.1.2. Experiment 2: Effect of 1 pre-irradiation administration of (b)(4) + three postirradiation administrations of G-CSF (on day+1, +2, and +3 postirradiation).

This experiment will evaluate hematological indices at periodic intervals and monitor animals for survival until 60 days postirradiation. In this experiment based on experimental design, (b)(4) will be administered (b)(4) prior to irradiation (Schematic 2). Filgrastim (G-CSF) administration will be initiated +24 h postirradiation and will also be administered on day +2 and day +3 postirradiation (Schematic 2). This experiment is based on mouse studies where in PI has demonstrated that 3 daily postirradiation injection of G-CSF alone are effective in increasing 30-day postirradiation survival (unpublished data).

Schematic 2



Experimental design
The following groups will be included in the study:

Group 1-Control – will receive PBS sc pre- and postirradiation, n = 3

Group 2- G-CSF alone- will receive G-CSF (+24 h, + day 2, and + day 3 postirradiation) n = 3

Group 3-(b)(4) -G-CSF- Will receive (b)(4) and
G-CSF (+24 h, + day 2, and + day 3 postirradiation) n = 3

Note: All other experimental conditions such as radiation dose, euthanasia etc will be similar to experiment 1 and hence are not described.

V.1.3. Experiment 3: Effect of 1 pre-irradiation administration of (b)(4) + three postirradiation administrations of (b)(4) (on day +1, +7, and +10): This experiment will evaluate pre and postirradiation (b)(4) administration on hematopoietic recovery and 60-day postirradiation survival. This concept is based on clinical use of (b)(4) (b)(4) in activity. (b)(4) (b)(4) is given once a week for treating idiopathic thrombocytopenia purpura (ITP). However, (b)(4) has not been evaluated as a radiation countermeasure.

Experimental design
The following groups will be included in the study:

Group 1-Control – will receive PBS sc pre- and postirradiation, n = 3

Group 2- (b)(4)
(-24 h, +1 day, +7 day, and +10 day) n = 3

Radiation dose: The dose in the present study will be 7.2 Gy @0.6 Gy/min representing approximately LD70/60 dose with minimal support care (unpublished preliminary data).

(b)(4) regimen: (b)(4) will be administered sc 24 h before irradiation and on day 1, day 7 and day 10 postirradiation.

Postirradiation monitoring: Animals will be monitored for 60 days postirradiation.

Euthanasia: Experimental animals will be euthanized at the end of 60 day observation period and all relevant tissues including bone marrow will be collected for histopathological and biochemical analysis.

V.2. Data Analysis

Comment: As mentioned in the non-technical synopsis, the experiments described in the protocol should be considered as pilot studies and hence statistical evaluation will be limited.

Statistician's remarks and approval:

Working Hypothesis for declaring a test agent effective; An observed 30% increase in survival over the vehicle-treated group exposed to a radiation dose producing ~ 70% (±10%) mortality, monitored over 60 days post irradiation. *(This criterion is based on data from previous extensive mouse studies/literature /scientific discussions & agency requirements to evaluate radiation countermeasures agents).*Means and SE with a significance level at 5% set for each test has to be performed. In order to evaluate the hypothesis set forth and establish a comparison of significance of deviation from the hypothesis, Fisher Exact Test to be used. The test can provide the significance and deviation of efficacy of the measure used. Type-1 error is controlled at 5%, type-2 error is 20% and sample sizes are equal between the different groups. The Exact Log Rank test (one-tailed) and the generalized Savage (Mantel-Cox) procedure (BMDP Statistical Software Inc., CA) will be used to compare survival of drug-treated and control groups in the radiation-survival experiments. The log-rank test or the Mantel-Cox test evaluates the test hypothesis of survival distribution statistics and potential effectiveness of drug response among the groups of samples at the established time points to ascertain the drug efficacy and effectiveness. It provides the statistics to differentiate dose-response efficacy based time distributions among groups of samples for potential new treatment evaluation comparison to controlled treatment with or without combination. This test is widely used in clinical trials to establish the efficacy of the drug or drug combinations and to establish the drug effectiveness. When inferential statistics is used such as t-test, Fisher's tests, ANOVA etc, to evaluate the outcome and comparisons, sample size (n) need to be determined to provide sufficient statistical capability for performance. Based on the power analyses the sample size proposed by the investigator is appropriately sufficient to deliver more than 80%. Although experiments with 8 NHPs (4 NHPs per group) have ~80% of power to detect significant differences between the drug-treated and vehicle controls, for the present protocol 3-6 animals per group (24 NHPs in total) are used since these studies are exploratory in nature (b)(4) has not been systematically analyzed in large animal model).

Demographic, hematology, vital signs, blood counts, chemistry, antibody levels, and cytokine estimations in both blood and urine will be compiled as summarized using mean, standard deviation, minimum, median and maximum values.

Vital signs will be summarized using descriptive statistics at each assessment time for each treatment group.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered The effectiveness of drugs such as (b)(4) against radiation cannot be adequately assessed using current *in vitro* technologies. The U.S. FDA recognizes the ethical considerations involved in testing the efficacy of radioprotective drugs in humans. Therefore, the FDA requires preclinical drug assessments (safety and efficacy) to be performed using both small and large animal models (FDA 2002) prior to granting approval for safety testing in humans. The proposed experiments will provide efficacy data with NHPs, one of the preferred large-animal models. If these studies are successful, full-fledged GLP studies will be conducted for safety and efficacy. GLP studies are very expensive and cannot be done with the grant for this study.

V.3.2. Animal Model and Species Justification NHPs are necessary for the pre-clinical development of a drug candidate intended for use in humans because drug

metabolism and physiology are very similar between humans and NHPs. Testing a drug with potential for human application in NHPs ensures safety and specificity prior to the drug entering into the clinic. This is true especially because of safety risks and the chance of unexpected autoimmune or hyper-immune reactions. Due to the species-specific nature of immune reactions, the drugs can only be studied *in vivo* in hosts whose immune systems are very closely related to that of humans. Rhesus macaques are the model of choice for investigations of radiation injury and countermeasures because of the large database available from the existing literature that allows for robust comparison. The FDA has accepted rhesus macaques as the appropriate animal model for pilot and pivotal efficacy testing of radiation countermeasures under the Animal Efficacy Rule, where efficacy testing cannot be performed *in* humans. The safety and efficacy of (b)(4) was demonstrated in mice. (b)(4) (b)(4) it is appropriate to conduct the present set of experiments to advance the drug for human use.

V.3.3. Laboratory Animals: *Macaca mulatta* (rhesus macaque) will be obtained from vendors registered with the USDA.

V.3.3.1. Genus/Species: *Macaca mulatta*

V.3.3.2. Strain/Stock: Preference will be given to animals of Chinese origin so that comparisons can be made with existing data on NHPs from AFRRI. Male and female *M. mulatta* are required that have been pole and collar trained, have normal cardiovascular parameters, no infections or wounds, and which have normal liver and kidney enzyme functions.

V.3.3.3. Source/Vendor: In general, the sources for these animals are commercial vendors registered with USDA. (b)(4) (b)(4)

V.3.3.4. Age: Animals of 3 to 8 years of age are requested for the present study.

V.3.3.5. Weight: Animals must weigh in the range of 3–8 kg.

V.3.3.6. Gender: Male and female

V.3.3.7. Special Considerations: NHPs shall test negative for Herpes B Virus (aka, *Macacine herpesvirus*), simian T-cell leukemia virus type 1 (STLV-1), simian immunodeficiency virus (SIV), and simian retrovirus (SRV) Types 1, 2, 3, and 5. Animals shall also test negative by means of virus isolation or polymerase chain reaction (PCR) for SRV Type 2. NHPs will either be vaccinated for measles or, in the case of previously measles-vaccinated NHPs, tested for the presence of measles antibodies.

NHPs coming from the vendor colony must test negative for Salmonella sp. and Shigella sp. On the advice of the attending veterinarian, in consultation with the PI, the exclusion criteria for certain viral and bacterial pathogens may be waived.

CBC and blood chemistry will be obtained from vendors for each primate and screened for abnormalities by an AFRRI veterinarian. Any abnormal findings will be discussed with the PI and a determination of the fitness of the animal for use on the protocol will be made. Individual medical records will be maintained during the entire length of the study. Tuberculosis testing will be performed in accordance with VSD SOPs.

Use of collared animals allows them to sit in special chairs for blood draw as well as for other examinations/treatments.

V.3.4. Number of Animals Required (by species): Total (24): 12 males and/or 12 females

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement: Pain is not expected from the irradiation *per se* but the sequelae of radiation exposure at the levels indicated in this protocol will cause pain and distress. Unexpected circumstances that elicit pain and/or distress will immediately be brought to the attention of the principal or co-investigator and attending staff veterinarian for appropriate action as detailed in AFRRI Protocols.

The use of pole and collar trained animals (preferably from vendor) will reduce the stress on the animals during blood draw and injections. Acclimatization to bottle for oral supplementation such as drinking water, juices, Gatorade and electrolytes will facilitate working with the animals and relieve the discomfort from radiation-induced vomiting or diarrhea. Prior to placement of the animals within comfortable radiation restraining chairs they will receive light immobilizing doses of ketamine (as determined by veterinarian) to reduce the anxiety of moving. The time in the chair will be kept to a maximum of 1 h. In addition, the animals will be transported to the radiation facility in a closed HEPA-filtered cart to reduce the excitement of moving from their home cages to the radiation area. Medications will be provided in case of fever, diarrhea, vomiting, and bloating.

Lastly, relative to a humane endpoint, moribund animals will undergo euthanasia. Euthanasia will only be provided by qualified veterinary staff. The endpoint of the studies will be either death from the squeal of radiation injury or euthanasia for moribund animals. To minimize animal suffering, pain, or distress, moribund animals will be considered to have arrived at the study endpoint. Such animals will be euthanized and recorded as "mortality".

Animals will be considered morbid when they display symptoms listed in V.4.5 Experimental animals that are morbid, but not moribund, will be allowed to continue in the experimental protocol with minimal supportive care in order to evaluate survival accurately.

Animals will be considered moribund when showing some or all of the signs of morbidity, the animals also display severely debilitated state and are in terminal distress. Animals will be monitored more frequently during this critical phase of the study.

V.3.5.2. Reduction: Further, we propose to use multiple sampling points for blood collection. Baseline data obtained from individual animals pre-irradiation (3 time points) will be used for assessing post-irradiation alterations in hematological parameters.

V.3.5.3. Replacement: None

V.4. Technical Methods

Animal procedures: NHPs will be pair-housed as much as possible after observing compatibility. Animals will be quarantined for 4-6 weeks prior to the inception of the experiment. Environmental enrichment will be in the form of fruit, vegetable, or additional dietary supplements, and various cage-enrichment devices. Fruits will be washed in fresh water, treated with dilute chlorine solution (200 ppm chlorine/5 gallons water) and rinsed before providing it to animals.

Chair training: Animal order request will include installation of collars and training in the technique of pole and collar with chair restraint. This training will be further reinforced at AFRRI by VSD staff and project personnel.

Irradiation: On the morning of irradiation, the NHPs will not be fed. Animals will be sedated, loaded into specialized restraint boxes, transported to the AFRRI cobalt facility using a self-contained breathing unit (HEPA-filter equipped) transport container and irradiated. Following radiations, animals will be returned to their original room and kept in clean cages. Since the NHPs will be sedated prior to restraining in the specialized boxes for radiation, "restraint" training in this instance is not mandatory.

Animals will be assigned to treatment groups using either a computerized randomization procedure or a physical randomization procedure depending upon the sample size. Randomization is designed to achieve balance in terms of neutrophil counts and body weights. Body dimensions (waist) will be measured prior to radiation. Animals may be removed from the study based on abnormal neutrophil counts or clinical signs (physical exam, clinical chemistry) during the weeks before irradiation, in which case backup animals will be randomized. The list of animals, with assigned groups and irradiation pairings, will be discussed with AFRRI dosimetrists in advance before the irradiation day to facilitate dosimetry.

Radiation dosimetry will be performed by the AFRRI dosimetrists using different sized cylindrical monkey phantoms inside monkey restraint boxes (b)(4) (b)(4)

General procedures:

Drug formulation:

(b)(4) will provide the drug. The drug will be endotoxin tested by Charles River laboratories and the drug will be diluted appropriately in sterile PBS prior to injection (within 1-2 h after preparation). The drug will be maintained undiluted at 4 ºC until injection. The drug dose will be (b)(4) administered sc.

Filgastrim (Neupogen®) recombinant human methionyl granulocyte colony stimulating factor (r-metHuG-CSF) will be purchased from Amgen, Inc. (Thousand Oaks, CA). The drug will be diluted appropriately in sterile 5% Dextrose prior to injection (within 1-2 h after preparation). The drug will be maintained undiluted at 4 ºC until injection. The drug dose will be 10 µg/kg administered *sc.*

Blood collection: Blood samples will be collected under appropriate SOPs. Blood (0.5 ml to 1.5 ml/day depending on the time point) will be collected from a peripheral vessel or femoral vein with a 22-25 G needle attached to a syringe to reduce the chances of bleeding after blood collection in irradiated animals. An aliquot (0. 5 ml) from each sample will be taken immediately for CBC/differential. Further, 0.5 ml will be used to prepare serum that

will be frozen at -70 °C for clinical chemistry and antibody measurements. If additional blood collection (extra 1 ml/collection) is required for bacteremia (in case of severe neutropenia) or for evaluation of other parameters such as antibody assays then amendments will be submitted to request such collections.

The area for the blood draw will be swabbed with disinfectant to include 70 % alcohol (isopropyl or ethanol) solution for routine collections and additionally with providone-iodine for microbial sample collections (left in place for 3 minutes) before inserting the needle. Providone-iodine, if used, will be swabbed off to reduce irritation to the tissue .The blood sample will be drawn as described above. While withdrawing needle, pressure will be applied at the same time with a clean gauze to reduce bleeding from the blood draw site. While making routine observation of animals, the blood-draw site will be checked for bleeding. If the site becomes infected, topical antibiotics will be applied, under supervision by VSD.

Urine collection:
Free Catch Method: Urine collection procedure will involve the use of standard trays with fiberglass mesh filters (Johnson *et al*., 2012).Trays will be placed under NHP cages after morning cleaning has been performed by VSD animal husbandry personnel (placed at approximately 10:00 am) and will remain under cages for approximately 4-6 h. Normal feeding and watering will be performed while trays are under cages. Urine will then be carefully removed from the tray with a disposable 2 ml pipette, aliquoted and stored at -80^0 C. Baseline urine collections will be performed at least 1 week before radiation. Urine collection will continue at every other from day 0-8 and then approximately once to twice per week through day 35. Samples will remain frozen at -80^0 C indefinitely until cytokine, trace element and metabolic analysis can be performed.
Alternative Methods: Other methods including the use of metabolic cages and diapers have also been described for urine collection in Non-human primates as reviewed by Kurien *et al* in 2004. The PI will reserve the initiative to explore other options if the free catch method is insufficient to obtain pure animals urine for qualitative and quantitative purposes.

Identification of pain: NHPs demonstrate remarkably little reaction to surgical procedures and injury in the presence of humans. An NHP in pain has a general appearance of misery and dejection. We will observe all irradiated animals for grimace and glassy eyes, loud and persistent vocalization, avoidance of social interaction, and grooming. Gastrointestinal injury may be associated with abdominal pain displayed as facial contortions, teeth clenching, and restlessness. Headaches manifest by pressing the head against the enclosure surface. Self-directed injurious behavior may be a sign of more intense pain. Since most primates in pain will refuse food and water, daily food and water intake will be closely monitored. Further, response to a familiar personnel and changes in willingness to cooperate may also indicate pain, which will be closely monitored and recorded.

Euthanasia: Animals will be sedated according to current American Veterinary Medical Association (AVMA, 2013) guidelines. All animals will first be sedated with ketamine (IM, 10 mg/Kg). Animals will then be euthanized with sodium pentobarbital (IV, 100 mg/kg). See item V.4.6 for euthanasia criteria before the end of the study.

Veterinary Care: All animal procedures performed will be in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and applicable VSD

SOPs. Our facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal usage will be approved by the Institutional Animal Care and Use Committee in accordance with approved protocols. Species specific environmental enrichment and dietary supplements are provided according to standard operating procedures.

Routine and emergency veterinary medical care: Animals will be quarantined for 4-6 weeks on arrival and could be assessed for pole and collar training during the quarantine period. Each animal will be observed twice daily for evidence of pain or distress. Body weights will be determined at least once prior to the start of the study, and on a regular basis when the animals are chaired for blood collections. Body temperature will also be determined at least once prior to the start of the study, and on a regular basis when the animals are chaired for blood collections using chips implanted by VSD trained staff. The time of each blood collection will be recorded for each NHP. Animals will be qualitatively assessed twice daily (a.m. and p.m.) for general behavioral status and food consumption. Appropriate standard operating procedures will be followed by staff veterinarians in the event of pain or distress.

Minimal supportive care: We define minimal supportive care as clinical care that does not involve platelet or blood transfusion. Clinical care will be minimal; platelet counts may drop below 20,000 cells/µl for several days, but whole blood will not be transfused, as this would negate the purpose of the study. Skin characteristics will be monitored daily for signs of dehydration, which may be possible if animals avoid drinking after exposure. In this case, supplemental fluids will be administered (Table 1).

Table 1. Summary of Minimal Supportive Care

Drug class	Allowed medication or supportive care agents	Indication and/or criterion for administration
Anesthetics	Ketamine 10 ± 5 mg/kg, IM	**Sedation during procedures:** • Administered before animal manipulations to minimize stress and anxiety.
Analgesics	Acetaminophen (Tylenol) 5–10 mg/kg PO BID-QID	**Pain Management:** • Acetaminophen can be administered when evidence of pain is present.
Antipyretic	Acetaminophen (Tylenol) 5–10 mg/kg PO BID-QID	**Fever [temperature ≥ 104 °F (40°C)]**
Supplemental Fluids	Hydrating fruits and pop sickles. PRANG 15 ± 5 ml/kg PO in consultation with VSD staff.	**Dehydration: (At the request of the investigator or veterinarian)** • Symptoms include diminished skin turgor, dry mucous membranes, dry nose, increased hematocrit, and sunken eyes **Fever [temperature ≥ 104 °F (40°C)]:**
Nutritional Support	BIO-SERV certified Rhesus Liquid Primate Diet	**Weight Loss (Anorexia):** Nutritional support can be administered when body weight is <85% of baseline and be continued as long as the body weigh remains <85% of baseline, and the animal is not eating.

Antibiotics (Topical)	As per veterinarian consultation	**Evidence of subcutaneous infections, open wounds:** Antibiotic cream can be administered (under supervision of VSD staff) to ameliorate discomfort due to skin lesions.
Anti-diarrheal	Loperamide hydrochloride (Imodium), Initial dose 0.04 mg/kg QID.	**Diarrhea:** At the first sign of soft to loose stools: Imodium will be administered up to twice daily and should continue for 3 days. It may be stopped earlier than 3 days if the diarrhea resolves.
Barbiturates	Pentobarbital sodium, 100 mg/kg IV.	**Euthanasia.**

If pain and/or distress are seen, the PI and VSD veterinarian will be notified immediately to evaluate and determine the appropriate treatment, even on the weekends and after normal business hours. Body temperature may be monitored daily. Hematology will be monitored one week before irradiation, and at appropriate times after irradiation. Symptomatic therapy will be provided with development of abnormal clinical signs. For example, fruit or treats may be given in case of anorexia. In cases of open wounds with significant defects following skin necrosis due to subcutaneous infections, the area will be thoroughly cleaned and appropriate care will be provided by technical staff and VSD veterinarian. If pain and/or distress are observed, affected NHPs will be evaluated by a staff veterinarian and will be treated as determined by the staff veterinarian.

Inappetence or dehydration with or without signs of gastrointestinal distress will be treated with either fluid supplementation (*per os* tubing) or Liquid Primate Diet at the request of the investigator or staff veterinarian. Antibiotic cream may be administered topically (under supervision by VSD staff) in order to ameliorate discomfort due to skin lesions.

Estimation of hematological indices: Blood (0.5-1.5 ml/day depending on time point (see Table 2)) will be obtained from the saphenous vein (or femoral vein) using a 22–25 G needle attached to a 3 ml (generally) heparinized syringe from non-anesthetized NHP, transferred immediately into pre-coated ethylenediaminetetra-acetic acid (EDTA, Sigma-Aldrich, St. Louis, MO, USA) tubes and mixed gently on a rotary shaker until analysis. The coded tubes will be analyzed for white blood cell (WBC), absolute neutrophil counts (ANC), monocytes, lymphocytes, reticulocytes, hemoglobin and platelet counts using the ADVIA 2120 (Siemens Medical Solutions Diagnostics, Dublin, Ireland) and data will be generated using the MS software, version 5.9.

Estimation of peripheral blood progenitor cells: The primary effect of (b)(4) is on the hematopoietic stem and progenitor cell (HSPC) proliferation. Since we have not included bone marrow collection during the 60 day observation period in these NHPs, assay of the peripheral blood for HSPCs will serve as a substitute for determining the effect of (b)(4) on the hematopoietic cells in normal and irradiated NHPs. Heparinized blood will collected as described above and total CFCs [i.e., granulocyte-macrophage colony-forming units (CFU-GM), erythroid burst-forming units (BFU-E), and multilineage (granulocyte, erythrocyte, macrophage, megakaryocyte) CFU (CFU-GEMM)] and high-proliferative potential CFC (HPP-CFCs) will be assayed.

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals 24

V.4.1.1.1.1. Column C ___0__ (# of animals)

V.4.1.1.1.2. Column D ___0__ (# of animals)

V.4.1.1.1.3. Column E ___24__ *(# of animals)*

V.4.1.1.1.4. Pain Category Assignments

Exp.	Experimental/Control Group	C	D	E	Totals
1	(b)(4) and G-CSF (G-CSF administered until neutrophil recovery	0	0	9	9
2	(b)(4) and G-CSF (G-CSF administered for only three days postirradiation)	0	0	9	9
3	(b)(4) pre and post irradiation			6	6
Totals:				24	24

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

Microchips will be implanted under ketamine (10 mg/Kg, IM) sedation or local (lidocaine) anesthesia and are placed before the initial start of the study. If necessary a drop of surgical glue will be placed at the chip insertion site to ensure that the chip stays in place.

Bone marrow collection will be made pre-euthanasia. It will be carried out under "Clean" and not "sterile" conditions. Animals will be euthanized after bone marrow collection before the animal is allowed to recover from anesthesia.

Bone marrow is expected to be a painful procedure. As such, the primates will be under general anesthesia at the time of marrow cell harvesting. NHPs will be sedated with 10mg/Kg ketamine IM in order to facilitate placement of an IV catheter, the animal is intubated and maintained on isoflurane at a surgical plane of anesthesia. Additional local anesthesia with Lidocaine (approximately 1 ml) will be provided at the marrow harvesting site at the recommendation of the staff veterinarian. Bone marrow sampling will be performed in the VSD under clean conditions and not aseptic conditions.

V.4.1.2.2. Pre- and Post-procedural Provisions Animals will be assessed for signs of pain and distress by either the veterinary or research staff at least twice per day or more often as needed according to VSDM SOP. Before procedures that require general anesthesia, NHPs will be fasted for 12 h prior to administration of anesthetic. However, terminal bone marrow collection to be carried out in the protocol would not require 12 h fasting. Clinical signs of acute radiation sickness can be treated with medications on the advice of a VSD veterinarian (e.g., acetaminophen, Imodium, lomotil, but not ibuprofen or drugs that may affect the immune system, or induce platelet aggregation). For any NHPs that are not taking in sufficient food and/or water, additional foods can be offered to entice them to eat. In addition, NHP will be prior-acclimated to feeding syringes and sipper bottles to allow for the use of supplemental oral fluids if dehydration is noted. Criteria for

euthanasia are listed in Section V.4.5. NHP will be assessed at least 2X daily and additional observational time point(s) will be added during critical phases of the animals undergoing procedures that are appropriate for the study.

V.4.1.2.3. Paralytics No paralytics will be used in this study.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures Because the principle purpose of this study is to determine the PK/PD and benefits on survival by treatment with (b)(4) against radiation-induced mortality, there are no alternatives to irradiation. Supporting information such as the clinical chemistry analyses, hematological analysis of the blood and bone marrow, bacterial studies, and histopathology analyses of selected tissues will help identify the usefulness or problems associated with the use of (b)(4) as prophylactic/therapeutic agent in irradiated animals.

V.4.1.3.1. Sources Searched Agricola, Pubmed, altweb

V.4.1.3.2. Date of Search 05-20-2014

V.4.1.3.3. Period of Search Agricola (1974-preent), Pubmed (1946-present), Altweb

V.4.1.3.4. Key Words of Search

Keywords	AGRICOLA 1974-present	PubMed 1946-present	Altweb
Alternatives to painful procedures (radiation)	6	14493/389*	3
primates+bone marrow harvesting	4	608 / 82**	1
radiation+(b)(4)+primate	0	2	0
primate+blood draw	0	722***	12
primate+pain	2	18058	11
Thrombopoietin +(b)(4)+ primate+pain	0	0	0
*From radiation exposure [all methods], **Other animals, ***Other animals only			

V.4.1.3.5. Results of Search: The search clearly demonstrated that there are no alternatives or pain relief in literature that could be used in the present study.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: There are no alternative procedures for irradiation because it is a unique stimulus/stress that cannot be otherwise duplicated. Radiation itself does not cause pain or distress. Nevertheless, the sequelae of nausea, vomiting, and diarrhea causes pain and distress in humans in the early post-irradiation period, when high doses (8 Gy and above) are used. Although radiation does not induce pain, animals in these experiments might experience pain and distress prior to death because of hematological and gastrointestinal damage.

Bacteria translocation from the alimentary canal may cause infections that result in discomfort either locally or systemically. Many opportunistic pathogenic bacteria have unusual or even unique virulent characteristics, but they have common attributes, including binary multiplication and penetration of tissues, and cause common responses and disease process in animals, which cannot be mimicked readily by substitutes. The investigators and animal care staff will provide as much comfort and well-being to the animals as is

consistent with the scientific integrity of the work plan. The principle purpose of these studies is to evaluate the efficacy of (b)(4) in enhancing survival of irradiated NHPs. As stated, bone marrow, peripheral blood, liver and kidney (liver and kidney analysis done by blood chemistry with the hematological studies) analyses will also be performed after (b)(4) and G-CSF administration. Moribund animals will be euthanized in consultation with VSD veterinarian.

We expect that (b)(4) as well as G-CSF will provide some relief from pain and or discomfort due to the sequelae of irradiation by its protective effect and by the possibility that it will advance hematopoietic recovery in some or all of the (b)(4) treated and irradiated primates. Microchip implantation is not considered a surgical procedure. Bone marrow extraction is considered a surgical procedure. Prior to general anesthesia for all surgical procedures, all NHP will be fasted for 12-18 h, however, terminal bone marrow collection described in the protocol does not require 12-18 h fasting.

V.4.2. Prolonged Restraint None

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions:

Microchip implantation is not considered a surgical procedure. Bone marrow extraction is considered a surgical procedure. Since the bone marrow collection in this protocol will occur prior to euthanasia, fasting of animals 12-18 h prior to anesthesia will not be required.

V.4.3.2. Procedure(s) NHPs may be sedated or given local anesthesia for microchip implantation. The chips are manufactured by Bio Medic Data Systems, Inc (BMDS) and used to monitor body temperature. They come pre-loaded in a specially designed needle/syringe device.

General Anesthesia:

Animals are sedated using ketamine (10mg/Kg IM) and chips are inserted subcutaneously between the shoulder blades. Once the chip is deposited, the needle is withdrawn and the skin is pinched between 2 fingers for about 10-20 seconds to ensure that the chip itself (about the size of a grain of rice) does not dislodge through the puncture site. If a chip fails, a second chip will be inserted. If necessary a drop of surgical glue will be placed at the puncture site to ensure that the chip stays in place.

Local Anesthesia:

Animals will be restrained in a chair. A small patch of fur on the left or right side of upper back may be shaved for microchip placement. Lidocaine ointment may be applied to the area, a 5 min time lapse will be allowed for the lidocaine to be effective and then a microchip will be inserted by a trocar. If necessary a drop of surgical glue will be placed at the chip insertion site to ensure the chip stays in place.

Bone marrow collection.

The method proposed for the marrow collection is adapted from those used by Fortman *et al.*, (2001) and Kushida *et al.*, (2002).

- General anesthesia
- Premedication-ketamine-5-15 mg/kg IM
- Induction – 3-5% Isoflurane in 100% Oxygen by Mask

- Maintenance-1-3 % Isoflurane in 100% Oxygen by endotracheal tube or mask
- Site of bone marrow sample: Iliac crest, trochanteric fossa of the femur, or proximal humerus.
- Procedure: Animal will be positioned appropriately (dorsal or ventral, or lateral recumbency, depending on site). A sterile drape will be used to cover the site.
- A Biospy or Aspirate sample will be collected as determined by the PI.
- Biopsy: A small 1-2 cm incision will be made in the skin at the site of bone marrow collection. A bone marrow biopsy needle will be advanced through the incision into the cortex of the bone. The stylet will be removed and the needle advanced 1-2 cm with gentle rotation and rocking of the needle. The needle is then retracted and advanced slightly before being gently removed from the bone. The included expeller is used to remove the sample from the needle and in to a cassette with a foam insert, if necessary, which is then put in 10% formalin for fixation and processing.
- Aspirate: A small 1-2 cm incision will be made in the skin at the site of bone marrow collection. A bone marrow aspiration needle will be advanced through the incision into the cortex of the bone. The stylet is removed from the bone marrow needle. A 2-5 ml syringe will be used to aspirate 2-3 ml of bone marrow.

V.4.3.3. Post-surgical Provisions N/A since animals after bone marrow collection will be euthanized.

V.4.3.4. Location All surgical procedures will take place in the VSD surgical suite.

V.4.3.5. Surgeon AFRRI VSD trained staff or veterinarian will perform implantation of microchip and the bone marrow collection.

V.4.3.6. Multiple Major Survival Operative Procedures None

V.4.3.6.1. Procedures

V.4.3.6.2 Scientific Justification

V.4.4. Animal Manipulations

V.4.4.1. Injections Trained personnel will carry out Injections within the scope of VSD SOPs.

Drug injection: The drug in sterile PBS will be freshly prepared from a liquid formulation of the drug on the day of injection. Depending on the drug concentration and dilution, drug injection volume may vary between 0.2 to 1.0 ml/animal/injection.

Needle: Drug will be administered in unanesthetized but restrained animals using 22-25G needle.

V.4.4.1.1. Pharmaceutical Grade Drugs: G-CSF is a pharmaceutical grade product and will be used in the study. It will be procured through USUHS pharmacy- (Neupogen® Filgrastim 300 mcg/1 ml), Amgen Inc., CA. The drug will be diluted in 5% dextrose and if required further diluted in PBS for *sc* injection.

V.4.4.1.2. Non-Pharmaceutical Grade Drugs (b)(4) (b)(4)

Sterile phosphate-buffered saline will be used as the vehicle. An aliquot of the solution will be sent to Charles River Laboratories for endotoxin testing; a copy of the results will be sent to IACUC. At the same time, pH will be tested to ensure that it falls in the range stipulated by IACUC (pH 6-8). A 23-25G needle will be used to administer subcutaneous injections.

V.4.4.2. Biosamples Chair restraint of NHPs for the purposes of phlebotomies was previously discussed as a technique to obtain blood samples over a short period of time that would reduce stress to the animals. In place of this procedure, or in addition to it, training may be provided to the NHPs such that they offer their limbs for the blood sampling procedure.

Table 2: Blood collection; time-points and volumes

Time of blood draw	PK (0.5 ml)	CBC (0.5 ml)	Colony forming Unit assay (CFU) (0.5 ml)	Total blood, ml
-7d	+	+	+	1.5
-3d		+		0.5
-2d	+	+	+	1.5
day -1 (Pre-TBI injection)				
Irradiation (DAY 0)				
Day 1 (post-TBI injection)				
Day 2	+	+	+	1.5
Day 4	+	+	+	1.5
Day 8	+	+	+	1.5
Day 10	+	+	+	1.5
Day 12	+	+		1.0
Day 14		+		0.5
Day 16		+		0.5
Day 18		+		0.5
Day 20	+	+	+	1.5
Day 22		+		0.5
Day 24		+		0.5
Day 26		+		0.5
Day 28	+	+	+	1.5
Day 35	+	+	+	1.5
Day 49	+	+		1.0
Day 60	+	+		1.0
Day 60	Bone	marrow	(terminal)	3.0 ml

V.4.4.3. Adjuvants N/A

V.4.4.4. Monoclonal Antibody (MAb) Production N/A

V.4.4.5. Animal Identification The animals are identified by indelible markings on their chest and/or leg. If the primate is not tattooed upon arrival, a VSD colony number will be assigned and tattooed onto the chest.

V.4.4.6. Behavioral Studies: None

V.4.4.7. Other Procedures None

V.4.4.8. **Tissue Sharing Tissue sharing will be allowed with appropriate paperwork filed with the IACUC.**

V.4.4.9. Animal By-Products N/A

V.4.5. Study Endpoint

Many of the primates that are administered (b)(4) G-CSF are expected to survive. However, vehicle-treated animals, may show 70% ±10% mortality. Moribund animals will be closely monitored and euthanized at the advice of attending veterinarian to minimize excessive pain. Moribundity will be used in place of mortality to relieve the animal from pain and distress. Euthanasia will be carried out when any one or more of the following signs of severe morbidity or moribundity are observed by AFRRI veterinarians and in consultation with the principal investigator (see below). Necropsies and histopathological analyses will be performed on all euthanized animals by the veterinary pathologist or pathology staff. The parameters to provide guidelines for moribundity have been described under 'study end-points' of 'General procedure' in the experimental design.

Treatment with (b)(4) and G-CSF may result in the survival of all irradiated animals, based on high level protection of mice from exposure to supra-lethal radiation dose and its potential to stimulate hematopoietic recovery. Therefore, an endpoint for individual animals will be related to success or failure of treatment with (b)(4) At the end of 60 days, bone marrow collection will be made and animals euthanized.

Study endpoints: Experimental animals will be euthanized based on an assessment and in consultation with attending veterinarian. Moribund animals will be euthanized. Animals will be declared moribund in consultation with a veterinarian when generally in poor condition and exhibiting one or more of the following signs:

1. Weight loss: loss of more than 20% body weight over a 3-day period when compared to baseline weight. Normally animals will be weighed twice a week, however, if inappetence sets in then we will monitor weight on a daily basis.
2. Inappetence: complete anorexia for 24 hours. This is defined as refusing regular food and all food enrichment items).
3. Weakness/inability to obtain food or water: Inability to stand would be a criterion for euthanasia and would not allow the animal to persist in this state for 24 hours.
4. Minimal or absence of response to stimuli (animal does not move when prodded), assuming the animal has recovered from anesthesia.

5. Core body temperature: below 35.9ºC following a period of febrile neutropenia (≤500x10^3 cells/µl) by way of microchip will be done on a daily basis.
6. Severe acute anemia: (<40 g/L hemoglobin, <13% hematocrit)
7. Infection documented by blood culture (septicemia) and accompanied by severe systemic signs of illness.
8. Other signs of severe organ system dysfunction with a poor prognosis as determined by a veterinarian that may need additional diagnostic tests:

8.1 Respiratory: severe dyspnea, severe cyanosis.

8.2 Gastrointestinal: severe vomiting or diarrhea, evisceration (immediate euthanasia required). NOTE: Transient vomiting and diarrhea are expected results of whole-body gamma irradiation. Therefore, as criteria for euthanasia, these symptoms should be severe and life-threatening per se, i.e., hemorrhagic diarrhea to the point of causing anemia as listed above or severe uncompensated dehydration.

8.3 Urogenital: renal failure characterized by elevated BUN and creatinine.

8.4 Neural: CNS depression, seizures, paralysis of one or more extremities.

8.5 Integumentary: Non-healing wounds, repeated self-trauma, severe skin infections, indicating severe organ system dysfunction with a poor prognosis.

V.4.6. Euthanasia Animals will be euthanized according to current American Veterinary Medical Association (AVMA, 2013) guidelines. All animals will first be anesthetized with ketamine (IM, 10 mg/Kg). Animals will be euthanized with sodium pentobarbital (IV 100 mg/kg). The animals will be euthanized only under the guidance and supervision of a staff veterinarian. After euthanasia solution has been administered, the animals will be examined by VSD veterinarian or technician to confirm death. A full body necropsy and histopathology analysis will be performed by a veterinary staff in consultation with a pathologist, after death has been confirmed.

V.5. Veterinary Care Animal procedures performed at AFRRI will be in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals. AFRRI facility is accredited by Association for Assessment and Accreditation of Laboratory of Animal Care (AAALAC) International.

V.5.1. Husbandry Considerations NHPs will be housed according to IAW VSD SOPs.

V.5.1.1. Study Room All animals will be assigned room(s) designated by VSD staff.

V.5.1.2. Special Husbandry Routine and emergency veterinary medical care: Animals will be quarantined for 4–6 weeks on arrival and chair-trained during the quarantine period. Each animal will be observed twice daily for evidence of pain or distress. Body weights will be determined at least once prior to the start of the study and at periodical intervals or when chaired for blood collection. Body temperature will be determined using a microchip installed in animals. Temperature will be measured during blood collection schedule when the animals are chaired. Animals will be qualitatively assessed twice daily (a.m. and p.m.) for general behavioral status and food consumption. Additional observation points will be added based on recommendation from attending veterinarian and will depend on the health of experimental animals. Appropriate standard operating procedures will be followed by staff veterinarians in the event of pain or distress.

V.5.1.3. Exceptions N/A

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care All primates will be quarantined for 4–6 weeks prior to any experiment. Animals will be tested for TB. Also, note the requirements given under V.3.3.2 and V.3.3.3 above.

All NHPs will be observed at least twice per day by veterinary staff (IAW VSDM SOP 2002, Animal Health Rounds). A veterinarian and a veterinary technician will provide 24 h emergency care to the animals if needed (IAW VSD SOPs). When animals become ill or debilitated, a veterinarian will be contacted immediately to assess the animal and provide adequate/emergency care. If the animal becomes moribund, it will be euthanized according to current American Veterinary Medical Association (AVMA, 2013) guidelines.

V.5.2.2. Emergency Veterinary Medical Care The on-call veterinary officer will be available via telephone 24 h daily.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy In these studies, all primates will receive regular enrichment (IAW VSDM SOPs).

V.5.3.2. Enrichment Restrictions N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1 with NHP handling only	(b)(6)
				Basic training	
				1, 3, 5	
				1 (handling only), 3b	
				1 (handling only)	
				1, 2, 3, 4	
				1,2,3,4,5,7(gavage,& Mandibular bleeds)	
				1-5	
				Basic training	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

In-protocol training of personnel: The presence of two trained vet techs in the protocol allows for an opportunity to train other staff including PI with procedures such as pole and collar, injections, blood draw on sedated animals and blood draw on physically restrained animals. For this purpose either naïve or experimentally used animals may be used. The

training will be certified by VSD staff (responsible for training) as well as approved by attending veterinarian.

VII. BIOHAZARDS/SAFETY N/A

References:

Bertho, J., Frick, J., *et al.* (2005). "Comparison of Autologous Cell Therapy and Granulocyte Colony Stimulating Factor (G-CSF) Injections vs. G-CSF Injection Alone for the Treatment of Acute Radiation Syndrome in Non-Human Primate Model." Int. J. Radiation Oncology Biol. Phys., Vol. 63, No. 3, 911–920,

Drouet M, Delaunay C, *et al.* (2008) Cytokines in combination to treat radiation-induced myelosuppresssion: evaluation of SCF + glycosylated EPO + pegylated G-CSF as an emergency treatment in highly irradiated monkeys. Haematologica 2008 Mar; 93(3):465-466.

Farese AM, Melanie V *et al.* (2012): A nonhuman primate model of the hematopoietic acute radiation syndrome plus medical management. Health Phys. 103(4):367–382.

FDA animal rule (2002): 21 CFR 314.610 and 21 CFR 601.91

Fortman JD, Hewett TA, Bennett BT (2001). The Laboratory nonhuman primate. Bone marrow aspiration and biopsy, Chapter 5, pp 170-174, CRC Press, Boca Raton, FL.

Johnson, CH *et al.* (2012): Radiation Metabolomics.5.Identification of Urinary Biomarkers of Ionizing Radiation Exposure in Nonhuman Primates by Mass Spectrometry-Based Metabolomics. Radiat Res. 178(4): 328–340.

Kurien, BT *et al.* (2004): Experimental animal urine collection: A review. Lab Anim. 38: 333-361.

Kushida T, Inaba M,*et al* (2002). Comparison of Bone Marrow Cells Harvested from Various Bones of Cynomolgus Monkeys at Various Ages by Perfusion or Aspiration Methods: A Preclinical Study for Human BMT. "Stem cells" **20**:155-162.

Kuter, D. J. and C. G. Begley (2002). "Recombinant human thrombopoietin: basic biology and evaluation of clinical studies." Blood" **100**(10): 3457-69.

Kuter, D. J. (2007). "New thrombopoietic growth factors." Blood **109**(11): 4607-16.

Kuter, D. J. (2008). "New drugs for familiar therapeutic targets: thrombopoietin receptor agonists and immune thrombocytopenic purpura." Eur J Haematol Suppl **69**: 9-18.

(b)(4),(b)(6)

Stickney, D. R., C. Dowding, *et al.* (2007). "5-androstenediol improves survival in clinically unsupported rhesus monkeys with radiation-induced myelosuppression." Int Immunopharmacol **7**(4): 500-5.

Wang, B., J. L. Nichol, *et al.* (2004). "Pharmacodynamics and pharmacokinetics of AMG 531, a novel thrombopoietin receptor ligand." Clin Pharmacol Ther **76**(6): 628-38.

VIII. ENCLOSURES : None

X. ASSURANCES

Protocol Title: Radiation countermeasure studies using (b)(4) and G-CSF in Nonhuman Primates (*Macaca mulatta*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM** / **AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)		7-15-2014
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

Revised 8-26-2014, revised 9-10-2014

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	**6/13/2014**	2nd Resubmission Date:	**9/10/2014**
Approved/**Returned for Revision:**	**7/8/2014**	**Approved**/Approval Withheld by IACUC:	**9/10/2014**
1st Resubmission Date:	**8/26/2014**	**EXPIRATION DATE:**	**9/9/2017**
Approved/**Returned for Revision:**	**9/4/201**	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
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- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Evaluation of CDX-301 as a radiation countermeasure in the laboratory mouse (*Mus musculus*); effects of neutrons, high and low dose rate gamma photons

IV. PRINCIPAL INVESTIGATOR:

(b)(6) PhD, Research Biologist, Radiation Countermeasures, Scientific Research Dept. tel (b)(6) fax: (b)(6) email: (b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, CDR, USN** Date

Head, Scientific Research Department, AFRRI

Telephone: (b)(6)

(b)(6)

- Or –

(b)(6) **PhD, LTC, USA** Date

Head, Scientific Research Department, AFRRI

Telephone (b)(6)

(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI

Telephone (b)(6) Fax (b)(6)

(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department, USUHS

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**

IACUC Chair, (b)(6) Scientific Research Department, AFRRI

Telephone (b)(6) Fax: (b)(6)

(b)(6)

PROTOCOL TITLE: Evaluation of CDX-301 as a radiation countermeasure in the laboratory mouse (*Mus musculus*); effects of neutrons, high and low dose rate gamma photons

PRINCIPAL INVESTIGATOR: (b)(6) PhD, Research Biologist, Radiation Countermeasures, Scientific Research Dept. tel: (b)(6) fax: (b)(6) (b)(6) email: (b)(6)

CO-INVESTIGATOR(S): (b)(6) PhD, Scientist (b)(6) Radiation Countermeasures, Scientific Research Dept. tel: (b)(6) fax (b)(6) email (b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS There is a pressing need for drugs to prevent ionizing radiation injury, for use in the field by military personnel, emergency responders and by the civilian population in harm's way. Radiation damages blood-forming tissues, resulting in mortality due to infection and hemorrhage (hematopoietic syndrome). The vast majority of studies on the acute radiation syndrome (ARS) and countermeasure efficacy have been performed using photon irradiation (gamma and x-rays). However, historical data from Hiroshima and Nagasaki, as well as the potential use of radiation dispersal devices underscore the need to understand the impact of radiation containing mixed neutrons and gamma photons, hereby called mixed fields (MF). Neutrons have different mechanisms of injury to cells and tissues compared to photons, with a higher relative biological effectiveness (RBE). Therefore, countermeasure efficacy may be different between gamma and mixed field exposures, depending on the mechanisms of action of the countermeasure. The present work will confirm and extend preliminary studies with CDX-301, a promising radiation countermeasure. Preliminary studies performed at AFRRI in CD2F1 mice demonstrated the efficacy of CDX-301 as a significant radiation prophylactic agent with 91-100% of mice administered a single dose of CDX-301 24 h prior to total body irradiation Further, CDX-301 demonstrated efficacy as a radiation mitigant; when administered at a single dose to CD2F1 mice 4 after TBI, CDX-301 improved 30-day survival by 25-33% compared to vehicle-treated group. The proposed studies will:

1). a. generate dose response curves for radiation dose versus mortality for two qualities of total body irradiation in mice: 2:1 neutron/gamma and pure gamma, b. optimize dosing schedule for CDX-301under both mixed 2:1 neutron/gamma field and pure gamma, and c. determine dose reduction factor (DRF) for CDX-301 with mixed neutron/gamma field. A DRF is the estimate of the relative degree of efficacy of a radiation countermeasure, based on a shift of the radiation-mortality dose-response curve to the right and is expressed as a ratio between vehicle and drug response at a dose of radiation that causes 50% mortality in both groups. DRF helps to compare various drug responses under standard conditions.

2) evaluate the effects of pure gamma and mixed neutron/gamma fields, with and without treatment with CDX-301, on core hematological parameters known to affect

recovery during the hematopoietic syndrome, such as numbers of circulating blood elements, numbers of blood progenitors in bone marrow, numbers of immune cells in spleen, liver and lungs, and concentrations of cytokines (hematological and immune system hormones) in blood.
3) Irradiate mice at a low dose rate with gamma photons to simulate radiation fall out conditions and measure the effect of CDX-301 on the above mentioned hematological parameters, as well as DNA damage and reactive oxygen species effects in tissues. The survival is expected to be 100% in low dose rate studies.

II. BACKGROUND

II.1. Background The risk of exposure to ionizing radiation from war, terrorism or nuclear accidents is thought to be increasing (1-3). A nuclear detonation in an urban environment could result in hundreds of thousands of casualties. These casualties would include a large proportion of people who would experience Acute Radiation Syndrome (ARS) from being exposed either to a mixture of neutrons and gamma rays initially, or to gamma rays from cutaneous fallout. In light of these facts, it would be extremely helpful to have safe and effective radiation countermeasures for treatment of casualties after exposure (4-7). Although radiation countermeasures generally have been studied in subjects exposed to pure photons (gamma- or X-rays), the mechanisms of injury of these low linear energy transfer (LET) radiations differ from those of high LET radiation such as neutrons, and these differences may affect countermeasure efficacy. (b)(4)

(b)(4)

(b)(4) In contrast, preliminary studies at AFRRI suggest that CDX-301 is effective against exposure to both MF and pure gamma-irradiation.

CDX-301 is the soluble recombinant human protein form of the Fms-related tyrosine kinase 3 ligand (Flt3L), a hematopoietic cytokine (10). It is expressed on multiple cell types in both soluble and membrane forms, and interacts with a receptor found on hematopoietic stem cells (HSC), early progenitor cells, and immature thymocytes, resulting in the proliferation, differentiation, development and mobilization of these cells in the bone marrow, peripheral blood, and lymphoid organs. CDX-301 represents the 153 N-terminal amino acids of the human Flt3L extracellular domain after the cleavage of the N-terminal signal peptide (Figure 1).
It is a clear, colorless liquid soluble in saline with a pH of 7.0. It has been produced by Celldex Therapeutics, Inc. (Needham, MA; (b)(6) under GMP conditions and with GLP toxicology studies. There is no detectible endotoxin or mycoplasma contamination. CDX-301 mobilized hematopoietic stem and progenitor cells (HSPC) in a Phase 1 Clinical Program leading towards the treatment of cancer patients requiring hematopoietic stem cell transplantation (HSCT) (11).

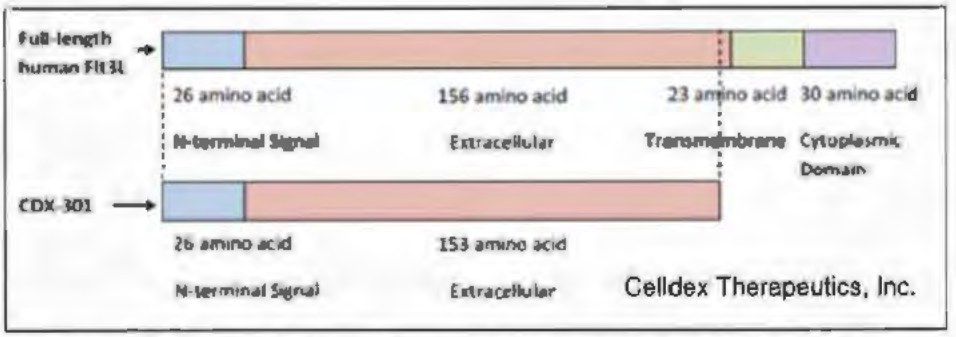

Figure 1

Early studies in the C57Bl/6J model demonstrated significant improvement in survival (40% with vehicle vs 100% with CDX-301) when mice irradiated at 7.76 Gy were treated with CDX-301 (25 μg) for 10 days (days -1, 0, 1, 2, 3, 4, 5, 6, 7, 8) (personal communication, Celldex Therapeutics Inc.) (b)(4)

(b)(4)

(b)(4) The proposed study builds on the reported and ongoing studies using CDX-301. Three main goals (specific aims) are:
a. optimize drug response in MF,
b. conduct parallel studies in Cobalt 60,
c. characterize drug response in animals exposed to prolonged low dose rate gamma radiation.
Mechanistic studies (tissue response) will be another focus in all these experiments.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched BRD, NIH Reporter, PubMed

II.2.2. Date of Search May 8, 2014

II.2.3. Period of Search All available years:
BRD: FY2005-FY2009
NIH Reporter: 1988-2013 (Projects and Publications)
PubMed: includes MEDLINE (1966-present) and OLDMEDLINE (1950-1965).

II.2.4. Key Words of Search neutron, gamma, ionizing radiation, Flt3 ligand, CDX-301, cytokine, protracted radiation, chronic radiation, low dose rate

II.2.5. Results of Search BRD: There were no results from: CDX-301, Flt3 ligand, Flt3 ligand + ionizing radiation, Flt3 ligand + gamma, Flt3 ligand + neutron, Flt3 ligand + protracted radiation, Flt3 ligand + chronic radiation, Flt3 ligand + low dose rate. When the search was generalized to cytokines, the following results were obtained:

1. Cytokine + ionizing radiation = 4 hits; consisted of AFRRI work on 5-Androstenediol (5AED) and work on herpes virus therapy
2. Cytokine + radiation = 11 hits; consisted of AFRRI work on 5AED and work on herpes virus therapy.
3. Cytokine + gamma = 3 hits; consisted of AFRRI work on 5AED and work on herpes virus therapy
4. Cytokine + neutron = 0 hits

5. Cytokine + protracted radiation = 0 hits
6. Cytokine + chronic radiation = 0 hits
7. cytokine + low dose rate = 0 hits

NIH Reporter: 1990-present

1. CDX-301 = 2 hits; CDX-301 and Plerixafor Combination in Stem Cell Mobilization for Transplantation
2. Flt3 ligand + ionizing radiation = 2 hits; consisted of work on pleitrophin and epidermal growth factor
3. Flt3 ligand + radiation = 33 hits; consisted of work on pleitrophin and epidermal growth factor; Flt3 ligand as a therapeutic against glioblastoma and acute myeloid leukemia. The closest body of work consisted of the use of Flt3 ligand as an immunoprotectant against ARS in a dog model.
4. Flt3 ligand + gamma = 34 hits. Of these only one referred to the use of Flt3 ligand as a radioprotectant against gamma photons, and this was in a dog model. Other references included immune cell studies, structure/function studies, Flt3 ligand as a therapeutic against chlamydia trachomatis, and Flt3 ligand in various cancer models.
5. Flt3 ligand + gamma radiation = 10 hits. These consisted of the use of Flt3 ligand and other cytokines as a radioprotectant in a dog model.
6. Flt3 ligand + neutron = 5 hits. These were structural studies were neutrons were discussed in the context of X-ray crystallography.
7. Flt3 ligand + neutron radiation = 5 hits. These were identical to those found in the Flt3 ligand + neutron search.
8. Flt3 ligand + protracted radiation = 0 hits
9. Flt3 ligand + chronic radiation = 4 hits. These consisted of treatments of the elderly with Flt3 ligand for acute myeloid leukemia (AML).
10. Flt3 ligand + low dose rate = 0 hits

Note: We did not use the generalized term cytokine in this search because the specific cytokine of interest, Flt3 ligand, produced results.

Pubmed:

1. CDX-301 = 0 hits
2. Flt3 ligand + ionizing radiation = 13 hits. These include Flt3 ligand as a biomarker, therapeutic against UV and gamma radiation (alone and in combination), role in stem cell expansion. These studies all use the full length form of Flt3 ligand. Our studies use the truncated form of Flt3 ligand, CDX-301.
3. Flt3 ligand + radiation = 73 hits. These include Flt3 ligand as a biomarker, as a therapeutic against radiation using in vitro and *in vivo* methods, effect of Flt3 ligand on bone marrow and hematopoietic recovery. These studies use the full length form of Flt3 ligand. Our studies use the truncated form of Fl3 ligand, CDX-301.
4. Flt3 ligand + gamma radiation = 12 hits. These include previously described studies as well as the effects of Flt3 ligand on tumor models. Note: Flt3 ligand + gamma alone yielded 140 hits but this search was too broad. It included correlations with Flt3 ligand and interferon gamma as well as immune cell development via interactions with receptor gamma chains for specific cytokines.
5. Flt3 ligand + neutron = 0 hits
6. Flt3 ligand + neutron radiation = 0 hits
7. 7. Flt3 ligand + protracted radiation = 1 hit. This described the use of Flt3 ligand as a rapid and simple method of triage, using human data from a radiation accident.
8. Flt3 ligand + chronic radiation = 1 hit. This described the use of serum Flt3 ligand as a biomarker after chronic ingestion of radiation in a mouse model.
9. Flt3 ligand + low dose rate = 4 hits.

Note: These describe Flt3 ligand involvement in tumor models, either in human or mouse studies.

III. OBJECTIVE / HYPOTHESIS The objective of this protocol is to develop CDX-301 as a countermeasure against gamma radiation and mixed neutron (MF)/gamma-radiation. This work involves optimizing CDX-301 administration (concentration and time of injection), as well investigating the effects on hematopoietic cells and tissues. In addition, our work will address the feasibility of CDX-301 as a countermeasure against prolonged exposure to gamma irradiation and address potential mechanisms of protection.

IV. MILITARY RELEVANCE Work performed under this protocol would directly affect the military response to a nuclear detonation. Development of CDX-301 as a countermeasure would provide radiation protection to first responders, those in zones of high danger where prompt radiation exposure would consist of neutrons and photons, as well as those exposed during radiation fallout. It is essential to understand the efficacy of this agent in these different scenarios, with the ultimate goal of making a responsible recommendation to our military personnel and the civilian population.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

Male CD2F1 mice will be used in all experiments. Irradiations in the AFRRI reactor and Cobalt-60 facility will be performed according to previously published methods, following AFRRI standard operating procedures (12-16). Irradiations in the Low Level facility will be performed in collaboration with AFRRI dosimetrists and based on consultation with AFRRI VSD. Mice irradiated in the reactor will be inserted into aluminum holders and placed onto a carousel that rotates the mice within the exposure beam to ensure uniform exposures to all subjects. Mice irradiated at the Cobalt-60 facility will be placed into well-ventilated Plexiglas boxes and irradiated bilaterally. Mice irradiated in the Low Level Cobalt facility will be housed in modified cages (3 animals/cage) approved for long-term housing. Food and water will be available *ad libitum*. Temperature, humidity, and standard 12 h light/dark cycles will be maintained during chronic exposure. Sham-irradiated mice will undergo the same procedures as the irradiated mice (transport to radiation facility, placement into aluminum cylinder, Plexiglas box, or modified cages), minus the radiation exposure. In the reactor, non-neutron components of the exposure are controlled by shielding outside of the reactor core area within the exposure room. All reactor-irradiated mice, their tissues, and their bedding will be considered radioactive unless declared otherwise as a result of environmental Health Services (EHS) surveys.

V. 1. 1. Experiment 1 Drug dose and schedule optimization studies with CDX-301 as measured by 30 day post-irradiation survival

Two concentrations of CDX-301 (400 μg/kg and 800 μg/kg) will be administered by subcutaneous (SC) injection using 25-30 gauge needles (17) at 0.1 ml/injection site. A 400 μg/kg dose of CDX-301 protected mice against MF radiation, and 800 μg/kg provided protection against gamma-radiation. Because of these results, both doses were selected for Experiment 1. Six drug dosing schedules at times between -24 h and +48 h after radiation will be studied using both radiation sources and qualities. Table 1 summarizes animal numbers. Animals will be monitored for 30 days for survival.

Table 1: Animal requirements for experiment 1 (30 day survival study)

3 sc drug doses:	0 (sterile saline vehicle), 400, or 800 μg/kg
6 drug schedules:	Schedule1: pre-irradiation (-24 h) Schedules 2-5: post-irradiation: +4 h; +12 h; +4 and +48 h; +12 and +48 h Schedule 6: pre-, and post-irradiation -24 h, and +48 h
1 radiation dose:	LD70/30*
2 radiation qualities:	MF (2:1 neutron:gamma); pure gamma
Mice/Group:	24
Iterations:	2

Total # of animals	1728

*5.71 Gy MF and 9.2 Gy gamma radiation

3 drug groups (0, 400, 800 µg/kg) x 6 drug administration schedules x 1 radiation dose x 2 radiation qualities (MF and gamma) x 24 animals/group x 2 iterations = 1728 mice

V. 1. 2. Experiment 2 Determine the DRF of CDX-301 on MF-irradiated mice

Five radiation doses will be chosen to establish probit curves ranging from near 5% mortality to near 95% mortality with CDX-301 administration. Table 2 summarizes animal numbers. The endpoint will be 30-day survival. (Note: DRF studies with Cobalt 60 gamma radiation will not be done since this has been completed (b)(6) (b)(6)

Table 2: Animal requirements for experiment 2 (DRF, only for MF, 30 day survival)

5 radiation doses	4.0 4.5, 5.0, 5.5, 6.0 Gy
2 sc drug doses	0 (sterile saline vehicle), optimal concentration (See Table 1)
2 drug schedules	-24 h before radiation; optimal dosing schedule given after radiation (See Table 1)
1 radiation quality	MF (2:1 neutron:gamma)
Mice/group	16
Iterations	1
Total # of animals	320

5 radiation dose x 2 drug doses (vehicle, drug) x 2 drug administration schedules (pre- and post-irradiation) x 1 radiation quality (MF) x 16 animals/group x 1 iteration = 320

V. 1. 3. Experiment 3 Conduct hematopoietic and functional studies

Determine the effect of CDX-301, administered after a sublethal dose of MF or gamma-irradiation on hematopoietic cells in circulation, circulating cytokine levels, bone marrow cellularity, morphology, and function, and hematopoietic cells populations and function from spleen, lung, and liver.

Mice will be exposed to a sublethal dose of gamma or MF radiation appropriate for hematopoietic syndrome hematology studies where measurable numbers of blood elements are desired throughout the experiment (3 Gy MF; 7 Gy gamma) (b)(4) Using a sublethal dose makes it possible to carry out a time course of hematological parameters undistorted by data censoring due to mortality, and to allow a sufficient number of surviving cells for analysis during the first two weeks. Previous experiments have shown that detection of radioactivity is below background in 24 h or less in mice administered a 3 Gy dose of MF in the AFRRI TRIGA. This allows samples to be collected at day +1 after MF radiation. Sublethally-irradiated mice, or non-irradiated controls, will be treated with CDX-301 at the optimal concentration and administration schedule (determined in Experiment 1). Blood for CBC/diff and cytokine studies, femurs for GM-CFC studies, and spleen, lung, and liver for analysis of cell populations will all be collected from the same mice, according to standard protocols (b)(4) Briefly, mice will be anaesthetized using Isoflurane at days 1, 3, 7, and 14 after radiation (20 mice/timepoint) and blood will be collected by cardiac puncture. An optimal volume of collection will be 1.0 ml, which will be divided into tubes for CBC/diff and serum collection. Removal of this volume of blood is exsanguination and will be done only on anesthetized mice (18). Mice will be euthanized humanely by cervical dislocation after

blood collection. Both femurs will be dissected from the adhering tissues and flushed with sterile IMDM to obtain bone marrow single cell suspension. Spleen, lung, and liver will be removed and placed on ice in sterile dishes containing appropriate media. The day of irradiation is considered day 0. Table 3 summarizes animal numbers. Please note that only one optimum drug dose post-irradiation will be used in the study.

Table 3: Animal requirements for Experiment 3 (hematology)

2 sc drug doses	0 (sterile saline vehicle), optimal drug concentration (determined in Expt. 1)
1 drug schedule	optimal dosing schedule (post-irradiation) (determined in Expt. 1)
2 radiation doses	sublethal dose (0 and 3 Gy MF; 0 and 7 Gy gamma)
2 radiation qualities	MF (2:1 neutron:gamma) and pure gamma
Mice/Group	20
4 collection time points	+1, +3, +7, +14 day after radiation
Iterations	2
Total # animals	**1280**

2 drug doses (vehicle, drug) x 1 drug administration schedule (post-irradiation) x 2 radiation doses x 2 radiation qualities (MF and gamma) x 4 time points x 20 animals/group x 2 iterations = 1280

V. 1. 4. Experiment 4: Low dose Cobalt 60 studies
NOTE: The proposed studies will not be performed without a feasibility study and detailed SOP, followed by an approved IACUC Amendment. Experimental details are for informational purposes.

Determine the effect of CDX-301, administered sc after chronic total body gamma-irradiation given at a low dose rate, on circulating blood cells, cells within blood forming tissues, circulating cytokines, and markers of oxidative stress in serum.

Facility: AFRRI Low Dose Cobalt facility is unique and will allow housing both animals to be irradiated (on a protracted basis) as well as appropriate controls in a shielded area but subjected to the same housing conditions. The macroenvironment will include a temperature range of 20-26°C (68-79°F), 30-70% relative humidity, and a 12 hour light/dark cycle as spelled out in *The Guide*. Radiation Safety personnel are on call 24 h in the Low Dose Facility, and the room is monitored by a camera which can be visualized outside the exposure area. Prior to performing any animal experiments, a feasibility study will be performed where the room itself will be monitored for 72 h to ensure proper environmental conditions (temperature, humidity, lighting). This will also involve testing the capability of the facility's current camera, to determine the level of monitoring that can be accomplished on camera. The results of this preliminary experiment (without animals) will be provided to IACUC for review. An SOP will be written based on this feasibility study. Upon completion of the feasibility study as suggested by AFRRI veterinary staff, an initial test experiment will be performed using 2 cages (6 mice) to monitor mice in the facility under non-irradiated conditions as well as irradiated conditions at the lowest dose for the study (3 Gy). Mice will be housed during irradiation (for extended period-initially 48 ± 4 h) in well-ventilated cages made specifically for chronic mouse irradiation (Appendix 1). Each cage holds 3 mice; each

animal compartmentalized in a space of 15 in^2 with food and water freely available in each compartment of the cage. Non-irradiated control mice will be housed in the Low Level facility in a shielded area to protect mice from radiation. These animals will be transferred to these specialized cages in the vivarium for a minimum of 72 h before being transported to the Low Dose Cobalt facility for radiation or sham exposure to allow acclimatization. During the acclimatization period we will monitor environmental enrichment, particularly nestlet size, to determine if further adjustments are necessary.
Irradiation: CD2F1 male mice will be subjected to irradiation doses up to 7 Gy at a low dose rate, which will be determined by the Operational Dosimetry Division prior to the commencement of animal exposure (total dose being delivered over 48 ± 4 h). This includes 48 h of radiation exposure and additional time (up to 4 h) that it may take to pause radiation and enter the facility for monitoring and husbandry. The objective of this experiment is chronic radiation exposure, so the "pause" time will be kept minimal. Investigators will temporarily pause the radiation once daily to monitor the mice. This includes replenishing food and water, and closely monitoring the animals for injury related or non-related to radiation. In addition, investigators will request that the radiation be temporarily paused to enter the exposure room if additional monitoring is required.
Drug Administration: CDX-301 will be administered at the optimal concentration determined in Experiment 1, 24 h before radiation and at the optimal dosing schedule determined in Experiment 1. If the optimal injection schedule coincides with the length of time that mice will be irradiated (i.e. time less than or equal to 48 h), a portable procedure hood will be transported to the facility, radiation paused, and injections administered in the Low Level facility as opposed to transporting animals back to VSD.
Table 4 summarizes animal numbers. At the end of the radiation period blood will be removed by cardiac puncture from isoflurane anesthetized mice, and after death is confirmed by cervical dislocation, femurs and organs (lung, liver, and spleen) will be removed from these mice as in the previous experiment (Experiment 3), as well as organs for fixation and possible histopathology, (femurs, trachea, thymus, esophagus, lungs, heart, liver, spleen, kidney, stomach, pancreas, small intestine, cecum, colon, urinary bladder, reproductive organs, endocrine organs, head). Blood and tissue harvest will be 1, 2, 4, 7, 10, 14, 21, 28, 35 and 42 d after completion of the irradiation.

Table 4: Projected animal requirements for experiment 4 (Low Dose Gamma Radiation Facility)*

NOTE: This is not included in V.4.1.1.1. Number of Animals or V.4.1.1.1.4. Pain Category Assignments

4 radiation doses	0, 3, 5, 7 Gy
2 sc drug doses	0 (sterile saline vehicle), optimal concentration
2 drug schedules	-24 h before radiation; optimal schedule after radiation
1 radiation quality	Gamma
Mice/timepoint	5
10 time points	1, 2, 4, 7, 10, 14, 21, 28, 35, 42 d after radiation
Iterations	1
Trial mice	6 (3 irradiated + 3 non-irradiated)

Total # of animals required	**6* (approved in this protocol)**

*Low dose radiation is not expected to lead to lethality and hence should be considered as "hematology"

~~4 radiation doses x 2 drug doses (vehicle, drug) x 2 drug administration schedule (pre- and post-irradiation) x 1 radiation quality (gamma) x 10 time points x 5 animals/group x 1 iteration = 800 + 6 trial mice, total = 806~~ (see Note in the beginning of this section).

V.2. Data Analysis

Means with standard errors, or percentages, will be reported if applicable. The significance level will be set at 5% for each test. All statistical tests will be two-sided. Multiple comparison tests will be used when appropriate. Drugs will be compared to vehicle injections, and irradiation will be compare to sham irradiation where appropriate. Statistical software, PC SAS, will be used for statistical analyses.

Experiment 1, 2: Log-rank tests will be used in comparing survival curves for each experiment. Fisher's exact tests will be used for comparisons of percent survival among groups. If those are significant, pair-wise comparisons will be done by Fisher's exact tests

Experiment 3: Probit analysis will be used. The DRF will be calculated as the ratio of the radiation LD50/30 for drug-treated and vehicle-treated groups; standard errors and 95% confidence intervals will be derived.

Experiment 4: Analysis of variance (ANOVA) will be used to detect significant differences among groups. If significant, then Tukey-Kramer pair-wise comparisons will be used to identify which groups are different from each other. These experiments involve multiple time points, and since the parameters are being followed over a period of time, in consultation with statistician, 5 animals/time point has been included in the protocol.

Note on requirement to repeat some experiments: It is a generally accepted principle of the scientific method that results must be repeated to evaluate a hypothesis. Scientific journals (e.g., Endocrinology) state that experiments must be repeated to qualify for publication.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered We considered computer models and tissue culture as alternatives to animals. Both alternatives lack the knowledge base and complexity needed to evaluate the effects of radiation and countermeasures on the hematological and immune systems. The phenomena under study involve complex information-processing networks comprising hundreds of cell types and biological signaling molecules. Responses to irradiation and drugs involve interactions between the central nervous system, the peripheral nervous system including the enteric nervous system, endocrine glands, the immune and inflammatory systems, hematopoietic organs, etc. We do not know all of the cell types and tissues involved and many of the signaling molecules have not been discovered yet. Even if all of the tissues, cell types and signaling molecules were known, the present state of the art of tissue culture would be incapable of reproducing the *in vivo* relationships. One would have to reproduce much of an entire mammalian organism in culture to study these phenomena in *vitro*, a scenario that would have its own ethical issues. As for computer models, the most

powerful supercomputers available in the foreseeable future would be incapable of analyzing interactions between so many elements in the network. Drugs and formulations will have to be evaluated as countermeasures in whole animal models. The purpose of the proposed research is to prove the efficacy of a countermeasure, as well as to begin to identify the cells and signaling molecules necessary to construct a model of radiation injury and countermeasure mechanisms.

V.3.2. Animal Model and Species Justification Evaluation of countermeasures in the animal model is the only option for identifying potential safe and efficacious countermeasures. The murine model is the most well-defined immunological animal model for this type of research. In addition, this model is preferred over other species due to readily available immunological reagents. Mice have significant hematological and immunological similarity to higher animals and humans. AFRRI staff has had extensive experience with mice, including male CD2F1 mice, in radiation injury and countermeasure research.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species: Mice (*Mus musculus*)

V.3.3.2. Strain / Stock: CD2F1

V.3.3.3. Source / Vendor: VSD approved multiple vendors.

V.3.3.4. Age: 10-14 weeks

V.3.3.5. Weight: 24-32 g

V.3.3.6. Sex: male

V.3.3.7. Special Considerations: The vendor's animal husbandry, breeding, and shipping practices will be in accordance with the Animal Welfare Act P.L. 89-544 and The "Guide" for the Care and Use of Laboratory Animals.

The animals must be adventitious disease free. All animals should be raised with restricted access and strict entry and exit policy in caging that reduces the exposure to pathogens such as in barrier condition, microisolation caging or isolators. They must be free of pathological lesions as assessed by gross exam.

Health surveillance monitoring must be performed regularly on the colonies and verified by an outside source. Health Surveillance reports should be sent with the animal shipments and must be current as well as accurate.

The mice must be pathogen-free, specifically for the following agents: Pseudomonas aeruginosa, Proteus morgani and Pasteurella. In addition, all mice need to be free of the following viral agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epizootic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's

Mouse Encephalomyelitis Virus (TMEV/GDVII), Encephalitozoon cuniculi, CAR bacillus, Mycoplasma pulmonis, and Clostridium piliforme. Endoparasite- and ectoparasite-free.

Genetic monitoring should be used to detect possible contamination and policies and procedures must be in place to reduce genetic drift.

Mice used in chronic radiation experiment (Expt 4) will be housed 3 per cage in special compartmentalized cages constructed specifically for the AFRRI Low Level Cobalt facility (please see the design attached as an enclosure to the protocol). Each compartment is 15 in^2, in accordance with AALAS regulations on space/animal and The Guide (48). Each compartment will contain standard bedding and free access to food and water.

V.3.4. Number of Animals Required (by species) Mice = 3334

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement In the mortality studies, in order to minimize discomfort when animals become morbid, investigators and animal care staff will examine the mice at least twice daily, including early morning and late afternoon with increased frequency of observations when animals are in the critical phase. Moribund animals will be considered to have arrived at the study endpoint and will be euthanized. Criteria for identifying morbidity and moribundity will be based on the current IACUC Operating Policy #10 with the accompanying Appendix A clinical observations score sheet (August 2014). This Policy is under review and an amendment will be submitted in the event of any policy changes. Analgesics were considered as a way of minimizing discomfort during the onset of opportunistic infections, which will occur during the survival experiments. However, analgesics and anesthetics are well-known to alter the functional properties of the immune system and resistance to infection (19-43), which would defeat the purpose of the study.

V.3.5.2. Reduction We will use the same mice for peripheral blood counts, analysis of cytokines in serum, and tissue collection for clonogenic and immune organ studies. We will determine the optimal dose of CDX-301 to administer in Expt 1, thus reducing the number of mice used for our studies.

V.3.5.3. Replacement None

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C 3 (# of animals)

V.4.1.1.1.2. Column D 83 (# of animals)

V.4.1.1.1.3. Column E 3248 (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1	Survival experiment – all irradiated			1728	1728
2	DRF study – all irradiated			320	320
3	Hematology		80	1200	1280
4	Hematology	3	3		
Totals:		3	83	3248	3334

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization Mice will be anesthetized as part of euthanasia (in cases of moribundity or for sample collection) using a portable metered Isoflurane system. There will be no recovery from anesthesia in this case and death confirmed by cervical dislocation. All euthanasia will be according to AVMA guidelines and performed by trained and technically proficient personnel (44).

V.4.1.2.2. Pre- and Post-procedural Provisions Mice will be housed and monitored in accordance with VSD standard operating procedures both before and after irradiation procedures (see Section V.5. "Veterinary Care"). For special Husbandry considerations post-procedural (see Section V.5.1.2)

V.4.1.2.3. Paralytics N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched AGRICOLA

V.4.1.3.2. Date of Search May 8, 2014

V.4.1.3.3. Period of Search All dates available

V.4.1.3.4. Key Words of Search ionizing radiation AND pain, injection AND pain, CDX-301 AND pain, Flt3L AND pain, neutron AND pain, gamma AND pain

V.4.1.3.5. Results of Search Agricola: Ionizing radiation and pain resulted in 0 hits. Radiation and pain resulted in 20 hits. These encompassed articles where radiation was used as a therapeutic against a number of tumors, mainly in dogs. Several studies address skin dermatitis in response to radiation and suggested increased monitoring. We propose to monitor all animals at least twice/day for any signs, including changes/roughness of fur.

To study the efficacy of any radiation countermeasure in modulating survival, and to investigate its mechanism of action, one needs to irradiate animals. A search using radiation related terms neutron AND pain and gamma AND pain yielded no relevant results. Irradiation itself is not a painful process, in fact, it can be analgesic (45), but it induces various changes in the body, and kills hematopoietic cells. In irradiated animals, the immune response is compromised, and opportunistic infections may ensue. As discussed earlier, anesthetic or analgesic drugs cannot be used. All drug injections will be administered by sc injection. A search on subcutaneous AND injection AND pain

yielded 51 hits. Most were irrelevant to the scope of this proposal. Gurney et al. demonstrated that an sc injection of an agent of interest was as effective as and less painful than an intramuscular injection (46). Subcutaneous injections also proved to be painless and most effective in a dog model (47). No references were found suggesting any effect of CDX-301 or Flt3L on pain or distress. Since the drug under investigation is being developed to enhance survival, and we believe the mechanisms involve modulating complex signaling in hemato-immune-endocrine networks, we do not believe alternatives to the use of whole animals are feasible. As discussed earlier, in vitro experiments do not serve our purpose. See section V.3.1.

V.**4.1.4. Unalleviated Painful or Distressful Procedure Justification** Irradiated animals die due to compromised immune responses and opportunistic infections. The percentage of surviving animals is the indicator of the efficacy of a countermeasure. As discussed above, we cannot give anesthetic and/or analgesic agents to animals since they are known to interact with the immune system, and would confound the interpretation, resulting in a waste of animals.

V.4.2. Prolonged Restraint N/A

V.4.3. Surgery N/A

V.4.3.1. Pre-surgical Provisions N/A

V.4.3.2. Procedure(s) N/A

V.4.3.3. Post-surgical Provisions N/A

V.4.3.4. Location N/A

V.4.3.5. Surgeon N/A

V.4.3.6. Multiple Major Survival Operative Procedures N/A

V.4.3.6.1. Procedures N/A

V.4.3.6.2 Scientific Justification N/A

V.4.4. Animal Manipulations Subcutaneous injections will be performed as described in V.4.4.1. Blood will be removed from anesthetized mice as described in V.4.4.2. Tissues required for the study will be removed after euthanasia, described in V.4.4.2.

V.4.4.1. Injections We will use 25-30 gauge needles for SC injections in the nape of the neck. Injections will consist of CDX-301 diluted in PBS, or PBS alone in a 0.1 ml volume.

V.4.4.1.1. Pharmaceutical Grade Drugs N/A

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

CDX-301 will be provided by Celldex Therapeutics, Inc. (Needham MA) in sterilized form. It was produced under GMP conditions and with GLP toxicology studies. There is no detectible endotoxin or mycoplasma contamination.

V.4.4.2. Biosamples Blood will be drawn from the caudal vena cava or by cardiac puncture using a 23 gauge needle in mice anesthetized by Isoflurane inhalation. A maximum of 1 ml of blood will be collected, and approximately 0.5 ml will be sent to hematology while 0.5 ml will be used for cytokine analysis after serum separation. After Isoflurane anesthesia and blood collection, animals immediately will be euthanized by cervical dislocation. Femur marrow for clonogenic assays, tissues processed for cell isolation (spleen, lung, liver, heart) and/or for fixation and possible histopathology, (femurs, trachea, thymus, esophagus, lungs, heart, liver, spleen, kidney, stomach, pancreas, small intestine, cecum, colon, urinary bladder, reproductive organs, endocrine organs, head), or bodies for disposal, will be collected after euthanasia as described under Euthanasia (V.4.6). All euthanasia will be according to AVMA 2013 guidelines by trained and technically proficient personnel (44).

V.4.4.3. Adjuvants N/A

V.4.4.4. Monoclonal Antibody (MAb) Production N/A

V.4.4.5. Animal Identification Cage cards

V.4.4.6. Behavioral Studies N/A

V.4.4.7. Other Procedures N/A

V.4.4.8. Tissue Sharing N/A

V.4.4.9. Animal By-Products N/A

V.4.5. Study Endpoint In Experiments 1, and 2 the endpoints are euthanasia of moribund animals. A score sheet provided in IACUC Policy #10 (attached) will be part of the criteria for euthanasia in the 30-day survival experiments. In Experiments 3 and 4, the endpoints are blood and tissue collection for further studies.

V.4.6. Euthanasia For sample collection, mice first will be anesthetized with Isoflurane and then cervical dislocation will be performed to cause death under a portable fume hood with charcoal filter. Mice to be euthanized at the end of the experiment will be exposed to CO_2 by investigative or VSD staff followed by cervical dislocation.

V.5. Veterinary Care

V.5.1. Husbandry Considerations No more than 4 mice will be placed in a cage during experiments. Animals will receive Harlan Teklad Rodent Diet (w) 8604 and water *ad libitum*. Acidified water (pH 2.5 – 3.0) is routinely provided to AFRRI mice. Cages will be regularly changed and cleaned by VSD staff in accordance with Standard Operating

Procedures and the most recent edition of the *Guide for the Care and Use of Laboratory Animals*. Animal rooms will be maintained under a 12 h light/dark cycle.

Animals housed in the Low Level Facility will receive identical care, diet, and water to those housed in VSD. Husbandry during this time will be provided by research staff. Husbandry for animals irradiated in the TRIGA Reactor will be provided by research staff until the study room is cleared by members of the AFRRI Radiation Safety Office.

V.5.1.1. Study Room Experiments 1-3: As assigned by VSD in the vivarium. Experiment 4: The radiation will occur over 48 h +/- 4 h in the Low Level Facility. A feasibility study will be done in the room prior to the actual experiments. This study will monitor the timing of the light dark cycle, temperature and humidity of the room over 48 h +/- 4 h It will also determine the feasibility of using the current monitoring camera (used to monitor the radiation source) to visualize cages, and to what level of detail. The results of this study will be presented to IACUC before animal research in this room will begin. Upon completion of the radiation, animals will be housed in a room assigned by VSD in the vivarium.

V.5.1.2. Special Husbandry Provisions Reactor-irradiated animals will be monitored for radiation levels by the Radiation Safety staff. (b)(6) (b)(6) The activated animals, room, and bedding will be considered radioactive until levels of radiation are not distinguishable from background. This is estimated to be one day when doses below an LD50/30 are given (less than 4.92 Gy) and no more than two days when doses above an LD50/30 (4.92 Gy and higher) are given. This estimate is based on past experience of investigators at AFRRI.

The room housing the irradiated mice will be designated a "radiation room" by RSD and EHS, labeled as such, and access will be restricted to properly trained staff until declared clear by RSD/EHS radiological surveys. Standard radiological room operating procedures will require removal of all PPE before exiting the room and surveying all equipment and materials for radioactivity with a hand held Fluke 451-B detector before allowing them to be removed from the room.

No irradiated, live mice will be moved out of VSD. Samples will be collected in VSD prep areas after mice have been declared non-radioactive by EHS. Following standard operating procedures, gloves will be worn at all times when handling the irradiated mice. Finger ring dosimeters will be worn by all personnel handling irradiated mice until they have been declared non-radioactive by Safety. Before mice have been declared non-radioactive by Safety, carcasses and bedding will be disposed of in the Rad Waste Facility area designated for such material. Irradiated mice remains will be disposed of in the Radiation Safety Storage area designated for such material until the mice can be surveyed for radiation hazard level before final disposal.

All personnel involved in these neutron irradiation experiments will remain current with respect to radiation safety training and will continue to receive annual radiation worker training. They will also undergo all required specialized training provided by SHD and RSD, including AFRRI reactor exposure room training given by the reactor facility staff. Appropriate dosimetry will be worn at all times when working with the reactor. This includes the standard TLD along with a self-reading pocket chamber to serve as a backup. Source accountability and ALARA principles will be adhered to at all times, and the SWIMS procedure will be followed in case of any spills of potentially radioactive materials.

Animals involved in Experiment 4 will be housed in the Low Level Cobalt Facility during chronic irradiation. During this time the temperature, humidity, and light cycle will be maintained as per The Guide for the Care and Use of Laboratory Animals.
V.5.1.3. Exceptions None

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care The animals will be observed twice daily by research staff of this protocol (morning and late afternoon). Animals in a critical phase (period of study when animals begin to show clinical signs of morbidity and increased mortality is expected) will be monitored three times/day, including a late evening check. Any moribund mice will be euthanized as soon as possible. If needed, a VSD veterinarian will be contacted for the decision to euthanize. The most current approved IACUC Policy Letter #10 will serve as a reference for the criteria for health evaluation/euthanasia.

V.5.2.2. Emergency Veterinary Medical Care AFRRI veterinary staff will be consulted as needed in an emergency veterinary medical care scenario.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy Nestlets and other enrichment items may be allowed in animals that have been separated for experimentation.

V.5.3.2. Enrichment Restrictions Nestlets will be allowed in experimental cages.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3, 4b, 5	(b)(6)
				1, 3, 4a, 5	
				7 – tail vein	
				1, 3, 3d, 4a, 4b, 5, 7	
				1, 2, 3, 4a, 5	
				7 – tail vein	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY N/A

VIII. ENCLOSURES Pdf of the new divider for holding animals over 48 + h in low dose facility

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41. (b)(4),(b)(6)
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X. ASSURANCES

Protocol Title: Evaluation of CDX-301 as a radiation countermeasure in the laboratory mouse (Mus musculus); effects of neutrons, gamma photons, and rate of exposure.

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM** / **AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)		9-10-2014
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

Appendix 1: Low dose cage divider for 48+ h low dose radiation

Appendix 2: Clinical Observations with Criteria for Euthanasia (Rodent)

DATE:	Time:	Animal ID#	Score
Appearance:			
	Normal (smooth coat, clear eyes/nose)		0
	Hunched and/or fluffed		1
	Ocular discharge, and/or edema		3
	Soft stools (fecal matter around anus)		5
	Pale, white mucus membranes/skin**		6
	Bloody diarrhea		9
	Blue mucus membranes/skin (cyanosis)*		12
Respiratory Rate:			
	Normal breathing		0
	Increased breathing (double normal rate, rapid, shallow)		6
	Abdominal breathing (gasping +/- open mouth breathing)*		12
General Behavior:			
	Normal (based on baseline observations)		0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)		1
	Decreased mobility		2
	Ataxia, wobbly, weak**		6
	Inability to stand*		12
Provoked Behavior:			
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))		0
	Subdued; responds to stimulation (moves away briskly)		1
	Subdued even to stimulation (moves away slowly)		3
	Unresponsive to gentle prodding **		6
	Does not right when placed gently on side within 5 seconds*		12
Weight Loss: (Only to be used if the score is 6 or more than 6 in other categories):			
	Normal < 20%		0
	20-25%		3
	26-30%		6
	31-34%		9
	≥35%*		12
TOTAL			_____

**** Regardless of total score, notify appropriate veterinary staff personnel immediately.**

*** Regardless of score, immediately euthanize (death is imminent)**

Total Score:

< 6 Normal

6 - 11 Morbid: See VI.D.2 and VI.D.4 in IACUC Policy #10 for guidelines

≥ 12 Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*). Any single criteria of 12* euthanize immediately; consider as 'found dead.

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (December 2013)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET
Revised 10-31-2014

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	7/15/2014	2nd Resubmission Date:	
Approved/Returned for Revision:	7/31/2014	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	10/31/2014	**EXPIRATION DATE:**	11/17/2017
Approved/Returned for Revision:	11/18/2014	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Screening radiation countermeasures for prophylaxis and mitigation of gastrointestinal syndrome (GI-ARS) in the lab mouse, *Mus musculus*.

IV. PRINCIPAL INVESTIGATOR:

(b)(6) **PhD,**
Research Biologist, SRD, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, LTC, USA** Date
Head, Scientific Research Department, AFRRI
Telephone (b)(6) Fax (b)(6)
(b)(6)

- Or –

(b)(6) **PhD, CDR, USN** Date

Head, Scientific Research Department, AFRRI
Telephone: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician
Telephone (b)(6) Fax (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI
Telephone (b)(6) Fax: (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Safety and Occupational Health Specialist, USUHS
Telephone (b)(6) Fax: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**

IACUC Chair (b)(6) Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: Screening radiation countermeasures for prophylaxis and mitigation of gastrointestinal syndrome (GI-ARS) in the lab mouse, *Mus musculus*.

PRINCIPAL INVESTIGATOR: (b)(6) PhD,
Research Biologist, AFRRI
Uniformed Services University of the Health Sciences
Phone: (b)(6) Fax (b)(6)

Co-Investigator: (b)(6) PhD,
Research Biologist, AFRRI
Uniformed Services University of the Health Sciences
Phone: (b)(6) Fax (b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS Nuclear proliferation, terrorist activity, and the distribution of nuclear and radioactive materials through underground networks make incidents involving radiation injuries increasingly likely. Scenarios involving radiological hazards include nuclear detonations, covert placement of radioactive substances, and dirty bombs. Depending upon the exposure dose, radiation can cause extensive damage to the rapidly dividing cell populations of the hematopoietic and the gastrointestinal systems leading to secondary infections and hemorrhage resulting in fatalities. At doses of 8 Gy and above in humans, there is severe damage of the gastrointestinal system, with accompanying weight loss, diarrhea, dehydration, and disruption of the gastrointestinal mucosa allowing large numbers of gut microorganism to enter the systemic circulation, causing opportunistic infections and mortality. The outcome is collectively termed Gastrointestinal Acute Radiation Syndrome (GI-ARS). Currently, there is no effective treatment for GI-ARS consequent to accidental radiation exposure.

The current protocol is funded by the (b)(4) (b)(4) to develop a rodent model with 5% shielding of the bone marrow, and to evaluate potential radiation countermeasures that can delay mortality and increase survival of lethally irradiated mice when administered after irradiation. We propose to 1) establish the baseline for GI injury using total body irradiation [TBI], 2) develop a radiation model for GI-ARS with 5% shielding of the bone marrow [partial body irradiation, PBI], 3) evaluate the efficacy of pharmacological countermeasures (CM) in both TBI and PBI, and 4) to study the pathophysiology of radiation-induced gastrointestinal injury and its time-course, as well as the effect of CM on these parameters.

II. BACKGROUND

II.1. Background

Gastrointestinal Syndrome

The three biological systems most affected by high levels of radiation are (1) the vascular system in the brain; (2) the mucosal cells lining the gastrointestinal tract, and (3) the hematopoietic (HP)/immune system. Damage to the brain's vasculature, which occurs only at the very highest levels of radiation exposure, is irreversible and lethal.

Gastrointestinal damage, including lesions within the gut followed by sepsis, is induced by high levels of radiation and is known as gastrointestinal acute radiation syndrome (GI-ARS). Exposure to high doses of radiation exceeding 10-12 Gy causes GI-ARS, which can result in death in 5-10 days. The extent of injury to the bone marrow and the GI tract are the principal determinants of survival after exposure to total body irradiation (TBI).

The classical radiobiological view presents radiation-induced tissue injury as the direct consequence of DNA damage and cell death induction in target cells, meaning that the severity of tissue damage would be directly related to cell depletion during the acute phase. In mouse models of the gastrointestinal syndrome, acute injury has been mainly attributed to apoptosis of epithelial stem cells and microvascular endothelial cells. The pathophysiological mechanisms of GI-ARS is complex and involves loss of clonogenic crypt cells with eventual depopulation of the intestinal villi, defective regeneration of the irradiated intestinal stem cells and a systemic inflammatory response syndrome from a host of cytokines and growth factors released in the serum, following exposure to radiation and gut microbes (Marxhman *et al* 2002, Potten *et al*, 1998). Survival from GI-ARS depends on the rate of the crypt depopulation and the efficiency and number of the residual clonogens, capable of regenerating crypt-villus units.

Partial body irradiation for GI-ARS

(b)(4)

(b)(4) has been in the forefront (MacVittie 2012) in developing animal models for evaluation of various countermeasures which are organ specific. One such model is the Partial body Irradiation with 5% bone marrow shielding (PBI/BM5 or PBI). In a bioterrorism event, typical victims would receive a nonhomogeneous radiation dose that preserves roughly 5% or more of their marrow. The PBI model protects approximately 5% of bone marrow [BM] and is sufficient to separate the effects of BM collapse from those of GI toxicity and to spare 100% of mice from death (LD100/30) at the hematological doses (Zhang *et al* 2010). (b)(4) has requested AFRRI to develop a PBI model to evaluate countermeasures for GI-ARS in mice at AFRRI). The current proposal addresses this request. It is necessary to develop specific jigs suitable to our radiation sources to isolate/shield one hind leg form exposure to irradiation. It is anticipated that 5% shielding of the BM will increase survival by a factor of 1.2 to 1.3.

Zhang et al 2010 Int. J. Radiation Oncology Biol. Phys., Vol. 77, No. 1, pp. 261–268, 2010
MacVittie TJ 2012 Health Physics 2012, Volume 103, Number 4

Countermeasures against GI-ARS

An intense area of research in radiopathology involves the prophylactic use of trophic growth factors such as keratinocyte growth factor (Dorr *et al*, 2002, Terry *et al*, 2004) and glucagon-like peptide 2 (Booth *et al* 2004). Keratinocyte growth factor and interleukin-11 and growth facts that inhibit cell cycle in regenerating crypts, such as, transforming growth factor-β1 (TGFβ1) promote radioresistance of crypt cells when administered prophylactically (Booth and Potten 2001, Booth *et al* 2000, Khan *et al* 1997).

Besides the intestinal crypts where the putative intestinal stem cell (ISC) reside, the stroma or the ISC niche has also been postulated to be a target in GI-ARS. Thus, endothelial cells residing in the ISC niche, is particularly vulnerable to radiation. Growth factors, such as, basic fibroblast growth factor (bFGF) that prevented radiation-induced endothelial (Okunieff *et al* 1998, Zhang *et al* 2010) cell death, also conferred radioprotection from GI-ARS. Recently, a peptide derived from Salmonella Flagellin, which is a ligand for TLR5, was found to be radioprotective against GI-ARS in murine and primate models, possibly via NFkB activation (Saha *et al* 2012).
Work at AFRRI is currently focused on the prophylactic use of vitamin isomers; Li *et al* (2013) reported that DT3 administered 24 h prior to 10-12 Gy irradiated CD2F1 mice improved survival, and protected the intestinal tissue by reducing inflammatory process. Singh *et al* (2012) reported alpha-tocopherol succinate protected against GI-ARS by inhibiting apoptosis, promoting regeneration of crypt cells, and inhibiting translocation of gut bacteria.

Currently, there are no reports of CMs effective when administered post-irradiation.

(b)(4)

References

Marshman *et al.*, The intestinal epithelial stem cell. Bioessays 2002.

Potten *et al.*, Stem cells in gastrointestinal epithelium: numbers, characteristics and death. Philos Trans R Soc Lond B Biol Sci 1998.

Dorr *et al.* Amelioration of acute oral mucositis by keratinocyte growth factor: Fractionated irradiation. Int J Radiat Oncol Biol Phys 2002.

Terry *et al.* Cellular kinetics of murine lung: Model system to determine basis for radioprotection with keratinocyte growth factor. Int J Radiat Oncol Biol Phys 2004.

Booth *et al.* Teduglutide ([Gly2]GLP-2) protects small intestinal stem cells from radiation damage. Cell Prolif 2004.

Booth and Potten, Protection against mucosal injury by growth factors and cytokines. J Natl Cancer Inst Monogr. 2001.

Booth *et al.*, Transforming growth factor-B3 protects murine small intestinal crypt stem cells and animal survival after irradiation, possibly by reducing stem-cell cycling. Int J Cancer 2000.

Khan *et al.*, Enhancement of murine intestinal stem cell survival after irradiation by keratinocyte growth factor. Radiat Res 1997.

Li *et al.* Delta-tocotrienol protects mice from radiation-induced gastrointestinal injury. Radiat Res. 2013.

Okunieff *et al.* In vivo radioprotective effects of angiogenic growth factors on the small bowel of C3H mice. Radiat Res 1998.
Singh *et al*, Alpha-tocopherol succinate protects mice against radiation-induced gastrointestinal injury. Radiat Res 2012.
Zhang *et al.* Mitigation effect of an FGF-2 peptide on acute gastrointestinal syndrome after high-dose ionizing radiation. Int J Radiat Oncol Biol Phys 2010.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched BRD, NIHRePORTER, PUBMED, Embase

II.2.2. Date of Search 05/10/2014-06/02/2014

II.2.3. Period of Search The search period extended from 1998 to 2005 for BRD, 2009-present for NIHReporter, 1970 to present for PubMed, 2006 to present for Embase.

II.2.4. Key Words of Search Radiation-induced gastrointestinal syndrome (GI-ARS), (b)(4) (b)(4) and radiation, AGS injury, symptoms, GI-ARS, GI-ARS countermeasures, surrogate markers, Partial body irradiation, total body irradiation

II.2.5. Results of Search Several references were found on PubMed and Embase (>500) for the individual terms; a few pertinent ones have been used in the background section for GI-ARS, countermeasures to GI-ARS and (b)(4) There were no hits under BRD. These are the results under NIHRePORT summarized here:
(b)(4) 1 hit.
Radiation: 3792 hits, 48 hits were relevant for (Radiation countermeasures).
Gastrointestinal syndrome and radiation: 34 hits.
The following are the most relevant.

Project Title	Project Number	Contact PI / Project Leader	Organization Name
CHARACTERIZATION OF TELOMERASE EXPRESSING INTESTINAL STEM CELLS	5R01DK084056-04	BREAULT, DAVID T	CHILDREN'S HOSPITAL CORPORATION
MEDICAL COUNTERMEASURE AFTER RADIATION EXPOSURE	1R43AI107953-01A1	CASTILLO, GERARDO M	PHARMAIN CORPORATION
BECLOMETHASONE POST EXPOSURE THERAPY FOR GASTROINTESTINAL ACUTE RADIATION SYNDROM	5R43AI100417-02	DONINI, OREOLA	SOLIGENIX, INC.
STEM CELL-BASED THERAPIES FOR RADIATION-INDUCED GASTROINTESTINAL SYDROME (RIGS)	5U19AI091175-04	GUHA, CHANDAN	ALBERT EINSTEIN COLLEGE OF MEDICINE
SOMATOSTATIN ANALOGS AS COUNTERMEASURES AGAINST INTESTINAL RADIATION TOXICITY	5U19AI067798-09	HAUER-JENSEN, MARTIN	DUKE UNIVERSITY
DEVELOPMENT OF MEDICAL COUNTERMEASURES TO MITIGATE OR TREAT GI-ARS	272201300021C-0-0-1	MCCOOL, SHANNON	RXBIO, INC.
DEVELOPMENT OF MEDICAL COUNTERMEASURES TO MITIGATE OR TREAT GI-ARS	272201300030C-0-0-1	RIVENBURG, THOMAS	SOLIGENIX, INC.
FGF-P TO TREAT POST-IRRADIATION GI SYNDROME	5R43AI098330-02	ZHANG, AIGUO	DIACARTA, LLC

However, none of the studies are duplicated here.

III. OBJECTIVE / HYPOTHESIS The objective of this protocol is to develop a rodent model for acute gastrointestinal syndrome with 5% shielding of the bone marrow tissue and to evaluate various drugs as radiation protectants and mitigators, as recommended by (b)(4) using survival as the endpoint. Further, we will determine the pathophysiology of GI-ARS following irradiation and the effect of countermeasures on these markers of GI injury to understand the mechanism of GI-ARS.

IV. MILITARY RELEVANCE In the event of a mass casualty event involving radiological/nuclear material, military personnel and first responders can be exposed to radiation doses ranging from mild, moderate to very high. At 6-8 Gy exposures (mild to moderate), GI-ARS or the gastrointestinal syndrome occurs, for which there is currently no FDA approved pharmacological intervention. GI-ARS cannot be managed with supportive care including antibiotics, hydration or cell therapies; eventually leading to mortality. Progress in understanding the differences in the mechanisms involved in response of the GI to high doses of radiation has led to the development of new rationales for selective protection of the normal cells, by suppressing death signals and increasing radioresistance of the normal cells. This protocol will address the critical gap in knowledge regarding GI-ARS and to develop strategies to protect, ameliorate, or treat armed personnel.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

V.1.1. Experiment 1.

V1.1.a Establish LD-30/10, LD-50/10, LD-70/10 and LD-100/10 for CD2F1 mice following total body irradiation.

1. We will use 6 radiation doses to determine the LD-50/10 for CD2F1 mice [radiation dose that results in 50% mortality of CD2F1 mice within 10 days post-TBI].
2. We will use n=10 mice per radiation dose for the preliminary study. The study will be reiterated prior to start of countermeasure screening.
3. Probit analyses will determine the LD-30, LD-50 and LD-70 radiation doses for the model.

6 radiation doses (10, 11, 12, 13, 14 and 15 Gy, 0.6 Gy/min)* x 10 mice x 2 iteration

Subtotal = 120 mice

*The radiation doses are based on literature. We will refine the dose based on preliminary data to arrive at the correct GI dose.

V.1.1.b. Determine the pathophysiology of GI-injury in mice irradiated with different GI total body radiation doses (TBI) to establish a radiation-dose response for GI injury.
4 radiation doses *x 4 time points post TBI (4 d, 6 d, 8 d and 10 d post-TBI) x 10 mice

Subtotal = 160 mice

* *Radiation doses will be determined based on V.1.1.a.*

Note: V.1.2. Experiment 2 has been shelved

V.1.3. Experiment 3

V.1.3.a. Test a promising radiation countermeasure against radiation injury (GI-ARS) after exposure to total body irradiation (TBI).

1. There will be 3 arms for the survival study: Prophylaxis (24 h pre-TBI), and two mitigation arms (post-TBI) starting at 4 h and 24 h post-TBI.
2. The radiation dose resulting in 100% mortality on day 10 will be selected for testing radiation countermeasures based on results from V.1.1.a.
3. Delay in mortality and improvement in survival (30% over vehicle) is the 'pass' criteria for a successful radiation countermeasure.
4. The countermeasure identified for preliminary screening in the GI model is (b)(4) [50 mg/kg, 4 doses, once a day by oral gavage].

For each drug, the animal requirement is calculated as follows:

Drug + vehicle (2 groups) x 3 regimen (one pre- and two post-TBI) x 1 rad dose (LD-100/10) x 1 route (*po*) x 24 CD2F1 mice per group **Subtotal = 144 mice/drug**.

Pilot study (Partial body radiation): It appears from conversation with JIG manufacturers for partial body radiation that Cobalt 60 gamma radiation cannot be used for such studies due to the scattering of the radiation. The alternate option is to use X-ray source. PI has identified the source as the unit available (b)(4) Additional discussions are in progress and hence PI is not requesting any animals for even a pilot study. **Subtotal = 0 mice**

Shelved experiments V1.2., V1.3.b, V1.4.,V.1.5., and V.1.6

Partial body radiation studies (place holder- no animals requested)

V.1.2. Experiment 2 [The details pertaining to partial body irradiation is under active discussion between the PI, VSD, and radiation dosimetry lead. We will submit an amendment at a later date describing the precise methodologies].

V.1.2.a. Establish LD-50/10 for CD2F1 mice following 5% shielding of bone marrow.
6 radiation doses (12, 13, 14, 15, 16 and 17 Gy, 0.6 Gy/min) x 10 mice x 2 iteration*

Subtotal = 0 mice^

**These radiation doses are projected based on a DRF of 1.3 for survival following 5% bone marrow shielding reported in literature. These will likely change based on the AFRRI model, radiation source, dose-rate and other variables.*

V.1.2.b. Determine the pathophysiology of GI-injury in mice irradiated with different GI partial body radiation doses (PBI) to establish a radiation dose response for GI injury.
4 radiation doses x 4 time points post TBI (4 d, 6 d, 8 d and 10 d post-TBI) x 10 mice*

Subtotal = 0 mice^

**Radiation doses will be determined based on V.1.2.a.*
^Mice will be requested for PBI studies in the amendment detailing methodologies for irradiation, anesthesia, and other logistics.

V.1.3.b. Test a promising** radiation countermeasure for GI efficacy after exposure to partial body irradiation (PBI). **[Details for anesthesia and irradiation will be submitted as an amendment once the details are determined by the PI, VSD and Radiation Department].

1. *As before, there will be 3 arms for the survival study: Prophylaxis (24 h pre-TBI), and two mitigation arms (post-TBI) starting at 4 h and 24 h post-TBI.*

2. *The radiation dose resulting in 100% mortality on day 10 with 5% bone marrow shielding will be selected for testing radiation countermeasures based on results from V.1.2.a.*
3. *Delay in mortality and improvement in survival (30% over vehicle) is the 'pass' criteria for a successful radiation countermeasure.*
4. (b)(4) *is the first countermeasure to be tested in the PBI model [50 mg/kg, 4 doses, once a day by oral gavage].*

For each drug, the animal requirement is calculated as follows:
Drug + vehicle (2 groups) x 3 regimen (one pre- and two post-TBI) x 1 rad dose (LD-100/10) x 1 route (po) x 24 CD2F1 mice per group **Subtotal = 0 mice/drug^**

^Mice will be requested for PBI studies in the amendment detailing methodologies for irradiation, anesthesia, and other logistics.

V.1.4. Experiment 4
***Optimization studies**: Optimization studies will be initiated in CD2F1 mice only if any of the drugs tested meets the AFRRI criteria of >30% survival than vehicle in the preliminary radiation survival screen. As a reduction strategy, we will use only PBI. Drug dose and schedule will be selected based on optimum survival by* (b)(4) *We project initiating the optimization studies with 50 mg/kg* (b)(4) *administered once a day by oral gavage for 10 days [see background].*

***Drug dose optimization study**: 6 groups (Five drug doses + 1 vehicle) x 1 radiation dose (LD-100/10) x 2 regimen (pre- and post-exposure) x 1 route x 20 animals per group* **Subtotal = 0 CD2F1 mice^**

***Time optimization study**: 4 different time points x 1 optimum dose + 1 vehicle (2 groups) x 1 radiation dose (LD-100/10) x 1 route x 20 animals per group* **Subtotal = 0 CD2F1 mice^**
^Mice will be requested for PBI studies in the amendment detailing methodologies for irradiation, anesthesia, and other logistics.

V.1.5. Experiment 5
Dose reduction factor study (DRF)
The dose reduction factor (DRF) studies will use the optimum drug dose and times determined by the optimization studies to evaluate the effectiveness of the drug against a range of GI radiation doses in CD2F1 mice for both TBI and PBI conditions.

DRF for total body irradiation (TBI)

6 TBI-irradiated vehicle groups (0.6 Gy/min) + 6 TBI-irradiated drug groups* x 24 mice per group*

Subtotal = 0 CD2F1 mice

**These radiation doses will be determined based on experiment V.1.1 and V.1.2 and are considered a guidance. The appropriate radiation doses will be determined using experiment V.1.1.*

DRF for partial body irradiation (PBI)

6 PBI-irradiated vehicle groups (0.6 Gy/min) + 6 PBI-irradiated drug groups* x 24 mice per group*

Subtotal = 0 CD2F1 mice^

**As above, data generated from experiment V.1.2 will determine the radiation dose range.*

^Mice will be requested for PBI studies in the amendment detailing methodologies for irradiation, anesthesia, and other logistics.

V.1.6. Experiment 6
*V.1.6.a. Determine the pathophysiology of GI-injury in mice irradiated with GI radiation doses (**PBI**) and treated with a countermeasure. Data from these studies will form the basis of understanding the mode of action of the drug. Experiment V.1.4. will determine the optimum dose and schedule for these studies.*

Naïve + vehicle + drug alone (3 groups) x 4 time-points x 10 mice per group x 1 route of administration x 1 drug **Subtotal = 0 CD2F1 mice^**

^Mice will be requested for PBI studies in the amendment detailing methodologies for irradiation, anesthesia, and other logistics.

2 experimental groups (vehicle-irradiated, Drug-irradiated) X 4 time-points X 10 mice per group x 1 route of administration X 2 radiation doses ***Subtotal = 0 CD2F1 mice^***
^Mice will be requested for PBI studies in the amendment detailing methodologies for irradiation, anesthesia, and other logistics.

V.2. Data Analysis

Note: THIS SECTION HAS NOT BEEN REVISED in the light of shelving certain experiments as shown above, because ultimately similar type of statistics will be used.

All data will be reported as means with standards errors or percentages where applicable.

Experiment V.1.1 and V.1.2: A 6 dose probit model to determine LD-50 will have a>80% power to detect significant difference if the effect size is 1.0 standard deviations and the sample size per dose is 10 animals. We request an iteration for each of these experiments to
Allow for additional radiation doses if the preliminary study fails to achieve the LD-5/10 to LD-100/10 range for survival.

Experiment V.1.3 and V.1.4: Assumptions for 30% improvement in 10-d survival in the radiation countermeasure group compared to none in the vehicle group. Based on a log-rank test with 80% power and 5% two-sided significance, we determined that this design has adequate power for screening countermeasures using a 10-day survival and N of 20 (Kodell *et al*, 2010).

Experiment V.1.5. 20 animals per dose with six radiation doses is sufficient to determine a probit line fit to mortality data for DRF calculations. At least three out of the six doses should sustain survival other than 100 or 0 percent. Finney's methods will be adhered to in making probit fits and estimating DRF.

Experiment V.1.6. For testing the overall difference between groups (averaging over time points), two-way ANOVA with 10 animals per group measured at 4 time points will have 80% power to detect a difference of 0.91 standard deviations if the within-subject correlation is 0.2. For comparisons between groups at a single time point, the proposed sample size will have 80% power to detect a difference of 1.8 standard deviations between groups, based on a t-test for independent samples with a 5%, two-sided significance level.
Prasanna and Devi (1993) observed standard deviations of ~35 crypts. A sample size of 8-10 per group will have 80% power to detect a difference of 3 crypts (2.38 std. dev) based on a 2 group t-test with 5%, 2-sided significance level. Observed differences are expected to exceed 10 crypts.

Kodell *et al*. Determination of sample size for demonstrating efficacy of radiation countermeasures. Biometrics. 2010.
Prasanna and Devi, Modification of WR-2721 radiation protection from gastrointestinal injury and death in mice by 2-mercaptopropionylglycine. Rad Res. 1993.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered: Although high throughput screening and computer modeling have advanced significantly in recent years in identification of promising drugs, it would be simplistic to assume that cell systems or artificial intelligence modeling can replace the data that can be generated by using an animal model. Irradiation of animals will result in a cascade of changes including death of stem and progenitor cells in bone marrow, alterations in signal transduction pathways, multiple organ dysfunctions etc. due to differing radiosensitivities of organs. Currently, there are no alternatives in existence that can predict or compute the complexity of the response to total body irradiation. The US FDA recognizes the ethical considerations involved in testing the efficacy of radioprotective drugs in humans. The FDA regulations concerning the approval of new drugs or biological products when human efficacy studies are not ethical or feasible are known as the "Animal Rule" (Crawford 2002). Therefore, FDA requires preclinical drug assessments (safety and efficacy) to be performed using both small and large animal models (FDA 2002) prior to granting approval for safety testing in humans. These regulations state that the pathophysiological mechanism of radiotoxicity and its prevention or substantial reduction by the product must be well understood and demonstrated in more than one animal species expected to react with a response predictive for humans. Further, the endpoint in such studies must be clearly related to the desired benefit in humans, generally the enhancement of survival or prevention of major morbidity. Finally, there must be supporting pharmacokinetic (pK) and pharmacodynamic (pD) data (USFDA 2009).

Crawford. New drug and biological drug products; evidence needed to demonstrate effectiveness of new drugs when human efficacy studies are not ethical or feasible. Federal Register 67: 37988<37998; 21 CFR parts 314 and 601.FDA, HHS; ACTION: Final Rule; 2002.
U.S. Food and Drug Administration. Guidance for industry:animal models, essential elements to address efficacy under the Animal Rule. Silver Spring, MD: CBER; 2009.

V.3.2. Animal Model and Species Justification Recent advances in molecular techniques have allowed significant improvements in understanding the cellular and biochemical processes. These advances are possible only due to whole animal modeling of human ailments. The mouse, in particular, has high sequence homology to humans at a genomic level as well as similar hematological and immunological response to radiation. Mouse models not only confirm primary pathological processes but have also provided a venue for studying basic molecular, cellular, biochemical, and cytological processes. Further, there are extensive data on the mouse for comparison, review and analysis. The practicalities of breeding and housing these small mammals have made the mouse model invaluable. The Principal Investigator's group at AFRRI has significant training and experience in handling mice in radiation countermeasure studies.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species Mouse, *Mus musculus*

V.3.3.2. Strain / Stock CD2F1

V.3.3.3. Source / Vendor CD2F1 from VSD approved vendors (Harlan Inc., Charles Rive laboratories, or Jackson Laboratories).

V.3.3.4. Age 7-8 weeks on purchase.

V.3.3.5. Weight 25-30 grams

V.3.3.6. Sex Males have been used historically and will continue to be used in this study. Females may be included as per the Program Manager's directive. In this case, an amendment will be submitted detailing the reason for inclusion of females.

V.3.3.7. Special Considerations Commercially procured animals must be adventitious disease free. Specific pathogen-free, including *Pseudomonas aeruginosa,* and *Pasteurella*: All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi,* CAR bacillus, *Mycoplasma pulmonis*, and *Clostridium piliforme.*
Endoparasite- and ectoparasite-free.

V.3.4. Number of Animals Required (by species) 424 CD2F1 mice

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement The PI will take necessary steps to assure that all personnel in the project are rigorously trained in handling techniques (ear-punch, injections, oral gavage, intraperitoneal and subcutaneous injections, and routine cage-side observations) to minimize distress and pain to the animals. Specifically, during capture and restraint of the rodents, care will be taken to ensure that handling does not contribute to distress. Topical ointment/lubrication gel will be used, if necessary, to prevent drying of the cornea as well as at the site of drug injection in consultation with attending veterinarian and veterinary staff.

V.3.5.2. Reduction The protocol will use a tiered approach to reduce the number of animals. In preliminary studies, if we achieve the LD-50 in the first attempt, we will repeat the study with 5 radiation doses and an n of 5 each to validate the data. Wherever possible we will reduce the number of repeats planned for the experiments above.

V.3.5.3. Replacement It is not feasible to use non-animal systems in place of actual animal models to address the questions in this project. Rodents are considered sentient and hence rodents will be used for screening large number of potential radiation countermeasures. Only those drugs that pass the AFRRI criteria for significant survival benefit will advance to studies in higher mammals.

V.4. Technical Methods
V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals 424

V.4.1.1.1.1. Column C __ (# of animals).

V.4.1.1.1.2. Column D __160___ (# of animals). These mice will not be irradiated. They will be deeply anesthetized, euthanized, and their tissue harvested as a terminal procedure.

V.4.1.1.1.3. Column E __264 (# of animals). These mice will be irradiated and will not receive any intervention for pain relief. However, they will be euthanized if found moribund.

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1	TBI probit			120	120
3	Countermeasure (CM) Screening [TBI]			144	144
6	Pathophysiology of GI injury		160		160
	Shelved experiments				
2	~~Partial body probit*~~			~~0~~	~~0~~
4	~~Partial body -Optimization for CM*~~			~~0~~	~~0~~
5	~~Partial and total body Dose Reduction Factor*~~			~~0~~	~~0~~
Totals:			160	264	424

** experiments shelved (all crossed out experiments)*

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization The protocol will not use analgesics during radiation since they will adversely affect the outcome of the experiments. The proposed study is to determine the effects of radiation and countermeasures on the function of the immune and blood forming systems; use of any analgesics and anesthetics would interfere with the function of the immune system and therefore compromise the interpretation of the results. It is anticipated that at high radiation doses, animals will demonstrate varying degrees of morbidity and moribund, which makes it critical to define the criteria for euthanizing the animals without undermining the study objective. Euthanasia will be performed by trained personnel in accordance to the current AVMA guidelines (AMVA Guidelines on Euthanasia, 2013).

For Experiment 6, mice will be placed in the Isoflurane chamber and delivered a metered amount of 5% Isoflurane mixed with 100% oxygen until all voluntary motor movement ceases and the animal is recumbent for 10 seconds. Animal's respiration rate will be closely monitored through the Plexiglas chamber during induction of anesthesia. When animals are recumbent, they will be removed from the chamber and their nose/mouth placed in the anesthesia apparatus breathing/gas administration cone for maintenance of anesthesia at 3% Isoflurane and 100% O_2. Blood will be collected by cardiac stick or from the inferior vena cava (as described under procedures). All

personnel will be extensively trained for efficient administration of the agents as well as to minimize handling stress.

Anesthesia for partial body irradiation [PBI]: This is under discussion with VSD based on the time projection for radiation at high doses (12-15 Gy; approximately 30 min). The PI will submit a detailed description for the procedure after preliminary studies under the aegis of VSD.

Association AVM. AMVA Guidelines on Euthanasia. http://www.avmaorg/issues/animal_welfare/euthanasiapdf; 2013.

V.4.1.2.2. Pre- and Post-procedural Provisions Mice will be housed under standard conditions both before and after radiation procedures in accordance with current VSD rodent husbandry Standard Operating Procedures (see section V.5).

V.4.1.2.3. Paralytics N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures
V.4.1.3.1. Sources Searched AGRICOLA, PubMed, EMBASE

V.4.1.3.2. Date of Search: 05/30/2014

V.4.1.3.3. Period of Search AGRICOLA: 1975-2004, EMBASE 1974-present PubMed~1950 to present.

V.4.1.3.4. Key Words of Search Gastrointestinal Radiation Syndrome and pain, (b)(4) and pain, anesthesia and radiation, analgesia, distress, humane.

V.4.1.3.5. Results of Search There are no alternatives to irradiation. While radiation itself does not cause pain or distress, it induces a number of changes in the body that alters immune response and destroys hematological and gastrointestinal tissues, resulting in opportunistic infections, and multiorgan dysfunction leading to death. It is anticipated that animals exposed to high lethal radiation doses will become moribund. Unfortunately, literature clearly demonstrates that use of analgesics alters radiation response and therefore leads to increase in animal number for better statistical results or for iteration of entire experiments. No references were found suggesting distress due to (b)(4) though some changes associated with the pathophysiology of biological response to the drug is expected, such as increase in fiber content of GI and therefore more stool formation.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification Because exposure to irradiation is potentially lethal or causes debilitating effects in humans, it is ethically impermissible to test the effectiveness of radiation countermeasures on human volunteers. Irradiation compromises the immune and blood forming systems leading to mortality. The radiation-induced mortality and potential percentage increase of survivors over 30 days using potential radiation countermeasures are study endpoints for this protocol. Administration of drugs and analgesics is not an option since it is well documented that they interact with the immune system which will

confuse data interpretation (Galley *et al*, 1997, 1998). Pain relieving measures are not used because such measures may compromise the experimental integrity of the study.

Galley *et al*., The effect of midazolam and propofol on interleukin-8 from human polymorphonuclear leukocytes. Anesth Analg 1998.
Galley and Webster. Effects of propofol and thiopentone on the immune response. Anaesthesia 1997.

V.4.2. Prolonged Restraint The experimental animals will receive radiation at a dose rate 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (dosimetry 02/28/2013;or the latest dosimetry map) using plastic racks. Mice will be inserted into the standard radiation boxes just prior to irradiation (~15-30 min of exposure time) and taken to the cobalt facility for irradiation. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period. They will be monitored daily (twice a day when necessary) for 30 days and euthanized at the completion of the observational period. Although the radiation dose results in mortality on day 10 post-irradiation, all surviving mice will be monitored for 30 day, to determine the effect of the countermeasure.

V.4.3. Surgery N/A
V.4.3.1. Pre-surgical Provisions
V.4.3.2. Procedure(s)
V.4.3.3. Post-surgical Provisions
V.4.3.4. Location
V.4.3.5. Surgeon
V.4.3.6. Multiple Major Survival Operative Procedures
V.4.3.6.1. Procedures
V.4.3.6.2 Scientific Justification

V.4.4. Animal Manipulations
V.4.4.1 Animal Identification: Individual animals will be identified by either ear punch or tattoos as per VSD policy.

V.4.4.1. Injections For the oral route, drug(s) will be administered using a 20 G gavage needle (Fisherbrand-Animal feeding needles, disposable-sterile, non-toxic and nonpyrogenic-20 G, 1 ½" length). Non-disposable, metal ball ended, oral gavage needles are included as an alternative to the disposable needles. Drug volume will be 0.2 ml (with a maximum of 0.5 ml). The animal will be restrained in a vertical position to align the spine straight. The bulb will be introduced into the mouth horizontally. Using the needle as lever, the syringe will be moved into a vertical position and the needle dropped down the esophagus into the stomach. The needle contents will be administered when the needle hub touches the animal's mouth. All precautions will be taken to ensure that the placement of the needle is properly positioned and that the animal is not struggling severely. No forceful feeding will be done to prevent perforation of the esophagus.

Irradiation: These will follow the standard AFRRI protocol with mice placed in restrainers shortly prior to exposure, irradiation at the cobalt facility and return to VSD for further monitoring. Briefly, the experimental animals will receive radiation at a dose

rate 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (dosimetry 02/28/2013 or current dosimetry map) using plastic racks. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period. They will be monitored daily (twice a day when necessary) for 30 days and euthanized at the completion of the observational period. In case of non-lethal exposures, mice will be harvested at specific time-points after irradiation, and tissues collected after terminal blood draw.

Irradiation for PBI is under discussion with (b)(6) Once the details are determined, the PI will submit an amendment describing the procedure for PBI.

V.4.4.1.1. Pharmaceutical Grade Drugs N/A

V.4.4.1.2. Non-Pharmaceutical Grade Drugs (b)(4) will be tested for endotoxin levels if this information is not supplied by the manufacturers. Physical characterization, including osmolality and pH of final solutions will be in the range stipulated by IACUC by its policy letter on testing non-pharmaceutical grade drugs (policy #9). We do not anticipate filtering the test agent due to the limited availability of drugs, and uncertainty as to how much of the drug will be filtered out. However, we will ensure that drug preparation is carried out under hygienic conditions (use of PPE, clean surface area for drug preparation, use of clinical grade solvents, sterile needles and syringes) to preclude contamination of test samples.

V.4.4.2. Biosamples

Blood collection: Mice will be anesthetized as described under section V.4.1.2.1 (anesthesia). Blood draw for hematology is collected either by cardiac stick or from the inferior vena cava (IVC). The animals will be deeply anesthetized in the rodent anesthesia machine; the tail will be pinched for reflexive movement, indicative of insufficient anesthesia. If there is no response, the animal will be moved to the station with individual nose cone for continued anesthesia. An incision will be made on the right side below the abdomen of the animal, closest to the IVC, the vein exposed and blood drawn with a 23 G needle. Similarly, cardiac stick will be performed on the animal positioned at the platform under anesthesia. The animals will be placed on the back, and the skin wet with 70% alcohol. The needle will be inserted under the sterum towards the mouse head, and negative pressure applied to the plunger very gently. If not blood appears, the needle will be slowly withdrawn so that the needle remains under the skin and then redirected in a slightly different direction. In both cases, the animals will be immediately euthanized on completion of blood draw by cervical dislocation.

V.4.4.3. Adjuvants N/A

V.4.4.4. Monoclonal Antibody (MAb) Production N/A

V.4.4.5. Animal Identification Generally, cage cards will be used to distinguish drug-treated and control groups. Some experiments might necessitate ear punch or tattooing by personnel to identify individual animals in a cage. This will be done as per VSD policy.

V.4.4.6. Behavioral Studies N/A

V.4.4.7. Other Procedures N/A

V.4.4.8. Tissue Sharing On approval by IACUC.

V.4.4.9. Animal By-Products N/A

V.4.5. Study Endpoint The study end-point for experiments 1-5 is 10 day mortality following irradiation. However, if the mice survive past 10-d post-TBI, we will monitor survival for 30 days to determine mortality at different radiation doses.

Since this is a screening protocol, the PI requests modification of policy#10 (revised July 2014)to score morbidity and determine mortality. The revised scoring sheet for the current study has been included as an appendix.

All scores (5, 6 and 9 from the "appearance" clinical signs) and the amended respiratory score are removed following consultation with the AV, because the former have never been observed in our studies and the latter, because we do see lowered respiratory rates. Mice will only be euthanized if there is a score of 12 in a single category. Body weights are not included since there is no clear correlation between body weight loss and mortality. Further, we do not anticipate significant body weight loss during the course of the study [10 days post-TBI]. Previous publications (b)(4) (b)(4) describing survival studies did not use body weight as a parameter to determine survival (b)(4) Further, other radiation researchers do not use body weight loss as a mortality criterion either (Plett *et al*, 2012).

However, to adhere to the policy of preventing animal suffering, the PI will facilitate the late PM and early AM checks (not more than 10 h apart) in addition to the twice a day checks during the critical lethality period. All morbid mice will be monitored carefully and their conditions scored for documentation.

The critical period spans between day 5 and 10 post-TBI.

(b)(4)

Plett *et al., 201*, Establishing a murine model of the hematopoietic syndrome of the acute radiation syndrome. Health Physics, Vol. 103, 343-355.

Animals will be will be immediately euthanized when they display abnormal breathing, are recumbent, or unable to respond to mild external stimulus by the research staff and scored as dead on removal, while recorded as euthanized in the medical records. VSD veterinarians will be consulted for all matters regarding the animal welfare.

V.4.6. Euthanasia Mice found moribund will be euthanized by CO_2 inhalation plus confirmatory cervical dislocation by investigator/ technician according to the directives of the VSD-SOP [Rodent Euthanasia Guidelines] and in accordance with current AVMA Guidelines on Euthanasia (AMVA 2013). At the conclusion of the experimental time frame, all animals that survived the procedures of irradiation, drug intervention and controls, will be sacrificed by CO_2 inhalation plus confirmatory cervical dislocation and their carcasses disposed of according to VSD regulations.

V.5. Veterinary Care

V.5.1. Husbandry Considerations Husbandary practices will be performed as per standard AFRRI VSD SOPs.

V.5.1.1. Study Room As assigned by VSD

V.5.1.2. Special Husbandry Provisions N/A

V.5.1.3. Exceptions Mice will be socially (group) housed as described above, except when there is a possibility that during survival studies cage mates may be lost through attrition and for a period of time the sole surviving mouse may remain singly housed until the end of the 30-day study.

V.5.2. Veterinary Medical Care We do not project routine veterinary medical care. However, in case of minor injuries arising from fighting, topical application of antibiotics is acceptable with consensus from the PI and VSD.

V.5.2.1. Routine Veterinary Medical Care Experimental animals will be observed daily by either the investigator or technician or both. VSD personnel will monitor all animals during their routine rounds as per VSD SOPs. In the course of the study, as animals approach morbidity, the research staff observations will be increased to a minimum of twice a day, early morning and late afternoon (includes weekends and holidays). There is no supportive therapy contemplated since this would compromise the experimental results (described earlier in the end point and pain category sections).

V.5.2.2. Emergency Veterinary Medical Care Moribund animals (gasping, recumbent, non-responsive to mild stimulus), will be euthanized by trained personnel.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy: Igloos and huts may be provided to animals but not on active experiments. Nestling pads will be provided in each cage during bedding changes as per AFRRI-VSD SOPs.

V.5.3.2. Enrichment Restrictions For active experiments, no igloos or huts will be provided, but nestlets will be used. Irradiated animals are considered immunosuppressed and hence PI is of the opinion that Igloos will not be appropriate.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3, 4b, 5	(b)(6)
				1, 3, 4a, 5	
				1, 3, 4a, 5	
				1, 3, 4a, 5	
				1,3,4a,4b, 5	
				1,3,4a,4b, 5	
				1,3,4a,4b, 5	

Procedure and manipulation codes:

Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY All personnel working in the protocol have undergone necessary training in handling experimental animals. The amount of drug used will be small and standard safety precautions will be taken while handling them in the laboratory and in the vivarium. All personnel listed in the present protocol have been trained as general radiation users on a regular basis. All investigators and personnel will use appropriate protective gear while in the vivarium as described by the VSD. All listed personnel are current in safety training and medical surveillance.

VIII. ENCLOSURES None

X. ASSURANCES

Protocol Title: Screening Radiation Countermeasures for prophylaxis and mitigation of gastrointestinal syndrome ((GI-ARS) in the lab mouse, *Mus musculus*.

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): [**AM**] / **AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / [**WILL**] **NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)		10-31-14
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

Clinical Observations with Criteria for Euthanasia (Rodent)

DATE:	Time:	Animal ID#	Score
Appearance:			
	Normal (smooth coat, clear eyes/nose)		0
	Hunched and/or fluffed		1
	Ocular discharge, and/or edema		3
	Blue mucus membranes/skin (cyanosis)*		12
Respiratory Rate:			
	Normal breathing		0
	Change in breathing (an increase or decrease in respiratory rate from normal baseline)		3
	Abdominal breathing (gasping +/- open mouth breathing)*		12
General Behavior:			
	Normal (based on baseline observations)		0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)		1
	Decreased mobility		2
	Ataxia, wobbly, weak**		6
	Inability to stand*		12
Provoked Behavior:			
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))		0
	Subdued; responds to stimulation (moves away briskly)		1
	Subdued even to stimulation (moves away slowly)		3
	Unresponsive to gentle prodding**		6
	Does not right when placed gently on side within 5 seconds*		12

Regardless of total score, notify appropriate veterinary staff personnel.
*** Regardless of score, immediately euthanize (death is imminent).**

Total Score:

< 6 Normal

6 - 11 Morbid: Continue to monitor at PM and AM checks

≥ 12 Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*).
Any single criteria of 12* euthanize immediately; consider as 'found dead.'

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (December 2013)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

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This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

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Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	10/17/2014	2nd Resubmission Date:	
Approved/**Returned for Revision**:	11/4/2014	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	11/12/2014	**EXPIRATION DATE:**	11/20/2017
Approved/Returned for Revision:	11/21/2014	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

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- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Re-Purposing (R)-3-Hydroxybutyl (R)-3-hydroxybutyrate, a ketone ester (or R3-KE) as a Radiation Mitigator in mice (*Mus musculus)*

IV. PRINCIPAL INVESTIGATOR: (b)(6) PhD

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, CDR, USN** Date
Head, Scientific Research Department, AFRRI
Telephone (b)(6) Fax (b)(6)
(b)(6)

- Or –

(b)(6) **PhD, LtCol, USA** Date
Head, Scientific Research Department, AFRRI
Telephone (b)(6)

(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician

Telephone (b)(6) Fax: (b)(6)

(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Environmental Health and Safety Department, USUHS

Telephone (b)(6) Fax (b)(6)

(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.

(Date)

(b)(6) **Ph.D.**

IACUC Chair, (b)(6) Scientific Research Department, AFRRI

Telephone (b)(6) Fax: (b)(6)

(b)(6)

PROTOCOL TITLE: Re-Purposing (R)-3-Hydroxybutyl (R)-3-hydroxybutyrate, a Ketone ester (or R3-KE) as a Radiation Mitigator in mice *(Mus musculus)*

PRINCIPAL INVESTIGATOR:

(b)(6) PhD
Senior Scientist, AFRRI
(b)(6)
SRD/AFRRI
(b)(6)

CO-INVESTIGATOR(S): (b)(6)
(b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS

Radiation mitigators are needed to protect military personnel from adverse effects of radiation exposure in the event of a nuclear weapon disaster or in a terrorist attack employing a dirty bomb. A safe and efficacious material that can be delivered after *radiation* exposure is needed and a variety of agents are being evaluated for their effectiveness (b)(4),(b)(6) (b)(4),(b)(6) (b)(4),(b)(6) is in the process of developing a medical counter measure mitigator, (R)-3-Hydroxybutyl (R)-3-hydroxybutyrate, a ketone ester or more simply called "R3-KE". Thus far R3-KE has been shown to be highly effective in preventing radiation-induced cell death and decreasing radiation-induced chromosomal damage in *in vitro* radiation studies when delivered pre or post exposure. An *in vivo* radiation lethality study is needed to confirm R3-KE's radiation damage mitigation ability. R3-KE is currently being studied in other laboratories for its potential to treat Alzheimer's disease (AD) and Parkinson's disease (PD) and its use resulted in an increase in mental acuity and physical performance in tested Special Operations soldiers. It has been categorized as generally regarded as safe (GRAS) by the FDA and possesses very low clinical toxicity in human clinical trials. Its low toxicity in humans and preliminary *in vitro* radiation results make it an excellent radiation mitigation candidate.

II. BACKGROUND

II.1. Background

Radiation mitigators are needed to protect military personnel from adverse effects of radiation exposure in the event of a nuclear weapon disaster or in a terrorist attack employing a dirty bomb. A safe and efficacious material that can be delivered rapidly and easily after radiation exposure is needed (b)(4),(b)(6) (b)(4),(b)(6) is in the process of developing a medical counter measure mitigator, (R)-3-Hydroxybutyl (R)-3-hydroxybutyrate, a ketone ester or more simply called "R3-KE". Thus far R3-KE has been shown to be highly effective in preventing radiation-induced cell death and decreasing radiation-induced chromosomal damage *in vitro* radiation studies when delivered pre or post exposure. An *in vivo* radiation lethality study is needed to confirm R3-KE's radiation damage mitigation ability.

History of R3-KE and Military Use

R3-KE is an organic ester product comprised of one unit of β-hydroxybutyrate and 1, 3-butane-diol. In the liver the 1, 3-butane-diol is rapidly converted to β-hydroxybutyrate. R3-KE has undergone a battery of safety tests in both human subjects and in animals (1, 2). It is a safe and efficacious compound and has received FDA approval and determined to be Generally Regarded As Safe (GRAS). R3-KE was developed for a specific intended military purpose. (b)(4) (b)(4)

(b)(4)

Metabolism of Ketone Bodies

Ketone bodies, in particular, 3-hydroxybutyrate and acetoacetate are intermediary metabolites typically produced from beta-oxidation of fatty acids in the liver. During periods of extended fast, as carbohydrate reserves become exhausted, fatty acids released from adipose tissue become partially oxidized in the liver and ketones, 3-hydroxy butyrate and acetoacetate are released into the blood. Generally blood ketones rise to a few millimolar (3-7 millimolar) during an extended fast. Aside from the liver, many physiological systems (e.g. skeletal and heart muscle, and particularly the nervous system) are capable of utilizing ketones effectively for highly efficient energy production (4). Unlike glucose, ketone metabolism is independent of blood insulin levels. Ketones are transported in the blood and enter into various tissues via the mono-carboxylate transporter (MCT) sparing glucose utilization and thereby aide in maintaining blood glucose levels. This allows ketones a notable metabolic advantage for maintaining system homeostasis during periods of heightened physiological stress (i.e. traumatic somatic wounds or burn injuries) as the glucose-insulin signaling becomes compromised.

Medical Uses of Ketones

Decades of independent medical research have shown that the metabolism of ketones, through administration of a ketogenic diet (very high in fat and devoid of carbohydrate), has had significant therapeutic advantage in treating cases of juvenile epilepsy (5). One possible mechanism is hypothesized to involve a form of modulating energy pathways (5). Ketones have also shown significant benefit in a Parkinson's disease model by limiting cellular damage through mitigating free radical injury (6). (b)(4)

(b)(4)

Ketone metabolism and the antioxidant environment.

Radiation is well known to involve the production of oxidant species to create reactive oxygen species leading to an oxidized cellular environment and oxidative phosphorylation. The metabolism of ketones enhances several metabolic outcomes increasing acetyl-coA and mitochondria NADH formation while at the same time restoring the NADPH/NADP+, ratio for the regeneration of GSH (8, 9). As an electron donor reduced glutathione (GSH) is a major cellular antioxidant which can neutralize oxygen radicals producing the oxidized GSSG. GSH is regenerated from GSSG via glutathione reductase and the reductant, NADPH. It is well known that ionizing radiation ionizes water and organic oxygen compounds producing oxygen free radicals. Organic oxygen radicals are the primary initiators of biochemical events leading to lipid and protein oxidation, and aberrations in nucleic acid structure in connection with radiation exposure. Therefore a compound like R3-KE should be tested for its ability to alter radiation-induced damage since mechanistically it appears to have significant benefit to increasing the anti-oxidant environment

Furthermore, the metabolism of ketones has recently been shown to increase both catalase and manganese super-oxide dismutase (MnSOD) in tissues, two noted enzymes that are important in

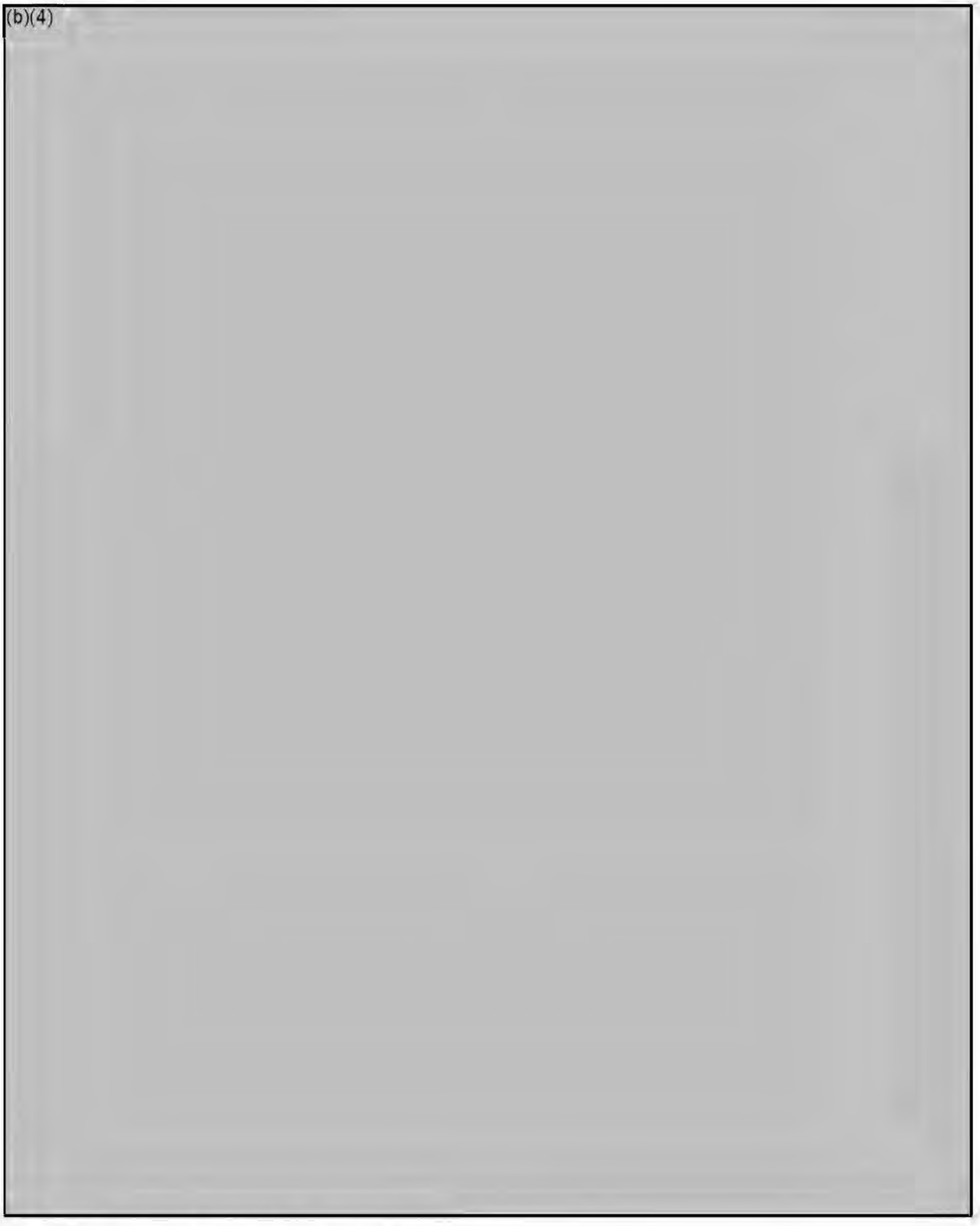
resolving oxygen free radicals (10). These increases have been attributed to increased levels of the transcription factor FOXO3A in cells treated with β-Hydroxybutyrate (10). Ketones have also been demonstrated to have histone deacetylase (HDAC) activity *in vitro* and *in vivo* (10).

(b)(4)

(b)(4) In addition to being formed by ionizing radiation, free radicals can be formed by the mitochondrial electron transport system, the quantitatively most important reaction being the non-enzymatic reaction of the free radical co-enzyme Q semi-quinone (13). A final mechanism of action may involve a decrease in the amount of Q semi-quinone by ketones, thus decreasing the baseline formation of free radicals (14).

(b)(4)


(b)(4)



II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

II.2.2. Date of Search Biomedical Research Database Jun 24, Jun 27 2014
Projectreporter: Jun 24, Jun 27 2014
PubMed; Jul 22, 2013; Aug 6, 2013; Jun 24 2014; Jun 27, 2014

II.2.3. Period of Search Biomedical Research Database 1998-2009
ProjectReporter 2009-2014
PubMed 2000-2014

II.2.4. Key Words of Search
Radiation, ketone ester, mitigator, protector, mouse, rodent, oral administration, injection, R3-KE, (R)-3-Hydroxyhutyl (R)-3-hydroxyhutyrate

II.2.5. Results of Search
BRD A search of the BDR database did not reveal any queries regarding the combination of the keywords, radiation, ketone, ester, and rodent. A search of the word radiation alone resulted in 325 entries but the combination of radiation and ketone yielded none. A search of the word ketone alone resulted in 14 entries. Several of these involved the studies I referred to in my background regarding the use of ketone ester as a means to increase physical efficiency and preserve cognitive function during extended periods of extreme stress.
Project Reporter: A search of the Project Reporter database showed that there are no studies funded to evaluate the ketone ester and radiation. There is one project in which a ketogenic diet is used to modify chemo and radio- tumor responses (based on tumor cell requirements for oxidants) but there are no publications on this new study in the PubMed database. As expected there are multiple studies to evaluate radiation countermeasures although none involve the ketone ester or ketone compounds.
PubMed: PubMed has been searched numerous times; these searches were conducted during the preparation of the grant proposal to DMRDP last summer and fall. However, I did conduct another PubMed search on Jun 24 and 27th. The recent searches again indicated that there are no publications on the use of ketone ester to modify radiation response in vivo or in clinical trials. The ketone ester is being tested in a clinical trial for Alzheimer's disease (AD) and Parkinson's disease (PD) based on the hypothesis that it could enhance cellular antioxidant capability. As stated in the background, ketone ester has been tested in rodent models for AD and PD. It was also tested in special operations soldiers for its ability to increase physical endurance and mental acuity.

III. OBJECTIVE / HYPOTHESIS
This project intends to determine whether R3-KE via oral administration post-radiation can mitigate radiation lethality in a mouse model, protect against radiation-induced chromosomal damage, ameliorate adverse radiation-induced hematopoietic system changes, and increase protective cytokine levels and expression. An *in vitro* study of the effects of R3-KE on gene expression using a microarray genomics approach will be done to investigate which gene pathways are affected by R3-KE but does not involve animal use.

IV. MILITARY RELEVANCE
Military personnel could be exposed to radiation due to nuclear weapons use, accidental exposure, or a terrorist attack. This type of radiation exposure could lead to the development of

acute radiation syndrome (ARS) and possibly result in death to an exposed individual. Currently the ability for medical treatment of personnel post-radiation injury is limited and there are no FDA-approved post-radiation countermeasures for ARS. The focus of this proposal is to test a candidate for post-exposure mitigation of radiation injury and would meet the Department of Defense need to test post-radiation countermeasures. The candidate R3-KE, is a safe, food supplement with a long shelf-life that could easily be disseminated to large numbers of potentially exposed military personnel and/or civilians. (b)(4)

(b)(4)

A two-fold approach to mitigation of radiation injury should be considered. The first approach involves the rapid and early delivery of a radiation mitigator to anyone suspected of being exposed; the second approach is undertaken after exposure-triage, in a hospital location of a second radiation therapeutic countermeasure. If this proposal is successful in determining that R3-KE is an effective radiation mitigator, this product could easily be distributed to large numbers of military personnel regardless of their radiation-exposure status but provide medical benefit to those actually exposed.

This proposal does not duplicate research that is being done in a BARDA or HHS funded project. (b)(4)

(b)(4)

Therefore the objective of this project is to progress the cell-based countermeasure studies (see preliminary results), to conduct animal-based radiation countermeasure and mitigation experiments. This will assist the development of safe and efficacious countermeasures for mitigation of radiation injury.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

V.1.1. Pilot Study

(b)(4) we will first do a pilot study with the R3-KE alone at each dosage to monitor for any toxic effects. We do not expect any toxic effects from the R3-KE based on its GRAS approval by the FDA and the observations that in human trials and rat studies, there we no reported or observed toxic effects (1, 2). For our mouse study, we will start with the lowest dosage (140 mg/kg) and proceed to increasing dosage groups (350, 750 mg/kg). R3-KE will be administered via oral gavage using AFRRI SOP for oral gavage (see attached SOP). After gavage animals will be monitored for 3hr continuously and then again within 4 hrs (assumed to be approximately 4 pm the day of gavage). Then they will be

monitored twice daily for the next 6 days; this will be done at approximately 9 am and 4 pm daily. This study will be completed before any further animal studies are initiated. After the toxicity study is completed, an assessment of the results will be done to determine if the dosages need to be altered from what is in this protocol. After 7 days animals will be euthanized as described in section V.4.6 and tissues collected at necropsy. Adverse effects are not anticipated at these doses based on the human and rat studies (1, 2). Ten animals per dose will be used (0, 140, 350, 750 mg/kg). Toxicity is not expected as R3-KE has been tested in humans and rats without any adverse effects. However, evidence of toxicity would include shivering, motionless, lack of drinking and eating, and diarrhea. To assess for toxicity at the time of euthanasia, 7 days post R3-KE administration, we intend to conduct serum biochemistry and necropsy in the mice. Organ weight will be measured and histological examination of liver and kidney will be conducted.

PILOT STUDY Dosage Response of Oral KE (NO RADIATION)	
Saline	*10*
Control Group	
KE (140 mg/kg)	*10*
KE (350 mg/kg)	*10*
KE (750 mg/kg)	*10*

Total # mice: **40 mice**
Pain Category C: **40 mice**
Euthanasia by isoflurane overdose (1-4%) followed by confirmation with cervical dislocation.

Experiment 1

Determine whether R3-KE (orally administered post-radiation) treatment can mitigate radiation lethality in a mouse model. This experiment will assess the following, a) optimum R3-KE dosage required for maximum radiation mitigation at 8.5 Gy; b) post-radiation administration of R3-KE and radiation mitigation at varying doses of radiation; c) optimum timing of R3-KE administration for optimum radiation mitigation; and d) dose-reduction factor for R3-KE.

Aim 1 will focus on whether R3-KE has potential as a radio-mitigator of acute radiation damage. For this radiation lethality mitigation study, mice will be exposed to ^{60}Co gamma radiation total body irradiation (TBI) and then treated (post- radiation) with R3-KE (oral gavage) and the endpoint will be radiation lethality/survival at 30 days. Radiation dose response effects will be examined as well as optimization of dose, dosage level, and timing of administration. A dose reduction factor (DRF) analysis will be conducted. This aim is divided into four sub-Aims. All animals will be weighed before the experiments begin and will be weighed 3x weekly during the experiments; animal may be weighed more than 3X weekly if their health status indicates it is warranted.

Purpose Aim1: To determine if R3-KE orally administered post-radiation can protect total body irradiated (TBI) mice from acute radiation lethality.

Aim 1a: Assess radiation mitigation potential of varying dosages of R3-KE at 8.5 Gy (0.6 Gy/min) when administered orally. AFRRI SOP for oral gavage will be used (REF SOP). To achieve Aim1a, mice (n = 15) will be given R3-KE (oral gavage; 140, 350, or 750 mg/kg body weight) (SOP V.4.4) in saline 24 hr after 8.5 Gy ^{60}Co whole irradiation (0.6 Gy/min); after radiation mice will be observed for 30 days for survival. After 30 days, surviving mice will be euthanized and tissues collected for analysis. This radiation dose was chosen based on the LD50/30 radiation dose of 8.2-8.5 Gy for DBA mice. The experiment will be repeated for a total of 30 mice/group. The results are expected to show which dosage of R3-KE is most efficacious at increasing radiation survival. This dosage will be used in further experiments. The R3-KE dosages chosen to be tested in this aim will be chosen based on the pilot study. They were also tentatively selected based on the safety profile in healthy adult subjects (1) and oral administration in rats (2). Euthanasia by isoflurane overdose (3-5%) followed by confirmation with cervical dislocation will be used.

Aim 1 Radioprotection of Acute Lethality by KE
Aim 1a

Dosage Response of Oral KE at 24 hrs Post-8.5 Gy	
Saline (No Rad, No R3-KE)	***15 (x2)***
Control Group	
KE (140 mg/kg)	***15 (x2)***
KE (350 mg/kg)	***15 (x2)***
KE (750 mg/kg)	***15 (x2)***
Radioprotection Study	
Radiation	**15 (x2)**
Radiation + KE (140 mg/kg)	**15 (x2)**
Radiation + KE (350 mg/kg)	**15 (x2)**
Radiation + KE (750 mg/kg)	**15 (x2)**
Total mice	**240**

Total # mice: 240 mice
Pain Category C: 120 mice
Pain Category E: 120 mice
Euthanasia by isoflurane overdose (3-5%) followed by confirmation with cervical dislocation.

Aim 1b: Assess post-radiation administration of R3-KE and radiation mitigation at varying doses of radiation. The response of the most efficacious dosage of R3-KE determined in Aim 1a will be used. To achieve Aim 1b, mice will be irradiated with ^{60}Co radiation (8.0, 8.5, and 9.0 Gy; 0.6 Gy/min; TBI) and orally administered R3-KE (optimum dosage in saline) post-radiation. Mice will be observed for 30 days after radiation for survival. These results are expected to determine the range of R3-KE effectiveness at various radiation doses. Euthanasia by isoflurane overdose (3-5%) followed by confirmation with cervical dislocation.

Aim 1b

Oral Administration of KE and Radiation Mitigation at Varying Doses of Radiation			
	8.0 Gy	8.5 Gy	9.0 Gy
Radiation + Saline	15 (x2)	15 (x2)	15 (x2)
Radiation + KE (optimum mg/kg)	15 (x2)	15 (x2)	15 (x2)
Total mice	180		

Total # mice: 180 mice
Pain Category E: 180 mice
Euthanasia by isoflurane overdose (3-5%) followed by confirmation with cervical dislocation

Aim 1c. Determine the optimum timing of R3-KE administration after 8.5 Gy. The optimum timing of oral R3-KE administration post-radiation will be tested using the radiation lethality model. Using the optimum dosage of R3-KE determined in Aim 1a, mice will be irradiated (^{60}Co, 8.5 Gy, 0.6 Gy/min) and administered R3-KE at 1, 8, 12, 24, or 48 hr) post-radiation. Mice will be observed for 30 days after radiation for survival. These results are expected to determine the optimum administration time of R3-KE at 8.5 Gy. Euthanasia by isoflurane overdose (3-5%) followed by confirmation with cervical dislocation.

Aim 1c

Optimum Timing of KE Oral Administration After 8.5 Gy					
	1 hr	8 hr	12 hr	24 hr	48 hr
Rad + Saline	15 (x2)	15 (x2)	15 (x2)	15 (x2)	15 (x2)
Rad + KE (optimum dose mg/kg)	15 (x2)	15 (x2)	15 (x2)	15 (x2)	15 (x2)
Total mice	300				

Total # mice: 300 mice
Pain Category E: 300 mice
Euthanasia by isoflurane overdose (3-5%) followed by confirmation with cervical dislocation

Aim 1d. Determine the dose-reduction factor (DRF) for R3-KE. Information regarding DRF is crucial to evaluating a potential radiation mitigator and provides a standard by which a quantitative comparison to other known radiation mitigators can be done. The DRF will be determined using the optimum dosage of R3-KE (Aim 1a) and the optimum scheduling of R3-KE administration (Aim 1c). Mice will be divided into 10 groups (15 each); 5 groups will be treated with R3-KE (optimum dosage) and 5 groups of saline as control. Mice will be irradiated (7.5, 7.75, 8.0, 8.5, and 9.5 Gy) and at the optimum time post-radiation (Aim 1c) will be orally administered R3-KE. These doses are based on preliminary results that the lowest radiation dose will result in a significant number of survivors (>80%) and the highest dose 100% lethality (at 30 days) (41-42). Three doses were chosen between these extremes. After radiation/R3-KE treatment animals will be monitored for 30 days for survival. For calculation of the dose

reduction factor, percent mortality versus radiation dose will be fitted using probit analysis. The DRF will be calculated as a ratio of the radiation LD50/30 with and without administration of R3-KE; the standard error and 95% confidence interval will be derived using error propagation. These results are expected to determine what the DRF for R3-KE is.

DRF Study					
	7.5 Gy	7.75 Gy	8.0 Gy	8.25 Gy	9.0 Gy
Rad + Saline	15 (x2)	15 (x2)	15 (x2)	15 (x2)	15 (x2)
	7.5 Gy	8.0 Gy	8.5 Gy	9.0 Gy	9.5 Gy
Radiation + KE (optimum dose mg/kg)	15 (x2)	15 (x2)	15 (x2)	15 (x2)	15 (x2)
Total mice	300				

Total # mice: 300 mice
Pain Category E: 300 mice
Euthanasia by isoflurane overdose (3-5%) followed by confirmation with cervical dislocation

The DRF is an important measure of the efficacy of a putative radioprotective drug. As represented hy the designs of this experiment, establishing efficacy of such a compound like R3-KE by demonstrating a statistically significant DRF is generally accomplished using 5–7 radiation doses with the lowest dose giving mortality near 0% and the highest dose giving mortality near 100% (11). Since the R3-KE group is expected to result in increased survival, higher radiation doses are needed for the statistical analysis. Since the promulgation of the New Animal Rule (16), it is imperative that these efficacy determinations be robust because the final tests are done only in animals; human efficacy studies are not ethical or feasible.

All animals will be euthanized by an overdose of isoflurane anesthesia (3-5%) followed by cervical dislocation. Euthanasia will be conducted as described in section V.4.6.

Each sub-part of Aim 1 involves 15 mice per group and will be repeated for a total of 30 mice/group.

V.1.2. Experiment 2

Assess whether R3-KE (orally administered post-radiation) treatment can protect bone marrow against radiation-induced chromosomal damage.

Purpose of Aim 2: To determine whether R3-KE (post-radiation) can prevent radiation-induced chromosomal damage

Aim 2: It is well known that significant biological consequences of exposure to radiation include cell death, gene mutations, and chromosome aberrations. Radiation-induced chromosomal damage has implications for both acute radiation survival and development of radiation-induced late effects. This aim will assess whether R3-KE can protect bone marrow from radiation damage. Chromosomal aberrations in hone marrow cells will be studied at twenty-four hours after R3-KE treatment (optimum R3-KE dosage, optimum time of administration). Mice will be divided into four groups (5 per group) as follows: Group1) No radiation-No R3-KE; Group 2)

R3-KE optimum dosage in saline; Group 3) Gamma radiation (0, 0.5, 1.0, 2.0, 4.0, and 6.0 Gy) followed by saline (optimum R3-KE timing); and Group 4) Gamma radiation (0, 0.5, 1.0, 2.0, 4.0, and 6.0 Gy) followed by R3-KE (optimum dosage, timing post-radiation) in saline. Twenty-two hours after R3-KE treatment animals will be injected i.p. (see Section V.4.4.1) with 0.01ml/g body weight of 0.025% colchicine and euthanized after 2 hrs. The 22 hrs post-R3KE administration was chosen based on previous studies by others which showed that this was the optimum time to allow for chromosomal aberrations to form post-radiation (17). All animals will be euthanized as described in Section V.4.6. Euthanasia will be under deep isoflurane anesthesia (3-5%); exsanguination by cardiac puncture, followed by cervical dislocation.

Metaphase plates will be prepared from femur bone marrow and approximately 1000 metaphases (200/animal) will be counted for each group. The same number of metaphases per animal (200) will be prepared. Aberrant metaphases and different aberrations, chromatid and chromosome breaks, fragments, rings and dicentrics, will be scored under a light microscope. The PI hypothesizes that different doses of radiation will induce different numbers of aberrations and that R3-KE will decrease the number of aberrations. Results will be presented as percentage aberrant metaphases. Statistical analysis is described below. These results are expected to determine whether R3-KE can protect bone marrow cells from radiation-induced chromosomal damage.

Aim 2 Protection of Bone Marrow against Radiation-Induced Chromosome Damage

Radiation-Induced Chromosome Damage 24 hrs After Optimum KE Administration Post-Rad						
	0.0 Gy	0.5 Gy	1.0 Gy	2.0 Gy	4.0 Gy	6.0 Gy
No Rad/No R3-KE	5 (x2)					
R3-KE	5 (x2)					
Rad + Saline	5 (x2)	5 (x2)	5 (x2)	5 (x2)	5 (x2)	5 (x2)
Rad + KE (optimum dose and timing)	5 (x2)	5 (x2)	5 (x2)	5 (x2)	5 (x2)	5 (x2)
Total Mice	140					

Total # mice: 140 mice
Pain Category D: 20 mice
Pain Category E: 120 mice
Euthanasia will be isoflurane anesthesia (3-5%) exsanguination by cardiac puncture, followed by cervical dislocation.
There will be 5 mice/group and the experiment will be repeated for a total of 10 mice per group.

V.1.3. Experiment 3

Measure the effect of R3-KE treatment (orally administered post-radiation) on hematological parameters including blood cell elements (circulating neutrophils, GM-CFC, and platelets) at times post radiation (8 hr to 28 days).

Purpose of Aim 3: To determine if R3-Ke treatment post-radiation can increase hematopoietic recovery from radiation.

This aim will assess if R3-Ke can enhance hematological cell recovery from radiation exposure. Mice will receive a sublethal radiation exposure (6.0 Gy) to increase survivability up to and at 30

days post-radiation. Mice will be divided into four groups: Group 1) No Radiation-No R3-KE; Group 2) R3-KE optimum dosage in saline; Group 3) Gamma radiation (6.0 Gy) + Saline; and Group 4) Gamma radiation (6.0 Gy) followed by R3-KE (optimum dosage, timing post-radiation) in saline. At the following times post-R3-KE treatment (0.3, 1, 3, 7, 14, 21, and 28 days) mice will be euthanized (10/group/day). Blood will be collected as described in Section V.4.4 and 4.4.2 using AFRRI SOP on Blood collection. Euthanasia will be under deep isoflurane anesthesia (3-5%); exsanguination by cardiac puncture, followed by cervical dislocation (section 4.4.6).

Hematological parameters (total and differential counts) will be determined using a Heska Hematology Analyzer (Heska, Boston, MA) which we have in our laboratory. Otherwise the VSD hematology lab may be used for total and differential counts.

Aim 3

Hematological Parameters Following R3-KE Treatment Post-Radiation: Blood Element Counts							
	0.3 d	1 d	3 d	7 d	14 d	21 d	28 d
No Rad	10 (x2)	10 (x2)	10 (x2)	10 (x2)	10 (x2)	10 (x2)	10 (x2)
KE	10 (x2)	10 (x2)	10 (x2)	10 (x2)	10 (x2)	10 (x2)	10 (x2)
Rad + Saline	10 (x2)	10 (x2)	10 (x2)	10 (x2)	10 (x2)	10 (x2)	10 (x2)
Rad + KE (optimum)	10 (x2)	10 (x2)	10 (x2)	10 (x2)	10 (x2)	10 (x2)	10 (x2)
Total Mice	560						

Total # mice: 560 mice
Pain Category D: 140 mice
Pain Category E: 420 mice
Euthanasia will be isoflurane anesthesia (3-5%) exsanguination by cardiac puncture, followed by cervical dislocation.
There will he 10 mice/group and the experiment will be repeated for a total of 20 mice/group.

V.1.4. Experiment 4

Investigate the cytokine profile *in vivo* indnced by R3-KE treatment (orally administered post-radiation) using a sandwich ELISA (serum) and multiplex Luminex (proteins) after radiation (6.0 Gy).

Purpose of Aim 4: To determine if R3-KE Treatment post-radiation has an effect on cytokines causing an increase in the levels of radiation-mitigating cytokines.

Cytokines and growth factors have shown efficacy against radiation injury. Therefore the effect of R3-KE administration on cytokines should be examined. Cytokine protein levels will be analyzed in mouse plasma at various time points in relation to R3-KE (optimum dosage) post oral administration (0, 4, 24, 48 hr) and/or radiation and will be quantified by multiplex Luminex. Cytokine gene expression will be evaluated by semi-quantitative RT-PCR in bone marrow and spleen cells at the same time points as well. These results will show whether R3-KE affects cytokine levels as a potential mechanism of its action in vivo. Mice will be sublethally irradiated with gamma radiation 6.0 Gy. At 4, 24, or 48 hr post oral R3-KE administration +/- radiation, blood will be collected as described in Section V.4.4 using AFRRI Blood collection SOP and mice will be euthanized as described in Section V.4.6. Controls (NO Rad NO R3-KE will be assessed as well. Euthanasia will be under deep isoflurane anesthesia (3-5%); exsanguination by cardiac puncture, followed by cervical dislocation.

Aim 4
Effect of R3-KE on Cytokines
(Cytokine Message and Serum Protein Levels)

	4 hr Post Injection	24 hr Post-Injection 48 hr Post-Rad	48 hr Post Injection 72 hr Post-Rad
No Rad No R3-KE	10 (x2)	10 (x2)	10 (x2)
R3-KE	10 (x2)	10 (x2)	10 (x2)
Rad (6.0 Gy)	10 (x2)	10 (x2)	10 (x2)
Rad + R3-KE (Optimum dosage/timing	10 (x2)	10 (x2)	10 (x2)
Total Mice	240		

Total # mice: **240 mice**
Pain Category D: **60 mice**
Pain Category E: **180 mice**
Euthanasia will be isoflurane anesthesia (3-5%) exsanguination by cardiac puncture, followed by cervical dislocation.
There will be 10 mice/group and the experiment will be repeated for a total of 20 mice per group.

For all aims, euthanasia will be confirmed by cessation of the heartbeat and breathing.

Total mice for all Aims/Experiments = 2000

V.2. Data Analysis

Statistical Analyses:

The statistical tests chosen were suggested by Dr. (b)(4),(b)(6) Means and SE with a significance level at 5% set for each test or aim has to be performed. In order to evaluate the hypothesis set forth and establish a comparison of significance of deviation from the hypothesis, Fisher Exact Test will be used. The test can provide the significance and deviation. The Fisher test for Variance or the ANOVA which provided the means to analyze the differences between group and their associated procedures is a suitable test to detect significance between sampling groups. ANOVA is a test of choice to compare group means and group variables. Bonferroni and Newman-Keuls methods (pairwise comparisons) and Tukey-Kramer comparison for unequal group size can be used for identifying which groups are different from each other. When inferential statistics is used such as t-test, F tests, ANOVA etc., to evaluate the outcome and comparisons, sample size (n) needs to be determined to provide sufficient statistical capability for performance. Based on the power analyses the sample size n=10 proposed by the investigator is appropriately sufficient to deliver more than 80%. Fisher's exact test will be used to detect if there is a significant difference across the groups in survival rates. For Aim1d to study the binomial response variables such as the drug efficacy or response for treated or non-treated groups studies, 'Probit analyses' can be used which is a powerful method to calculate probits, regression coefficient, and confidence intervals. Probit analysis is used to analyze many kinds of dose-response or binomial response experiments in a variety of fields. Here the probit can be used to record the response of the drug at various time points and concentrations and response is always binomial and the relation between response and concentration/time points is

sigmoidal. By plotting the response at different time points and doses. ORF can be calculated as the ratio of the radiation for drug-treated and vehicle-treated groups.

Pilot or Aim #	Number per group	Endpoint	Test Used
Pilot (Assess Toxicity)	10	Euthanasia	ANOVA
Aim 1a (Assess Dosage of R3KE on Single Dose Rad Survival)	15	Survival at 30 days	ANOVA, Fishers Test
Aim 1b (Assess Effect of Optimum Dosage on Varying Rad Doses)	15	Survival at 30 days	ANOVA, Fishers Test
Aim 1c (Assess Effect of Optimum Dosage on Timing of R3KE Administration on Rad Response)	15	Survival at 30 days	ANOVA, Fishers Test
Aim 1d (DRF study with optimum R3KE dosage and optimum timing)	15	Survival at 30 days	ANOVA, Fishers Test
Aim 2	5 (2 femurs per animal)	Planned euthanasia at 24 hrs post rad/R3KE	ANOVA, Fishers Test
Aim 3	10	Planned euthanasia	ANOVA, Fishers Test
Aim 4	10	Planned euthanasia	ANOVA, Fishers Test

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

The development of a medical countermeasure requires that it be tested *in vitro* and *in vivo*. Prior to the development of this DMRDP funded project and this animal use proposal R3-Ke was tested in vitro for its potential effectiveness as a radiation mitigator (b)(4)

(b)(4)

(b)(4) *In vitro* studies do not consider physiological factors which could affect the response to a potential radiation mitigator. Therefore an animal model is necessary to test the effectiveness of R3-KE as a radiation mitigator. It is the goal of this project however to always keep animal welfare as a priority and to use as few as rodents as possible to achieve scientific and statistical accuracy.

V.3.2. Animal Model and Species Justification

The murine model has been widely used for radiation survival studies in my laboratory to evaluate radiation exposure and a variety of endpoints including survival, radioprotection, mitigation, carcinogenesis, and biomarker development. (b)(4)

(b)(4)

(b)(4) The rodent model is the least sentient model that can be used to assess the efficacy of a radiation biomarker. Use of an invertebrate model will not provide the

necessary information regarding physiological responses i.e. cytokine induction, that a mouse model will provide. (b)(4)

(b)(4)

V.3.3. Laboratory Animals
DBA/2 murine model

V.3.3.1. Genus / Species
Mus musculus

V.3.3.2. Strain / Stock
DBA/2

V.3.3.3. Source / Vendor
Taconic, or Charles River (USDA # 14-R-0144)
Multiple vendors available.

V.3.3.4. Age
6 -12 weeks

V.3.3.5. Weight
15-30 g

V.3.3.6. Sex
Male and female.

V.3.3.7. Special Considerations
None

V.3.4. Number of Animals Required (by species) 1960

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement

(b)(4)

V.3.5.2. Reduction (b)(4)

(b)(4) Previously published in vivo studies evaluating R3-KE delivered in a ketogenic diet to rodents has enabled us to assess the least toxic dosage of R3-KE

to deliver in our studies thus reducing the number of toxicology experiments that would need to be done. It is also important to note the FDA has already designated R3-KE as generally regarded as safe ("GRAS") which also supports the reduced use of animals to establish safety and toxicology. This information has enabled us to reduce the number of animals we need to use. This information also enables us to avoid dose-ranging experiments which would require the use of larger numbers of animals.

V.3.5.3. Replacement The use of an animal model is necessary to determine if a mitigating agent can protect against acute radiation effects and increase survival. We have already conducted *in vitro* experiments with immortalized human cell lines to better understand the mechanisms of how R3-KE works; however, tissue culture systems do not consider physiological factors which could affect survival. Using *in vitro* studies with the human cell lines has already replaced the use of some animals in this study as I described above.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals 1960

V.4.1.1.1.1. Column C ___160___ (# of animals)

V.4.1.1.1.2. Column D ___220___ (# of animals)

V.4.1.1.1.3. Column E ___1620___ *(# of animals)*

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
Pilot	Toxicity Pilot of KE alone (no radiation)	40			40
1a	Dosage response of KE +/- Rad	120		120	240
1b	Dose response of Rad +/- KE			180	180
1c	Timing of KE +/- Rad			300	300
1d	DRF Study Rad + KE			300	300
2	Bone marrow Protection (Rad +/- KE)		20	120	140
3	Hematology Study (Rad +/- KE)		140	420	560
4	Cytokine Study (Rad +/- KE)		60	180	240
Totals:		160	220	1620	2000

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

AFRRI SOP on blood collection via cardiac stick will be done for Aims 2, 3 and 4. First, mice will be deeply anesthetized for this procedure using isoflurane (3-5%). Their reflexes will be tested to ensure they are deeply anesthetized before proceeding. After blood collection, mice will be euthanized as described in Section V.4.6.

V.4.1.2.2. Pre- and Post-procedural Provisions
No surgery is being conducted.

V.4.1.2.3. Paralytics
None will be used.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched AltWeb
PubMed
Agricola

V.4.1.3.2. Date of Search
Jun 30 2014
Jun 30 2014
Sept 26, 2014

V.4.1.3.3. Period of Search

V.4.1.3.4. Key Words of Search Radiation, mitigator, pain, alternatives, death as an endpoint, survival study, DBA/2, lethality, survival, hematopoietic syndrome, LD50/30, mouse, model, pain, suffering, gavage, oral administration, ketone esters, R3-KE, (R)-3-Hydroxybutyl (R)-3-hydroxybutyrate

V.4.1.3.5. Results of Search
Acute radiation exposure can be a painful process due to the decrement of digestive and hematological systems based on human studies. The literature verifies that developing symptoms of radiation illness appears to be painful and is distressful in humans and animals. As was indicated in the justification of animal use section, development of a post radiation mitigator that is safe and efficacious in humans required testing an animal model. This is partially based on FDA regulations and the use of an animal model in testing a potential radio-mitigator is beyond our control and is necessary if we are to develop mitigators for humans. At the earliest sign that the animals are in pain or distress, they will be immediately be considered for a humane euthanized and the criteria for euthanasia will be carefully monitored. In this project we are assessing the animals multiple times daily to decrease the amount of time that they might be in distress; if we observe any animal assessed by the scoring sheet to be moribund, they will be euthanized. As was indicated earlier, we have exhausted all preliminary non-animal alternatives including *in vitro* survival and radioprotection studies using human cell models.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification
IACUC Policy 10 will be used to establish early endpoints in experiments with early mortality. Experiments 1(a, b, c, and d) are the acute radiation syndrome studies; the doses used in aims 2-4 (0.5-6 Gy) are not expected to induce death (b)(4) (b)(4) In experiment 1 a significant number of mice will be irradiated alone or will be

irradiated and then receive R3-KE to test its mitigation abilities. These radiation doses 7.5 to 9.5 are expected to cause death in approximately 50 to 100% of the animals by day 30 post radiation (without any R3-KE countermeasure). There is no pharmacological intervention/support planned for the radiation alone groups as these groups are needed to provide a radiation control for the experiment. With or without R3-KE administration, this exposure could cause more than momentary unalleviated pain or distress. To decrease any pain or period of distress, after the radiation or radiation + R3-KE, animals will be monitored multiple times daily with at least 3 planned monitoring times for 30 days; additional monitoring times are anticipated to include a monitoring time in the late evening so that the next monitoring time will be 10 hr or less after that late evening monitoring. Animals that appear morbid will be monitored 3-4X daily. Monitoring will include weighing the animals since baseline weights will have already been established at the start of the experiment. These animals cannot be given any prophylactic pain medication for two reasons. 1) There is no specific information indicating what dose of analgesic would be effective to alleviate the discomfort/distress/pain of hematopoietic ARS in mice and 2) Prophylactic analgesia could be contraindicated and detrimental to the protocol purpose.

V.4.2. Prolonged Restraint N/A

V.4.3. Surgery N/A

V.4.3.1. Pre-surgical Provisions No Surgery

V.4.3.2. Procedure(s) N/A

V.4.3.3. Post-surgical Provisions N/A

V.4.3.4. Location N/A

V.4.3.5. Surgeon N/A

V.4.3.6. Multiple Major Survival Operative Procedures N/A

V.4.3.6.1. Procedures N/A

V.4.3.6.2 Scientific Justification N/A

V.4.4. Animal Manipulations

1) Permanent individual identification for each rodent will be done prior to the start of any experiments. IF tattooing is available in VSD and is done by VSD in co-ordination with (b)(6) (b)(6) tattooing will be used. If tattooing is not available, permanent individual identification using the ear notching method will be used. This method require a special tool to punching or notching holes at various positions in the ears, this technique is commonly used in rodents. Procedure will take place under sterile conditions in VSD after released from quarantine. Manual restraining is required to perform this technique
2) Animals will receive either administration of sterilized R3-KE via gavage or an administration of sterile water via gavage. Administration will take place under sterile conditions in VSD. See next section for additional details.

3) Animals will be placed in a plastic holder for radiation. The time in the holder will be approximately ten minutes prior to radiation, ten to twenty minutes in the radiation facility, and ten minutes in the transfer back to VSD. The total time will be approximately 40 minutes. Previous experience indicates that this is a good time estimate. The radiation doses in this project range from 0.5 Gy to 9.5 Gy.
4) Oral gavage will be used to administer R3-KE. Prior to the initiation of oral gavage, a VSD veterinarian or designee will review the oral gavage technique of the staff person conducting the oral gavage. The AFRRI GLP oral gavage protocol will be used to administer the R3-KE. An 18-20 gauge feeding tube will be selected. Isoflurane anesthesia may be used after consultation with VSD staff. The volume of the R3-KE will be 0.15 ml.
5) Blood collection will be done using AFRRI SOP for cardiac puncture blood collection (see attached SOP).

V.4.4.1. Injections

The purpose of aim 2/experiment 2 is to measure chromosomal aberrations at 24 hrs post-radiation. In aim 2/experiment 2, at 22 hrs post-radiation +/- R3-KE, mice will be injected i.p. using a 26 gauge needle with 0.01ml/g body weight of 0.025% colchicine and euthanized as described above at 2 hr after colchicine injection. Colchicine is used to stop cell mitosis in the metaphase stage to allow for chromosomal assessment. It is during this stage of nuclear division that the chromosomes are most condensed and, as a result, visible with a light microscope.

V.4.4.1.1. Pharmaceutical Grade Drugs None

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

(R)-3-Hydroxybutyl (R)-3-hydroxybutyrate, a Ketone ester (or R3-KE) is rated GRAS by the FDA and is available from the NIH, NIAAA. It will be sterilized by filter (0.2 micron filter) prior to gavage administration. R3-KE is solubilized in sterile water (pH 7.4).

Colchicine (Sigma-Aldrich Co.) will be used in Aim 2. It is solubilized in sterile water and is pH 7.2. It will be sterilized by filter (0.2 micron filter).

V.4.4.2. Biosamples

Blood and tissue collection in Pilot study:
The AFRRI GLP Blood collection SOP will be used. Animals will be deeply anesthetized using isoflurane 3-5%. Using a 23-25 gauge needle, the cardiac blood will be collected to exsanguination (1 ml expected). After exsanguination, the animal will undergo cervical dislocation as a secondary euthanasia method.

Blood collection in Aims 2-4.
The AFRRI GLP Blood collection SOP will be used. Animals will be deeply anesthetized using isoflurane 3-5%. Using a 23-25 gauge needle, the cardiac blood will be collected to exsanguination (1 ml expected). After exsanguination, the animal will undergo cervical dislocation as a secondary euthanasia method.

V.4.4.3. Adjuvants N/A

V.4.4.4. Monoclonal Antibody (MAb) Production N/A

V.4.4.5. Animal Identification Tattoo, Ear notch, and cage cards. Tattoo will be used if available in AFRRI VSD.

V.4.4.6. Behavioral Studies None

V.4.4.7. Other Procedures None

V.4.4.8. Tissue Sharing At necropsy, tissues will be shared with (b)(4),(b)(6) including brain and blood. A tissue sharing form will be completed.

V.4.4.9. Animal By-Products None

V.4.5. Study Endpoint -
This project is divided into 4 aims or experiments with different endpoints.

1) For the pilot study to evaluate R3-KE toxicity, the endpoint will be euthanasia at 7 days post R3-KE gavage administration. We do not expect to observe any signs of toxicity after R3-KE administration, however the animals will be euthanized upon observation of morbidity (not expected) or at 7 days. Criteria for toxicity include: shivering, motionless, lack of drinking and eating, diarrhea.
2) For aim1/experiment 1, the endpoint for the radioprotection parts of the study is survival at 30 days or euthanasia based upon morbidity and moribundity criteria at any point post-radiation for up to 30 days. The purpose of Aim 1/ experiment 1 is to determine optimum R3-KE dosage, optimum timing of R3-KE administration, and the DRF for R3-KE. The endpoint of experiment 1 is survival or euthanasia after 6.5 to 9.5 Gy gamma radiation +/- R3-KE. We hypothesize that a portion of the animals receiving the R3-KE (via gavage) will survive the radiation and will survive until 30 days post-radiation (8.1 Gy which is the LD50/30 for this strain). It is also expected that a portion (approximately 80%) of the animals that do not receive R3-KE (only sterile water via gavage) will not recover from radiation (8-9.5 Gy) and will not survive for 30 days post-radiation exposure. Any surviving animals will be eutbanized at 30 days post radiation for necropsy and tissue collection. It is our intention to euthanize any animal post-radiation at an earlier endpoint to minimize pain and distress. Research staff will be trained to differentiate between animals showing signs/symptoms of morbidity (affeeted with radiation syndrome) and those in a state of moribundity (an irreversible condition leading to death).
3) For aim 2/experiment 2 the endpoint is planned euthanasia at 24 hrs post-radiation. The purpose of aim 2 is to determine whether R3-KE (post-radiation) can prevent radiation-induced chromosomal damage. Chromosomal damage will be measured at 24 hrs post radiation after the i.p. administration of colchicine to arrest cellular mitosis. The doses used are 0, 0.5, 1.0, 2.0, 4.0, and 6.0 Gy +/- R3-KE and are not expected to induce morbidity/moribundity within 22-24 hrs. Animals will be closely monitored for the 24 hrs post-radiation (3 x during the 24 hr period).
4) For aim 3/ experiment 3 the endpoint is planned euthanasia at (0.3, 1, 3, 7, 14, 21, and 28 days) post-radiation +/- R3-KE treatment. The purpose of Aim 3 is to determine if R3-KE treatment post-radiation can increase hematopoietic recovery from radiation. A single sublethal radiation dose of 6.0 Gy will be used and it is not expected that any animals will

show morbidity or moribundity at that dose for the duration of the experiment 0 to 28 days post-radiation. However, animals will be monitored as previously detailed each day.

5) For aim 4/ experiment 4, the endpoint is planned euthanasia at (0, 4, 24, 48 hr) post-radiation +/- R3-KE treatment. The purpose of aim 4 is to determine if R3-KE Treatment post-radiation has an effect on cytokines causing an increase in the levels of radiation-mitigating cytokines. A single sublethal radiation dose of 6.0 Gy will be used and it is not expected that any animals will show morbidity or moribundity at that dose for the duration of the experiment 0 to 48 hr post-radiation. However, animals will be monitored as previously detailed each day.

V.4.6. Euthanasia

All animals in the Pilot study and Aim 1 will be euthanized by an overdose of isoflurane anesthesia (3-5%) given via chamber followed by secondary confirmation with cervical dislocation. For aims 2-4 animals will be deeply anesthetized with isoflurane (3-5%), then euthanized by exsanguination by intra-cardiac blood withdrawal followed by secondary confirmation with cervical dislocation. If necessary, euthanasia by CO_2 followed by cervical dislocation may he used as an alternative method. In all cases, death will be confirmed by cessation of the heart beat and breathing.

V.5. Veterinary Care

V.5.1. Husbandry Considerations

Animals will be housed no more than 4 per cage in a micro-isolator cage. Animals will receive food and water ad libitum. Cages are changed three times weekly in accordance with the current VSD Rodent Hushandry SOP. Animals will have a 12 hour/12 hour light and dark cycle.

V.5.1.1. Study Room

Study room is provided and assigned by AFRRI VSD.

V.5.1.2. Special Husbandry Provisions

None

V.5.1.3. Exceptions

None

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care

Research sStudy personnel will monitor all animals (Exps 1-4) 3X daily however as stated above, animals will be more frequently monitored during critical periods. The animals will be individually observed at each observation period and weighed three times weekly or daily during the critieal period. A necropsy will be performed by research study personnel and hematological and molecular assessment will be made.

If an animal becomes ill or debilitated during the course of the study (prior to a planned euthanasia), the principle investigator and study personnel will use the score sheet to assess condition of the animal. A determination of morbidity or moribundity will be made by study

personnel; VSD staff may be consulted. When an animal is determined to be moribund it will be euthanized by research study staff according to section "XI. C. 7 Euthanasia will be done as described in section V.4.6.\

Routine veterinary care will be provided by VSD as per the standard SOPs.

Animals will be evaluated as per the score sheet in Policy 10 and moribund mice will be euthanized as described in section V.4.6.

V.5.2.2. Emergency Veterinary Medical Care

Emergency veterinary medical care will include euthanasia (and necropsy) if the animal meets the criteria described in section V.5.2.1 after consultation with a member of the research study staff.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

Animals can be given items in accordance with VSD/IACUC policy.

V.5.3.2. Enrichment Restrictions

None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1,3,5,	(b)(6)
				1,2,3,4,5,7	
				1	
				1,3,5	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY N/A

VIII. ENCLOSURES

X. ASSURANCES

Protocol Title: Re-Purposing (R)-3-Hydroxybutyl (R)-3-hydroxybutyrate, a Ketone ester (or R3-KE) as a Radiation Mitigator in mice (*Mus musculus*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) PhD _____ _____ _____

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

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(b)(4),(b)(6)

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DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	1/12/2015	2nd Resubmission Date:	
Full Committee Review:	1/29/2015	Final Approval:	3/30/2015
1st Resubmission Date:	2/27/2015	**EXPIRATION DATE:**	3/29/2018
1st Tier Approved:	3/16/2015	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:	3/18/2015	2nd Tier Approved:	3/30/2015
Returned for Revision:	3/20/2015	2nd Resubmission Date:	
1st Resubmission Date:	3/24/2015	Approved/Approval Withheld:	

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I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER (b)(6)

III. PROTOCOL TITLE: Advanced Development of Gamma-tocotrienol as a Radiation Countermeasure in Nonhuman Primates (*Macaca mulatta*)

IV. PRINCIPAL INVESTIGATOR:

(b)(6) Ph.D. Date

(b)(6)

Radiation Countermeasures Program, AFRRI, (b)(6)

Tel (b)(6) fax (b)(6)

(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, LTC, USA** Date
Head, Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date
Statistician
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, LTC, VC, USA** Date
Head, Veterinary Sciences Department, (b)(6)
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date
Safety and Occupational Health Specialist, USUHS
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**
IACUC Chair, (b)(6), Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: Advanced Development of Gamma-tocotrienol as a Radiation Countermeasure in Nonhuman Primates (*Macaca mulatta*)
PRINCIPAL INVESTIGATOR:

(b)(6) Ph.D. Date
(b)(6)
Radiation Countermeasures Program, AFRRI (b)(6)
Tel (b)(6) fax (b)(6)
(b)(6)

CO-INVESTIGATOR(S): N/A

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS

In the event of radiological or nuclear exposure (i.e. a "dirty" bomb, or a nuclear accident), medical care would be focused on treatment of acute radiation syndrome (ARS), also known as radiation toxicity or radiation sickness. Exposure of mammals to total body ionizing radiation (TBI) can lead to lethal ARS. The cause of death depends on the radiation dose. The lowest lethal doses cause death by severe hematopoietic injury which leads to increased risk of sepsis due to a suppressed immune system, and hemorrhagic events can occur due to depletion of platelets (thrombocytopenia). As the radiation dose escalates, symptoms of a compromised immune and coagulation system persist, along with addition of damage to gastrointestinal (GI) tract. The impaired integrity of the GI epithelium further increases the risk of bleeding, dehydration and sepsis. The increase in radiation dose involves injury to other tissues (lung, skin, kidney, etc.) making the situation less and less medically manageable, and ultimately incurable. We plan to assess gamma-tocotrienol (GT3) as a radiation countermeasure against the hematopoietic and GI syndromes of ARS. This study will employ a well-defined, nonhuman primate (NHP) model in order to assess the countermeasure's efficacy (increased survival) against different doses of radiation and mechanism(s) of action for hematopoietic and GI rescue.

II. BACKGROUND

II.1. Background

Although the search for radiation countermeasures for ARS was initiated more than half a century ago, no safe and effective radiation countermeasure has been approved by the United States Food and Drug Administration (US FDA) for the ARS (b)(4) (b)(4) Most recently, natural products have been investigated for prevention and therapy of human diseases because they are 'generally recognized as safe' and appropriate for medicinal purposes. Unlike their synthetic analogs, they are well tolerated and minimally toxic, even in the upper ranges of dietary intake (*3*,) Vitamin E represents a family of (b)(4) compounds that is divided into two subgroups called tocopherols and tocotrienols, which act as important antioxidants that regulate peroxidation reactions and control free-radical production within the body (*5, 6*). This family of compounds has eight

different isoforms that belong to two categories: four saturated analogues (α, β, γ, and δ) called tocopherols and four unsaturated analogues referred to as tocotrienols. These 8 components are collectively known as tocols. Tocopherols and tocotrienols share common structural features of a chromanol ring and a 15-carbon tail at the C-2 position derived from homogenistate and phytyl diphosphate, respectively. Tocotrienols differ structurally from tocopherols by the presence of three trans-double bonds in the hydrocarbon tail. The isomeric forms of tocopherol and tocotrienol are distinguished by the number and location of methyl groups on the chromanol rings. Recent studies suggest that both the molecular and therapeutic targets of the tocotrienols are distinct from those of the tocopherols. A number of studies have shown that tocotrienols are superior antioxidants compare to tocopherols (*1, 7-10*).

Gamma-tocotrienol (GT3), one of the eight isomers (tocols) of vitamin E, is a potent inhibitor of HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase (*11, 12*). Its antioxidant activity was a compelling reason to evaluate it for radioprotective efficacy; in recent years, it has received a great deal of attention by researchers and appears to be one of the most promising radioprotective tocols tested to date. GT3 has been shown to increase survival in rodents, through the amelioration of the hematopoietic and GI syndromes. When administered 24 h before ^{60}Co γ-irradiation, GT3 significantly protected mice against radiation doses as high as 11.5 Gy, and its dose reduction factor (DRF) as a radioprotector (24 h before irradiation, 200 mg/kg, subcutaneous route, sc) was 1.29 (b)(4) GT3 demonstrated dose response against different doses of radiation used. GT3 treatment accelerated hematopoietic recovery as judged by higher numbers of total white blood cells, neutrophils, monocytes, platelets, and reticulocytes in peripheral blood (b)(4) and enhanced hematopoietic progenitors (b)(4) in the bone marrow (BM) of irradiated mice. Additionally, GT3-treated irradiated mice had higher numbers of colony-forming cells, more regenerative microfoci for myeloid and megakaryocytes, higher cellularity in BM, and reduced frequency of micronucleated erythrocytes compared to vehicle treated mice compared to irradiated vehicle controls (b)(4) Mouse survival studies with GT3 suggested the most efficacious time for drug administration was 24 h prior to irradiation, possibly due to the induction of key hematopoietic cytokines during that time frame. GT3 treatment resulted in significant induction of granulocyte colony-stimulating factor (G-CSF) and several other cytokines in mice; administration of G-CSF antibody completely abrogated the radioprotective efficacy of GT3 in mice (b)(4) GT3 mobilized progenitors in peripheral blood (b)(4) and transfusion of such blood, enriched progenitor cells also was capable of protecting mice against γ–irradiation (b)(4)

These results prompted us to evaluate GT3 against ionizing radiation in NHP model in a pilot study. We evaluated GT3 pharmacokinetics and efficacy using different doses of drug and radiation. Our results demonstrate promising radioprotective efficacy of GT3 in NHPs.

Three drug doses (9.375, 18.75 and 37.5 mg/kg, n = 4 for each group) were selected for pharmacokinetics (PK) studies based on earlier efficacy and toxicity studies conducted in the murine model. Each dose was administered sc to 4 NHPs and blood samples were collected at various time points following GT3 injection. Studies were terminated at the end of d 20 based on the previous observations with other drugs. PK study data are presented below displaying maximum blood plasma concentrations (C_{max}), the time at which C_{max} is obtained (T_{max}), half-life ($T_{1/2}$), area under the curve (AUC), clearance rate (Cl) and mean retention time (MRT) for the various doses of GT3 that were administered.

Pharmacokinetic parameters of GT3 in rhesus macaques

Dose (mg/kg)	C_{max} (ng/ml)	T_{max} (h)	$T_{1/2}$ (h)	AUC (µg*h/ml)	Cl (ml/h/kg)	MRT (h)	MRT (d)
9.375	734.9	2	30.4	26.192	0.358	48.6	2.0
18.75	997.4	8	34.6	43.714	0.429	48.0	2.0
37.5	1142	12	40.9	82.225	0.456	273.9	11.4

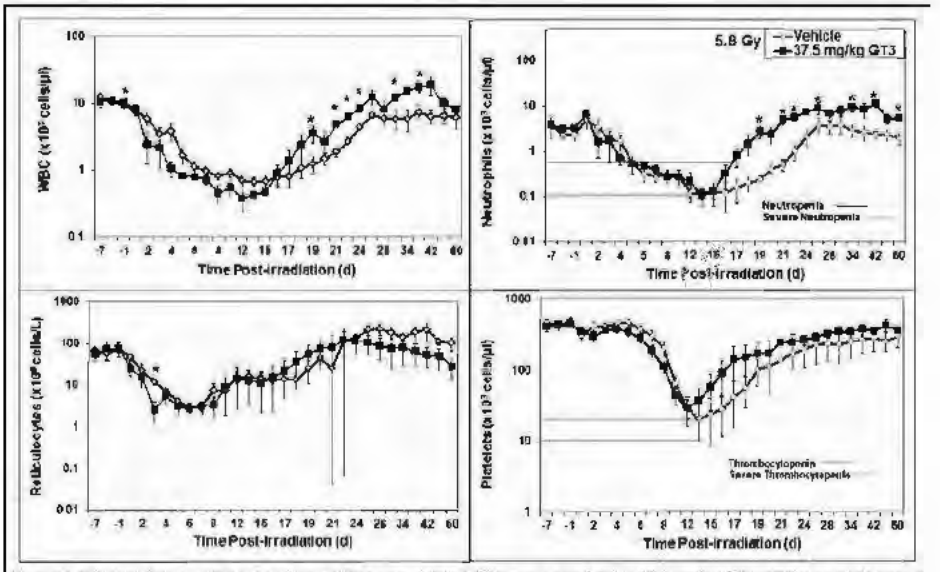


Figure 1. Effect of GT3 on CBC in irradiated NHPs exposed to 5.8 Gy and administered 37.5 mg/kg GT3. GT3 was administered sc 24 h prior to irradiation and blood samples were collected at various time points in relation to irradiation for CBC analysis.

Pilot efficacy study in NHP: The NHPs (n = 4) treated with 37.5 mg/kg GT3 had a higher level of WBCs beginning at d 16 post-irradiation compared with vehicle control, this difference became significant at d 21 post-irradiation and continued inconsistently until d 38, by which both groups average WBC levels returned to pre-exposure levels (figure 1). These same NHPs had a higher number of neutrophils beginning at 15 d post-irradiation and had a faster recovery from neutropenia. The GT3-treated group was considered neutropenic from d 6-16; the vehicle-treated group was neutropenic from d 6-20. The reticulocyte response curve for the 37.5mg/kg NHPs closely resembles the response curve for those treated with the vehicle. Only at 3 days post irradiation was there a significant difference between treatment groups. Though, the platelet counts for the GT3-treated NHPs were also higher, there was no significant difference between the two treatments.

NHPs treated with 75 mg/kg GT3 (n = 4), had higher levels of WBCs than the vehicle treated NHPs at day 14; this improvement was significant at day 15 and continued until day 24 when both groups' counts returned to normal levels (figure 2). Additionally, GT3-treated NHPs had an accelerated recovery from neutropenia and remained in a state of severe neutropenia for one day compared to the vehicle-treated NHPs (6 days). The GT3-treated group recovered from neutropenia on d 15 compared to the vehicle-treated NHPs recovery from neutropenia on d 20. NHPs treated with 75 mg/kg clearly had improved reticulocytes recovery compared to the vehicle treated group by d 16 post-irradiation; significant difference by treatments ranged from d 19 to 22 post-irradiation. The GT3-treated NHPs had significantly higher platelet counts than the vehicle-treated NHPs from d 15-28. The GT3-treated group did not become thrombocytopenic whereas the vehicle group was in a state of thrombocytopenia from d 12-16 post-irradiation.

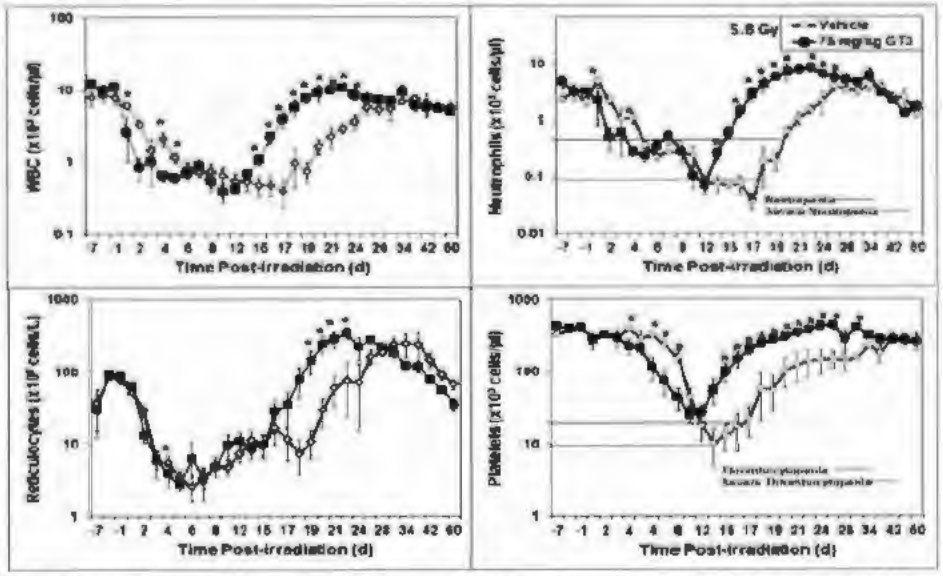


Figure 2. Effect of GT3 on CBC in irradiated NHPs exposed to 5.8 Gy and administered 75 mg/kg GT3. GT3 was administered sc 24 h prior to irradiation and blood samples were collected at various time points in relation to irradiation for CBC analysis.

The trend of accelerated recovery of GT3-treated NHPs at 5.8 Gy was also noted at the higher radiation dose, 6.5 Gy (n = 8). Figure 3 shows that WBC was significantly higher in GT3-treated animals from d 14 to 28 post-irradiation compared to vehicle group. GT3-treated NHPs had significantly higher neutrophil counts than the

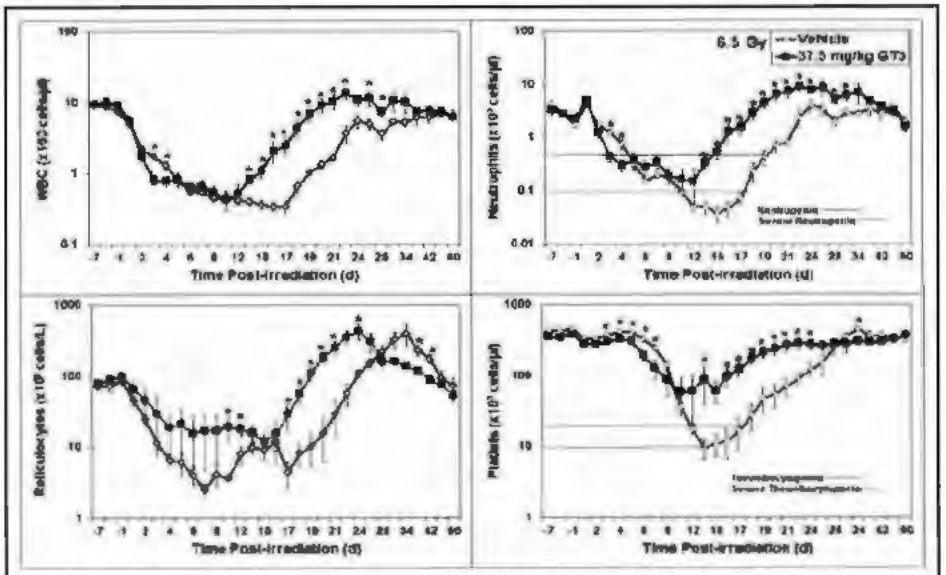


Figure 3. Effect of GT3 on CBC in irradiated NHPs exposed to 6.5 Gy and administered 37.5 mg/kg GT3. GT3 was administered sc 24 h prior to irradiation and blood samples were collected at various time points in relation to irradiation for CBC analysis.

vehicle-treated NHPs continuously from d 14-30. The GT3-treated group was considered neutropenic from d 3-14, whereas the vehicle-treated group was in a state of neutropenia from d 5-19 and was considered severely neutropenic from d 10-17.

These exciting preliminary results of the pilot study demonstrate an unprecedented promise of GT3 as safe, well-tolerated, highly effective radioprotector (i.e. given before irradiation). Therefore, we decided to develop GT3 as a FDA approved radiation countermeasure for human use. The ultimate goals of this proposal are (1) to characterize in detail the radioprotective potential of GT3 against radiation exposure in NHPs; (2) to evaluate GT3's capacity to accelerate hematopoietic and GI recovery in irradiated animals exposed whole-body as well as with partial-body exposures and, (3) to evaluate in the NHP model a select number of potential biomarkers for GT3's radioprotective efficacy.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

BRD: DoD Biomedical Research Database
PubMed: United States National Library of Medicine
FEDRIP: Federal Research in Progress Database

II.2.2. Date of Search

9/23/2014, 1/2/2015 (latest search results are given)

II.2.3. Period of Search

BRD: 1998-2014
Pubmed: 1965-2014
FEDRIP: 1992-2014

II.2.4. Key Words of Search

1. Gamma-tocotrienol
2. Gamma-tocotrienol and radiation
3. Gamma-tocotrienol and irradiation
4. Gamma-tocotrienol and drug delivery
5. Gamma-tocotrienol and cytokines
6. Gamma-tocotrienol and radiation countermeasure
7. Gamma-tocotrienol and radioprotection
8. Gamma-tocotrienol and primates
9. Gamma-tocotrienol and primates and radiation
10. Gamma-tocotrienol and pancytopenia
11. Gamma-tocotrienol and hematopoietic cells
12. Gamma-tocotrienol and radiation and hematopoietic injury
13. Gamma-tocotrienol and bone marrow
14. Gamma-tocotrienol and crypt count
15. Gamma-tocotrienol and GI syndrome
16. Gamma-tocotrienol and neutropenia
17. Gamma-tocotrienol and thrombocytopenia
18. Gamma-tocotrienol and biomarker
19. Gamma-tocotrienol and biomarker and G-CSF
20. Gamma-tocotrienol and G-CSF and non-human primate

21. Gamma-tocotrienol and acute radiation syndrome
22. Gamma-tocotrienol and GI syndrome

II.2.5. Results of Search

Literature Search for Duplication				
#	Keywords of Search	Literature Sources and Periods of search		
		BRD: 1998 - 2014	**PubMed: 1965-2014**	**FEDRIP: 1992 - 2014**
		Results of Search		
1	Gamma-tocotrienol	18	298	1
2	Gamma-tocotrienol and radiation	17	31	4
3	Gamma-tocotrienol and irradiation	17	18	1
4	Gamma-tocotrienol and drug delivery	2	6	0
5	Gamma-tocotrienol and cytokines	7	29	0
6	Gamma-tocotrienol and radiation countermeasure	11	4	0
7	Gamma-tocotrienol and radioprotection	10	11	2
8	Gamma-tocotrienol and primates	2	126	0
9	Gamma-tocotrienol and primates and radiation	2	6	0
10	Gamma-tocotrienol and pancytopenia	2	1	0
11	Gamma-tocotrienol and hematopoietic cells	9	7	1
12	Gamma-tocotrienol and radiation and hematopoietic injury	9	5	1
13	Gamma-tocotrienol	15	8	3
14	Gamma-tocotrienol and radiation	2	0	0
15	Gamma-tocotrienol and irradiation	3	1	0
16	Gamma-tocotrienol and drug delivery	3	3	0
17	Gamma-tocotrienol and cytokines	3	1	0
18	Gamma-tocotrienol and radiation countermeasure	4	17	0
19	Gamma-tocotrienol and radioprotection	1	1	0
20	Gamma-tocotrienol and primates	1	0	0
21	Gamma-tocotrienol and primates and radiation	5	2	0
22	Gamma-tocotrienol and pancytopenia	3	1	0
None of these searched references address the questions to be tested in this protocol.				

Some related studies with GT3 have been conducted in mice however; none have been conducted with the refined formulation proposed in this protocol or using the NHP model. Studies in mice are helpful in that they have proven the principle that GT3 is prophylactically useful in managing morbidity and mortality in lethally irradiated animals. These results seem to demonstrate an unprecedented promise of GT3 as safe, well-tolerated, highly effective radioprotector (i.e. given before irradiation). Therefore, we decided to develop GT3 as a FDA approved radiation countermeasure for human use. There are no references of using gamma-tocotrienol in the NHP model for radiation protection.

III. OBJECTIVE / HYPOTHESIS Tocols protect mice against lethal γ-irradiation when administered by sc injection 24 h prior to irradiation. It is also found to be efficacious in a pilot study in NHPs against different doses of radiation when administered 24 h prior to radiation exposure. Specifically, we hypothesize that: 1) GT3 will enhance survival in NHPs exposed to different doses of radiation since we have observed significant improvement in neutropenia and thrombocytopenia in a pilot study using NHPs exposed to 5.8, 6.5, and 7.2 Gy whole body radiation. 2) GT3 is effective through sc route 3) GT3

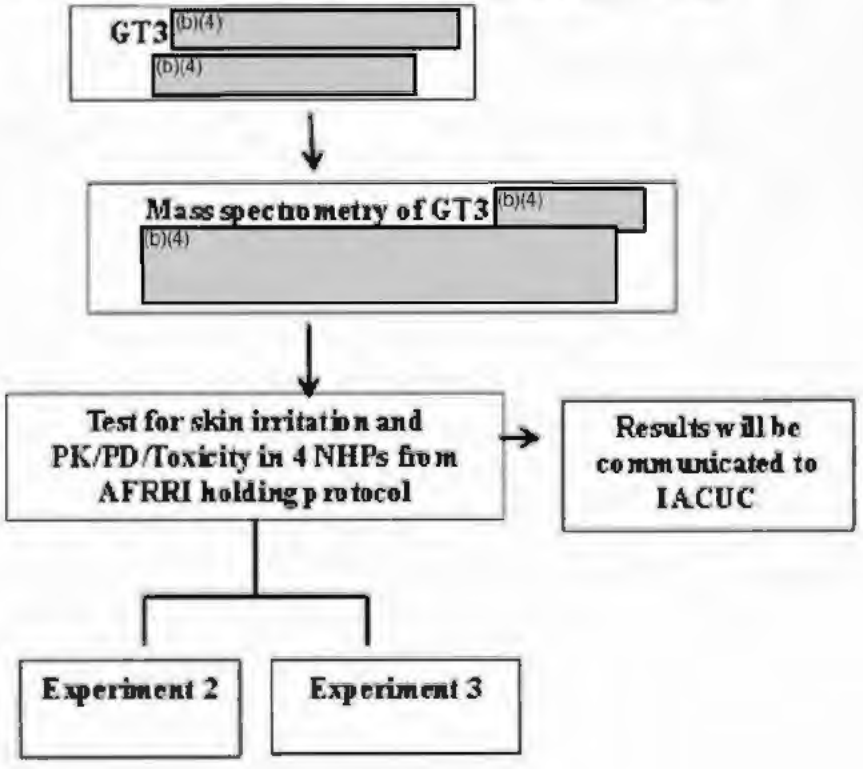
will be effective against partial-body radiation exposure similar to its efficacy against whole body radiation exposure. GT3 will accelerate recovery against radiation injury to hematopoietic as well as GI injury-induced by whole body and partial body radiation exposures. G-CSF (and interleukin-6; IL-6) in conjunction with CBC may serve as efficacy biomarkers for GT3 in NHP.

IV. MILITARY RELEVANCE

Currently, there are no FDA approved pharmaceutical agents that can prevent or treat injury from external ionizing radiation. The problem has become more acute in recent times due to the unpredictable nature of terrorist attacks. Recognizing this urgent need, the U.S. Department of Defense has assigned top priority to the "development of medical countermeasures to radiation exposure" against both early and late arising health effects. Advanced development of radiation countermeasures are currently an unfunded requirement. These concerns imply the urgent need to develop an appropriate countermeasure for radiation injuries potentially sustained by war fighters during combat operations and enable commanders to make judicious decisions in troop deployment. There are several drugs at different stages of the development. This study will perform essential research and development of a promising agent (gamma-tocotrienol (GT3), a component of vitamin E, also known as a tocol) as a prophylaxis for potential injuries sustained by military personnel who have been accidentally exposed to ionizing radiation.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures



We propose to carry-out the following experiments to test new preparation of GT3 from another vendor, identify biomarkers for GT3, investigate efficacy and also investigate hematopoietic and GI accelerated recovery of irradiated animals by treatment with GT3. Details of subsequent experiments will depend on results obtained in previous experiment.

V.1.1. Experiment 1. Investigate the pharmacokinetics/pharmacodynamics/toxicity of new batch of GT3 from (b)(4) when administered sc

The new batch of GT3 (prepared in 5% Tween 80 and saline) (b)(4) will be tested in four NHPs (obtained from Veterinary Science Department holding/training protocol) for PK/pharmacodynamics (PD)/toxicity before initiating efficacy study in next experiment. This study will be conducted in un-irradiated NHPs to determine the complete blood chemistry (CBC), biochemistry, cytokine and protein micro array (proteomics). Blood draw schedule for PK study has been given in table # 1. GT3 levels in blood will be determined by mass spec as performed earlier in our collaborator's laboratory. Skin irritation will be closely monitored. After the last bleed on day 20 and completion of experiment 1, animals will be transferred to the holding protocol of the Veterinary Science Department (VSD).

Table 1 Blood collection schedule for tests discussed above

GT3 dose mg/kg	Time of blood draw	For PK 0.5 ml	For CBC, 0.5ml	For Cyto 0.5 ml	For Chem 0.5 ml	For Gene array 0.5 ml	Total blood, ml
0	-7d	+	+				1.0
0	-3d	+	+		+	+	2.0
0	-1d	+		+			1.0
37.5	0 day of injection)						
	0.25 h	+		+			1.0
	0.5 h	+		+			1.0
	1.0 h	+		+			1.0
	2 h	+		+			1.0
	4.0 h	+		+			1.0
	8.0 h	+		+			1.0
	12.0 h	+		+			1.0
	1 d	+	+	+	+		2.0
	2 d	+	+	+		+	2.0
	3 d	+		+			1.0
	4 d	+	+	+			1.5
	5 d	+		+			1.0
	6 d	+		+			1.0
	7 d	+	+	+			1.5
	10 d	+	+	+			1.5
	14 d	+	+	+			1.5
	20 d	+	+	+	+		2.0
On day 0, the blood collection volume will be 7 ml, over 1% of the animal's total blood volume.							

We will be well within the acceptable published blood collection volumes (*19*) that are followed at AFRRI. According to these guidelines 1% blood (2.2 ml per day for 4 kg NHP – minimum body weight of experimental NHP) can be withdrawn every 24 h. Up to 15% of circulating blood volume can be drawn in

a single day repeated every 4 weeks in normal healthy animal. For the entire span of the study, the total blood drawn (26 ml) will be well below the recommended maximum limits (32 ml total, 15% of total blood volume in a 4 kg animal over a period of 28 days). Each NHP weight will be different and that fact will be kept in mind while blood drawing.

V.1.2. Experiment 2. Investigate the radioprotective efficacy of GT3 in NHPs using different doses of whole body radiation exposure using large sample size

Following experimental set up and NHP distribution scheme will be used:

Two groups of NHPs (16 NHP per treatment group; 8 males + 8 females, totaling 32 NHPs) will be irradiated with 5.8 Gy total body irradiation (expected to be $LD_{30/60}$) 24 h after single sc injection of either vehicle (5% Tween 80 in saline) or 37.5 mg/kg GT3. In another experiment, other two groups using, the same number of NHPs will be exposed to 6.5 Gy (expected to be $LD_{50/60}$) total body irradiation, 24 h after single sc injections of 37.5 mg/kg GT3 or vehicle. NHPs will be observed for 60 d following irradiation, while monitoring survival and multiple clinical and laboratory parameters such as CBC, blood chemistry, citrulline, and cytokine level. Total number of animals: 64 NHPs (16 NHPs in each group x 2 groups x 2 radiation doses).

Table 2. Efficacy of GT3 in NHPs using large sample size

NHP#	Drug	Route	Dose	Frequency	Irradiation dose (Gy)
16	GT3	sc	37.5 mg/kg	24 h prior to irradiation	5.8
16	Veh	sc	37.5 mg/kg	24 h prior to irradiation	5.8
16	GT3	sc	37.5 mg/kg	24 h prior to irradiation	6.5
16	Veh	sc	37.5 mg/kg	24 h prior to irradiation	6.5

For GT3 or vehicle, the administration volumes will be in accordance with Diehl et al, *(19)*, sc injection volume for rhesus macaques is 2 and 5 ml/kg (good practice and maximum volume, respectively). Exact dose will be calculated based on animal body weight and drug concentration. We expect that drug administration volume will be within good practice limits and we may not need to use maximum volume allowed.

Table 3. Blood collection schedule for experiment 2

GT3 dose mg/kg	Time of blood draw	CBC 0.5 ml	Cytokine/Bio markers 1.0 ml	Biochemis try 0.5 ml	Bacteremia 1.0 ml	Citrulline 0.5 ml	Total blood, ml
	-7d	+			+		1.5
	-3d	+	+	+			2.0
	-1d	+		+	+		2.0
37.5 mg/kg	GT3/vehicl e injection day -1						0
Radiation Dose 5.8 or 6.5 Gy	Day 0						(Total 2.0 ml on day 0, see below)
	4 h		+				1.0
	8 h		+				1.0
	12 h		+	+			1.5
	Day 1	+	+			+	2.0
	Day 2	+	+	+			2.0
	Day 4	+				+	1.0
	Day 6	+	+				1.5
	Day 8	+			+	+	2.0

	Day 10	+					0.5
	Day 12	+					0.5
	Day 14	+					0.5
	Day 16	+					0.5
	Day 18	+					0.5
	Day 20	+				+	1.0
	Day 22	+					0.5
	Day 24	+			+		1.5
	Day 26	+					0.5
	Day 28	+		+		+	1.5
	Day 30	+					0.5
	Day 34	+			+		1.5
	Day 38	+		+		+	1.5
	Day 42	+			+		1.5
	Day 50	+		+		+	1.5
	Day 60	+		+		+	1.5

We will be well within the acceptable published blood collection volumes (*19*) that are followed at AFRRI. According to these guidelines 1% blood (2.2 ml per day for 4 kg NHP – minimum body weight of experimental NHP) can be withdrawn every 24 h. Up to 15% of circulating blood volume can be drawn in a single day repeated every 4 weeks in a normal healthy animal. For the entire span of the study, the total blood drawn (33 ml) will be well below the recommended maximum limits.

V.1.3. Experiment 3. Study of hematopoietic and GI injury, accelerated recovery, and efficacy biomarkers in NHPs administered GT3

The GT3 used in experiment 2 will be used for this experiment. Two different experiments will be conducted for studying hematopoietic and GI injury accelerated recovery by GT3 administration. For both experiments, 8 NHPs will be used for each treatment (GT3 and vehicle: 5% Tween 80 in saline). These NHPs are being sub-distributed into smaller groups as stated in table 4. The experiment will be initially conducted using whole body exposure and then repeated with partial body exposure with LINAC. Total 64 NHPs for this specific aim (8 animals per group x 2 groups x 2 experiments for GI and hematopoietic injury models x 2 experiments for whole body and partial body radiation exposures).

Table 4.Hematopoietic and GI injury in NHPs administered GT3 or vehicle

Hematopoietic Study (Whole body irradiation)					
NHP#	Drug	Route	Dose	Frequency	Irradiation Dose (Gy)
4	GT3	Sc	37.5 mg/kg	24 h prior to irradiation	4.0
4	Veh	Sc	37.5 mg/kg	24 h prior to irradiation	4.0
4	GT3	Sc	37.5 mg/kg	24 h prior to irradiation	5.8
4	Veh	Sc	37.5 mg/kg	24 h prior to irradiation	5.8
Hematopoietic Study (Partial body irradiation)					
4	GT3	Sc	37.5 mg/kg	24 h prior to irradiation	4.0
4	Veh	Sc	37.5 mg/kg	24 h prior to irradiation	4.0

4	GT3	Sc	37.5 mg/kg	24 h prior to irradiation	5.8
4	Veh	Sc	37.5 mg/kg	24 h prior to irradiation	5.8
GI Study (Whole body irradiation)					
8*	GT3	Sc	37.5 mg/kg	24 h prior to irradiation	12.0
8*	Veh	Sc	37.5 mg/kg	24 h prior to irradiation	12.0
GI Study (Partial body irradiation)					
8*	GT3	Sc	37.5 mg/kg	24 h prior to irradiation	12.0
8*	Veh	Sc	37.5 mg/kg	24 h prior to irradiation	12.0

*Out of 8 NHPs in each group, 3 will be euthanized on day 4, 3 on day 7, and the remaining 2 will be euthanized on day 10 post-irradiation.

For GT3 or vehicle, the administration volumes will be in accordance with *Diehl et al, (19)*, sc injection volume for rhesus macaques is 2 and 5 ml/kg (good practice and maximum volume, respectively). Exact dose will be calculated based on animal body weight and drug concentration. Since GT3 is highly soluble in its proposed vehicle, we expect that drug administration volume will be within good practice limits and we may not need to use maximum volume allowed.

Hematopoietic injury and accelerated recovery by GT3 administration

To assess radiation damage and recovery by GT3 administration a total of 16 NHPs (8 receiving vehicle and 8 receiving GT3) will be exposed to either whole body or partial body radiation. Each treatment group will be split evenly in half. Each sub-group will then receive one of two radiation doses 4 Gy (sublethal) and 5.8 Gy ($LD_{30/60}$) for studying hematopoietic injury at two different radiation doses and accelerated recovery by GT3 administration.

BM aspiration

BM aspiration will be carried out on days -8, 2, 7, 14, and 30. BM cells will be used for various colony forming (CFU) assays as previously described (*20*) and the BM plasma will be used to obtain information about the BM microenvironment. The procedure for BM aspiration has been detailed under section V.4.3.2. 'Procedure(s)'. Cytokines/chemokines analysis by multiplex Luminex will also be carried out in each sample (the protein concentration in each sample will be measured with a modified Bradford reaction, Coomassie Plus Protein Assay, Thermo Scientific, Rockford, IL). It has been reported for mice that radiation and GT3 affect various cytokines/growth factors in BM plasma (*20, 21*). BM cells (mononuclear cells) will also be analyzed for apoptosis by TUNEL assay (b)(4)

For CBC, whole blood will be collected in EDTA coated tubes and complete blood count will be obtained to assess hematopoietic injury. Animals will be euthanized around 30 d post-irradiation (period when there is maximum hematologic difference between GT3- treated and control group) for necropsy, histopathology of various organ and additional BM analysis.

Table 5. BM and blood sample collection for hematopoietic recovery in irradiated animals treated with GT3

GT3 dose mg/kg	Time of blood collection/BM aspiration	BM 2 ml	Blood for CBC 0.5 ml	Total volume
	-8 d	+	+	2.5 ml
37.5 mg/kg (-24 h)				
	Radiation 5.8 or 6.5 Gy			
	Day 2	+	+	2.5 ml
	Day 7	+	+	2.5 ml
	Day 14	+	+	2.5 ml
	Day 16		+	0.5 ml
	Day 18		+	0.5 ml
	Day 20		+	0.5 ml
	Day 22		+	0.5 ml
	Day 24		+	0.5 ml
	Day 26		+	0.5 ml
	Day 28		+	0.5 ml
	Day 30	+	+	2.5 ml

This table will be applicable to both experiments (whole-body and partial-body irradiation) and two different radiation doses (4 and 5.8 Gy). We will be well within the acceptable published blood collection volumes (*19*) that are followed at AFRRI. According to these guidelines 1% blood (2.2 ml per day for 4 kg NHP – minimum body weight of experimental NHP) can be withdrawn every 24 h. Up to 15% of circulating blood volume can be drawn in a single day repeated every 4 weeks in a normal healthy animal. For the entire span of the study, the total blood drawn (16 ml) will be well below the recommended maximum limits.

GI injury and accelerated recovery by GT3 administration

This study will include evaluation of the crypt microcolony assay, where crypt survival will be quantified at 7 days after high-dose radiation exposure (rather than 3 days in mice), coupled with necropsy and histopathology of various organs. Our proposed experimental model is based upon those developed and published by MacVittie and colleagues, in which rhesus macaques were exposed bilaterally to 10.0-14.0 Gy at a 0.80 Gy/min (*23*). For this study, a high radiation dose of 12 Gy (approx. $LD_{75/15}$ for whole body, lethality will be lower with partial body) will be used as this dose induces a classic radiation GI syndrome consistent with depletion of GI stem cells and subsequent organ failure. Animals exposed to whole body radiation will duly succumb within the expected 20 day period to GI syndrome and hematopoietic injury. NHPs exposed to partial body radiation will experience GI injury and hematopoietic injury, however, due to residual BM cells in specific body area (5% sparing of BM – hind limbs fibula, tibia, and feet not irradiated – in LINAC shielding is not required for sparing) these animals are expected to rapidly recover.

Out of 8 NHPs in each group, 3 will be euthanized on day 4, 3 on day 7, and the remaining 2 will be euthanized on day 10 post-irradiation. Such study has been carried out in [redacted] with another radiation countermeasure with remarkable results.

GI study end points

The primary end point will be crypt count in jejunum samples on d 4, 7, and 10 post-irradiation. Samples of small and large intestine will be collected at necropsy and fixed in formalin, embedded in paraffin, cut, mounted, and stained with hematoxylin and eosin (H&E). Microcolony crypt cell survival assay will be performed as described by Withers and Elkins (*24*). The number of crypts will be calculated according to this method described by MacVittie (*23*).

Secondary endpoints will include key GI- and hematologic-associated parameters. These parameters will include a) histological analysis of radiation damage in the jejunum and colon, b) Ki-67 analysis of proliferating cells, c) mucosal surface area (measured in longitudinal H&E-stained sections of the jejunum using projection cycloid method described by Baddeley (*25*) which was validated in the [redacted] d) body weight loss, and e) the incidence, severity, and duration of diarrhea and dehydration. Hematologic analysis will include neutropenia and thrombocytopenia related parameters, incidence of febrile neutropenia, and incidence of positive blood cultures. Serum citrulline levels will be determined. Blood samples will also be tested for gut bacterial translocation.

Assessment of vascular oxidative stress

Pre-and post-irradiation peroxynitrite measurements provide information about the severity of radiation-induced oxidative/nitrosative stress. Peroxynitrite readily oxidizes dihydrorhodamine 123. The peritoneal aorta from NHPs euthanized on days 4 and 7 will be used for this assay to indicate the level of peroxynitrite production. It has been demonstrated in mouse model that vascular peroxynitrite production was increased by radiation exposure and administration of GT3 inhibited such radiation-induced peroxynitrite production (*21*).

Table 6. Euthanasia schedule of experiment for GI injury and accelerated recovery experiment

Groups	Day 4 post-irradiation	BM Day 7 post-irradiation	Day 10 post-irradiation	Total
GT3 + 12 Gy	3	3	2	8
Vehicle + 12 Gy	3	3	2	8

This table will be applicable to both experiments (whole-body and partial-body irradiation).

Table 7. Blood sample collection schedule for GI injury and accelerated recovery experiment

GT3 dose mg/kg	Time of blood draw	CBC 0.5 ml	Cytokine/Bio markers 1.0 ml	Biochemis try 0.5 ml	Bacteremia 1.0 ml	Citrulline 0.5 ml	Total blood, ml
	-7d	+			+	+	2.0
	-3d	+	+	+			2.0
37.5 mg/kg	GT3/vehicl e injection day -1						0
Radiation Dose 12 Gy	Day 0						(Total 2.0 ml on day 0, see below)
	4 h		+				1.0
	8 h		+				1.0
	12 h		+	+			1.5
	Day 1	+	+			+	2.0
	Day 2	+	+	+			2.0
	Day 4	+				+	1.0
	Day 6	+	+				1.5
	Day 7	+			+	+	2.0

This table will be applicable to both experiments (whole-body and partial-body irradiation). We will be well within the acceptable published blood collection volumes (*19*) that are followed at AFRRI. According to these guidelines 1% blood (2.2 ml per day for 4 kg NHP – minimum body weight of experimental NHP) can be withdrawn every 24 h. Up to 15% of circulating blood volume can be drawn in a single day repeated every 4 weeks in a normal healthy animal. For the entire span of the study, the total blood drawn (16 ml) will be well below the recommended maximum limits.

V.2. Data Analysis

In order to evaluate the hypothesis set forth and establish a comparison of significance of any deviation from the hypothesis, Fisher Exact Test will be performed. The test can provide the significance and deviation. The Fisher test for Variance or the ANOVA which provides the means to analyze the differences between group and their associated procedures is a suitable test to detect significance between sampling groups. ANOVA is a test of choice to compare group means and group variables. Means and standard deviations will be calculated. A $p<0.05$ will be considered statistically significant. When inferential statistics is used such as *t*-test, Fisher's tests, ANOVA etc, to evaluate the outcome and comparisons, sample size (n) needs to be determined to provide sufficient statistical capability for performance.

For experiment 2, to investigate the radioprotective efficacy of GT3 in NHPs using different doses of whole body radiation, we will be using n=16 (8 males and 8 females in each group) which should give more than 80% power for calculation (*27*). We expect a 30% difference (30% mortality in control and 0% mortality in drug treated group) at 5.8 Gy. Similarly at 6.5 Gy we would expect 50% mortality in control and 0% mortality in drug treated group.

For survival data, a Kaplan-Meier plot and log-rank test will be used to visualize and compare survival data, respectively. Fisher's exact test will be used to compare survival rates at the end of 60 d, with a Bonferroni correction used to control for type I error if multiple comparisons are made. For additional efficacy/mechanistic data, mean

values with standard errors (SE, when applicable) will be reported. Independent samples *t*-tests or analysis of variance (ANOVA) will be used to detect if there are significant differences between experimental groups. All statistical tests will be two-sided, with a 5% significance level. Statistical software SPSS version 22 (IBM, Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) will be used for analyses.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

Both computer models and tissue culture have been considered as alternatives to animals. These alternatives lack the knowledge base and complexity needed to evaluate the effects of radiation and radiation countermeasures on the immune system. The phenomena under study involve complex information-processing networks comprising large number of cell types and biological signal transduction molecules. Responses to irradiation and radioprotective agents involve interactions between the central nervous system, the peripheral nervous system including the enteric nervous system, endocrine glands, the immune and inflammatory systems, hematopoietic system, GI system etc. We do not know all of the cell types and tissues involved and many of the signal transduction molecules have not been discovered yet. Even if all of the tissues, cell types and signaling molecules were known, the present state of tissue culture would be incapable of reproducing *in vivo* relationships. One would have to reproduce much of an entire mammalian organism in culture to study these phenomena *in vitro*. As for computer models, the most powerful supercomputers available in the near future would be incapable of analyzing interactions between so many elements in the network. Thus these drugs necessitate investigation in whole animal models to see overall outcome.

V.3.2. Animal Model and Species Justification

As described above, preliminary studies have shown that GT3 is effective in mice when administered sc as a radioprotector. Demonstrating efficacy in a large animal species (LAS) is a critical requirement of the FDA Animal Rule. *Rhesus macaque* has 95+% DNA sequence identity with humans. NHPs are necessary for the pre-clinical development of a drug candidate intended for use in humans, because drug metabolism and physiology are so similar between NHPs and humans. Rhesus macaques are the model of choice for investigations of toxicity, pharmacokinetics, biomarkers, radiation injury, and countermeasures, because the large database available from the existing literature. The FDA has accepted rhesus macaques as the appropriate animal model for pivotal efficacy testing of radiation countermeasures under the Animal Efficacy Rule, where efficacy testing cannot be performed in humans. The biomarker and efficacy of the drug to be tested in this protocol have already been demonstrated in mice. Previous experience with other radiation countermeasures (e.g. Neummune or HE 2100 or 5-AED) has shown that testing in dogs is not always relevant to pharmacokinetics and efficacy in primates. Metabolism of 5-AED in dog hepatocytes was much different than in NHPs and human hepatocytes. Animal model of pig (specifically Gottingen minipig) is currently under initial stage of the development for studying radiation injury. This model is not well defined and mature enough to evaluate radiation countermeasures. Our ultimate goal is to generate data which will be submitted to US FDA and our data should be acceptable to them for approval of this radiation countermeasure (GT3). Minipig

model has not reached to that stage of acceptance yet. (b)(4) Therefore, this study in rhesus macaque is being undertaken to guide future development of this class of radiation countermeasures. There are several reports for use of NHPs in radiation research and countermeasure development (*31-34*).

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species

*Macaca mulatta (*Rhesus macaques*)*

V.3.3.2. Strain / Stock Chinese or Indian origin

V.3.3.3. Source / Vendor

AFRRI VSD will procure animals from vendors registered with the USDA or DoD.

V.3.3.4. Age 2.5 to 7 years

V.3.3.5. Weight 4 to 8 kg

V.3.3.6. Sex Male and female

V.3.3.7. Special Considerations

Animals should test antibody negative for Herpes B virus (aka *Macacine herpesvirus*), Simian T-cell leukemia virus type 1 (STLV-1), Simian Immunodeficiency virus (SIV) and Simian Retrovirus (SRV) Types 1, 2, 3, and 5. Animals shall also test negative by means of virus isolation or polymerase chain reaction (PCR) for SRV Type 2. NHPs will either be vaccinated for measles or, in the case of previously measles-vaccinated NHPs, tested for the presence of measles antibodies. NHPs shall come from the vendor colony negative for Salmonella, Shigella and Yersenia. Vendor must provide the health reports indicating the pathogen free status of the NHPs for the agents listed in this section. Animals will be pole and collar trained before use in experiment.

V.3.4. Number of Animals Required (by species)

Macaca mulatta: 132 (66 males and 66 females)

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1.

For biomarker and hematopoietic/GI injury recovery studies, end point will be measurement of BM cell and GI crypt cell analysis till predetermined days. For this purpose blood, BM, and GI samples (only from euthanized animals) samples will be collected at predetermined time points and analysis of relevant parameters will be carried out.

For efficacy study, the endpoint currently mandated by the FDA for approval of radiation countermeasures is mortality. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress, using an extensive set of criteria (listed under section V.4.5). The actual irradiation procedure does not cause pain or distress. Animal care and use procedures will only be performed by trained and certified personnel.

V.3.5.2. Reduction

As we move forward with experiments, plans will be reevaluated at every stage of the experiments, in light of incoming data in an attempt to reduce the numbers of experiments and groups, if possible. If deemed not necessary based on incoming data, some investigations may not be carried out to optimize the use of experimental animals.

V.3.5.3. Replacement

There are no *in vitro* techniques available to demonstrate that this drug will counter the effects of whole-body irradiation in humans. Efficacy and safety have already been demonstrated in rodents. The preferred large animal model for preclinical evaluation of radiation countermeasures intended for use in humans is NHP. This study represents the next step necessary to develop this drug for FDA approval. According to FDA scientists and the radiobiology community, the large database of radiation studies in *Macaca mulatta* makes this the most useful large animal model to evaluate interactions of radiation injury and drugs intended for use in humans.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C 0

V.4.1.1.1.2. Column D 4

V.4.1.1.1.3. Column E *128*

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1	PK/PD/toxicity		4		4
2	Efficacy study			64	64
3	Accelerated hematopoietic /GI injury recovery			64	64
Totals:			4	128	132

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

Microchips will be implanted to animals during first sedation prior to irradiation (experiment 2 and 3) with the help of veterinary department staff. For experiment 1, microchips will be implanted before first blood draw on -8 d. BM will be collected under anesthesia in experiment 3. This procedure has been described in detail under section V.4.3.2 (not elaborated here to avoid duplication). Since anesthesia, analgesia, and tranquilizers are known to affect functions of the immune system of animals, we plan to minimize their use during course of the experiment after irradiation (*35-37*).

Pain is not expected from actual irradiation process. The sequelae of high dose of radiation may result in pain but the animals treated with GT3 will experience reduced infections observed in immunosuppressed animals. As observed in mice and a recent pilot study conducted in NHPs, we expect GT3 will enhance hematopoietic recovery

after radiation. This should lead to an accelerated recovery and improved health. Use of other bactericidal agents to combat unresolved infections during the experiments is not warranted in this study as the scientific end points identified above will be abridged. If pain and/or distress are observed, the PI and attending veterinarian staff will be notified to evaluate and determine the appropriate course of treatment. If pain and/or distress cannot be alleviated, the animal will be evaluated for euthanasia.

Some animals may feel temporary discomfort such as nausea, emesis, and diarrhea that may occur after irradiation. In such situation, administration of flavored fluids using a water bottle will be considered. No treatments for radiation-induced behavioral manifestations will be provided as they may interfere with the evaluation of GT3 in post-irradiation recovery.

Use of ketamine and Isoflurane for BM aspiration and LINAC partial body irradiation has been elaborated under section V.4.3.1. Pre-Surgical Provisions and V.4.4.7.

V.4.1.2.2. Pre- and Post-procedural Provisions

Animals will be observed for signs of pain and distress by either the veterinary or research staff at least twice daily. After surgical procedure (such as BM aspiration), NHPs will be monitored at least twice daily for signs of surgical site complication such as infection or trauma.

V.4.1.2.3. Paralytics

No paralytic agent will be used in this study.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched

AGRICOLA: AGRICultural OnLine Access
PubMed: United States National Library of Medicine
IVIS: International Veterinary Information Service

V.4.1.3.2. Date of Search

9/25/2014 – 9/26/2014, 2/20/2015

V.4.1.3.3. Period of Search

AGRICOLA: 1966-2014
Pubmed: 1965-2014
IVIS 1998-2014

V.4.1.3.4. Key Words of Search

1. Pain and radiation
2. Ionizing radiation and pain
3. Ionizing radiation and alternatives
4. Pain and primate and radiation
5. Pain and primate and irradiation
6. Bone marrow aspiration and pain
7. Gamma-tocotrienol and pain
8. Gamma-tocotrienol and pain and radiation
9. Gamma-tocotrienol and side effect

10. Gamma-tocotrienol and toxicity
11. Gamma-tocotrienol and bone marrow aspiration and pain

V.4.1.3.5. Results of Search

#	Keywords of Search	Literature Sources and Periods of search		
		AGRICOLA: 1966 - 2014	**PubMed: 1965-2014**	**IVIS: 1998 - 2014**
		Results of Search		
1	Pain and radiation	24	10966	339
2	Ionizing radiation and pain	0	509	22
3	Ionizing radiation and alternatives	0	159	2
4	Pain and primate and radiation	0	9146	7
5	Pain and primate and irradiation	0	9385	4
6	Bone marrow aspiration and pain	0	280	101
7	Gamma-tocotrienol and pain	0	0	0
8	Gamma-tocotrienol and pain and radiation	0	0	0
9	Gamma-tocotrienol and side effect	0	5	0
10	Gamma-tocotrienol and toxicity	0	28	0
11	Gamma-tocotrienol and bone marrow aspiration and pain	0	0	0

No citations for pain were associated with exposure to ionizing radiation, the use of analgesia in conjunction with sub-lethal doses of ionizing radiation, or the use of analgesia in conjunction with GT3 in all of the searched databases. The use of GT3 was not associated with any reports of pain. Studies conducted in mice show GT3 to be well tolerated and low toxicity even when administered at a high dose (400 mg/kg). No alternatives were cited as being a replacement for irradiation. BM aspiration can be a painful procedure; however, appropriate pain management will be used to minimize the discomfort to animals undergoing BM aspiration.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification

(a): BM aspiration: Animals will get proper analgesics for this procedure and they will not be in a painful or distressful condition.

(b) Irradiation: As stated above, irradiated animals finally succumb due to a compromised immune response and microbial infections. In the event that there is pain and distress for animals, as we have already discussed under above headings, we cannot give anesthetic/analgesic agents to the animals (except at the time of BM aspiration which can't be performed without anesthesia) since these agents interact with the immune system, and in turn will affect experimental results.

There are conflicting reports regarding effects of buprenorphine on the immune system (*38-45*). A majority of reports suggest that buprenorphine influences immune response. Since buprenorphine has been shown to influence various arms of cellular and humoral immune response (*40, 43-45*), we have decided not to use it in our efficacy study. This agent will be administered to NHPs of experiment 3 for biomarker study as

stated above. Non-steroidal anti-inflammatory drugs are not suitable for use in survival study experiments (*46*). Such drugs are being used after BM aspiration.

V.4.2. Prolonged Restraint

Animals to be irradiated will be placed in restraint devices for irradiation in the cobalt facility. Animals will be sedated for this purpose (ketamine 10-20 mg/kg, im).

For blood collection, NHPs will be placed in a primate restraint chair. NHPs will not be left in the restraint chair for more than 30 minutes.

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions

BM aspiration proposed in experiment 3 is considered a surgical procedure. Animals will be assessed for signs of pain and distress by either the veterinary or research staff at least twice per day or as often as needed. Antibacterial prophylaxis may be administered: Cefazolin iv or sc (20 - 25 mg/kg, one time before BM aspiration) or ampicillin (15 - 20 mg/kg, iv or im one time before BM aspiration). Animals will be fasted for 8-12 h before sedation. Animals will be fed a snack (1 full banana or one apple or other fresh fruit) the night (around 8-9 pm) before BM aspiration is scheduled to avoid prolonged fasting Water will not be restricted and animals will have free access to water at all times. The NHPs will be chemically sedated with ketamine (5-15 mg/kg im) or Telazol (5-10 mg/kg im) with (needle 22-25 G). For induction, 3-5% isoflurane in 100% oxygen by mask will be used. For maintenance, 1-3% isoflurane will be administered in 100% oxygen via endotracheal tube or mask. While the animal is under the anesthesia, vital signs will be monitored including SpO_2, pulse, respiration rate and body temperature. Bair Hugger heated surgical table will be used for keeping animal warm. Animal body temperature will be recorded frequently using a rectal thermometer or via microchip during the procedure.

Before BM aspiration, a catheter (20-25 G, 1" long) will be placed in saphenous or cephalic vein. We will administer 100-200 ml bolus of 5% dextrose in normal saline through iv catheter during the procedure to take care of loss of appetite because of fasting, analgesia, and anesthesia.

V.4.3.2. Procedure(s)

The site of BM aspiration may be the iliac crest, scapula, ischial tuberosity, trochanteric fossa of the femur, tibial crest, or the proximal humerous. The site will be prepared as a surgical site: hair will be clipped using # 40 surgical blade and the site scrubbed at least 3 times using either betadine or chlorhexidine and 70% alcohol. Animal will be positioned appropriately (dorsal or ventral or lateral recumbency) depending on the site being used for the BM aspiration. A sterile drape will be used to cover the site. One dose of buprenorphine (0.005 – 0.03 mg/kg, im or sc or iv) will be administered before procedure. A small stab incision (just enough for a 16-20G needle to go through) will be made in the skin at the site of BM aspiration. We will infuse 0.1 - 0.2 ml analgesic (0.5% Marcaine or 2% lidocaine or 4% Articaine) at the BM aspiration site. Then, BM aspiration needle will be advanced through the incision into the cortex of the bone. The stylet is removed from the BM needle. A –5-10 ml syringe will be used to aspirate the BM. The needle will be removed and incision will be closed with tissue glue or a suture after BM aspiration. Either non absorbable nylon or absorbable monocryl

suture will be used to close the skin. Only autoclaved or disposable sterile instruments will be used. Only one attempt will be made per site and only two sites will be attempted at any one sampling point. No more than 3 ml of BM will be collected per animal.

V.4.3.3. Post-surgical Provisions

Animals will be monitored at least twice per day for signs of surgical complications such as infection or trauma. If treatment for such conditions is needed, a VSD veterinarian will provide the appropriate medical intervention: After the procedure Rimadyl (2-4 mg/kg, sc or po every 12-24 h for 1-2 d or longer as determined by the veterinarian) will be administered.

V.4.3.4. Location

The procedure will occur in the VSD surgical suite at the veterinarian's discretion.

V.4.3.5. Surgeon

VSD personnel trained in the specific procedure and aseptic technique will perform surgical procedures.

V.4.3.6. Multiple Major Survival Operative Procedures

V.4.3.6.1. Procedures

N/A

V.4.3.6.2 Scientific Justification

Though BM aspiration is not a major procedure per se, we are conducting repeated aspiration s which may make it fall into this section. Hematopoietic recovery is an important objective of this protocol. To accomplish these objectives, we need to collect BM and blood samplings at different time points in relation to radiation exposure. (stated above in table 5).

Same site can be used for BM aspiration again after a rest period of 14 days in a specific study. Same animal will be used to aspirate BM at specified time points (applicable only for experiment 3). We have identified several sites for BM aspiration in NHPs of experiment 3. There will be no BM aspiration in experiment 1 and 2.

V.4.4. Animal Manipulations

V.4.4.1. Injections

NHPs will be dosed with GT3 or vehicle in the dorsal scapular region (midline). Injections will be performed with a sterile 21 - 25 gauge needle. The site for GT3 injection will be prepared as a surgical site before the injection: hair will be clipped using # 40 surgical blade and the site scrubbed at least 3 times using either betadine or chlorhexidine and 70% alcohol. Injections will be performed by appropriately trained staff listed in this protocol under section VI. Injections for ketamine (5-15 mg/kg) will be administered *im* with 22-25 gauge needle length of ¾-1". The injection volume for this will be 0.1-1.0 ml.

V.4.4.1.1. Pharmaceutical Grade Drugs

N/A

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

Gamma-tocotrienol will be purchased as a formulation in 5% Tween 80 (in saline) (b)(4),(b)(6) (b)(4) Since we are planning to obtain this product from another vendor, we plan to test this agent in 4 NHPs in experiment 1 before proceeding to efficacy study in experiment 2.

Excipient proposed (5% Tween 80 in saline) for the drug formulations is approved by FDA for animal oral and parenteral administration and has been supplied (b)(4) (b)(4) in the past. This excipient is used regularly in parenteral injections in animals at AFRRI. GT3 will be formulated in this sterilized excipient under aseptic conditions to deliver a dose of 200 mg/kg in 0.1 ml. Such formulation has been used parenterally in mice and in NHPs in the past. The supply of new GT3 batch will be tested for endotoxin before use. The pH and osmolality are within the acceptable range as discussed in the earlier protocols. The NHP protocol in which GT3 and this formulation was used is (b)(6) (b)(6)

V.4.4.2. Biosamples

Blood withdrawals will be carried out according to the schedule presented above in experiment 1. Blood will be collected from a peripheral vessel rather than a femoral vein with a 21-25 gauge sterile needle attached to a 2-3 ml syringe, to reduce the chances of bleeding after blood collection in irradiated animals.

The area for the blood draw (samples for bacterial culture) will be swabbed with providone-iodine twice before inserting the needle. Providone-iodine will be swabbed off to reduce irritation to the tissue. For samples collected for other tests, only alcohol swab will be used. The blood sample will be drawn as described above. While withdrawing needle, pressure will be applied at the same time with a sterile bandage to reduce bleeding from the blood draw site. While making regular routine observation of animals, the blood-draw site will be checked for bleeding. In the event the site becomes infected, topical triple antibiotics (bacitracin, neomycin, and polymyxin) or similar agent will be applied.

BM samples will be collected as stated above under section V.4.3.2. GI tissue and other organs will be collected from euthanized animals as discussed under experimental methods above.

V.4.4.3. Adjuvants

No adjuvants will be used.

V.4.4.4. Monoclonal Antibody (MAb) Production

N/A

V.4.4.5. Animal Identification

Animals arrive tattooed and such tattoo will be used for identification. Additionally cage tags or ID's written on cages with permanent markers are used for identification.

V.4.4.6. Behavioral Studies

No behavioral studies will be performed in this protocol.

V.4.4.7. Other Procedures

Management of skin irritation following sc administration

Before the GT3 injection, the site will be prepared as a surgical site: hair will be clipped using # 40 surgical blade and the site scrubbed at least 3 times using either betadine or chlorhexidine and 70% alcohol. Following the sc administration of GT3, the injection site will be closely monitored (manually palpating the site to ascertain any increased sensitivity or mild increases in swelling that aren't visually readily apparent). A VSD veterinarian will be notified if mild redness, skin wrinkling, alopecia or other abnormal clinical signs are observed. If severe redness, irritation, or ulceration of the skin develops, the VSD veterinarian will initiate treatment. Treatment may include topical antibiotics and/or topical anti-inflammatory drugs. The veterinarian will be notified immediately, if the injection site develops into an abscess. If the abscess is less than 1 cm in diameter and remains the same or regresses in size and the NHP exhibits no other adverse clinical signs, continued monitoring is all that is needed. If the abscess continues to increase in size, then the animal may be restrained in a chair, sedated, and the abscess drained then flushed with betadine/chlorhexidine. A diagnostic culture swab will be taken prior to draining the site. The treatment regimen may include lancing the abscess and/or surgical placement of a penrose drain. Additional treatment may include local/oral/parenteral analgesics as suggested by veterinarian. Development of recurring abscess: Animal will be sedated for a more surgically invasive procedure including debridement of the dead necrotic infected tissue (antibiotics and/or anti-inflammatories may be used as suggested by veterinarian).

Transport plan

For whole body irradiation, the sedated animals will be placed in positioning aide devices and will be transported inside a cart to the High Level Gamma Radiation (HLGR) facility or LINAC facility. HEPA filtered transport carts will be used for transportation of animals through common hallways/areas. At no time will the animals be removed from the restraint boxes while outside of the VSD vivarium. At least two personnel will accompany the animals. Additional ketamine may be administered as needed for chemical restraint (animals are not awake during irradiation), and provide animal handling support. After irradiation, the NHPs in their restraint boxes will be removed from the irradiation platform, returned to the transport cart and returned to the vivarium.

For partial body irradiation with LINAC, there will be no shielding since irradiation is carried out after CT scan. Both legs will not be irradiated. This procedure has not been executed at AFRRI before. It is our best guess that it will take 30-45 min. Animals will be sedated with ketamine (details given under section V.4.4.1. Injections) and maintained with isoflurane (3-5%) via mask and they will be intubated with endotracheal tube of appropriate size. Animals will be transportated to LINAC. Then they will be maintained with isoflurane (1-3%) during irradiation procedure. Anesthesia will be monitored as stated under V.4.2.1. Anesthesia/Analgesia/Tranquilization throughout the procedure. During entire procedure veterinarian/veterinarian designee will be available.

V.4.4.8. Tissue Sharing

This is a collaborative research project between AFRRI PI and (b)(6) (b)(6) Tissue samples will be shared (b)(6) (b)(6) and for this purpose tissue sharing form will be completed after approval of the protocol and prior to tissue sharing. Unused biological samples will be retained for additional studies and stored in the PI's laboratory. Studies planned will depend upon additional funding for supplies and staff in future.

V.4.4.9. Animal By-Products

No animal by-products will be used in the proposed study.

V.4.5. Study Endpoint

The primary endpoint used in the efficacy study will be mortality. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress. Moribundity will be used as the endpoint (tracking the symptoms listed in this section) as a surrogate for mortality. Euthanasia will be carried out when one or more of the following signs and symptoms of moribundity are observed as determined by or in consultation with AFRRI veterinarians. Necropsy and histopathological analysis will be performed on all euthanized animals. The following parameters will provide the guidelines for moribundity (all signs will be taken together along with the recommendation of a veterinary science department veterinarian for euthanasia decision):

1. Weight loss: loss of more than 20% body weight from the baseline (NHPs will be weighed whenever we will put animal on the restraint chair attached to scale for blood collection),

2. Inappetance: complete anorexia for 2 days with deteriorating conditions based on clinical examination,

3. Weakness/inability to obtain feed or water: Inability or extreme reluctance to stand which persists for 1 h,

4. Minimal or absence of response to stimuli (animal does not move when prodded),

5. Core body temperature: Below 96.6 ºF following a period of febrile neutropenia (such as >103 0F and <500 neutrophils/ml),

6. Severe acute anemia: <13% hematocrit or <40 g/L hemoglobin (decision in consultation with AFRRI veterinarians),

7. Severe thrombocytopenia (<10,000 platelets/µl) (decision in consultation with AFRRI veterinarians),

8. Other signs of severe organ system dysfunction with a poor prognosis as determined by a veterinarian:
   - 8.1 Respiratory: any dyspnea or severe cyanosis.
   - 8.2 Gastrointestinal: sustained vomiting or diarrhea, obstruction, intussusceptions; peritonitis (transient vomiting and diarrhea are expected results of whole-body gamma irradiation, therefore, as criteria for euthanasia, these symptoms should be severe and life-threatening per se, i.e., hemorrhagic diarrhea to the point of causing anemia as listed above or severe uncompensated dehydration).
   - 8.3 Urogenital: renal failure

8.4 Nervous: sustained CNS depression, seizures, paralysis of one or more extremities.
8.5 Integumentary: non-healing wounds, repeated self-trauma, severe skin infections, indicating severe organ system dysfunction with a poor prognosis.

Surviving animals at the end of the study (60 d post-irradiation) will be euthanized for gross necropsy and tissue collection as described under section V.4.6. Histopathology of various organs will be conducted to study the effects of radiation and drug.

Endpoint for hematopoietic recovery will be analysis of BM cells and endpoint for GI recovery will be analysis of crypt in GI tissue.

V.4.6. Euthanasia

Animals will be humanely euthanized using American Veterinary Medical Association (2012 AVMA) guidelines. These animals will be given sodium pentobarbital intravenously (saphenous or cephalic), needle size 20-25 gauge ¾-1" length, (100 mg/kg, 1-5 ml). Prior to pentobarbital administration, animal will be sedated using ketamine (5-15 mg/kg im). Intracardiac administration will be performed if unable to administer sodium pentobarbital through peripheral veins. The animal will be deeply anesthetized by Isoflurane (1-5%) via mask before giving intracardiac injection. The animals will be euthanized only under the guidance of a staff veterinarian. After sodium pentobarbital administration, the animals will be examined (heart auscultation and pulse) to confirm death. A full body necropsy and histopathology analysis will be performed, after confirmation of the death.

V.5. Veterinary Care

V.5.1. Husbandry Considerations

All non-human primates will be housed individually in cages with at least 4.3 square feet of floor space per NHP in accordance with the Guide for the Care and Use of Laboratory Animals. In addition, every cage will be equipped with built in perches. Excreta and food waste will be removed from inside each indoor primary enclosure, once per day, to prevent the primates from becoming soiled, and to reduce disease hazards, insects, pests, and odors. Care will be exercised when washing the primary enclosure so that the animals remain dry at all times. Primary enclosures will be sanitized every two weeks. The secondary enclosure (room) will be sanitized once per month. Foraging boards as well as other enrichment devices will follow the same schedule as the primary enclosures of the primate.

Special care will be given to animals that have been irradiated as their natural immunity will be suppressed (more prone to infections). Irradiation boxes will be sanitized after every use or more often as needed.

V.5.1.1. Study Room

Study rooms will be assigned by VSD.

V.5.1.2. Special Husbandry Provisions

None

V.5.1.3. Exceptions

None

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care

All animals will be quarantined for 45 days prior to any experiment. For this study, NHPs will arrive at AFRRI after completing quarantine at suppliers's facility. As per Standard Operating Procedures, VSD staff will monitor all animals at least twice daily during morning and afternoon rounds. Any abnormalities will be reported immediately to the on-call veterinarian and PI. In addition, animals will be observed daily by this protocol's research staff. Any sick animal will be observed at least twice a day (early morning and late afternoon/night) by the research staff. When animals become ill or debilitated, a veterinarian will be contacted immediately to assess the animal and provide adequate/emergency care. If the animal becomes moribund, it will be euthanized via intravenous administration of pentobarbital sodium at a dose of 100 mg/kg body weight under the guidance of the veterinarian. Prior to pentobarbital administration, animal will be sedated using Ketamine (5-15 mg/kg intramuscularly) (needle 25 G).

V.5.2.2. Emergency Veterinary Medical Care

The on-call veterinary officer will be available via telephone 24 hours a day. The on-call roster is located outside the VSD conference room (Room # (b)(6) on the bulletin board and at the security watch desk. These procedures are in accordance with VSD SOPs

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

In these studies, all primates will receive regular enrichment [IAW VSDM SOP VS0226 (Environmental Enhancement Plan for NHP)]. Enrichment provided will include sanitizable plastic toys, contact with husbandry and research workers, and visualization of conspecifics. To facilitate the consumption of food post-irradiation, the primates may be provided with an array of drinking and food products that will heighten their interest. Such items will be kool-aid type drinks, jello and yogurt as examples of soft type foods, and bananas, sweet potatoes, and celery with peanut butter as examples of harder type foods. This brief listing is not meant to exclude any drink or food product that will aid in the consumption of diet designated for the animals.

V.5.3.2. Enrichment Restrictions

Fruits or vegetables containing citrulline such as watermelons, cantaloupes, curcubits (squash, pumpkin, zucchini) or cucumbers will be avoided since it will interfere with the study objective (citrulline will be measured in experiment 2 and 3).

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

On-the-job training for AFRRI employees is completed and assessed by the supervisor or designee and documented. When a trainee is judged competent in a given function, a training certification describing on-the-job training is completed by the person responsible for the training and approved by the supervisor or appropriate level of

departmental management. This training is documented in each individual employee's training record at AFRRI. The qualifications indicated below are only applicable for NHP procedures. The personnel listed have also been trained and have experience working with rodents.

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				7 (off-hour observations)	(b)(6)
				1,3 (b,d),4,5,7 (off-hour observation	
				1,3 (b,d),4,5,7 (off-hour observation	
				7 (off-hour observations)	
				7 (off-hour observations)	

The training indicated in the above table represents training that has been completed, (b)(6) will be trained further for performing injections and blood draws. All staff members have received annual training including refresher for 2015. In the table above, initial training years have been listed.

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY

There is no plan to use any potential biohazard. Standard laboratory safety precautions will be observed throughout this study.

VIII. ENCLOSURES

Form 310

List of references

X. ASSURANCES

Protocol Title: Advanced Development of Gamma-tocotrienol as a Radiation Countermeasure in Nonhuman Primates (*Macaca mulatta*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): (**AM**) / **AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / (**WILL NOT**) be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

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(b)(4),(b)(6)

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(b)(4)

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(b)(4),(b)(6)

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(b)(4) (b)(6)

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DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	3/17/2015	2nd Resubmission Date:	5/22/2015
Approved/Returned for Revision:	4/6/2015	Approved/Approval Withheld by IACUC:	5/26/2015
1st Resubmission Date:	4/22/2015	**EXPIRATION DATE:**	5/25/2018
Approved/Returned for Revision:	5/13/2015	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Radioprotective efficacy of new formulations of tocotrienols in mice, *Mus musculus*

IV. PRINCIPAL INVESTIGATOR:

(b)(6) **Ph. D**
Research Biologist, AFRRI-SRD
Office: (b)(6) Fax: (b)(6)
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD, LTC, USA** Date

Head, Scientific Research Department, AFRRI
Telephone: (b)(6)
(b)(6)

- Or –

(b)(6) **PhD, CDR, USN** Date

Head, Scientific Research Department, AFRRI
Telephone: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACVPM, DACLAM** Date

Head, Veterinary Sciences Department
Armed Forces Radiobiology Research Institute
(b)(6)
Office: (b)(6)
Fax: (b)(6)
Email: (b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Safety and Occupational Health Specialist, USUHS
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**
IACUC Chair, (b)(6) Scientific Research Department, AFRRI
Telephone (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: Radioprotective efficacy of new formulations of tocotrienols in mice, *Mus musculus*.

PRINCIPAL INVESTIGATOR:
(b)(6) Ph. D
Research Biologist (AFRRI – SRD)
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(b)(6)

CO-INVESTIGATOR: (b)(6) PhD,
Research Biologist (AFRRI – SRD)
Office: (b)(6) Fax (b)(6)
(b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBERS: (b)(6)

I. NON-TECHNICAL SYNOPSIS

Protection of first responders and military personnel deployed in a radiation scenario is a major concern for both the military and the homeland defense. We have shown that various analogs of vitamin E (tocopherol and tocotrienol), also designated as tocols protect and enhance survival in mice exposed to lethal doses of irradiation. The present proposal will compare the beneficial effects of delta- and gamma-tocotrienol (DT3 and GT3) with a commercially available dietary supplement (b)(4) (b)(4) (b)(4) We will compare the 30 day survival efficacy of three specially formulated emulsions in (b)(4) DT3 and GT3 in mice exposed to gamma-radiation (Cobalt-60). We will conduct basic toxicity studies after a subcutaneous injection of the drugs, followed by survival studies. Depending on experimental results, additional studies such as a. optimization studies (drug route, concentration, multiple administrations and time of drug administration), b. Dose reduction factors (DRF) studies (testing efficacy of promising candidates using various radiation doses), and c. hematological and mechanistic studies (to elucidate drug response) will be conducted with (b)(4) representing mostly DT3) and GT3 formulations.

II. BACKGROUND

II.1. Background: Threat characterization for radiologic and nuclear events (intentional or accidental) is defined by the nature of the incident [1]. Threat devices or sources include radiologic dispersal devices, radiologic exposure devices, a sophisticated nuclear weapon, improvised nuclear devices, nuclear power plants, and stolen or mishandled medical radiologic sources [2-5]. AFRRI has been in the forefront in evaluating radiation countermeasures against the deleterious effects of ionizing radiation (b)(4)

Tocols as radiation countermeasures: AFRRI investigators and their collaborators have extensively studied tocols including tocopherols, tocotrienols, esters such as tocopherol succinate, and found those to be radioprotective when administered prior to the total body radiation exposure (b)(4) The current protocol will allow us to test several formulations of tocols with the support of a commercial partner (b)(4) As mentioned in the nontechnical summary, (b)(4) and the company has interest in advancing tocols as radiation countermeasure.

Product Development Considerations:

T3 Sources/Manufacture: DT3 and GT3 are both derived from natural plant sources, the three most common sources being palm, rice bran, and annatto seeds [13, 14].

(b)(4)

(b)(4) is manufactured through a molecular distillation process. As a dietary supplement for oral use, it has a Technology Readiness Level (TRL) of 9 using the Portfolio Tracking Tool used by the Public Health Emergency Medical Countermeasures (PHEMCE), the Dept. of Health and Human Services (HHS), the department of Defense (DoD), and other United State government agencies (b)(4),(b)(6) Acquiring highly purified single T3s from the available natural sources requires a more complicated manufacturing process. This could have significant fiscal impacts in product development for bulk-level manufacturing (b)(4),(b)(6) (b)(4),(b)(6) is already commercially available as an oral dietary supplement and food additive, manufactured under cGMP conditions. Neither highly purified DT3 nor GT3 has been produced at bulk-scale to date (b)(4) has a Generally Regarded as Safe (GRAS) status (GRAS notice # (b)(4) with a letter from the U.S. Food and Drug Administration (FDA) dated May 13, 2014 stating that there were no questions about the product regarding its intended use "as a source of vitamin E in food at a level of up to 100 milligrams of tocotrienols per serving". There is one IND on file for oral use of (b)(4) in a clinical trial for osteoporosis at up to 600 mg/day.

(b)(4)

Tocols in the clinic: Tocotrienols are being tested for multiple medical indications. Promising results have been demonstrated for these vitamin E isomers (or vitamers) for multiple chronic diseases or conditions including cardiovascular disease, hypertension, hypercholesterolemia, diabetes, osteoporosis and in cancer prevention and treatment [16-26].

(b)(4) as well as to pursue additional funds from (b)(4) (b)(4) has been in consultation with the (b)(4) (b)(4) (use of GRAS emulsifiers) for human use. These formulations

(proprietary) based on [redacted] on behalf of [redacted] and will be tested at AFRRI using the current protocol.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched BRD, NIHRePORTER, PUBMED , Embase
II.2.2. Date of Search 02-02-2015 to 02-06-2015
II.2.3. Period of Search The search period extended from 1998 to 2005 for BRD, 2009-present for NIHReporter, 1970 to present for PubMed, 2006 to present for Embase.

II.2.4. Key Words of Search Each individual term Gamma-tocotrienol, Delta-tocotrienol, [redacted] + (emulsifier) + (radiation), + (radioprotection), + (radioprotection+mice)

II.2.5. Results of Search
Table 1. Literature search resulted the following hits as tabulated below:

KEY WORDS	PUBMED	NIHRePORTER	BRD	Embase
Gamma tocotrienol + emulsifier	310	408	30	418
Gamma tocotrienol +radiation	31	366	26	41
Gamma tocotrienol + radioprotection+mice	8	358	9	5
Delta tocotrienol + emulsifier	161	372	17	192
Delta tocotrienol +radiation	8	166	13	16
Delta tocotrienol +radioprotection+mice	2	363	5	4
[redacted] + emulsifier	0	0	0	0
[redacted] + radiation	0	0	0	0
[redacted] + radioprotection+mice	0	0	0	0

Table 2 provides a summary of these searches from NIHRePORTER database

Table1. Project Title	Project Number	Contact PI / Project Leader
Vitamin E Neuroprotection:Novel molecular mechanisms	5R01NS042617-08	SEN,CHANDAN K
Gamma tocopherol as an effective anti cancer agent for colon cancer	5R21CA133651-02	JIANG,QUING
Somatostatin analogs as countermeasure against intestinal radiation toxicity	5U19A1067798-10	HAUER-JENSEN,MARTIN
Intervention of pancreatic oncogenic pathways with dietary tocotrienols	5R01CA129227-05	MALAFA,MOKENGE P
Predicting liver function after irradiation	5R01CA132834-03	CAO,YUE
Radioprotection of the immune system	5U19A1067769-05	MCBRIDE, WILLIAM H
Lung radioprotection by inhibition of TNF-alpha and	5R01CA127477-04	ZHANG, MING

TNF-beta1		
Organismal radioprotection through pharmacological quiescence	5R44A1084284-05	STRUM,JAY COPELAND
Evaluating radiation medical countermeasures	Y01AI1759-1-0-2	Unavailable
Hematopoietic targets of Radiation: Identification and mitigation	5RO1A1080401-05	PALIS,JAMES
Mitochondrial targeted small molecule radiation mitigators	5U19A1068021-10	GREENBERGER, JOEL S
Functional characterization of ATM gene product	5RO1CA071387-14	KASTAN,MICHEAL BARRY
Cell based small molecule screens of novel radioprotective agents	5U19A1067751-05	D'ANDREA,ALAN D
Enhancing cancer treatment by normal tissue protection	1RO1CA184168-01	ROSEN,ELIOT M

Summary of the Search: None of these published references are duplicative of the current protocol. (b)(4) (b)(4)

III. OBJECTIVE / HYPOTHESIS: The objective of the present protocol is to compare the radioprotective efficacy of the following compounds with GT3 in Tween-80 used previously at AFRRI:
a. (b)(4) in a formulation containing emulsifying and stabilizing agents,
b. DT3 formulation containing (b)(4) and emulsifying agents,
c. GT3 formulation in (b)(4) and emulsifying agents
It is hypothesized that (b)(4) (b)(4)

IV. MILITARY RELEVANCE: In the event of a nuclear related accident or terrorist activity, the first responders including military, fire and police personnel will be at risk for exposure. The risks include mortality at higher doses such as 10 Gy and above, while exposure to 2-8 Gy may result in severe hematopoietic ablation, GI injury and ensuing infectious diseases (b)(4) Additional constraints in a mass casualty scenario such as significant delays in transport, disruption of communication and overfilling of limited hospital resources would increase the risk associated with exposure to ionizing radiation (b)(4) The availability of safe and effective countermeasures would greatly reduce the radiation risk. The efficacy of tocols, administered prophylactically in mice will allow us to advance one of the tocols (to be recommended after comparing the efficacy data from this study and programmatic hurdles) in large animals for FDA approval and national stock pile.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

General experimental design: The experimental design for this protocol is divided in to 5 subsections and each subsection covers a specific area:

Section v.1.1.: Basic toxicity
Section v.1.2.: Proof of principle - survival studies
Section v.1.3.: Drug optimization – survival studies
Section v.1.4.: Dose reduction factor - survival studies
Section v.1.5.: Hematological studies - sublethal dose of radiation

Section V.1.1.: Basic toxicity

We will adopt the modified toxicity protocol Optimal (non-toxic) Drug Dose for screening (ODDS) which has been used in our previous protocols (b)(6) (b)(6) at AFRRI to determine maximum tolerated dose (MTD). The purpose of using the ODDS method is that it allows testing in one gender (males in the present protocol) and requires fewer animals when compared to the FDA protocol. If the drug doesn't show any signs of basic toxicity (using the clinical sign discussed in Policy#10, Appendix 1), then the drug dose will be escalated 2 to 3 fold. However, if toxicity is observed, then the drug dose will be reduced by 2 to 3 fold (b)(4) Finally, for survival studies we will use $1/4^{th}$ of the observed non-toxic dose.

In our previous experiments using a protocol P# (b)(6) tocols in 5% Tween-80 did not show systemic toxicity although injection site inflammation was noted at a dose of 800 mg/kg (b)(4) Here we propose to start the toxicity study with 400 mg/kg dose (4 times the dose proposed for survival studies, section V.1.2). The dose will be reduced to 200 mg/kg if we observe inflammation in local injection site. However, if there are no observable inflammation in the injection site with 400 mg/kg drug, we will scale up the dose to 800 mg/kg (the new formulation with better emulsification of fat soluble tocols, may still be non- toxic at this high dose). We do not intend to increase the drug dose any further (b)(4)

The drug will be administered as a single subcutaneous (sc) administration under the nape of the neck. A vehicle with a similar composition but without the drug will be used as control. A maximum of six animals per drug dose will be used to evaluate toxicity. Animals will be monitored daily following IACUC Policy#10 for acute (first hour and then every hour up to 4 h) and chronic (up to 14 days) signs of toxicity using the observation codes listed in Appendix 1. Signs of acute toxicity include decreased activity, squinting eyes, hunching, labored breathing or injection site swelling [29]. If there is a sign of toxicity, the PI will facilitate the late PM and early AM checks (not more than 10 h apart) in addition to two times a day checks following Policy#10 to prevent animal distress. All morbid mice will be monitored carefully and their conditions scored for documentation (Please see section V.4.5). If animals treated with the 400 mg/kg dose show any sign of skin inflammation, one group of mice will be euthanized on day 14 and the other group of mice will be monitored up to day 30 after drug administration to allow the skin inflammation site to recover (Table 3). However,

animals treated with either 200 or 800 mg/kg of the test articles (TA) will be euthanized on day 14 after drug administration.

Table 3: Acute toxicity study (14 and 30 day) after a single sc drug administration

Drug Dose (mg/kg)	Groups (day 14 after drug administration)	Groups (day 30 after drug administration)	# of groups	# of animals per group	End point (whole blood)
400	Vehicle (AO + E), (b)(4) GT3(AO+E), DT3(AO+E), and GT3 (5% Tween-80 in PBS)	Vehicle (AO + E), (b)(4) GT3(AO+E), DT3(AO+E), and GT3 (5% Tween-80 in PBS)	10	6	CBC & Serum chemistry
200 or 800	(b)(4) GT3(AO+E), DT3(AO+E)	None	3	6	CBC & Serum chemistry
	Total no. of animals required = 13 groups x 6				78

AO+E-(b)(4) + emulsifying agents

Note: Injection site skin will be stored in formalin for histopathological analysis if needed.

Body weight changes: Each animal in the study will be weighed prior to drug treatment and weight loss/gain will be followed individually on days 2, 4, 7, 10, and 14 for groups terminated on day 14 and on day 21 and 30 for animals to be euthanized on day 30 (b)(4) Animals will be weighed at a specific time of the day (will be determined later). It should be noted that ODDS method only uses day 14, however, for this study, based on discussions with the supplier and the tocol team at AFRRI, an additional time point of 30 day is included in order to observe any delayed recovery of hematological parameters, body weight changes and recovery (if any) of the injection site inflammation. However, for an additional dose, 200 or 800 mg/kg, animals will be monitored only for 14 days (b)(4) following the standard protocol.

Hematological parameters and serum biochemical analysis: Blood will be collected under anesthesia by cardiac stick or inferior vena cavae (see section V.4.4.1 for more description about the procedure) on either day 14 or 30 after drug administration. Syringes will be wetted with Heparin (1000 units/ml solution). Part of the collected blood will be transferred to EDTA tubes (200 µl) for hematological analysis. The rest of the blood will be transferred to serum tubes (300-400 µl) for serum samples, and will be stored at -20C until analysis (b)(4) Hematological parameters (CBC/differential) and serum biochemistry will be analyzed using an Advia 2120 hematology system from Siemens Corporation or any other validated system at AFRRI.

Procedure: There will be six animals per group, and will be housed three per box. Each mouse will be identified by standard ear punch or tattoo system. Each cage will have an animal with a right ear punch, left ear punch and no punch. In the case of tattoo system, each animal will be tattooed on the tail prior to starting the experiment. The procedure is described under section V.4.4.1 (surgical methods or procedures). Individual mouse will be monitored for body weight loss and clinical signs of morbidity.

All formulations will be tested via subcutaneous route.

Dose 1 (400 mg/kg)
Day 14: 5 groups (Table 1) x 1 route (*sc*) x 6 mice per group = 30
Day 30: 5 groups (Table 1) x 1 route (*sc*) x 6 mice per group = 30

Dose 2 (200 or 800 mg/kg)
Day 14: 3 groups (Table 1) x 1 route (*sc*) x 6 mice per group = 18

Subtotal = 78

All mice will be placed in Column E (V.4.1.1.1.4. Pain Category Assignments, Table 6) since they might experience unalleviated experimental pain or distress due to drug or vehicle administration.

Section V.1.2. Radiation survival study:

There will be16 animals per group. Drug will be administered (sc) prophylactically 24 hour prior to irradiation. Animals will be exposed to Cobalt 60 gamma radiation (9.75 Gy at 0.6 Gy/min). This dose represents the LD90/30 dose for CD2F1 male mice (12-14 weeks old). Table 2 provides details of the experimental design. There will be four test article-treated (TA) groups and one control group (CA). 100 mg/kg will be used because it will be the ¼ MTD if 400 mg/kg is found to be non-toxic. GT3 in Tween-80 will be considered the positive control for this study. Animals will be monitored for 30 days post-irradiation.

Table 4: Survival efficacy of (b)(4) DT3 and GT3 in special formulation as compared to GT3 in Tween-80

Radiation dose 9.75 Gy (app. $LD_{90/30}$)	1 rad dose
Vehicle (AO+E), (b)(4) GT3(AO+E), DT3(AO+E), and GT3 (5% Tween-80 in PBS)	5 groups
Drug administration, -24 h, sc	1 treatment
Drug dose ~100 mg/kg*	1 drug dose
Mice/group	16
30 day survival	1 time point
Iterations	2
Total no. of animals required	1 X 5 x1 x 1 x 16 x 1 x 2 = 160
AO+E=(b)(4) + emulsifying agent(s)	
*The dose was selected based on our previous published work on DT3 and GT3. This may change based on the toxicity data of (b)(4) GT3 and DT3 formulations	

Sub-Total CD2F1 mice for efficacy studies = 160

All mice will be placed in Column E (V.4.1.1.1.4. Pain Category Assignments, Table 6) since they might experience unalleviated experimental pain or distress due to exposure to radiation.

Section V.1.3.

Optimization studies: Optimization studies will be initiated with all three new formulations (b)(4) DT3 in AO+E, and GT3 in AO+E). A vehicle group (AO+E) will be included in the study as required. Animals for three vehicle groups are included since each experiment may consist of only one formulation. All drug doses (3

per formulation, 50,100 and 200 mg/kg, please note that these doses were selected based on our previous published work on DT3 and GT3, these may change based on the toxicity data of (b)(4) GT3 and DT3 formulations) will be administered at -24 h for the drug optimization study. An amendment will be submitted to the IACUC if doses other than 50, 100, 200 mg/kg are to be used. However, for time optimization study only one of the drug doses from the drug optimization study will be used. The time optimization will include 3 different pre-irradiation time points (-24 h, -12 h, and -4 h). The radiation dose will be 9.75 Gy at 0.6 Gy/min for all of these optimization studies. Survival will be monitored for 30-day post-irradiation.

Drug dose optimization study:

Three (3) experimental groups (new formulations of (b)(4) DT3, and GT3) X 3 drug doses (50, 100, and 200 mg/kg) x 1 radiation dose (LD 90/30) x 1 route (*sc*) x 16 animals/ group Subtotal = **144**

Three (3) vehicle groups (assuming each formulation is tested separately (on different days) x 1 dose (original formulation) x 1 radiation dose x 1 route x 16 animals/group Subtotal = **48**

Time optimization study:

Three (3) different time points (-24 h, -12 h, -4 h) x 4 treatment groups (1 optimum drug dose for 3 drugs + 1 vehicle) x 1 radiation dose (LD90/30) x 1 route (*sc*) x 16 animals per group
Subtotal = **192**

All mice will be placed in Column E (V.4.1.1.1.4. Pain Category Assignments, Table 6) since they might experience unalleviated experimental pain or distress due to exposure to radiation.

Section V.1.4. Determination of Dose reduction factor (DRF):

Among the new formulations, two will be used for the DRF study. Since the (b)(4) formulation (b)(4) either (b)(4) formulation (b)(4) or DT3 (100% DT3) along with the GT3 formulation (100% GT3) will be used to conduct DRF studies, since these formulations may be advanced to large animal studies. Another group that will be included for comparison will be the vehicle (AO+E) group. Drug or vehicle will be administered 24 h prior to radiation (time of administration based on our (b)(4) administration time may change if the proposed time is not the optimum time in experiment V.1.3 Time Optimization Study). Six radiation doses will be selected for the drug groups. Similarly 6 radiation doses will be chosen for the vehicle group, but these may change depending on the survival profile of vehicle groups at higher radiation doses. Radiation doses will be selected so that the lowest dose will give 100% protection and the highest dose will give 100% mortality. Four additional intermediate doses will be selected, which will provide varying degrees of protection. From the probit analysis, LD50/30 radiation dose will be calculated for (b)(4) vehicle and tocols From these data, the DRF will be calculated as follows:
DRF = LD50/30 radiation dose for tocol / LD50/30 radiation dose for vehicle

Six radiation doses (9, 10, 10.5, 11, 11.5 and 12Gy)* x 2 drug groups (b)(4) DT3, or GT3) x 16 animals/rad dose Sub total (6 x 2 x 16) = **192**

Six radiation doses (8.5, 9, 9.5, 9.75, 10 and 10.5 Gy)* x 1 vehicle group (AO +E) x 16 animals/group Subtotal = (6 x 1 x16) = **96**

*These radiation doses are projected based on an earlier probit study and are considered a guidance. The appropriate radiation doses will be determined using the new dosimetry map (07/09/2014). An amendment will be submitted to the IACUC if the radiation doses change from what is stated here.

All mice will be placed in Column E (V.4.1.1.1.4. Pain Category Assignments, Table 6) since they might experience unalleviated experimental pain or distress due to exposure to radiation.

Section V.1.5. Amelioration of peripheral blood pancytopenia.
One of the earliest expressions of radiation effects is pancytopenia in peripheral blood as a result of shut-down of cell production in the bone marrow. Particular attention will be given to radiation-induced neutropenia (innate immunity and infectious complications) and thrombocytopenia (hemorrhagic complications).In this experiment one radiation dose will be used – 7Gy (hematopoietic range). The experimental design (b)(4) will be followed as described previously (b)(4) Drug or vehicle will be administered SC 24 h prior to radiation (time of administration based on our published data on GT3 and DT3, administration time may change if the proposed time is not the optimum time in experiment V.1.3 Time Optimization Study). An amendment will be submitted to the IACUC for any proposed changes to the time of drug administration. Blood will be drawn from deeply anesthetized mice at 0 time, 1 day, 4 days, 7 days, 10 days, 20 days, and 30 days after irradiation. Blood will be collected from the inferior vena cava or cardiac stick (as described in section V.4.4) into heparinized syringes, transferred to EDTA tubes, and total leukocytes, erythrocytes, neutrophils, platelets, lymphocytes, and monocytes will be counted using Advia 2120 cell counter (Siemens) (b)(4)

Without radiation:
Three drug formulations (b)(4) or DT3, GT3, vehicle (AO+E)) x 7 time-points x 6 mice/ group x 1 route of administration x no radiation Subtotal (3 x7 x 6) = **126**

With radiation
Three drug formulations (b)(4) or DT3, GT3, vehicle (AO+E)) x 7 time points x 6 mice/group X 7 time-points X 6 mice/group x 1 route of administration 1 radiation (7 Gy)

Subtotal (3 x7 x6) = **126**

All mice will be placed in Column E (V.4.1.1.1.4. Pain Category Assignments, Table 6) since they might experience unalleviated experimental pain or distress either due to exposure to radiation or deep anesthesia during blood draw.

V.2. Data Analysis All data will be reported as means with standards errors or percentages where applicable.

Experiment V.1.1 (ODDS) – This is a qualitative study so no additional statistics will be conducted.
Experiments V.1.2 (preliminary survival study) and V.1.3 (Optimization study)- Using calculations based on log rank test with 80% power and 5% two-sided significance level, we have determined that this protocol design has adequate power based on our previously published studies [redacted] Differences in 30-day survival (Experiments V.1.2 and V.1.3) will be analyzed using Fisher's exact test. Experiments V.1.2 will be repeated a maximum of 2 times with 16 mice per group, thus providing adequate statistical power for any conclusions. (b)(4)
Experiment V.1.4. (Dose Reduction Factor study) - Probit analysis will be used to generate the LD50 of the drugs under study using PASW Statistics (version 18). In our past experience, 16 animals per dose with six radiation doses has been sufficient to determine a probit line fit to mortality data for DRF calculations [redacted] At least three out of the six doses should sustain survival other than 100 or 0 percent. Finney's methods will be used to make probit fits and estimating DRF [redacted] 30]. (b)(4)
Experiment V.1.5: For testing the overall difference between groups (averaging over time points), a repeated measures ANOVA with 6 animals per group measured at 7 time points will have 80% power to detect a difference of 0.91 standard deviations if the within-subject correlation is 0.2. For comparisons between groups at a single time point, the proposed sample size will have 80% power to detect a difference of 1.8 standard deviations between groups, based on a t-test for independent samples with a 5%, two-sided significance level. Previous studies have yielded differences of this magnitude [redacted] (b)(4)

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered Although high throughput screening and computer modeling have advanced significantly in recent years in identification of promising drugs, it would be simplistic to assume that cell systems or artificial intelligence modeling can replace the data that can be generated by using an animal model. Irradiation of animals will result in a cascade of changes including death of stem and progenitor cells in bone marrow, alterations in signal transduction pathways, multiple organ dysfunctions etc. due to differing radiosensitivities of organs. Currently, there are no alternatives in existence that can predict or compute the complexity of the response to total body irradiation. The US FDA recognizes the ethical considerations involved in testing the efficacy of radioprotective drugs in humans. Therefore FDA requires preclinical drug assessments (safety and efficacy) to be performed using both small and large animal models [31] prior to granting approval for safety testing in humans.

V.3.2. Animal Model and Species Justification Recent advances in molecular techniques have allowed significant improvements in understanding the cellular and biochemical processes. These advances are possible only due to whole animal modeling of human ailments. The mouse, in particular, has high sequence homology to humans at a genomic level as well as similar hematological and immunological response to radiation. Mouse models not only confirm primary pathological processes

but have also provided a venue for studying basic molecular, cellular, biochemical, and cytological processes. Furthermore, there are extensive data on the mouse for comparison, review and analysis. The practicalities of breeding and housing these small mammals have made the mouse model invaluable. The Principal Investigator's group at AFRRI has significant training and experience in handling mice in radiation countermeasure studies. In this protocol, we will use male CD2F1 mice since this a historical strain AFRRI has been using for last 30 years. The PI also has used this strain in previous studies. To compare the previous data with this study, PI will use only male CD2F1 mice.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species: Mouse, *Mus musculus*

V.3.3.2. Strain / Stock: CD2F1,

V.3.3.3. Source / Vendor: CD2F1 from Harlan Inc. or any other approved vendors

V.3.3.4. Age: (procurement) 7-8 weeks

V.3.3.5. Weight (delivery) 22-26 grams

V.3.3.6. Sex Males have been used historically and will continue to be used in this study.

V.3.3.7. Special Considerations Commercially procured animals must be adventitious disease free. Specific pathogen-free, including *Pseudomonas aeruginosa*, and pasteuralla sp. All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus, *Mycoplasma pulmonis*, and *Clostridium piliforme*. Endoparasite- and ectoparasite-free.

V.3.4. Number of Animals Required (by species) (Table 5)

Animal (genus, species and common names)	**Original**
CD2F1	1162

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement: PI will take necessary steps for all personnel in the project to be rigorously trained in handling techniques (ear-punch, injections, and routine cage-side observations) to minimize distress and pain to the animals. Specifically, during capture and restraint of the rodents, care will be taken to ensure that handling does not

contribute to distress. Topical ointment/lubrication gel will be used, if necessary, to prevent drying of the cornea as well as at the site of drug injection in consultation with attending veterinarian and veterinary staff.

V.3.5.2. Reduction Although basic toxicity and survival will be carried out on all three formulations, additional studies will be done with the best formulation. Positive controls and negative controls will be used to confirm robustness of experimental conditions. In addition, control groups (such as vehicle) will be shared whenever possible between formulations that are dissolved in similar vehicles. In classical toxicology testing, groups of 10-20 mice of both sexes are routinely used. In using the ODDS method (current protocol), we will reduce the number of animals to 6 per group, and use only males, thereby reducing animal numbers significantly.

V.3.5.3. Replacement It is not feasible to use non-animal systems in place of actual animal models to address the questions in this project. Rodents are considered sentient and hence rodents will be used for screening large number of potential radiation countermeasures.

V.4. Technical Methods
V.4.1. Pain / Distress Assessment
V.4.1.1. APHIS Form 7023 Information
V.4.1.1.1. Number of Animals 1162

V.4.1.1.1.1. Column C 0 (# of animals)

V.4.1.1.1.2. Column D 0 (# of animals)

V.4.1.1.1.3. Column E 1162 (# of animals). These mice will be either treated with drug or vehicle or irradiated and will not receive any intervention for pain relief. However, they will be euthanized if found moribund. The mice (#126, expt V.1.5) not receiving any radiation, will be under deep anesthesia for blood collection.

V.4.1.1.1.4. Pain Category Assignments: (Table 6)

Exp. #	Experimental/Control Group	C	D	E	Totals
V.1.1.	ODDS safety/toxicity study			78	78
V.1.2.	Radiation survival studies			160	160
V.1.3.	Optimization studies			384	384
V.1.4.	Dose reduction factor studies			288	288
V.1.5.	Hematology			252	252
Totals:				1162	1162

V.4.1.2. Pain Relief / Prevention. Animals can experience pain and distress. Minimizing and alleviating pain in laboratory animals without compromising the methodological integrity of a research project is important both ethically and legally. Mice often exhibit pain and distress with only subtle changes in their behavior [32]. Potential signs associated with pain and distress in mice includes decreased food and water consumption, weight loss, self-imposed isolation/hiding rapid breathing, opened-

Mouth Breathing, abdominal Breathing, decreased movement, abnormal posture, muscle rigidity, twitching, trembling, tremor etc as included in Appendix 1 (sample scoring sheet for clinical observations with criteria for rodent euthanasia) in IACUC policy#10. PI and her staffs are trained to assess the presence or absence of pain or distress in animal and score them appropriately.

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization The protocol will not use anesthetics or analgesics during radiation since they will adversely affect the outcome of the experiments. The proposed study is to determine the effects of radiation and countermeasures on the function of the immune and blood forming systems. Use of any analgesics and anesthetics would interfere with the function of the immune system and therefore compromise the interpretation of the results [33]. Topical antibiotics will be applied if wounding occurs via in-cage fighting, under the guidance of the clinical veterinarian in accordance with standard treatment measures.

Anesthesia using standard Isoflurane apparatus under the guidance of the VSD will be carried out in mice for blood collection. All personnel will be extensively trained for efficient administration of the agents as well as to minimize handling stress.

Animals will be placed in the Isoflurane chamber and delivered a metered amount of 3-5% Isoflurane mixed with 100% oxygen until all voluntary motor movement ceases and the animal is recumbent for 10 seconds. Animal's respiration rate will be closely monitored through the Plexiglas chamber during induction of anesthesia. When animals are recumbent, they will be removed from the chamber and their nose/mouth placed in the anesthesia apparatus breathing/gas administration cone for maintenance of anesthesia at 1-3% Isoflurane and 100% O_2.

V.4.1.2.2. Pre- and Post-procedural Provisions Mice will be housed under standard conditions both before and after radiation procedures in accordance with current VSD rodent husbandry Standard Operating Procedures (see section V.5).

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched AGRICOLA, PubMed, EMBASE

V.4.1.3.2. Date of Search 02/09/2015

V.4.1.3.3. Period of Search AGRICOLA: 1975-2004, EMBASE 1974-present PubMed ~1970 to present.

V.4.1.3.4. Key Words of Search

Radiation and pain, [Gamma tocotrienol, Delta tocotrienol, (b)(4) toxicity and pain, analgesia, alternative, humane, alternative methods to blood collection by cardiac stick and inferior vena cava.

In order to increase specificity of literature review, several combinations such as radiation plus specific drug, radiation plus specific drug plus mice, alternatives to blood collection, Delta-tocotrienol, Gamma-tocotrienol, (b)(4) AND toxicity AND pain were evaluated.

Results of Search:

Table 2:Summary of Database Keyword Searches			
Keywords	Hits		
	PubMed	AGRICOLA	EMBASE
Gamma-tocotrienol AND radiation AND pain	0	0	0
Delta-tocotrienol AND radiation AND pain	0	0	0
(b)(4) AND radiation AND pain	0	0	0
Gamma-tocotrienol AND analgesia AND pain	0	0	0
Delta tocotrienol AND analgesia AND pain	0	0	0
(b)(4) AND analgesia AND pain	0	0	0
Radiation AND mouse AND pain	234	0	224
Gamma-tocotrienol AND toxicity AND pain	0	0	4
Delta-tocotrienol AND toxicity AND pain	0	0	2
(b)(4) AND toxicity AND pain	0	0	0
Alternatives to blood collection by cardiac stick and inferior vena cava	0	0	0
Pain AND analgesia And mice	1873	37	2236
Radiation AND mouse AND pain AND alternative	5	0	11
Radiation AND humane	12	1	34

There are no alternatives to irradiation. While radiation itself does not cause pain or distress, it induces a number of changes in the body that alters immune response and destroys hematological tissues, resulting in opportunistic infections, and multiorgan dysfunction leading to death. It is anticipated that animals exposed to high lethal radiation doses will become moribund. Unfortunately, literature clearly demonstrates that use of analgesics alters radiation response and therefore leads to increase in animal number for better statistical results or for iteration of entire experiments. Since the purpose here is to compare the efficacy of the three experimental drugs we will be unable to use analgesics and anesthetics because they may interfere with the innate system [33].

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: Because exposure to irradiation is potentially lethal or causes debilitating effects in humans, it is ethically impermissible to test the effectiveness of radiation countermeasures on human volunteers. Irradiation compromises the immune and blood forming systems leading to mortality. The radiation-induced mortality and potential percentage increase of survivors over 30 days using potential radiation countermeasures are study endpoints for this protocol. Administration of drugs and analgesics is not an option since it is well documented that they interact with the immune system which will confuse the interpretation [34-36]. Pain relieving measures are not used because such measures may compromise the experimental integrity of the study. All moribund animals will be euthanized by overdose of CO_2 inhalation and cervical dislocation.

V.4.2. Prolonged Restraint : The experimental animals will receive radiation at a dose rate 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (dosimetry 07/09/2014; or the latest dosimetry map) using plastic racks. Mice will be inserted into

the standard radiation boxes just prior to irradiation (~15 min of exposure time) and taken to the cobalt facility for irradiation. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period. They will be monitored daily following policy 10 (3-4 times a day during critical period with no more than 10 h interval between late night and morning check and at least twice a day during non-critical period) for 30 days and euthanized at the completion of the observational period.

V.4.3. Surgery: N/A
V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure(s): N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon : N/A

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.4. **Animal Manipulations:**
V.4.4.1 Ear punch: Three animals/group will be housed per shoe box during the toxicity study (section V.1.1.). They will be identified by ear punch (one animal will have a right punch, the second will have a left punch and the third will have no punch).

Procedure for ear-punch
a. Soak the ear punch (Fine Science Tools, Cat# 24212) in a disinfectant (100% ethanol) before use and between animals.
b. Place the device on the pinna of the ear (external ear) where the skin is thin avoiding the cartilage.
Press firmly to punch a circular hole through the ear

Tattoo procedure: VSD SOP will be used.

Irradiation: These will follow the standard AFRRI protocol with mice placed in restrainers shortly prior to exposure, irradiation at the cobalt facility and return to VSD for further monitoring. Briefly, the experimental animals will receive radiation at a dose rate of 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (dosimetry 07/09/2014) using plastic racks. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period. They will be monitored daily following policy 10 (3-4 times a day during critical period with no more than 10 h interval between late night and morning check and at least twice a day during non-critical period) for 30 days and euthanized at the completion of the observational period. In case of non-lethal exposures, mice will be harvested at specific time-points after irradiation, and tissues collected after terminal blood draw.

Blood collection: Mice will be anesthetized as described under section V.4.1.2.1 (anesthesia). Blood draw for hematology is collected either by cardiac stick or from the inferior vena cava (IVC). The animals will be deeply anesthetized in the rodent anesthesia machine; the tail will be pinched for reflexive movement, indicative of insufficient anesthesia. If there is no response, the animal will be moved to the station with individual nose cone for continued anesthesia. An incision will be made on the right side below the abdomen of the animal, closest to the IVC, the vein exposed and blood drawn with a 1 ml syringe with 23 G needles. Similarly, cardiac stick will be performed on the animal positioned at the platform under anesthesia. An one ml syringe with 23 G needle will be inserted underneath the Xyphoid at a 45 degree angle towards the left side of the thoracic cavity into the heart and blood will be drawn. In both cases, the animals will be immediately euthanized on completion of blood draw by cervical dislocation.

V.4.4.2. Injections: Subcutaneous injections will be given with either a 23 or 25 G needle to the nape of the neck of 0.1 ml (maximum volume 1 ml for 25 to 38 g animals). A disposable sterile needle will be used for all *sc* injections. One needle will be used per cage containing generally 4 animals. Endotoxin testing for all drugs will be completed prior to any animal testing at AFRRI and a report submitted for IACUC/VSD review and approval. The endotoxin tests are conducted by Charles River Laboratories. At the same time, pH will be tested to ensure that it falls in the range (6-8 for sc administration) stipulated by IACUC. It is expected that the osmolarity will not be more than AFRRI IACUC's recommended levels (Policy#9).

V.4.4.1.1. Pharmaceutical Grade Drugs

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

Research grade DT3, GT3, and (b)(4) formulations will be supplied by (b)(4) These (b)(4) (b)(4) and will be tested at AFRRI using the current protocol (using AFRRI intramural funds for animal studies). All formulations will be tested for endotoxin by Charles River Laboratory before using in the animals. pH and osmolality will be tested at AFRRI. Pure GT3 also will be supplied by (b)(4) and will be used as positive control (formulated in 5% Tween-80 at AFRRI) based on our previously published studies. We are not expecting any toxic effects in animals for any of the formulations. The following formulations will be supplied by (b)(4)

(b)(4)

V.4.4.2. Biosamples: N/A

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAb) Production : N/A

V.4.4.5. Animal Identification Generally, cage cards will be used to distinguish drug-treated and control groups. This will be a blinded study. Three drug groups will be designated as X, Y, and Z. Only vehicle group will be identified. Animals will be randomly distributed in groups. Some experiments might necessitate ear punch or notch or tattoos by personnel to identify individual animals in a cage. Ear punch will be performed as described in V.4.4.1.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: N/A

V.4.4.8. Tissue Sharing All tissues from the animals used in toxicity study will be preserved for histopathological analysis by the VSD pathologist. The pluck method will be used to harvest all the organs from tongue to rectum, brain, sternum, femur and blood may be collected at euthanasia. Tissue from irradiated mice (brain, GI, spleen, lung, liver, kidney) will be collected for histopathology at intervals.

V.4.4.9. Animal By-Products: N/A

V.4.5. Study Endpoint: The time frame for experiment V.1.1 (basic toxicity) will be 14 and 30 days after final drug dose administration.
The radiation survival studies will span 30 days after radiation exposure (experiments V.1.2, V.1.3. and V.1.4.). Serial sampling by terminal procedures is projected on 7 different time points after drug or irradiation for experiment V.1.5.
It is anticipated that the animals involved in these studies may succumb to death either due to a drug administration or radiation or a combination of the two. To maintain a suitable baseline for humane treatment of the animals while adhering to the study objective, the IACUC recommendations stipulated in Policy Letter #10 with some minor modification (as stated later in the paragraph) will be implemented for judging morbidity and moribundity. The moribund condition is defined as a clinically irreversible condition leading inevitably to death. Signs of morbidity in the mouse include difficulty in breathing, ruffling of hair, drying of lacrimal fluid, fall in body temperature, loss of appetite with/without diarrhea. A modified IACUC policy #10 will be used to record the scoring of clinical signs of pain in animals (attached Appendix 1). PI would like to modify the "Increased breathing" to "Changes in breathing" under "Respiratory rate" and change the score as 3 as used in the ongoing NIAID screening protocol P#(b)(6) Body weights are not included since there is no clear correlation between body weight loss and mortality. The objective of the present protocol is to compare the radioprotective efficacy of(b)(4) with GT3 in Tween-80 studied previously at AFRRI for last 8 years in five protocols(b)(6) (b)(6) . The PI is concerned that if the morbidity scoring, particularly weight loss scoring (which was not used in previous experiments) in combination with other morbidity criteria will lead to increased euthanasia and hence potentially affect the outcome of the results.(b)(4)

(b)(4) [redacted] Further, other radiation researchers do not use body weight loss as a mortality criterion [37]. However, body weights will be recoded and action for humane endpoints will be taken as follows (please see attached modified score sheet):
a) Body weights will be recorded prior to radiation.
b) Once animals reach a score of 6, weights will be recorded and monitored with each subsequent scoring change.
c) Animals who exceed 35% body weight loss with any other clinical signs are scored at a 12 and will be euthanized immediately.
To adhere to the policy of preventing animal distress, the PI will facilitate the late PM and early AM checks (not more than 10 h apart) in addition to two times a day checks during the critical lethality period (early morning, late morning, and late afternoon and evening). During non-critical periods, we will continue to check animals except late night. All morbid mice will be monitored carefully and their conditions scored for documentation (Appendix 1, revised scoring sheet).
PI will use a scoring card for each animal in the critical period and scored in each observation. Scores below 6 will not be recorded in the scoring sheet. Mice will be euthanized if there is a score of 12 in a single category or a cumulative score of 12 in multiple categories.

No supportive care is proposed, however, moribund animals will be immediately euthanized. VSD veterinarians will be consulted for all matters regarding the animal welfare.

V.4.6. Euthanasia
It is anticipated that at high radiation doses, animals will demonstrate varying degrees of morbidity and moribund, which makes it critical to define the criteria for euthanizing the animals without undermining the study objective.
In toxicity studies, if any animal shows discomfort (hunching, decreased activity, inappetence, separation from cage-mates), that animal will be under careful observation on consultation with the attending VSD veterinarian. Mice found moribund (defined in V.4.5.) or meeting the criteria for euthanasia following IAUC poloicy#10 (score sheet attached) will be euthanized by CO_2 inhalation plus confirmatory cervical dislocation by investigator/technician according to the directives of the VSD-SOP and in accordance with current AVMA Guidelines on Euthanasia [38, 39]. At the conclusion of the experimental time frame, all animals that survived the procedures of irradiation, drug intervention and controls, will be euthanized by CO_2 inhalation plus confirmatory cervical dislocation and their carcasses disposed of according to VSD regulations. An alternate method of euthanasia by cervical dislocation after exsanguination (by cardiac stick or inferior vena cava) under Isoflurane will be applied in experiment V.1.5.

V.5. Veterinary Care
V.5.1. Husbandry Considerations The AFRRI vivarium follows stringent protocols for the housing and care of all animals. The PI and designated team members will adhere to all the policy and guidelines set forth by standard operating protocols of the VSD.

The specific requirements for mice housing should meet the following criteria of opportunity for social interaction, opportunity to carry out normal behavior and the opportunity to rest and withdraw from each other [40]. Mice are social animals and hence will be caged together in groups of 4 or 8. For ODDS testing, mice will be housed in groups of 3 or 5 mice per cage and identified individually as described. Nestling pads will be provided in each cage during bedding changes. In cage shelters will also be provided as an enrichment strategy at the discretion of the PI depending on experimental conditions. Animals will receive Harlan Teklad Rodent Diet (w) 8604 and water *ad libitum*. Acidified water (pH 2.5 – 3.0) is routinely provided to AFRRI mice used in radiological experiments. Cages will be regularly changed and cleaned by VSD staff in accordance with Standard Operating Procedure. Animals will have 12 h light/dark cycle. Animals will be habituated to their surroundings and routine procedures prior to experimentation. In the event of procedures that last into the night cycle, care will be taken to minimize exposure to light.

V.5.1.1. Study Room As assigned by VSD

V.5.1.2. Special Husbandry Provisions N/A

V.5.1.3. Exceptions Mice will be socially (group) housed as described above, except when there is a possibility that during survival studies cage mates may be lost through attrition and for a period of time the sole surviving mouse may remain singly housed until the end of the 30-day study.

V.5.2. Veterinary Medical Care We do not project routine veterinary medical care of animals for injuries/inflammation related to injection site inflammation. In case of minor injuries arising from fighting, topical application of antibiotics is acceptable with consensus from the PI and VSD

.

V.5.2.1. Routine Veterinary Medical Care Experimental animals will be observed daily by either the investigator or technician or both. VSD personnel will monitor all animals during their routine rounds as per VSD SOPs. In the course of the study, as animals approach morbidity, the research staff observations will be increased to a minimum of at least three times a day, early morning and late afternoon (includes weekends and holidays). The timing between the late evening and early morning check will not exceed ten hours during the critical period. There is no supportive therapy contemplated since this would compromise the experimental results (described earlier in the end point and pain category sections).

V.5.2.2. Emergency Veterinary Medical Care. Moribund animals (gasping, recumbent, non-responsive to mild stimulus) will be euthanized by trained personnel.

V.5.3. Environmental Enrichment: As provided by the husbandry staff to animals not on active experiments.

V.5.3.1. Enrichment Strategy Nestling pads will be provided in each cage during bedding changes as per AFRRI-VSD SOPs.

V.5.3.2. Enrichment Restrictions

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING (Table 10)

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3, 4b, 5	(b)(6)
				1, 3, 4b, 5	
				1, 3, 4b, 5	
				1, 3, 4b, 5	
				1, 3, 4b, 5	
				1, 3, 4b, 5	
				1, 3, 4b, 5	
				1, 3, 4a, 5	

Procedure and manipulation codes:
Code 1 = Animal handling and euthanasia
Code 2 = Surgery (aseptic technique) pre- and post-operative care
Code 3 = Injections (3a–*ip*, 3b-*sc*, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-*iv*
Code 4 = Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5 = Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)
*Trained in retro-orbital injection, tail vein injection and tumor implantation.

VII. BIOHAZARDS/SAFETY All personnel working in the protocol have undergone necessary training in handling experimental animals. The amount of drug used will be small and standard safety precautions will be taken while handling them in the laboratory and in the vivarium. All personnel listed in the present protocol have been trained as general radiation users on a regular basis. All investigators and personnel will use appropriate protective gear while in the vivarium as described by the VSD. All listed personnel are current in safety training and medical surveillance.

VIII. ENCLOSURES none

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(b)(4)

(b)(4),(b)(6)

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X. ASSURANCES

Protocol Title: Radiation Countermeasure Screening for Protection and Mitigation in the lab Mouse, *Mus musculus*.

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): **I AM** / **AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)		04-22-2015
Principal Investigator (Printed Name)	**Principal Investigator (Signature)**	**(Date)**

Appendix 1.
Clinical Observations with Criteria for Euthanasia (Rodent)

Criteria:	Description:	Score
Appearance:		
	Normal (smooth coat, clear eyes/nose)	0
	Hunched and/or fluffed	1
	Ocular discharge, and/or edema	3
	Soft stools (fecal matter around anus)	5
	Pale, white mucus membranes/skin	6
	Bloody diarrhea	9
	Blue mucus membranes/skin (cyanosis)*	12
Respiratory Rate:		
	Normal breathing	0
	Change in breathing (an increase or decrease in respiratory rate from normal baseline)	3
	Abdominal breathing (gasping +/- open mouth breathing)*	12
General Behavior:		
	Normal (based on baseline observations)	0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)	1
	Decreased mobility	2
	Ataxia, wobbly, weak	6
	Inability to stand*	12
Provoked Behavior:		
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))	0
	Subdued; responds to stimulation (moves away briskly)	1
	Subdued even to stimulation (moves away slowly)	3
	Unresponsive to gentle prodding	6
	Does not right when placed gently on side within 5 seconds*	12
Weight loss		
	0-35% with no other clinical signs	0
	≥35% weight loss	12

*** Regardless of score, immediately euthanize (death is imminent)**

Individual or Cumulative Score:

< 6	Normal
6 - 11	Morbid: See VI.D.2 and VI.D.4 in IACUC Policy #10 for guidelines
≥ 12	Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*). Any single criteria of 12* euthanize immediately; consider as 'found dead.'

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (April 2015)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

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Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

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DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	**6/25/2015**	2nd Resubmission Date:	**7/30/2015**
Approved/**Returned for Revision:**	**7/14/2015**	**Approved**/Approval Withheld by IACUC:	**7/31/2015**
1st Resubmission Date:	**7/21/2015**	**EXPIRATION DATE:**	**7/30/2018**
Approved/**Returned for Revision:**	**7/29/2015**	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

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I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Regulation of radiation-induced damage by microRNAs in laboratory mice, *Mus musculus*

IV. PRINCIPAL INVESTIGATOR:

(b)(6) **Ph. D**
Research Biologist, AFRRI-SRD
Office: (b)(6) Fax: (b)(6)
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD** Date
Head, Scientific Research Department, AFRRI
Telephone (b)(6)
(b)(6)

- Or –

(b)(6) **PhD, LTC, USA** Date
Head, Scientific Research Department, AFRRI
Telephone: (b)(6)
(b)(6)

- Or –

(b)(6) **PhD, CDR, USN** Date
Head, Scientific Research Department, AFRRI
Telephone: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date
Statistician
Telephone (b)(6) Fax (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, LTC, VC, USA** Date
Head, Veterinary Sciences Department, AFRRI
Telephone (b)(6) Fax (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date
Safety and Occupational Health Specialist, USUHS
Telephone (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and aproved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**
IACUC Chair (b)(6) Scientific Research Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: Regulation of radiation-induced damage by microRNAs in laboratory mice, *Mus musculus*

PRINCIPAL INVESTIGATOR: (b)(6) Ph. D
Research Biologist (AFRRI – SRD)
Office: (b)(6) Fax: (b)(6)
(b)(6)

CO-INVESTIGATOR(S):

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS Ionizing radiation exposure presents an increasing threat to the global population. Therefore, it is critical that we develop drugs against ionizing radiation. One of the AFRRI missions is to develop radiation countermeasures to shield first responders, military personnel and civilians who are exposed to high amounts of radiation. Over the past few years, several promising drugs from AFRRI have already been advanced to Investigation New Drug (IND) status. We have extensively evaluated Gamma tocotrienol (GT3), a vitamin E isomer, in mice previously at AFRRI for last 8 years in five protocols (P# (b)(6) (b)(6) .Another drug of interest is (b)(4) (currently active in (b)(6) , a modified cytokine that acts as a growth factor for regenerating bone marrow after exposure to ionizing radiation. This drug has advanced to the clinic (successful phase 1 trials) and is promising against both low linear energy transfer (LET), as represented by Cobalt-60 γ-radiation, and high LET mixed-field radiation (neutron and gamma from AFRRI reactor). In recent years, micro Ribonucleic acids (miRNAs) have been implicated in cellular differentiation, carcinogenesis, and modulation of signaling pathways. Thus, to characterize the underlying mechanism of radioprotection, we studied the miRNA profile in spleen from irradiated mice with or without GT3 treatment. Our studies indicate that several miRNAs are differentially regulated in mice spleen after radiation exposure. Specifically, miR-34a is significantly increased (15-fold on day 15) in spleen after 8 Gy exposure. We have also shown that a single dose of GT3 administration 24 h prior to irradiation suppresses miR-34a expression. Therefore, we propose to extend our preliminary observations to blood, bone marrow, kidney, thymus, and liver. Network analysis in molecular biology can predict signaling pathways, molecular networks, and biological functions for genes. Such analysis of our preliminary data suggests that ERK and p38MAPK pathways may play a significant role in the mechanism of radioprotection. We will use CD2F1 male mice as *in vivo* models to study this signaling pathway. Our broad objective is to understand radiation-induced damage regulation by GT3 and (b)(4) modulated miRNAs. The study will involve: **a.** Confirmation of the effect of GT3 on ERK/p38MAPK in spleen, bone marrow, kidney, thymus, and liver and **b.** Evaluation of signaling pathways of the promising (b)(4) radiation countermeasure.

II. BACKGROUND

II.1. Background Ionizing radiation exposure presents an increasing threat to the global population. Therefore, it is critical that we develop drugs against ionizing radiation. One of the AFRRI missions is to develop radiation countermeasures to shield first responders, military personnel and civilians who are exposed to high amounts of radiation. Over the past few years, several promising drugs from AFRRI have already been advanced to Investigation New Drug (IND) status (b)(4) We have extensively evaluated Gamma tocotrienol (GT3), a vitamin E isomer, in mice. Another drug of interest is (b)(4) (b)(4) after exposure to ionizing radiation (b)(4) This drug has advanced to the clinic (successful phase 1 trials) and is promising against both low linear energy transfer (LET), as represented by Cobalt-60 γ-radiation, and high LET mixed-field radiation (neutron and gamma from AFRRI reactor). To meet the Food and Drug Administration's (FDA) animal rule for radiation countermeasures, it is essential to carry out studies to understand the mechanisms of radiation protection.

Survival from radiation lethality by GT3 and possible mechanism

We reported that gamma-tocotrienol, a naturally occurring vitamin E analog, protects mice from radiation-induced pancytopenia, restores bone marrow progenitor cells (b)(4) reduces intestinal and vascular oxidative stress, and protects 100% mice exposed to lethal dose of whole body γ-radiation (b)(4) CD2F1 mice treated with GT3 showed increased numbers of spleen colonies compared to vehicle treated animals at 12 days after 8.5 Gy of γ-radiation (b)(4) GT3 reduces radiation-induced vascular oxidative stress in mice through an HMG-CoA reductase dependent manner post-radiation treatment [4]. GT3 may exert its beneficial inhibitory effects on post-irradiation free-radical production by improving the availability of the endothelial nitric oxide synthase (eNOS) cofactor BH4 through downregulation of GTP-cyclohydrolase 1 regulatory protein (GFRP), thereby enhancing the production of NO (nitric oxide) and reducing the production of ONOO- (peroxynitrite) by eNOS [5]. GT3 has also been shown to improve the post-irradiation recovery of plasma markers of endothelial function, an effect that is not dependent on HMG-CoA reductase inhibition [5]. However, the signaling pathway through which GT3 protects mice from hematopoietic damage is not known. We propose here to study the molecular pathways involved in radioprotection by GT3.

Survival from radiation lethality by (b)(4) and possible mechanism

Recently a preliminary study by an (b)(4) (b)(4) (b)(4) in the CD2F1 mouse model when administered sc 24 before or 4 h after exposure to Co-60 γ-radiation. (b)(4) (800 μg/kg) demonstrated 100 % survival administered 24 h before total-body irradiation to CD2F1 mice and protected ~50% of the animals when administered 4 h after radiation. In another preliminary study an (b)(4) (b)(4) demonstrated ~80-100% survival in mice administered 4 h or 4 h and 48 h after mixed-field (65% neutron and 35% gamma) radiation. Based on this novel finding, (b)(4) approved funding to develop

this drug for mixed-field radiation, which has been accepted as a more realistic scenario for a nuclear accident or terrorist activity using an enhanced radiation weapon (ERW). In a preliminary study, (b)(4) mice from radiation-induced neutropenia and thrombocytopenia, and bone marrow cellularity after exposure to total-body radiation. However, the mechanism of radioprotection by (b)(4) (b)(4) is unknown. Previously authors have demonstrated that (b)(4) (b)(4)

Since GT3 and (b)(4) were found to be promising radiation countermeasures in mice models exposed to γ-radiation (preliminary studies), it is essential to understand the mechanism of action of these drugs according to Food and Drug Administration's (FDA) animal guidelines for the Investigational New Drug (IND) application process. This project will provide valuable information on microRNA and mRNA regulation by GT3 and (b)(4) in protecting mice from hematopoietic damage after radiation.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

To find any possibility for duplication, the following databases have been searched:
PubMed
BRD - Biomedical Research Database
Reporter (CRISP) - Computer Retrieval of Information on Scientific Projects, now known as 'Reporter'

II.2.2. Date of Search PubMed – 06/12/2015
BRD - 06/12/2015
Reporter (CRISP) - 06/12/2015

II.2.3. Period of Search

PubMed – 1960-2015
BRD – 1998-2015
Reporter (CRISP) – 1972-2015

II.2.4. Key Words of Search

1. Micro RNA AND Radiation
2. Gamma-tocotrienol AND micro RNA
3. (b)(4) AND micro RNA
4. Gamma-tocotrienol AND Radiation
5. (b)(4) AND Radiation

II.2.5. Results of Search

Table 1 includes a summary of the keywords and keyword combinations used for searching the RePORTer, BRD, and PubMed databases.

Table 1. Summary of Database Keyword Searches

Keywords	PubMed	BRD	RePORTer
Micro RNA AND radiation	852	1312	154
Gamma-tocotrienol AND micro RNA	1	2	1

(b)(4) AND micro RNA	0	0	0
Gamma-tocotrienol AND Radiation	32	83	399
(b)(4) And Radiation	0	20	0

The searches indicated that there have been no previous or current studies have been done on the role of microRNA in the regulation of DNA damage after radiation injury. Therefore, the proposed study to determine the mechanism of radioprotection by (b)(4) (b)(4) or GT3 and the role of microRNA in regulating DNA damage in mouse after whole body radiation is not a duplication of a previous experiment. While there were some hits, the studies were not relevant to the mechanism of radiation protection involving microRNA and GT3 or (b)(4)

III. OBJECTIVE / HYPOTHESIS The overall objective of this study is to decipher the molecular pathways involved in the protection from radiation-induced hematopoietic failure by two promising radiation countermeasures, Gamma tocotrienol (GT3) and (b)(4)

Specific Aim 1: Determine radiation-induced differential mRNA expression modulated by GT3 and confirm activation of ERK/p38MAPK signaling by GT3 in mice exposed to Cobalt-60 γ-radiation

In a preliminary study, we have shown that miR-34a, miR-15b, miR-150, miR-99a, miR-99b, miR-130, miR-143, and miR-145 were differentially regulated post-irradiation from day 1 to day 14 in spleen of GT3 treated mice compared to vehicle treated ones. Ingenuity pathway analysis (IPA) with these miRNAs identified predicted mRNAs and target signaling pathways of GT3 through ERK/p38MAPK. We propose to identify radiation induced mRNA targets that are regulated by GT3, and confirm the predicted target pathway of action of GT3 using pathway analysis. Mice will be treated with GT3 and total-body irradiated (TBI) to a sublethal dose of 7 Gy and spleen, bone marrow and blood will be harvested after irradiation. Whole genome expression will be done using the Illumina BeadChip array.

Specific Aim 2: Identify the signaling pathways modulated by (b)(4) through differential regulation of miRNAs and target mRNAs in irradiated mice

We hypothesize that mice exposed to a sublethal dose of TBI will produce a time-dependent differential expression of miRNAs during hematopoietic recovery, and pre-treatment with (b)(4) will significantly modulate these genes to rescue mice from radiation injury. Preliminary studies show that (b)(4) was found to be an effective radiation countermeasure administered before or after radiation, protecting mice from radiation-induced pancytopenia and lethality. MiRNA and mRNA analysis will be done in circulating blood, as well as in bone marrow and spleen. This data will determine the role of miRNAs in (b)(4) irradiated tissues and organs. To further understand the signaling pathway involved in the mechanism of action of (b)(4) we will identify and validate the predicted signaling targets of the (b)(4) regulated miRNAs.

This project will deliver a systematic approach to identifying signaling targets of radiation countermeasures using the complex network interactions of radiation induced mRNA and miRNA.

IV. MILITARY RELEVANCE Military operations are seriously impaired by radiation exposures depending on the dose and dose rate. Currently, there is no FDA approved preventive measure available for military personnel to protect them from the hazards of radiation exposure. The problem is more acute in recent times due to the unpredictable nature of terrorist attacks. Recognizing this urgent need, United States Department of Defense has assigned top priority in "Defense Technology Objectives (DTO)" to the "development of medical countermeasures to radiation exposure" against both early effects and late arising health effects. These concerns address the urgent need to develop an appropriate radiation protective agent (prophylactic or therapeutic) to sustain immediate warfighter capabilities and to enable the commanders to make judicious decisions in troop deployment. Currently, there are several drugs at various stages of development. This study will focus on revealing the mechanism of action of a prophylactic radiation countermeasure, gamma tocotrienol (GT3) and (b)(4) (b)(4)

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

General Experimental Design for animal studies: **Experiments 1 and 2:** Ten to twelve weeks old male CD2F1 mice (Harlan Labs, Indianapolis, Indiana) will be used in this experiment. GT3 will be supplied by (b)(4) (b)(4)

Mice will be injected subcutaneously (see section V.4.4.1) with either vehicle (5% Tween-80 for GT3 or PBS for (b)(4) or GT3 (100 mg/kg) or (b)(4) (400 µg/kg) administered 24 h before exposure to Co-60 radiation (0.6 Gy/min). Mice will be irradiated to 7 Gy resulted in the hematopoietic syndrome where measurable numbers of blood elements are desired throughout the experiment. Animals will be euthanized (see section V.4.5 and 6) from each experimental group on days 1, 4, 7, 14, and 21 and blood will be collected from anesthetized mice while they are alive from the inferior vena cava in serum separator tubes (see section V.4.4). Serum will be separated and stored at -80C. Bone marrow will be harvested following a standard protocol [3]. Spleen and other tissues (kidney, liver, and thymus) will be collected and snap frozen for storage at -80C. Both the drugs have been tested for 30-day survival in various protocols at AFRRI. There was no reaction observed at the injection site for the doses proposed in this protocol.

Table 2. Experimental design

Groups	Drug	Collection days	Radiation dose (Gy)	No. of mice/day	Total no. of mice
1	0 (naïve control)	1,4,7,14,21	0	5	25
2	PBS/GT3 veh	1,4,7,14,21	0	5	50

3	(b)(4) GT3	1,4,7,14,21	0	5	50
4	PBS/GT3 veh	1,4,7,14,21	7	5	50
5	(b)(4) GT3	1,4,7,14,21	7	5	50

We will repeat the study to confirm the data. Total no. of mice that will be used in this proposed study = 450. Tissues from naïve control animals will be shared between the two experiments.

V.1.1. Experiment 1. ***Determine radiation-induced differential mRNA expressions modulated by GT3 and confirm activation of ERK/p38MAPK/p53 signaling by GT3 in mice exposed to Cobalt-60 γ-radiation***

Total RNA will be extracted from blood, bone marrow and spleen collected at days 1, 4, 7, 14, and 21 post-radiation by a standard protocol (miRVana isolation kit, Ambion). Whole genome expression will be determined using Illumina Beadarray (MouseRef-8 version 2.0) BeadChips using the iscan system and BeadScan software, Illumina, San Diego) [7]. Data will be validated using RT-PCR and western blot analyses.

A total of 250 mice will be used for this experiment.
Groups = 5 (Naïve, 0 Gy Veh, 0 Gy GT3, 7 Gy Veh, 7 Gy GT3)
No. of time points = 5
No. of animals per group = 5
No. of repeat = 2

V.1.2. Experiment 2. Identify the signaling pathways modulated by (b)(4) through differential regulation of miRNAs and target mRNAs in irradiated mice

Total RNA will be extracted from blood, bone marrow and spleen collected at days 1, 4, 7, 14, and 21 post-radiation. RNA will be extracted using mirVana total RNA isolation kits (Life Technologies) following the manufacturer's protocol. Microarray hybridization and data analyses will be performed by a commercial service provider, LC Sciences in Houston, TX (www.lcsciences.com) and miRbase version 19.0 (http://mirbase.org/) will be used to study the expression profiling of 1265 unique mature mouse miRNAs using a µParaflo® microfluidic technology [8, 9]. Data will be analyzed and computed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression methods) [10] by LC Sciences, Houston TX. Array will be performed only using spleen RNA. Data will be validated using RT-PCR and western blot analysis.

A total of 200 mice will be used for this experiment.
Groups = 5 (0 Gy Veh, 0 Gy CDX-301, 7 Gy Veh, 7 Gy (b)(4)
No. of time points = 5
No. of animals per group = 5
No. of repeat = 2

A grand total of 450 animals will be used in this study.

Rationale for selecting the time of collecting spleen after radiation: Our published data demonstrate that regeneration of bone marrow progenitor cells starts after day 4 post-irradiation and the cells are recovered by day 13 (100%) with GT3 compared to vehicle (40%) (b)(4) Peripheral blood cells recovery was observed by day 21 after

radiation for both GT3 and (b)(4) Therefore, we chose five time points, days 1, 4, 7, 14, and 21 post-irradiation to study the miRNA regulations after radiation and its modulation by GT3 or (b)(4) during hematopoietic injury and consequent recovery.

Rationale of selecting sublethal dose (7 Gy): LD50/30 (lethal dose of radiation that results in the lethality of 50% of the exposed animals in 30 days) of CD2F1 mice that will be used in this study is 8.6 Gy (b)(4) A sublethal dose of 7 Gy will be used in order to keep the mice alive in vehicle-treated group to collect samples on specific time points after irradiation. These samples will be compared to the samples collected from either (b)(4) or GT3 treated mice.

V.2. Data Analysis

Statistical software PC SAS will be used for statistical data analysis. Ten animals per group will be used to provide statistical power >90% for two-tailed student's T-Test. We will report the mean with standard error or percentage when applicable. ANOVA (Analyses of Variance) with mixed-effect model will be used when testing one dependent variable and Multivariate analysis of variance (MANOVA) will be used when testing more than one dependent variable to detect if there is significant difference among the groups.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered Our ultimate goal is to find a suitable radioprotectant for human use. We have already established that GT3 can be good prophylactic radiation countermeasures in male CD2F1 mice, protecting both the hematopoietic and GI systems from radiation injury. (b)(4) was also found to protect mice from lethal dose of radiation and protect mice from hematopoietic damage. To advance the drug to clinical trial, we have to understand the mechanism of action of these radiation countermeasures. For this purpose, we want to know the role of microRNA in regulating DNA damage after radiation injury. For that purpose we have to use an animal model as proof-of-concept.

V.3.2. Animal Model and Species Justification As discussed in the previous section, animals must be used to study the mechanism of action of a radioprotectant *in vivo*. Mouse model will be used as a proof-of-concept because approximately 99% of human DNA is similar to that of mice, and mouse models are well characterized to study the mechanism of action of a radioprotectant (b)(4) Zebrafish and Drosophila is not considered for this study because their physiology is largely different from mammalian systems.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species Mus musculus

V.3.3.2. Strain / Stock CD2F1

V.3.3.3. Source / Vendor Harlan Labs, Indianapolis, Indiana or similar approved vendors (Ph# 800-793-7287)

V.3.3.4. Age 7 - 8 wks old

V.3.3.5. Weight 22-30 g

V.3.3.6. Sex Male
Justification of using only male mice: Previous studies have been conducted with male mice, so male mice are needed for data comparison. Although, it is important to confirm observations in females, such studies may be carried out at a later date.

V.3.3.7. Special Considerations Commercially procured animals must be adventitious, disease free, and pathogen-free—including *Pseudomonas aeruginosa,* and *Pasteurella.* All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus, *Mycoplasma pulmonis*, and *Clostridium piliforme.* They must also be endoparasite and ectoparasite-free.

V.3.4. Number of Animals Required (by species)

V.3.5. Refinement, Reduction, Replacement (3 R's)
V.3.5.1. Refinement PI will take necessary steps to all personnel in the project to be rigorously trained in handling techniques (injections, and routine cage monitoring) to minimize distress and pain to the animals. Specifically, during capture and restraint of the rodents before and after radiation, care will be taken to ensure that handling does not contribute to distress.
Animals will be irradiated at a sublethal dose of 7 Gy. We do not expect to see morbidity in the course of the study. However, for any reason, if we observe morbid animals, we will increase monitoring and euthanize animals following policy 10 at early time points.

V.3.5.2. Reduction Previously published and unpublished data have established which radiation dose and time points are important to get the information for hematopoietic regeneration after radiation injury. This information has enabled us to reduce the number of animals needed for this study. We are also reducing the animal numbers (n = 10) by sharing tissues for determining different parameters from the same experiment. Based on our previous experiments, we will use the minimum required number of animals for statistical validity. This will reduce the number of animals used in this protocol.

V.3.5.3. Replacement None

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C _____ (# of animals)

V.4.1.1.1.2. Column D ___50__ (# of animals)

V.4.1.1.1.3. Column E ___400__ (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
Expt 1	Naïve, 0 Gy Veh, 0 Gy GT3, 7 Gy Veh, 7 Gy GT3	0	50	200	250
Expt 2	0 Gy Veh, 0 Gy (b)(4) 7 Gy Veh, 7 Gy (b)(4)	0	0	200	200
Totals:			50	400	450

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization The protocol will not use anesthetics or analgesics during radiation since they will adversely affect the outcome of the experiments. The proposed study is to determine the effects of irradiation and countermeasures on the function of the immune and blood forming systems. Use of any analgesics and anesthetics would interfere with the function of the immune system and therefore compromise the interpretation of the results [10]. Topical antibiotics will be applied if wounding occurs via in-cage fighting, under the guidance of the clinical veterinarian in accordance with standard treatment measures.

Anesthesia using standard Isoflurane apparatus under the guidance of the VSD will be carried out in mice for blood collection. All personnel will be extensively trained for efficient administration of the agents as well as to minimize handling stress. Animals will be placed in the Isoflurane chamber and delivered a metered amount of 3-5% Isoflurane mixed with 100% oxygen until all voluntary motor movement ceases and the animal is recumbent for 10 seconds. Animal's respiration rate will be closely monitored through the Plexiglas chamber during induction of anesthesia. When animals are recumbent, they will be removed from the chamber and their nose/mouth placed in the anesthesia apparatus breathing/gas administration cone for maintenance of anesthesia at 1-3% Isoflurane and 100% O_2.

V.4.1.2.2. Pre- and Post-procedural Provisions Mice will be housed under standard conditions both before and after radiation procedures in accordance with current VSD rodent husbandry Standard Operating Procedures (see section V.5).

V.4.1.2.3. Paralytics No paralytic agents will be administered

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched
Altweb
AGRICOLA
PubMed

V.4.1.3.2. Date of Search
Altweb – 06/10/2015
AGRICOLA – 06/10/2015
Pubmed – 06/10/2015

V.4.1.3.3. Period of Search
1970-2015

V.4.1.3.4. Key Words of Search
Pain, analgesia, anesthesia, radiation, radioprotection, mouse, humane, alternative, gamma-tocotrienol, (b)(4)

V.4.1.3.5. Results of Search
We are studying the mechanism of radioprotection of (b)(4) and GT3 in this protocol. We will be using a subletha**l** dose 7 Gy in this study based on results from our previous studies. Irradiation itself is not a painful process but it induces various changes in the body (i.e., vomiting and nausea, changes in hematology cells numbers, etc.). Although radiation does not induce pain, animals in these experiments might experience pain and distress prior to death because of sequelae. Radiation compromises the immune system. As a result of a compromised immune response, various types of infections can initiate and become painful. Table 3 includes a summary of the keyword combinations used for searching and the results of the search. While the literature indicates that cell models have been used to study radiation damage, such models cannot alone predict mechanism of radioprotection of a specific drug. Although radiation-induced pain was alleviated during some studies [11-13], such pain relief was the aim of the studies in question, which focused on patients receiving radiotherapy for cancer treatment. We do not have the option of using alternatives such as a cell culture model. If animals show sign of pain or distress (please see section V.4.1), all moribund animals will be euthanized immediately to minimize potential pain or distress following policy#10.

Table 3. Summary of Database Keyword Searches

Keywords	Hits		
	AGRICOLA	Altweb	PubMed
Analgesia AND Radiation	4	6	553
Pain AND Radiation	37	12	11330

Pain AND Analgesia AND Radiation	4	6	368
Gamma-tocotrienol AND pain	0	0	0
(b)(4) AND pain	0	0	0
Radioprotection AND pain	0	0	154
Radiation AND humane	1	38	12
Pain AND analgesia AND mice	49	26	1904
Radiation AND mouse AND pain	0	9	239
Radiation AND mouse AND pain AND alternative	0	9	6

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification Since the purpose of these studies is to determine the mechanism of radioprotection of the immune system by (b)(4) or GT3, we will be unable to use analgesics and anesthetics because they may interfere with the innate system [14].

V.4.2. Prolonged Restraint
The experimental animals will receive radiation at a dose rate 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (dosimetry 07/09/2014; or the latest dosimetry map) using plastic racks. Mice will be inserted into the standard radiation boxes just prior to irradiation (~15 min of exposure time) and taken to the cobalt facility for irradiation. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period.

V.4.3. Surgery
N/A
V.4.3.1. Pre-surgical Provisions
N/A

V.4.3.2. Procedure(s)
N/A
V.4.3.3. Post-surgical Provisions
N/A
V.4.3.4. Location
N/A
V.4.3.5. Surgeon
N/A
V.4.3.6. Multiple Major Survival Operative Procedures
N/A
V.4.3.6.1. Procedures
N/A
V.4.3.6.2 Scientific Justification
N/A
V.4.4. Animal Manipulations

Irradiation: These will follow the standard AFRRI protocol with mice placed in restrainers shortly prior to exposure, irradiation at the cobalt facility and return to VSD

for further monitoring. Briefly, the experimental animals will receive radiation at a dose rate of 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (dosimetry 07/09/2014) using plastic racks. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period. They will be monitored daily following policy#10. There is no critical period for the animals in this protocol since all animals irradiated at 7 Gy (sublethal dose) are expected to survive based on our published manuscript on peripheral blood recovery post-irradiation at 7 Gy[(b)(4)] Tissue from mice will be harvested at specific time-points after irradiation after terminal blood draw.

Blood collection: Mice will be anesthetized as described under section V.4.1.2.1 (anesthesia). Blood will be collected from anesthetized mice while they are alive from the inferior vena cava (IVC). The animals will be deeply anesthetized in the rodent anesthesia machine induction chamber; the tail will be pinched for reflexive movement, indicative of insufficient anesthesia. If anesthesia is insufficient, animal will be replaced in induction chamber. If there is no response, the animal will be moved to the station with individual nose cone for continued anesthesia. An incision will be made on the right side below the abdomen of the animal, closest to the IVC. This will expose the vein and blood will be drawn with a 1 ml-syringe with 23 G needles. Animals will have cervical dislocation performed immediately upon completion of blood draw to confirm euthanasia.

V.4.4.1. Injections
Subcutaneous (SC) injections will be given with either a 23 or 25 G sterile needle to the nape of the neck. Animals will be injected once 24 h before radiation.

V.4.4.1.1. Pharmaceutical Grade Drugs

V.4.4.1.2. Non-Pharmaceutical Grade Drugs
[(b)(4)] is formulated as a sterile buffered solution (pH 7) intended for single-use parenteral administration under GMP conditions. The drug will be provided to AFRRI via an MTA[(b)(4)] GT3 formulation will be supplied by [(b)(4)] Endotoxin testing for all drugs will be completed prior to any animal testing at AFRRI and a report submitted for IACUC/VSD review and approval. The endotoxin tests are conducted by Charles River Laboratories. At the same time, pH will be tested to ensure that it falls in the range (6-8 for SQ administration) stipulated by IACUC. It is expected that the osmolarity will not be more than AFRRI IACUC's recommended levels (Policy#9). Both [(b)(4)] and GT3 have been tested previously in other protocols at AFRRI. PBS will be used as diluent foir [(b)(4)] A diluent (developed by [(b)(4)] will be used for GT3.

V.4.4.2. Biosamples Animals will be euthanized from each experimental group on days 1, 4, 7, 14, and 21 and blood will be collected from anesthetized mice while they are alive from the inferior vena cava (using a 23 G needle) in serum separator tubes. Serum will be separated and stored at -80C. Bone marrow will be harvested following a standard protocol. Spleen and other tissues (kidney, liver, and thymus) will be collected and snap frozen for storage at -80C.

V.4.4.3. Adjuvants

V.4.4.4. Monoclonal Antibody (MAb) Production

V.4.4.5. Animal Identification Generally, cage cards will be used to distinguish drug-treated and control groups. Animals will be randomly distributed in groups.

V.4.4.6. Behavioral Studies N/A

V.4.4.7. Other Procedures N/A

V.4.4.8. Tissue Sharing At present there are no plans for tissue sharing.

V.4.4.9. Animal By-Products N/A

V.4.5. Study Endpoint In experiments 1 and 2, at specific time points, experimental mice will be euthanized before tissue collection. Different tissues will be used for different experiments. If we see any morbidity or moribundity, frequency of examinations including late-night checks will be increased and animals will be scored following policy 10 (score sheet attached, appendix 1).

V.4.6. Euthanasia All moribund animals (see section V.4.5) (if any) will be euthanized by overdose of CO_2 inhalation as described in VSD SOP VS0211, by trained investigators or technicians in accordance with current AVMA Guidelines on Euthanasia. Compressed CO_2 gas in a cylinder with a regulator will be used for euthanasia in a euthanasia chamber. After blood draw (please see section V.4.4), animals will have cervical dislocation performed immediately to confirm euthanasia.

V.5. Veterinary Care

V.5.1. Husbandry Considerations The AFRRI vivarium follows stringent protocols for the housing and care of all animals. The PI and designated team members will adhere to all the policy and guidelines set forth by standard operating protocols of the VSD. The specific requirements for mice housing should meet the following criteria of opportunity for social interaction, opportunity to carry out normal behavior and the opportunity to rest and withdraw from each other [15]. Mice are social animals and hence will be caged together in groups of 4 or 8. For ODDS testing, mice will be housed in groups of 3 or 5 mice per cage and identified individually as described. Nestling pads will be provided in each cage during bedding changes. In cage shelters will also be provided as an enrichment strategy at the discretion of the PI depending on experimental conditions. Animals will receive Harlan Teklad Rodent Diet (w) 8604 and water *ad libitum*. Acidified water (pH 2.5 – 3.0) is routinely provided to AFRRI mice used in radiological experiments. Cages will be regularly changed and cleaned by VSD staff in accordance with Standard Operating Procedure. Animals will have 12 h light/dark cycle. Animals will be habituated to their surroundings and routine procedures prior to experimentation. In

the event of procedures that last into the night cycle, care will be taken to minimize exposure to light.

V.5.1.1. Study Room As assigned by VSD

V.5.1.2. Special Husbandry Provisions N/A

V.5.1.3. Exceptions Mice will be socially (group) housed as described above, except there might be time when few animals from the cage will be removed for blood and tissue collection.

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care Experimental animals will be observed daily by either the investigator or technician or both. VSD personnel will monitor all animals during their routine rounds as per VSD SOPs.

V.5.2.2. Emergency Veterinary Medical Care We do not expect animals will be moribund in this protocol. If moribund animals (gasping, recumbent, non-responsive to mild stimulus) are found, those will be euthanized by trained personnel.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy The enrichment strategy for mice will be according to VSD Standard Operating Procedure, which involves socially grouped housing and providing nestlets and hiding tubes in the cage.

V.5.3.2. Enrichment Restrictions

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3, 4b, 5	(b)(6)
				1, 3, 4b, 5	
				1, 3, 4b, 5	
				1, 3, 4b, 5	
				1, 3, 4b, 5	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY

All personnel working in the protocol have undergone necessary training in handling experimental animals. The amount of drug used will be small and standard safety precautions will be taken while handling them in the laboratory and in the vivarium. All personnel listed in the present protocol have been trained as general radiation users on a regular basis. All investigators and personnel will use appropriate protective gear while in the vivarium as described by the VSD. All listed personnel are current in safety training and medical surveillance.

VIII. ENCLOSURES None

References:

(b)(4),(b)(6)

5. Berbee, M., et al., *Mechanisms underlying the radioprotective properties of gamma-tocotrienol: comparative gene expression profiling in tocol-treated endothelial cells.* Genes Nutr, 2012. **7**(1): p. 75-81.

(b)(4)

2010. **285**(47): p. 36275-84.

7. Bhattacharyya, S., et al., *Elevated miR-155 promotes inflammation in cystic fibrosis by driving hyperexpression of interleukin-8.* J Biol Chem, 2011. **286**(13): p. 11604-15.
8. Gao, X., E. Gulari, and X. Zhou, *In situ synthesis of oligonucleotide microarrays.* Biopolymers, 2004. **73**(5): p. 579-96.
9. Zhu, Q., et al., *microParaflo biochip for nucleic acid and protein analysis.* Methods Mol Biol, 2007. **382**: p. 287-312.
10. Colucci, D.G., N.R. Puig, and R. Hernandez-Pando, *Influence of anesthetic drug on immune response: from inflammation to immunosuppression.* OA Anaesthetics, 2013. **21**(3): p. 1-7.
11. Ciezki, J. and R.M. Macklis, *The palliative role of radiotherapy in the management of the cancer patient.* Semin Oncol, 1995. **22**(2 Suppl 3): p. 82-90.
12. Desai, D.C., et al., *Dilatation of radiation-induced esophageal strictures under sublingual buprenorphine analgesia: a pilot study.* Indian J Gastroenterol, 1997. **16**(1): p. 14-5.
13. Sonoo, H., et al., *[Systemic therapy, pain relief and quality of life of breast cancer patients with bone metastasis].* Gan To Kagaku Ryoho, 1995. **22 Suppl 1**: p. 10-5.

14. Galley, H.F. and N.R. Webster, *Effects of propofol and thiopentone on the immune response*. Anaesthesia, 1997. **52**(9): p. 921-3.
15. AWIC, *http://awic.nal.usda.gov/nal_display/index*. 2010.

X. ASSURANCES

Protocol Title: Regulation of radiation-induced damage by microRNAs in laboratory mice, *Mus musculus*

As the Principal Investigator on this protocol, **I** acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)		7-30-2015
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

Appendix 1.
Clinical Observations with Criteria for Euthanasia (Rodent)

Criteria:	Description:	Score
Appearance:		
	Normal (smooth coat, clear eyes/nose)	0
	Hunched and/or fluffed	1
	Ocular discharge, and/or edema	3
	Soft stools (fecal matter around anus)	5
	Pale, white mucus membranes/skin	6
	Bloody diarrhea	9
	Blue mucus membranes/skin (cyanosis)*	12
Respiratory Rate:		
	Normal breathing	0
	Change in breathing (an increase or decrease in respiratory rate from normal baseline)	3

	Abdominal breathing (gasping +/- open mouth breathing)*	12
General Behavior:		
	Normal (based on baseline observations)	0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)	1
	Decreased mobility	2
	Ataxia, wobbly, weak	6
	Inability to stand*	12
Provoked Behavior:		
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))	0
	Subdued; responds to stimulation (moves away briskly)	1
	Subdued even to stimulation (moves away slowly)	3
	Unresponsive to gentle prodding	6
	Does not right when placed gently on side within 5 seconds*	12
Weight loss		
	0-35% with no other clinical signs	0
	≥35% weight loss	12

*** Regardless of score, immediately euthanize (death is imminent)**

Individual or Cumulative Score:

< 6	Normal
6 - 11	Morbid: See VI.D.2 and VI.D.4 in IACUC Policy #10 for guidelines
≥ 12	Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*). Any single criteria of 12* euthanize immediately; consider as 'found dead.'

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (April 2015)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

***** DELETE THIS PAGE PRIOR TO PROTOCOL SUBMISSION *****

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	6/26/2015	2nd Resubmission Date:	8/27/2015
Approved/**Returned for Revision:**	7/14/2015	**Approved**/Approval Withheld by IACUC:	8/27/2015
1st Resubmission Date:	7/20/2015	**EXPIRATION DATE:**	8/26/2018
Approved/**Returned for Revision:**	7/29/2015	Previous Protocol Number *(if related)*	(b)(6)
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER (b)(6)

III. PROTOCOL TITLE (b)(4) Protection from Radiation-Induced (b)(4) Injuries in a Murine Model (*Mus musculus*)

IV. PRINCIPAL INVESTIGATOR:

(b)(6) **Ph.D.** Date
Associate Professor (b)(6)
(b)(6) office: (b)(6) fax: (b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **Ph.D.** Date
Professor and Chairman (b)(6)
(b)(6) office: (b)(6) fax: (b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician
Telephone (b)(6) Fax (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI
Telephone (b)(6) Fax: (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Safety and Occupational Health Specialist, USUHS
Telephone (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**

IACUC Chair (b)(6) Scientific Research Department, AFRRI
Telephone (b)(6) Fax (b)(6)
(b)(6)

PROTOCOL TITLE: (b)(4) Protection from Radiation-Induced (b)(4) (b)(4) Injuries in a Murine Model (*Mus musculus*)

PRINCIPAL INVESTIGATOR:

(b)(6) Ph.D. Date
Associate Professor, (b)(6)
(b)(6) office; (b)(6) fax; (b)(4)

CO-INVESTIGATOR(S): None

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER:

I. NON-TECHNICAL SYNOPSIS The terrorist attack of September 11, 2001 against the United States has increased the concern that terrorists may acquire and use radioactive sources (e.g. dirty radioactive bombs) to expose military and civilian populations to ionizing radiation. However, despite this concern, there are currently no FDA approved drugs that can be used for the general population to prevent death that is likely to result from exposure to ionizing radiation. (b)(4) rradiation in humans can result in both (b)(4) (b)(4) injuries. However, because rodents are more resistant to (b)(4) injuries than *humans*, there is currently no murine model that has been developed to study both (b)(4) (b)(4) radiation injuries in the same animal.
(b)(4),(b)(6)

II. BACKGROUND

II.1. Background The acute toxic effects of exposure to high doses of ionizing radiation in humans have been shown to occur due to two sequential effects: first the ablation of immune cells, resulting in loss of immune function and increasing likelihood of pathogenic infection (b)(4) (b)(4) and subsequently the loss of epithelial progenitor cells of the gastrointestinal (GI) crypt (b)(4) Both of these events have been shown to be due to the loss of fast-growing pluripotent and/or progenitor cell types. The time course of the acute injuries correlates with the life span of the mature cell types of the tissue, which are not replaced because of radiation damage to the adult pluripotent cells and adult stem cells. There is currently no FDA approved prophylactic or treatment for prevention of acute radiation injuries following high-dose (b)(4) radiation exposure.

In some cases supportive measures can be used to treat acute radiation syndrome, and survival from (b)(4) radiation exposure is now possible (b)(4) This results in current efforts to mitigate or treat later stage effects of ionizing radiation exposure. (b)(4)

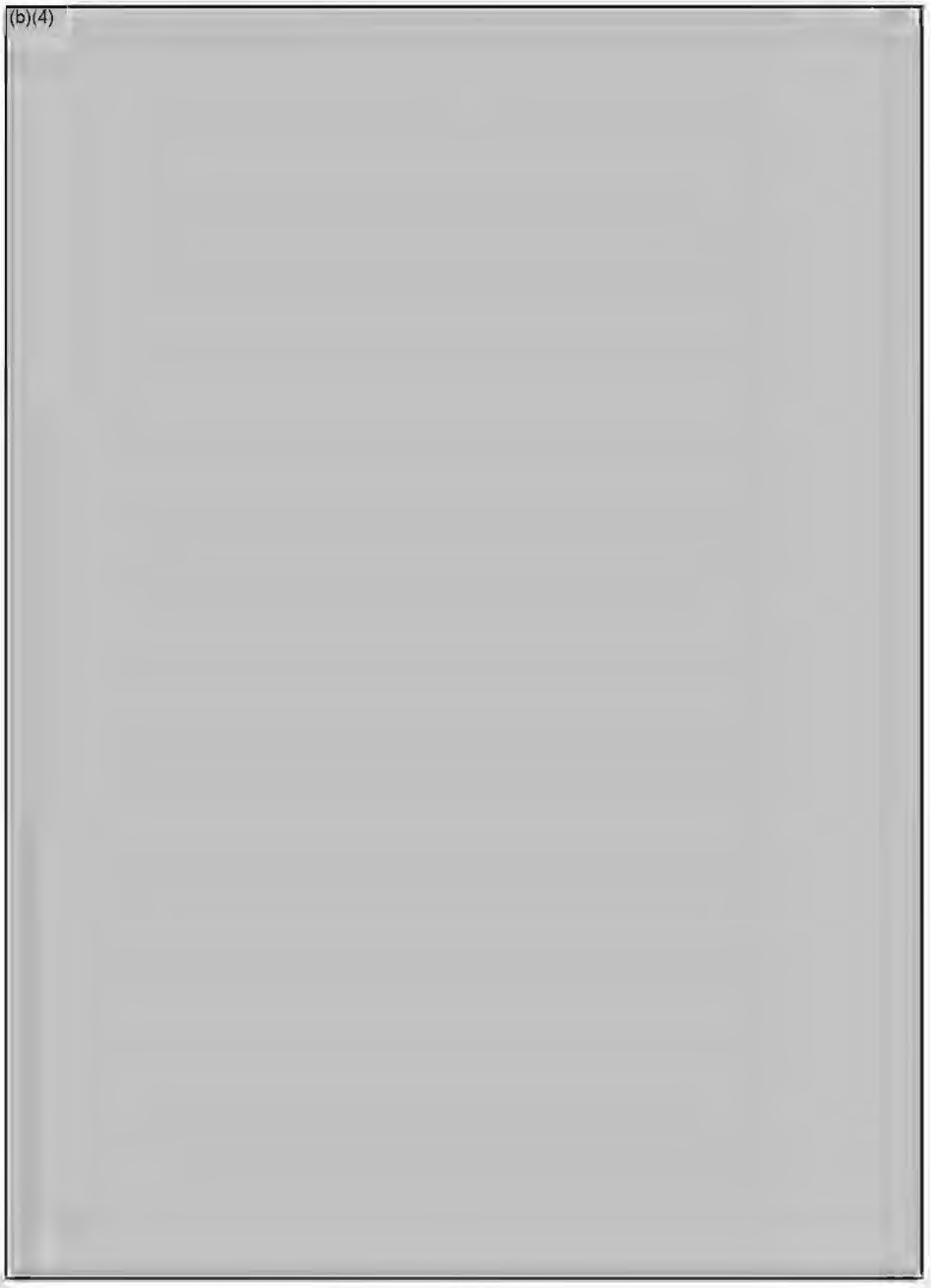
(b)(4)

In humans, death from (b)(4) can occur following survival from (b)(4) Cumulative evidence from a number of laboratories, including our own, shows that there is a threshold dose of radiation of (b)(4) to induce sufficient (b)(4) for the (b)(4) (b)(4) phases to occur in mice; below these doses, no permanent injury manifests, although some short-term effects are observed (b)(4) However, in humans, there is currently no accepted protocol for preventative treatment of radiation-induced (b)(4) and morbiditiy as high as 50% is associated with each phase (b)(4) (b)(4) (b)(4) injuries from radiation is considered to be a serious problem in the field for the development of agents to prevent these radiation injuries.

(b)(4) **as a radioprotectant and an antifibrotic agent:** (b)(4) (b)(4) and its proteolytic product (b)(4) are believed to have their primary biological functions in blood pressure homeostasis. However (b)(4) have been demonstrated to be upregulated in fibrotic tissues. Inhibition of (b)(4) or blockade of the (b)(4) receptors on cells has been shown to ameliorate drug-induced (b)(4) in murine and rat models (b)(4) (b)(4) receptor antagonists also prevent cardiac remodeling following ischaemia/reperfusion in rat models of cardiac remodeling diseases (b)(4) Several studies have also shown that inhibition of (b)(4) (b)(4) signaling mitigates radiation-induced injuries, including to the heart and (b)(4) (b)(4) One study shows that the (b)(4) reduces acute damage to the jejunal mucosa in mice following exposure to (b)(4) This work examined only initial tissue injury and did not extend findings past 5 days, and did not examine mortality. A second study found a reduction in (b)(4) and (b)(4) in rats exposed to (b)(4) (b)(4) radiation (b)(4)

Preliminary Studies:

(b)(4)


(b)(4)

(b)(4)

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched 1) Biomedical Research Database (BRD) (http://www.dtic.mil/biosys/org/brd/)
2) Computer Retrieval of Information of Scientific Projects (RePORTER) database (http://projectreporter.nih.gov/reporter.cfm)
3) PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed)

II.2.2. Date of Search Data base searches conducted on 6/03/15.

II.2.3. Period of Search Searches conducted for the entire time periods available in each data base. For BRD, 1998-2015; for RePorter, 1979-2015. For PubMed, 1970-2015.

II.2.4. Key Words of Search Searches were performed using the following key word combinations:

(b)(4)

II.2.5. Results of Search 1) BRD: (b)(4)
Two grants were funded to examine the activity of (b)(4)

(b)(4)

2) A RePORTER search revealed 2 current grants studying the effects of (b)(4) radiation induced injuries for (b)(4)

(b)(4)

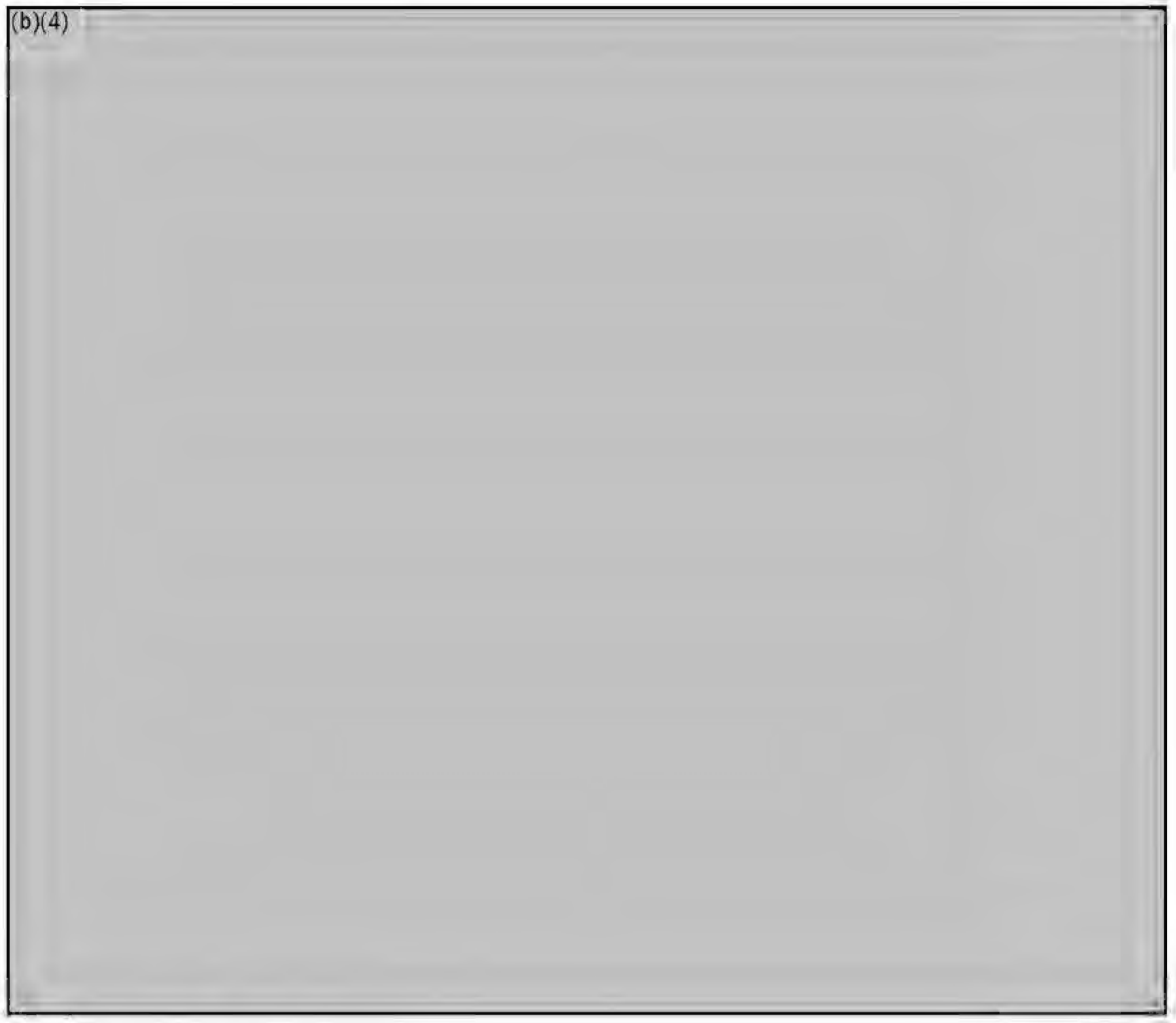
(b)(4)

None of the funded studies duplicate our research (b)(4)
(b)(4)

3) PUBMED Search *6/3/15*
None of the publications available described a murine model for both (b)(4)
(b)(4) injuries from radiation.

(b)(4)

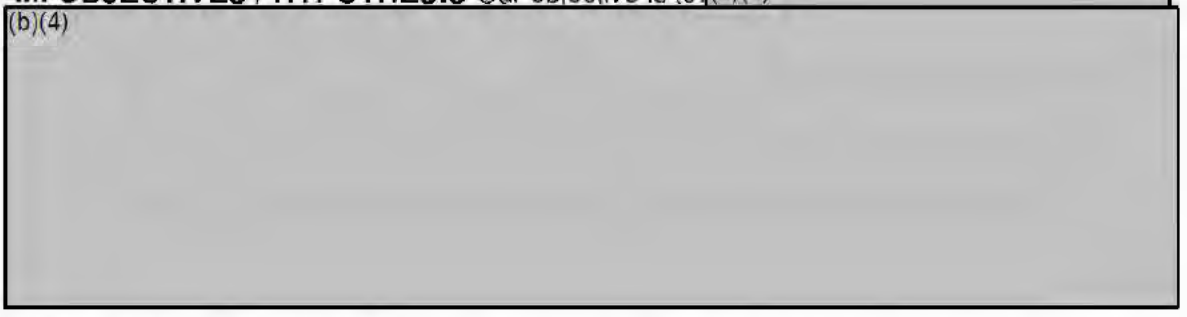
(b)(4)



Summary of other publications:

(b)(4)

III. OBJECTIVES / HYPOTHESIS Our objective is to: (b)(4)



IV. MILITARY RELEVANCE The Department of Defense is prepared to execute military missions within radiation environments, to manage radiation crises associated with terrorist activities, and to manage consequences in the event of nuclear weapons detonation. Recent studies indicate that there is an increasing risk of exposure to ionizing radiation (IR) to military personnel due to increasing terrorist activities, including potential use of stolen nuclear weapons or dispersal of radioactive material in a "dirty bomb" (b)(4) Because of potential exposure of military personnel to radiation, the DoD is committed to protect members of the Armed Services from the adverse health effects of ionizing radiation. High dose IR causes short term, acute injuries including hematopoetic and GI syndromes. However, both high and low dose IR (as may result from a radiation dispersal device) can cause (b)(4) as demonstrated by Japanese atomic bomb survivor data as well as accidental exposures to Russian populations (b)(4) While early effects of radiation injury now have treatment/ prophylactic strategies, the delayed effects currently have no treatment. Lack of

treatment for late effects of radiation, including (b)(4) will likely lead to delayed morbidity and mortality for personnel exposed to IR.

The Department of Defense (DoD) Chemical and Biological Defense Program 2007 Annual Report to Congress, Chapter 2.7.6 Medical Radiological Defense, addresses the requirements for the development of radioprotectants. This priority is outlined for the development of protectants for both acute and delayed radiation injuries, with an emphasis on (b)(4) (b)(4) According to *this* report, the mitigation of (b)(4) is a major research thrust area. (b)(4) (b)(4)

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

(b)(4)

V.1.1. Experiment 1 Examine the mechanism of radioprotection from (b)(4) (b)(4) (b)(4) An understanding of the mechanism(s) of both sensitization and protection will help in the rational design of radioprotectants in the future.

1.1. Mature and progenitor blood cell effects of (b)(4) after (b)(4) irradiation

One dose of radiation will be used (b)(4) (b)(4) Groups will be: Radiation only, (b)(4)

(b)(4) Time points will be (b)(4) (b)(4) days postirradiation for euthanasia to obtain (b)(4) Mice needed for experiment: need (b) to reach statistical significance for (b)(4) Note: the effects of (b)(4) (b)(4) (b)(4) The sham group will receive no treatment, and will be euthanized on day 0 to establish normal (b)(4) levels.

(b)(4) mice/group) x (3 conditions) x (6 time points) = (b)(4)
(b)(4) nice/group x sham group, no treatment = (b)(4)

(b)(4)

One dose of radiation will be used (b)(4) (b)(4) Groups will be: Radiation only, (b)(4) (b)(4) (b)(4) Time points will be: (b)(4) (b)(4) postirradiation. Mice needed for experiment: need to reach (b)(4) statistical significance for (b)(4) (b)(4) (b)(4) The sham group will receive no treatment, and will be euthanized on day 0 to establish normal (b)(4) levels.

(b)(4) mice/group) x (3 conditions) x (4 time points) = (b)(4)
(b)(4) mice/group x sham group, no treatment = (b)(4)

Endpoint: Endpoints for the experiment will be taken at (b)(4) days postirradiation for experiment 1a, and (b)(4) days postirradiation for experiment 1b. . Early time points will be if any animal exhibits signs or symptoms of morbidity or moribundity in accordance with AFRRI IACUC Policy #10. Our score sheet is also attached. Such animals will be euthanized at the time of observation.

V.1.2. Experiment 2 (b)(4)

(b)(4)

(b)(4) Mice needed for experiments: group to reach statistical significance for survival. (2 regimens of (b)(4) administration) x (5 doses of radiation) x mice/group) x (2 (b)(4) expt) (b)(4)

(b)(4)

(1 no treatment group) x (5 doses of radiation) x [redacted] mice/group) x (2 expt) = (b)(4) [redacted]

(b)(4) [redacted]

Endpoint: Endpoint for the experiment will be survival at [redacted] days. Animals suriving at (b)(4)

(b)(4) [redacted] days will be euthanized. Early time points will be if any animal exhibits signs or symptoms of morbidity or moribundity in accordance with AFRRI IACUC Policy #10. Our score sheet is also attached. Such animals will be euthanized at the time of observation.

V.1.3. Experiment 3 (b)(4) [redacted]

(b)(4) [redacted]

(b)(4) [redacted] mice/group) x (3 doses of (b)(4) [redacted] x (2 times of administration) = (b)(4) [redacted]

(b)(4) [redacted]

Endpoint: Endpoint for the experiment will be survival at [redacted] days. Animals surviving to (b)(4)

(b)(4) [redacted] days will be euthanized and tissue will be obtained. Early time points will be if any animal exhibits signs or symptoms of morbidity or moribundity in accordance with AFRRI IACUC Policy #10. Our score sheet is also attached. Such animals will be euthanized at the time of observation.

V.2. Data Analysis Experiment 1: Analysis of variance (ANOVA) will be used to detect significant difference among the groups. If significant, then the pair-wise comparison by Tukey-Kramer will be used to identify which group is different from the other. For experiment 1.1, N =

(b)(4) [redacted] mice per group could have 80% power to detect any significant difference between groups if any, given type I error of 5% and delta/sigma has at least 1.4, where the delta is the estimated mean differences between the two groups and sigma is the pooled standard deviation of the two groups (b)(4) [redacted] For experiment 3, we only need N = [redacted] based on our current data from (b)(4)

radiation experiments and induction of apoptotic and senescence markers.

Experiment: 2 and 3: Log-Rank test will be used for comparison among groups. If significant, Fisher's exact test will be used to detect significant differences across the groups in

(b)(4) survival rates at day [redacted] Kaplan Meier curves will be displayed. N = [redacted] mice per group will (b)(4) have 80% power in detecting a significant difference between two groups if any, given type I error of 5% where the treatment group survival rate is at least 68%, compared to a control group survival rate of 20%. Similar statements would apply, if the treatment group survival rates are at least 83%, 78%, or 73%, compared to vehicle group survival rates of 35%, 30%, and 25%, respectively [redacted]

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered For the last 9 years, we have utilized primary cell culture systems to examine anti-apoptotic mechanisms; however, we must determine whether our culture findings are relevant in whole animals. We will continue to use primary cell culture whenever possible to avoid unnecessary use of animals. We have also considered computer models as alternatives to animals. However, this alternative also lacks the knowledge base and complexity needed to evaluate radioprotective and radiotherapeutic agents.

V.3.2. Animal Model and Species Justification Tissue culture conditions cannot fully substitute for the complexities of whole organisms in response to radiation injury. Such injury involves the interactions between the [redacted] system and other organ systems. In order to develop viable treatments for humans, we find that our research must be conducted in whole animals.

[redacted] also advocates the mouse as the best species to use for radiation protection and radiotherapy protocols: 1) mice provide the least sentient species available whose response to radiation has been clearly identified, with physiological responses to radiation and radioprotectants that are similar to human physiology, 2) extensive literature is available on responses of mice to radiation and countermeasures, 3) more reagents are available to study the relevant cell types and molecules in mice than in any other species except humans, and the availability of transgenic and knockout mice make this model extremely attractive for further research into molecular mechanisms, and 4) [redacted] have extensive experience with this model in radiation injury and radioprotection research [redacted]

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species *Mus musculus*

V.3.3.2. Strain / Stock C57BL/6 mice, female. C57BL/6 mice have been used by [redacted] for the study of radiation-induced [redacted] injuries. These mice are also used by other laboratories. They provide consistent, reliable data for [redacted] injury and recovery.

CBA, female. CBA mice have previously been demonstrated to be sensitive to radiation-induced [redacted] in a shorter time course than C57BL/6 mice, and in a pattern that more closely resembles [redacted] in humans [redacted]

V.3.3.3. Source / Vendor [redacted] for both C57BL/6 and CBA (fully accredited by AAALAC Int)

V.3.3.4. Age 6-10 weeks (mice are ordered 6-10 weeks of age and irradiated from 12-14 weeks of age)

V.3.3.5. Weight Normal adult weight range for these strains: 17-21 g

V.3.3.6. Sex Female. Only females will be used in this study for the following reasons: 1) we have so far utilized female mice to lay the foundation of our studies for radiation-induced (b)(4) (b)(4) injuries; 2) because of long housing periods, it is advantageous to use female mice that are less aggressive toward each other.

V.3.3.7. Special Considerations Commercially procured animals must be adventitious disease free. Mice- Pathogen-free, including *Pseudomonas aeruginosa*, and Pasteurella: All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus; *Helicobacter* spp. *Mycoplasma pulmonis*, and *Clostridium piliforme*. Endoparasite- and ectoparasite-free.

V.3.4. Number of Animals Required (by species)

Mice C57BL/6 strain (b)(4)
Mice CBA strain
Total mice: (b)(4)

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement Animals demonstrating moribundity due to radiation exposure (b)(4) will be considered to have arrived at the study endpoint and will be immediately euthanized (See sections V.4.5 Study Endpoint and V.4.6 Euthanasia). The literature search revealed that, at present, there are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. In some cases radiation has been demonstrated to reduce pain in laboratory mice (b)(4) and has been in humans reported to alleviate cancer related bone pain (b)(4) (b)(4) A recent report showed that administration of botulinum toxin A was effective for reduction of pain and muscle spasms after clinical radiation for treatment of tumors (b)(4) (b)(4) Application of natural honey 20 min prior to radiation exposure was also shown to reduce mucositis with associated pain in humans following head/neck radiotherapy (b)(4) it has not been determined whether honey acts as a local radioprotectant. Spinal cord stimulation has also been reported in humans to alleviate pain resulting from radiotherapy (b)(4) however, this method would not be practical for use in mice because of the extensive spinal cord manipulation required. Human administration of morphine also relieves pain (b)(4) but again this would not be practical for mice in our experiments because of side effects of the drug. In humans, exposure to (b)(4) irradiation may induce vomiting and diarrhea, causing pain and distress. However, mice are not susceptible to vomiting.

V.3.5.2. Reduction Whenever possible our laboratory used shared control groups between experiments. Tissues will be collected for multiple assays from the same animals further reducing the total number of animals needed. At every stage of the research, planned experiments will be reevaluated, in an attempt to reduce the numbers of experiments and eliminate repeats of experiments where statistical significance has already been obtained. We also plan to take as many tissues as we can reasonably analyze for experiments examining protein oxidation.

V.3.5.3. Replacement Our laboratory has made extensive use of tissue culture techniques to study the effects of captopril, Ang II, and radiation on cells. While these studies have provided valuable data, it is not feasible to use non-animal systems in place of animal models to address the research questions in this protocol.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C (b)(4) # of animals)

V.4.1.1.1.2. Column D (# of animals)

V.4.1.1.1.3. Column E _ (# of animals)

V.4.1.1.1.4. Pain Category Assignments

(b)(4)

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization We will be unable to use analgesics since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4) Some anesthetics and sedatives have been reported to contribute to postoperative (b)(4) which likely reduce some functions of radiation. Additionally, it has been shown that the biometabolism of analgesics can induce protein oxidation, which would interfere with sample analysis (b)(4)

(b)(4)

Animals in experiments involving blood collection will be deeply anaesthetized immediately prior to sample collection. For euthanasia, animals will be given 0.1-0.2 ml/mouse pentobarbital (Fatal Plus 50 mg/ml) given i.p. with a 25 G needle.

An anesthesia protocol has been developed for use during the SARRP irradiation. Mice will first be placed in a chamber to deliver isoflorane (3-5% isoflurane in 100% oxygen), until deep anesthesia is obtained. Animals will then be placed in position in the SARRP with a nose cone properly positioned to deliver inhaled isoflurane (1-3% isoflurane in 100% oxygen). During irradiaition, animals will be monitored in real time by camera. If any animal appears to be out of alignment, experiments will be paused to allow repositioning of the animal.

V.4.1.2.2. Pre- and Post-procedural Provisions Animals will be housed and cared for as described in Section V.5. Veterinary Care both before and after dose administrations and irradiation procedures.

V.4.1.2.3. Paralytics None

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures
V.4.1.3.1. Sources Searched Sources searched: DoD Bioedical Research Data Base (BRD), CRISP, PubMED

V.4.1.3.2. Date of Search 6/03/2015

V.4.1.3.3. Period of Search Search covered published manuscripts from ~1965-present. (BRD search included all available years 1998 -2015).

V.4.1.3.4. Key Words of Search Pain, analgesia, mice, radiation, ionizing radiation, humane

V.4.1.3.5. Results of Search The literature search revealed that, at present, there are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. In some cases radiation has been demonstrated to induce opioid-mediated analgesia in laboratory mice (b)(4) and has been in humans reported to alleviate cancer related bone pain (b)(4) A report showed that administration of botulinum toxin A was effective for reduction of pain and muscle spasms after clinical radiation for treatment of tumors (b)(4) Application of natural honey 20 min prior to radiation exposure was also shown to reduce mucositis with associated pain in humans following head/neck radiotherapy (b)(4) it has not been determined whether honey acts as a local radioprotectant. Spinal cord stimulation has also been reported in humans to alleviate pain resulting from radiotherapy (b)(4) (b)(4) however, this method would not be practical for use in mice because of the extensive spinal cord manipulation required. However in a separate study in mice, low dose focal irradiation in a mouse model of bone cancer was associated with spinal changes in neuro-mediators of nociception, the sensory neurons that respond to potentially damaging stimuli by sending pain signals (b)(4) Human administration of morphine also relieves pain (b)(4) but again this would not be practical for mice in our experiments because of side effects of the drug. In humans, exposure to (b)(4) irradiation may induce vomiting and diarrhea, causing pain and distress. However, mice are not susceptible to vomiting.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification The literature search revealed that, at present, there are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. In some cases radiation has been demonstrated to reduce pain in laboratory mice (b)(4) and has been in humans reported to alleviate cancer related bone pain (b)(4) A recent report showed that administration of botulinum toxin A was effective for reduction of pain and muscle spasms after clinical radiation for treatment of tumors (b)(4) (b)(4) Application of natural honey 20 min prior to radiation exposure was also shown to reduce mucositis with associated pain in humans following head/neck radiotherapy (b)(4) it has not been determined whether honey acts as a local radioprotectant. Spinal cord stimulation has also been reported in humans to alleviate pain resulting from radiotherapy (b)(4) however, this method would not be practical for use in mice because of the extensive spinal cord manipulation required. Human administration of morphine also relieves pain (b)(4) but again this would not be

practical for mice in our experiments because of side effects of the drug. In humans, exposure to (b)(4) irradiation may induce vomiting and diarrhea, causing pain and distress.

V.4.2. Prolonged Restraint
No prolonged restraint is required for (b)(4) irradiation (b)(4) irradiation will require restraint in the SARRP for 20-45 min to receive X-ray exposure (b)(4) (b)(4) animals will be anesthetized using inhaled isoflurane during this period (see Anesthesia above).

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions Not applicable

V.4.3.2. Procedure(s) None

V.4.3.3. Post-surgical Provisions Not applicable

V.4.3.4. Location Not applicable

V.4.3.5. Surgeon

V.4.3.6. Multiple Major Survival Operative Procedures Not applicable

V.4.3.6.1. Procedures Not applicable

V.4.3.6.2 Scientific Justification Not applicable

V.4.4. Animal Manipulations

V.4.4.1. Injections Intra-peritoneal injections of pentobarbital for euthanasia 0.1-0.2 ml/mouse Fatal Plus (39-78 mg pentobarbital/mouse), will be performed with a 25 G needle.

V.4.4.1.1. Pharmaceutical Grade (b)(4) (b)(4)

V.4.4.1.2. Non-Pharmaceutical Grade Drugs Not applicable

V.4.4.2. Biosamples
Blood will be taken under deep anesthesia with pentoparbital. All other biosamples will be taken after euthanasia (b)(4) or analysis will removed *en bloc*. Intestinal tissue will be taken in smaller amounts (the first segment of the small intestine). All other tissues will be available to other researchers for tissue sharing.

For some experiments, blood will be obtained from the animals. Blood will be taken by percutaneous intra-cardiac collection using a 23 – 22 g needle. All other tissues will be available for tissue sharing with other investigators. Intra-cardiac puncture must be performed under deep anesthesia and is considered a non-survival procedure. The mouse is deeply anesthetized and toe pinch response will be used to determine the depth of anesthesia. The ventral chest will be wiped with 70% ethanol. A 23 – 22 g needle will then be inserted at the base of the sternum, bevel up (b)(4) at a 15-20^0 angle directed to the left of the midline. Once the needle is inserted, the blood is slowly aspirated. If no blood is observed, the needle is repositioned with slow aspiration. Once the blood

volume is collected, the mouse is euthanized by cervical dislocation while still under deep anesthesia. Typically 0.5-0.75 ml of blood is collected; sometimes less blood is available after radiation exposure.

V.4.4.3. Adjuvants Not applicable

V.4.4.4. Monoclonal Antibody (MAb) Production Not applicable

V.4.4.5. Animal Identification
All cages will have cage cards identifying the treatment groups for each experiment. For experiment 3, each animal within a cage will receive ear punches for individual identification (1 = no cut; 2 = right ear upper corner; 3 = left ear upper corner; 4 = both ears). For experiments 1 and 2, we have found that tails can be marked with an indelible marker that remains visible for the duration of the experiment for the purpose of scoring pain on each individual animal (1 = 1 line; 2 = 2 lines; 3 = 3 lines; 4 = no lines.).

V.4.4.6. Behavioral Studies Not applicable.

V.4.4.7. Other Procedures
(b)(4) (b)(4) Mice will be placed in well-ventilated Plexiglas boxes (standardized for the cobalt irradiation facility) for up to 45 min, but usually less time, during acute irradiation procedures. Following radiation exposures mice will be immediately moved to standard sized cages that house either 4-8 animals per cage. Holders will be sanitized after each use.

(b)(4) (b)(4) this dose of radiation is currently under determination in another experiment. Mice will first be placed in a chamber to deliver isoflurane (3-5% isoflurane in 100% oxygen), until deep anesthesia is obtained. Animals will then be placed in position in the SARRP with a nose cone properly positioned to deliver inhaled isoflurane (1-3% isoflurane in 100% oxygen). During irradiaition, animals will be monitored in real time by camera. If any animal appears to be out of alignment, experiments will be paused to allow repositioning of the animal. Following radiation exposure, mice will be allowed to recover from anesthesia under supervision, with heating pads. Immediately upon recovery, animals will be returned to standard cages that house 4-8 animals per cage. Holders will be sanitized after each use.

V.4.4.8. Tissue Sharing For most studies (b)(4) will be removed *en bloc*, and (b)(4) (b)(4) will be obtained. (b)(4)
All other tissues will be available for tissue sharing with other investigators. Our laboratory has made extensive use of shared tissues for training and technique development for (b)(4 tissue.

V.4.4.9. Animal By-Products Not applicable

V.4.5. Study Endpoint Endpoints will be taken at (b)(4) days postirradiation for experiment 1a, and at (b)(4) days postirradiation for experiment 1b. Animals will be

euthanized at the endpoints and tissues will be obtained. Early time points will be taken if any animal exhibits signs or symptoms of morbidity or moribundity in accordance with AFRRI IACUC Policy #10. (b)(4) (b)(4) (b)(4) Early endpoints will be used in the case of morbidity and morbundity. Specific health assessment criteria including rapid weight loss, ruffled fur, dried lacrimal fluid accumulated in the cul-de-sac of their eyes, are cool to the touch, decreased appetite, diarrhea, difficulty breathing or panting (b)(4) (b)(4) will used to determine early study endpoints for euthanasia. Mice exhibiting these signs will be considered morbid and will be monitored by the PI or other members of the investigative team at least twice daily, early morning and late afternoon. Mice will be considered moribund when, in addition to showing some or all of the signs of morbidity, they exhibit labored breathing plus either an inability to remain upright or are unmotivated to move. Morbid or moribund mice will be automatically euthanized as described in accordance with IACUC Policy 10. As mice will be checked twice daily, the longest period of time that any animal may exhibit signs of moribundity is ~16 h (the overnight period).

V.4.6. Euthanasia Moribund animals, as defined by AFRRI Policy #10, and animals at the stated endpoints of experiment 2 will be euthanized using CO_2, followed by cervical dislocation to confirm euthanasia. Animals at the stated endpoints of experiments 1 and 3 will be euthanized by injection of 0.1-0.2 ml/mouse Fatal Plus (39-78 mg pentobarbital/mouse), i.p. using a 25 G needle. Euthanasia will be confirmed by cervical dislocation. Animals which appear to be experiencing pain or distress as a result of radiation exposure or are found moribund will also be euthanized by pentobarbitol and necropsied. Animals used for blood collection will be deeply anesthetized with pentobarbital and after blood draw will be euthanized by cervical dislocation or by thoracotomy. At the end of experiments, unused animals will be transferred to other protocols or euthanized by exposure to 100% CO_2 from a compressed gas cylinder in accordance with the current American Veterinary Medical Association (AVMA) Guidelines for Euthanasia. Exsanguination, cervical dislocation, and/or (b)(4) will be used as a secondary measure to ensure euthanasia.

V.5. Veterinary Care

V.5.1. Husbandry Considerations All of the animals used in this study will be housed in the VSD facility in cages of 4-8 mice. Food and acidified (2.5 – 3.0 pH) water (to minimize *Pseudomonas* infection) and Harlan Teklad Rodent Diet #8604 will be available *ad libitum*. The 12:12 hr (lights off at 6 pm) VSD room light cycle is fine for all the studies. (b)(4) (b)(4) Additional medicated water will be present in the room, in a labeled and dated bottle in case a bottle needs to be refilled and staff cannot be reached.

V.5.1.1. Study Room (b)(4),(b)(6) (b)(4),(b)(6)

(b)(4),(b)(6)

V.5.1.2. Special Husbandry Provisions (b)(4)
(b)(4)
(b)(4) Additional medicated water will be present in the room, in a labeled and dated bottle in case a bottle needs to be refilled and staff cannot be reached. No food or fluid restrictions are required.

V.5.1.3. Exceptions Mice will be group housed except during the irradiation exposure as described above or in the event that their cage mates have had to be removed from the study. Under these circumstances they may be housed individually.

V.5.2. Veterinary Medical

V.5.2.1. Routine Veterinary Medical Care Experimental animals will be observed twice a day during the critical period by one of the study investigators or technicians and scored according to Policy 10. Animals will be observed once a day during the critical period by VSD personnel, for a total of three visits a day during the critical period in accordance with AFRRI Policy 10. VSD personnel will also monitor the animals twice a day during their rounds. As described in another section (study endpoint) morbid animals will be monitored at least twice daily, morning and afternoon. The experimental design does not contemplate supportive therapy. The justification for not providing supportive care has been described in the study end point section as well as in the pain category section. Criteria to be used for health evaluation while the animals are on study are as stated in Policy 10: weight loss, appearance, general behavior, provoked behavior, and respiratory rate. Animals exhibiting adverse signs may be euthanized, and considered to be at the study endpoint.

V.5.2.2. Emergency Veterinary Medical Care Emergency veterinary medical care is available 24 hours a day, 7 days a week, including weekends and holidays.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy Environment enrichment for the mice will be provided with the addition of nestlets and/or hiding tubes to each cage. Group housing of the mice also provides social enrichment.

V.5.3.2. Enrichment Restrictions None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)	M.S.	AFRRI	Multiple dates	1,3a,4,5,7a, 7b, 7c	8+yrs
	Ph.D.	USUHS/AFRRI	(b)(6)	1, 3a, 5	5 yr
	Ph.D.	AFRRI		1, 3a, 5	<1 yr
	B.S.	AFRRI		1, 3a, 5	5 yr
	Ph.D.	USUHS/AFRRI		1, 3a, 5, 7b	<1 yr

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others

7a. Ear punch for animal identification
7b.. Cobalt irradiation
7c. SARRP irradiation

VII. BIOHAZARDS/SAFETY

All the personnel will be trained as radiation users on a regular basis. All investigators and technicians while in the vivarium will use appropriate procedures as described by VSD, include the wearing of mask and gloves during observation of mice. No additional biohazards will be used for these experiments.

VIII. ENCLOSURES

I. (b)(4)
II. Score sheet from Policy 10
III. References

References:

(b)(4)

(b)(4)

(b)(4)

X. ASSURANCES

Protocol Title: (b)(4) Protection from Radiation-Induced (b)(4) (b)(4) Injuries in a Murine Model (*Mus musculus*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)		6/9/2015
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (August 2015)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

***** DELETE THIS PAGE PRIOR TO PROTOCOL SUBMISSION *****

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	9/3/2015	2nd Resubmission Date:	10/9/2015
Approved/**Returned for Revision**:	9/22/2015	**Approved**/Approval Withheld by IACUC:	10/13/2015
1st Resubmission Date:	9/29/2015	**EXPIRATION DATE:**	10/12/2018
Approved/**Returned for Revision:**	10/9/2015	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE:

Minimally Invasive Radiation Biodosimetry and Evaluation of Organ Responses in laboratory mice, *Mus musculus*

IV. PRINCIPAL INVESTIGATOR:

(b)(6) **Ph. D**
Research Biologist, AFRRI-SRD
Office: (b)(6) Fax: (b)(6)
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD** Date
Head, Scientific Research Department, AFRRI
Telephone (b)(6)
(b)(6)

- Or –

(b)(6) **PhD, LTC, USA** Date
Head, Scientific Research Department, AFRRI
Telephone (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date
Statistician
Telephone (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, LTC, VC, USA** Date
Head, Veterinary Sciences Department, AFRRI
Telephone (b)(6) Fax (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date
Safety and Occupational Health Specialist, USUHS
Telephone: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____,
(Date)

(b)(6) **Ph.D.**
IACUC Chair (b)(6) Scientific Research Department, AFRRI
Telephone (b)(6) Fax (b)(6)
(b)(6)

PROTOCOL TITLE:
Minimally Invasive Radiation Biodosimetry and Evaluation of Organ Responses in laboratory mice, *Mus musculus*

PRINCIPAL INVESTIGATOR:
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(b)(6) PhD
Scientist (b)(6) (AFRRI – SRD)
Tel: (b)(6)
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AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER:
CDMRP grant has been approved. Sponsor needs the approved animal protocol before funding approval. USU account number is pending.

I. NON-TECHNICAL SYNOPSIS

II. BACKGROUND

II.1. Background

Acute Radiation Syndromes (ARS) and Delayed Effects of Acute Radiation Exposure (DEARE)

Exposure to ionizing radiation from nuclear events or radiological accidents often leads to major harmful consequences to human health. Therefore, a critical need exists in the area of radiation countermeasures to diagnose and treat victims of radiation exposure. To this end, lack of reliable biomarkers that provide organ specific readout of biological response to radiation poses a major problem in identifying individuals who might be at risk for developing Acute Radiation Syndromes (ARS) and Delayed Effects of Acute Radiation Exposure (DEARE) following a radiological event. Depending on the dose, the symptoms of ARS can appear within hours to weeks. It is a challenge to triage individuals who are minimally exposed compared to those who received high radiation dose and need immediate treatment [1-4]. As such, ARS follows a deterministic effect whereby dose effects have distinct clinical outcomes: generally <2 Gy exhibit mild symptoms, hematologic effects (HE) are prominent at doses between 2 and 6 Gy, and gastrointestinal (GI) effects are prominent at doses >5 Gy and progress more rapidly at higher doses of >10 Gy where neurovascular collapse and death occur promptly [5]. Rapid diagnosis is essential as demonstrated by the dramatic increase in survival rates if treatments are administered early [6]. Currently, three criteria are mainly used to determine radiation exposure levels: clinical observation, lymphocyte depletion kinetics, and the dicentric chromosome (DC) assay [7, 8]. Patient to patient variability greatly decreases the accuracy of clinical diagnosis, lymphocyte depletion kinetics requires 3

days of analysis for a crude dosimetry reading. Moreover, the DC assay is highly technical and labor intensive.
A major issue that impacts the decision making in triage after radiation accidents is the heterogeneity due to variations in exposures [1, 3, 8, 9]. Depending on the geography of exposure, one or more organs may be affected at varying degrees. Damage to the GI system should be evident within days, which, however, requires a relatively higher dose than that needed to affect the HE system. The lung is a relatively sensitive organ; but the effects will not be apparent for weeks or even months [10, 11]. So far, there are no biomarkers that can accurately identify the levels of damage to the individual organs and the overlapping effects and multi-organ dysfunction. Current biodosimeters (lymphocyte depletion kinetics/dicentric chromosome assays) read only the response in the hematopoietic system. Because of the differences in the kinetics and latency period, it is difficult to detect and/or distinguish the effects on non-HE systems. In addition, the threshold and latency period could differ due to differences in immune status, inherent differences in the radiation sensitivity of the individual and other underlying conditions. Unfortunately, available diagnostics do not adequately meet these requirements, particularly in the case of partial body exposure scenario. This study addresses this critical gap of knowledge in the field through discovery and development of biomarkers that could be easily measured providing the readout of distinct organ specific and physiological response to radiation.

Circulating microRNAs (miRNAs) as radiation biodosimeters, biomarkers of organ injury/damage and early predictors of delayed effects:
miRNAs are small RNA molecules of 18-24 nucleotide length, originally identified as regulators of gene expression [12]. They are abundant in body fluids such as plasma, urine, tears, and breast milk. They are protected in exosomes, stable at room temperature for days, and ensure stability for long-term preservation and multiple freeze-thaw cycles [13]. Levels of specific miRNAs in blood can change after radiation, as do the expression levels of mRNAs [14, 15]. miRNAs can be released as exosomes and apoptotic bodies and/or by active secretary pathways. It has been shown that processing of the precursors of miRNAs can directly or indirectly be regulated by cytokines like TNFα and TGFβ1 [16-18] that are altered after radiation. Moreover, reduction in particular radiosensitive cell population (e.g. lymphocytes) will result in reduced circulating cell-free exosomes derived from those cells. There are specific signatures with regard to miRNA expression in different cells and their changes in circulating system can provide readout of organ-specific responses [19, 20]. Particularly, the HE system and lung constantly release exosomes associated with their respective signatures. We propose to exploit this unique tissue specific signature that can be read in exosomes released to the circulating system for evaluating the tissue responses. This can provide readout of changes in organs such as lung for which no biomarkers are currently available.

Survival from radiation lethality by gamma-tocotrienol (GT3)
We reported that gamma-tocotrienol, a naturally occurring vitamin E analog, protects mice from radiation-induced pancytopenia, restores bone marrow progenitor cells (b)(4) reduces intestinal and vascular oxidative stress, and protects 100% mice exposed to lethal dose of whole body γ-radiation (b)(4) CD2F1 mice treated with GT3 showed increased numbers of spleen colonies compared to vehicle treated animals at 12 days

after 8.5 Gy of γ-radiation (b)(4) GT3 reduces radiation-induced vascular oxidative stress *in* mice through an HMG-CoA reductase dependent manner post-radiation treatment (b)(4) GT3 has also been shown to improve the post-irradiation recovery of plasma markers of endothelial function, an effect that is not dependent on HMG-CoA reductase inhibition [24].

The proposed studies will allow us to develop a panel of circulating miRNAs for evaluation of organ responses/damages following partial body exposure and test the effect of GT3 on tissue-specific miRNA. miRNA biomarkers have several advantages over protein and metabolite biomarkers.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

To find any possibility for duplication, the following databases have been searched:
PubMed
Reporter (CRISP) - Computer Retrieval of Information on Scientific Projects, now known as 'Reporter'

II.2.2. Date of Search PubMed – 08/12/2015
Reporter (CRISP) - 08/13/2015

II.2.3. Period of Search
PubMed – 1960-2015
Reporter (CRISP) – 1972-2015

II.2.4. Key Words of Search
1. Micro RNA AND Radiation
2. Gamma-tocotrienol AND micro RNA
3. Gamma-tocotrienol AND Radiation
4. Lung and Radiation
5. Gastrointestinal system and Radiation

II.2.5. Results of Search
Table 1 includes a summary of the keywords and keyword combinations used for searching the RePORTer and PubMed databases.

Keywords	PubMed	RePORTer
Micro RNA AND radiation	852	154
Gamma-tocotrienol AND micro RNA	1	1
Gamma-tocotrienol AND Radiation	32	399
Lung and Radiation	30557	293
Gastrointestinal system and Radiation	25659	297

The searches indicated that there have been no previous or current studies have been done on developing a panel of microRNA biomarkers and to study the effect of GT3 in response to lung and gut injury. Therefore, the proposed study to develop a panel of circulating miRNAs for evaluation of organ responses/damages following partial body

exposure is not a duplication of a previous experiment. While there were some hits, the studies were not relevant to study the effect of GT3 following radiation.

III. OBJECTIVE / HYPOTHESIS

The objective of the project is to understand the acute, delayed and late effect of ionizing radiation and test the effects of radioprotectors and mitigators.

One of the major challenges in radiation countermeasure development is lack of biomarkers that provide minimally invasive and rapid readout of the recovery of the acute and delayed effects. As a proof-of-the-concept, we plan to compare the changes in multi-marker panel in mice treated with the tested agent, GT3, a novel prophylactics radiation countermeasure effective for HE-ARS and possibly for GI-ARS and lung DEARE. The vitamin E analogue, GT3 has been shown to exhibit significant radio-protective activities on hematopoietic stem cells and progenitors in multiple animal models [25].

(b)(4)

(b)(4) We will investigate the tissue versus plasma miRNA responses in evaluating the effect of gamma-tocotrienol (GT3), a novel radiation countermeasure in the mouse model.

IV. MILITARY RELEVANCE

Military operations are seriously impaired by radiation exposures depending on the dose and dose rate. Currently, there is no FDA approved preventive measure available for military personnel to protect them from the hazards of radiation exposure. The problem is more acute in recent times due to the unpredictable nature of terrorist attacks. Recognizing this urgent need, United States Department of Defense has assigned top priority in "Defense Technology Objectives (DTO)" to the "development of medical countermeasures to radiation exposure" against both early effects and late arising health effects. These concerns address the urgent need to develop an appropriate radiation protective agent (prophylactic or therapeutic) to sustain immediate warfighter capabilities and to enable the commanders to make judicious decisions in troop deployment. Currently, there are several drugs at various stages of development. This study will focus on comparing the changes in multi-marker panel in mice treated with the tested agent, GT3, a novel prophylactics radiation countermeasure effective for HE-ARS and possibly for GI-ARS and lung DEARE.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

V.1.1. Experiment 1

(b)(4)

Experimental Design: We will use C3H mice exposed to two sub-lethal doses of gamma-rays (4 and 7 Gy) (TBI from Cobalt-60), which will ensure that the mice will be

available for studies throughout the period of study, and the biomarkers will have dose response (LD 50/30 is 7.6 Gy) [27]. We will use 12-14 weeks old male C3H mice. We will collect plasma and tissues (spleen, bone marrow sternum and intestine) at the indicated time points and (b)(4) (b)(4) Tissues and blood will be collected at acute (day 1) and delayed (day 14 and 30) post-radiation. A part of the tissue spleen, bone marrow sternum and jejunum will be fixed in formalin for histopathological analysis. (b)(4) (b)(4)

V.1.2. Experiment 2

(b)(4) (b)(4) A next generation microirradiator, SARRP (small animal radiation research platform) X-ray with real time CT imaging [28, 29] will be used for organ targeted irradiation. Lung and blood will be collected at acute (day 1) and delayed (days 14, 30, and 90) post-radiation from animals exposed for lung radiation; and jejunum and blood will be collected at 3.5 and 14 days from animals exposed for gut radiation. A part of the tissue will be fixed in formalin for histopathological analysis; (b)(4)

Experimental Design: Cumulative evidence from a number of laboratories shows that a threshold dose of radiation (10-20 Gy) induces significant lung injury [30, 31]. Depending on the mice strains, radiation induced delayed and late effects on lung (alveolitis, pneumonitis and subsequent fibrosis) occur between 3 to 12 weeks [30, 31]. (b)(4)

General Methods: A single dose of 200 mg/kg GT3 (b)(4) or vehicle (80% Miglyol 812, 9% Span 80, 1% Solutol HS-15, 10% Phosphate Buffered Saline, pH 7.4) or saline will be injected subcutaneously in a volume of 0.1 ml per mouse at the nape of the neck [22] 24 hours before irradiation. Organ/tissue specific partial body exposure to lung, and intestine in mice will be done with SARRP radiation facility at AFRRI to accurately deliver low LET radiation very specifically to tissue as established at the Johns Hopkins University. It will provide orthovoltage X-ray irradiation of mice with beams that can be as narrow as 0.5 mm in diameter [29]. Real-time CT imaging of animals positioned on the exposure table immediately before the radiation will provide reliable pinpoint targeting of organs of interest. Whole blood will be collected under anesthesia and plasma will be prepared, aliquoted and stored in -80C. A part of the tissues (lung and jejunum) will be collected and transferred to 10% formalin for

histopathology analysis and residual tissues will be flash frozen in liquid nitrogen. (b)(4) [redacted]

Histological and Biochemical Assays: Tissue (jejunum and lung) histopathology (e.g. H & E) will be evaluated by a veterinary pathologist at AFRRI. Since several of the (b)(4) [redacted] are linked to immune responses, the effects of the agent in reducing lung toxicity will be evaluated by comparing changes in pro- *versus* anti-inflammatory cytokines in plasma samples (Millipore mouse 32-plex kit, Luminex bead-based assay).

Mice Needed for Experiment 1 (Co-60 whole body radiation):

Radiation doses: 4 and 7 Gy
Groups = 3 (saline, vehicle, GT3)
No. of time points = 3 (Day 1, 14, and 30)
No. of animals per group = 6
Naïve control = 10
No. of repeat = 2

Total no. of mice: (2 x 3 x 3 x 6 +10) x 2 = 236

Mice Needed for Experiment 2 (SARRP partial body body radiation):

Radiation doses: 12 and 16 Gy
Parts of the body: Lung and gut
Groups = 3 (saline, vehicle, GT3)
No. of time points for lung = 4 (Days 1, 14, 30, and 90)
No. of time points for gut = 2 (Days 3.5 and 14)
No. of animals per group = 6
No. of repeat = 2

Total no. of mice for lung: (2 x 3 x 4 x 6) x 2 = 288

Total no. of mice for gut: (2 x 3 x 2 x 6) x 2 = 144

Naïve control will be shared between experiment 1 and 2.

Total number of mice requesting for this protocol = 236+288+144 = 668

Adding a 10% increase to the total number of mice requested to account for unexpected losses = 70

Grand total = 668+70 = 738

V.2. Data Analysis

All analysis will be done by our collaborator (Ohio State University). Six animals per group (N=6) will be used for both experiments 1 and 2. Six animals per group would have 80% power to detect a significant difference between two groups if any, given type I error of 5% and delta/sigma of the differences between two group at least 2.4, where sigma is the common standard deviation, and delta is the mean difference [34].

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

In vitro studies using cell culture models have established the radioprotective and radiomitigating effect of certain anti-oxidents. However, animals study is required for understanding the systemic and tissue specific effects of radiation and counter effects of these agents. These studies will potentially lead to development of novel non-invasive assays to evaluate risk of ionizing radiation, therapeutic response as well as prevention of cancers and fibrosis in human beings. These can be achieved only by prior studies in live animals.

V.3.2. Animal Model and Species Justification

As discussed in the previous section, animals must be used to study the mechanism of action of a radioprotectant *in vivo*. Mouse model will be used as a proof-of-concept because approximately 99% of human DNA is similar to that of mice, and mouse models are well characterized to study the mechanism of action of a radioprotectant [22, (b)(4) [redacted] Zebrafish and Drosophila are not considered for this study because their physiology is largely different from mammalian systems.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species *Mus musculus*

V.3.3.2. Strain / Stock
C3H

V.3.3.3. Source / Vendor
Harlan Labs, Indianapolis, Indiana or similar approved vendors

V.3.3.4. Age
7 - 10 wks (age at acquisition). 12-14 weeks will be used in experiment.

V.3.3.5. Weight
22-30 g

V.3.3.6. Sex
Male

Justification of using only male mice: Previous studies have been conducted with male mice, so male mice are needed for data comparison. Although, it is important to confirm observations in females, such studies may be carried out at a later date.

V.3.3.7. Special Considerations
Commercially procured animals must be adventitious, disease free, and pathogen-free—including *Pseudomonas aeruginosa*, and *Pasteurella*. All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse

Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus, *Mycoplasma pulmonis*, and *Clostridium piliforme*. They must also be endoparasite and ectoparasite-free.

V.3.4. Number of Animals Required (by species)
C3H mice, 738
V.3.5. Refinement, Reduction, Replacement (3 R's)
V.3.5.1. Refinement
Animals will be irradiated at sublethal doses of 4 and 7 Gy for whole body Co-60 radiation. For SARRP exposure, animals will be irradiated at 12 and 16 Gy. We do not expect to see morbidity in the course of the study. However, if we observe morbid animals, we will increase monitoring and euthanize animals following policy 10 (score sheet attached, appendix 1) at early time points. PI will take necessary steps for all personnel in the project to be rigorously trained in handling techniques (injections, and routine cage-side observations) to minimize distress and pain to the animals. Specifically, during capture and restraint of the rodents, care will be taken to ensure that handling does not contribute to distress.

V.3.5.2. Reduction
Previously published and unpublished data have established which radiation dose and time points are important to get the information for miRNA after radiation injury. This information has enabled us to reduce the number of animals needed for this study. We are also reducing the animal numbers (n = 6) by sharing tissues for determining different parameters from the same experiment. Based on our previous experiments, we will use the minimum required number of animals for statistical validity. This will reduce the number of animals used in this protocol.

V.3.5.3. Replacement
N/A

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C _____ (# of animals)

V.4.1.1.1.2. Column D ___20__ (# of animals)

V.4.1.1.1.3. Column E ___718__ (# of animals)

V.4.1.1.1.4. Pain Category Assignments
Table 2.

Exp. #	Experimental/Control Group	C	D	E	Totals
1.	Naïve, 4 Gy veh, 4 Gy GT3, 7 Gy veh, 7 Gy GT3		20	216	236
2.	12 Gy veh, 12 Gy GT3, 16 Gy veh, 16 Gy GT3			432	432

Exp. #	Experimental/Control Group	C	D	E	Totals
	10% animals added due to unexpected loss			70	70
Totals:			20	718	738

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

The protocol will not use anesthetics or analgesics during whole body Co-60 radiation (Experiment 1) since they will adversely affect the outcome of the experiments. The proposed study is to determine the effects of irradiation and countermeasures on the function of the immune and blood forming systems. Use of any analgesics and anesthetics would interfere with the function of the immune system and therefore compromise the interpretation of the results [36]. Topical antibiotics will be applied if wounding occurs via in-cage fighting, under the guidance of the clinical veterinarian in accordance with standard treatment measures.

For partial body SARRP exposure (Experiment 2), inhaled isoflurane anesthesia will be used. Anesthesia will be induced in an induction chamber using 3-5% Isoflurane in 100% oxygen (flow rate 1000 cc/min initially and then reduced to 500 cc/min). The tail and hind paw will be pinched for reflexive movement, indicative of insufficient anesthesia. If anesthesia is insufficient, animal will be returned to the induction chamber. Once the mice are deeply anesthetized as indicated by slow and deep respiration, they will be transferred to a mouse platform. On the platform, the isoflurane (1-3%) will be delivered by a nose cone. The top incisors of the mouse will be positioned in a small hole of the nose cone for the proper delivery of the anesthesia. During the SARRP exposure, the mice will be continuously monitored via a camera. If the mice seem to be getting lighter or deeper as indicated by the breathing or movement, the exposure will be stopped to adjust the anesthesia rate.
A heating pad will be provided to keep mice warm during the period of recovery from anesthesia. The temperature in room where the mice will be irradiated will be measured, and if the temperature gets below 68-70 F, an alternate heating device (such as Bair hugger to blow warm air into the room) will be used to increase the temperature.

Anesthesia of mice will be carried out for blood collection using standard Isoflurane apparatus. All personnel will be extensively trained for efficient administration of the agents as well as to minimize handling stress. Animals will be placed in the Isoflurane chamber and delivered a metered amount of 3-5% Isoflurane mixed with 100% oxygen until all voluntary motor movement ceases and the animal is recumbent for 10 seconds. The tail and hind paw will be pinched for reflexive movement, indicative of insufficient anesthesia. If anesthesia is insufficient, animal will be returned to the induction chamber. Animal's respiration rate will be closely monitored through the Plexiglas chamber during induction of anesthesia. When animals are recumbent, they will be removed from the chamber and their nose/mouth placed in the anesthesia apparatus breathing/gas administration cone for maintenance of anesthesia at 1-3% Isoflurane and 100% O_2.

V.4.1.2.2. Pre- and Post-procedural Provisions

Mice will be housed under standard conditions both before and after radiation procedures in accordance with current VSD rodent husbandry Standard Operating Procedures (see section V.5).

V.4.1.2.3. Paralytics

No paralytic agents will be administered

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched

Altweb
AGRICOLA
PubMed

V.4.1.3.2. Date of Search

Altweb – 08/15/2015
AGRICOLA – 08/15/2015
Pubmed – 08/16/2015

V.4.1.3.3. Period of Search

1970-2015

V.4.1.3.4. Key Words of Search

Pain, analgesia, anesthesia, radiation, radioprotection, mouse, humane, alternative, gamma-tocotrienol, SARRP, Cobalt-60

V.4.1.3.5. Results of Search

Table 3 includes a summary of the keyword combinations used for searching and the results of the search. While the literature indicates that cell models have been used to study radiation damage, such models cannot alone predict mechanism of radioprotection of a specific drug. Although radiation-induced pain was alleviated during some studies [37-39], such pain relief was the aim of the studies in question, which focused on patients receiving radiotherapy for cancer treatment. We do not have the option of using alternatives such as a cell culture model.

Table 3. Summary of Database Keyword Searches

Keywords	Hits		
	AGRICOLA	Altweb	PubMed
Analgesia AND Radiation	4	6	553
Pain AND Radiation	37	12	11330
Pain AND SARRP	0	0	0
Pain AND Cobalt-60	0	0	20
Pain AND Analgesia AND Radiation	4	6	368
Gamma-tocotrienol AND pain	0	0	0
Radioprotection AND pain	0	0	154

Radiation AND humane	1	38	12
Pain AND analgesia AND mice	49	26	1904
Radiation AND mouse AND pain	0	9	239
Radiation AND mouse AND pain AND alternative	0	9	6

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification

Since the purpose of these studies is to determine the (b)(4) (b)(4) (b)(4) (spleen, bone marrow sternum, jejunum and lung), we will be unable to use analgesics and anesthetics because they may interfere with the innate immune system [40]. If we see any morbidity or moribundity, frequency of examinations including late-night checks will be increased and animals will be scored and euthanized following policy 10 (score sheet attached, appendix 1).

V.4.2. Prolonged Restraint

The experimental animals (Experiment 1) will receive radiation at a dose rate 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (dosimetry 07/09/2014; or the latest dosimetry map) using plastic racks. Mice will be inserted into the standard radiation boxes just prior to irradiation (~15 min of exposure time) and taken to the cobalt facility for irradiation. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period.
For partial body SARRP exposure (Experiment 2), mice will not be restrained since inhaled isoflurane anesthesia will be used during exposure.

V.4.3. Surgery

N/A

V.4.3.1. Pre-surgical Provisions

N/A

V.4.3.2. Procedure(s)

N/A

V.4.3.3. Post-surgical Provisions

N/A

V.4.3.4. Location

N/A

V.4.3.5. Surgeon

N/A

V.4.3.6. Multiple Major Survival Operative Procedures

N/A

V.4.3.6.1. Procedures

N/A

V.4.3.6.2 Scientific Justification
N/A

V.4.4. Animal Manipulations
Irradiation:
Experiment 1. These will follow the standard AFRRI protocol with mice placed in restrainers shortly prior to exposure, irradiation at the cobalt facility and return to VSD for further monitoring. Briefly, the experimental animals will receive radiation at a dose rate of 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (dosimetry 07/09/2014) using plastic racks. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period. They will be monitored daily following policy#10. There is no critical period for the animals in this protocol since all animals irradiated at 4 and 7 Gy (sublethal doses) are expected to survive based on our published manuscript on peripheral blood recovery post-irradiation at 7 Gy (b)(4) Tissue from mice will be harvested at specific time-points after irradiation after terminal blood draw. Bilateral irradiation will be delivered in the cobalt-60 facility of AFRRI at a dose rate of 0.6 Gy/minute using established protocol. Dose rate will be determined with the alanine/EPR system (b)(4) directly traceable to national standards of the US (NIST) and UK (NPL).

Experiment 2. A next-generation microirradiator for small animals, SARRP (Johns Hopkins Univ. and Gulmay, Inc.) will be used for partial body radiation in gut and thorax. It will provide orthovoltage x-ray irradiation of mice with beams that can be as narrow as 0.5 mm in diameter. Real-time CT imaging of animals positioned on the exposure table immediately before the irradiation will provide reliable pinpoint targeting of organs of interest. The SARRP uses a dual-focal spot. constant voltage X-ray source mounted on a gantry with a source-to-isocenter distance of 35 cm. X-rays of 80–100 kVp from the smaller 0.4-mm focal spot are used for imaging. Both 0.4-mm and 3.0-mm focal spots operate at 225 kVp for irradiation. Robotic translate/rotate stages are used to position the animal. Cone-beam computed tomography is achieved by rotating the horizontal animal between the stationary X-ray source and a flat-panel detector.The radiation beams range from 0.5 mm in diameter to 60 x 60 mm^2.

Blood collection: Mice will be anesthetized as described under section V.4.1.2.1 (anesthesia). Blood will be collected from anesthetized mice while they are alive from the inferior vena cava (IVC). The animals will be deeply anesthetized in the rodent anesthesia machine induction chamber; the tail and hind paw will be pinched for reflexive movement, indicative of insufficient anesthesia. If anesthesia is insufficient, animal will be returned to the induction chamber. If there is no response, the animal will be moved to the station with individual nose cone for continued anesthesia. An abdominal incision will be made on the right side of the animal, closest to the IVC, the vein exposed and blood drawn with a 23 – 25 G needle. Animals will have cervical dislocation performed immediately upon completion of blood draw to confirm euthanasia. All tissues such as bone marrow sternum, spleen and other tissues (lung and jejunum) will then be harvested from the animals after euthanasia.

V.4.4.1. Injections
Subcutaneous (SC) injections will be given with a 23 – 25 G sterile needle to the nape of the neck. Animals will be injected once 24 h before radiation. There will be 4 animals per cage. One needle will be used to puncture the vial once to draw the drug for 4 animals per cage and it will be injected to 4 animals.

V.4.4.1.1. Pharmaceutical Grade Drugs

V.4.4.1.2. Non-Pharmaceutical Grade Drugs
Research grade GT3 formulations will be supplied by (b)(4) All formulations will be tested for endotoxin by Charles River Laboratory before using in the animals. pH and osmolality will be tested at AFRRI. We are not expecting any toxic effects in animals for any of the formulations since we have tested the toxicity of the drug at 400 mg/kg dose (2 times higher than the proposed dose in this protocol) previously at AFRRI.

V.4.4.2. Biosamples
Animals will be euthanized from each experimental group on days 1, 3.5, 14, 30, and 90 as specified in section V.1 and blood will be collected from anesthetized mice while they are alive from the inferior vena cava (using a 23 – 25 G sterile needle) in EDTA tubes for plasma separation. Plasma will be separated and stored at -80C. Bone marrow will be harvested following a standard protocol. Spleen and other tissues (lung and jejunum) will be collected from the carcasses and snap frozen for storage at -80C.

V.4.4.3. Adjuvants

V.4.4.4. Monoclonal Antibody (MAb) Production

V.4.4.5. Animal Identification
Generally, cage cards will be used to distinguish drug-treated and control groups. Animals will be randomly distributed in groups. Some experiments might necessitate ear punch or notch or tattoos by personnel to identify individual animals in a cage. Tattoo will be done by VSD personnel using labstamp.

V.4.4.6. Behavioral Studies
N/A
V.4.4.7. Other Procedures
N/A
V.4.4.8. Tissue Sharing
We will be sharing tissues (plasma and all tissues collected in the protocol) with Dr. (b)(6)
V.4.4.9. Animal By-Products
N/A
V.4.5. Study Endpoint
1. The endpoint for experiment 1 using whole body Co-60 radiation will be day 1, 14, and 30 after radiation.
2. The end point for experiment 2 using SARRP partial body radiation in thorax will be day 1, 14, 30, and 90 after irradiation.

3. The end point for experiment 2 using SARRP partial body radiation in gut will be day 3.5 and 14 after irradiation.

In experiments 1 and 2, at specific time points, experimental mice will be euthanized before tissue collection. Different tissues will be used for different experiments. If we see any morbidity or moribundity, frequency of examinations including late-night checks will be increased and animals will be scored and euthanized following policy 10 (score sheet attached, appendix 1). Animals will be weighed the day prior to injection, animals outside ±10% of the mean weight excluded, and randomized into groups of four animals per box. Morbid animals will be weighed, if the weight loss is 35% or more, animals will be euthanized immediately according to policy 10 (Appendix 1).

V.4.6. Euthanasia

All moribund animals (see section V.4.5) (if any) will be euthanized by overdose of CO_2 inhalation as described in VSD SOP VS0211, by trained investigators or technicians in accordance with current AVMA Guidelines on Euthanasia. We will follow the same method in weekends and after hours. Compressed CO_2 gas in a cylinder with a regulator will be used for euthanasia in a euthanasia chamber. Blood will be collected from anesthetized mice while they are alive from the inferior vena cava (IVC). The animals will be deeply anesthetized in the rodent anesthesia machine induction chamber; the tail and hind paw will be pinched for reflexive movement, indicative of insufficient anesthesia. If anesthesia is insufficient, animal will be returned to the induction chamber. If there is no response, the animal will be moved to the station with individual nose cone for continued anesthesia. An abdominal incision will be made on the right side of the animal, closest to the IVC, the vein exposed and blood drawn with a 23 – 25 G needle. Animals will have cervical dislocation performed immediately upon completion of blood draw to confirm euthanasia. All tissues such as bone marrow sternum, spleen and other tissues (lung and jejunum) will then be harvested from the animals after euthanasia.

V.5. Veterinary Care

V.5.1. Husbandry Considerations

The AFRRI vivarium follows stringent protocols for the housing and care of all animals. The PI and designated team members will adhere to all the policy and guidelines set forth by standard operating protocols of the VSD. The specific requirements for mice housing should meet the following criteria of opportunity for social interaction, opportunity to carry out normal behavior and the opportunity to rest and withdraw from each other [41]. Mice are social animals and hence will be caged together in groups of 4 or 8. Nestling pads will be provided in each cage during bedding changes. In cage shelters will also be provided as an enrichment strategy at the discretion of the PI depending on experimental conditions. Animals will receive Harlan Teklad Rodent Diet (w) 8604 and water *ad libitum*. Acidified water (pH 2.5 – 3.0) is routinely provided to AFRRI mice used in radiological experiments. Cages will be regularly changed and cleaned by VSD staff in accordance with Standard Operating Procedure. Animals will have 12 h light/dark cycle. Animals will be habituated to their surroundings and routine procedures prior to experimentation. In the event of procedures that last into the night cycle, care will be taken to minimize exposure to light.

V.5.1.1. Study Room
As assigned by VSD

V.5.1.2. Special Husbandry Provisions
N/A

V.5.1.3. Exceptions
Mice will be socially (group) housed as described above, except there might be a time when few animals from the cage will be removed for blood and tissue collection. Animals may be singly housed if all the co-housed animals are euthanized because of moribund status.

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care
Experimental animals will be observed daily by either the investigator or technician or both. VSD personnel will monitor all animals during their routine rounds as per VSD SOPs.

V.5.2.2. Emergency Veterinary Medical Care
We do not expect animals will be moribund in this protocol. If moribund animals (gasping, recumbent, non-responsive to mild stimulus) are found, those will be euthanized by trained personnel.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy
The enrichment strategy for mice will be according to VSD Standard Operating Procedure, which involves socially grouped housing and providing nestlets and hiding tubes in the cage.

V.5.3.2. Enrichment Restrictions

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3, 4b, 5	(b)(6)
				1, 3, 4b, 5	
				1, 3, 4b, 5	
				1, 3, 4b, 5	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a-ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-inferior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY

All personnel working in the protocol have undergone necessary training in handling experimental animals. The amount of drug used will be small and standard safety precautions will be taken while handling them in the laboratory and in the vivarium. All personnel listed in the present protocol have been trained as general radiation users on a regular basis. All investigators and personnel will use appropriate protective gear while in the vivarium as described by the VSD. All listed personnel are current in safety training and medical surveillance.

VIII. ENCLOSURES

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X. ASSURANCES

Protocol Title:

As the Principal Investigator on this protocol, **I** acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that **I** have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

_____	_____	_____
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

Appendix 1.
Clinical Observations with Criteria for Euthanasia (Rodent)

Criteria:	Description:	Score
Appearance:		
	Normal (smooth coat, clear eyes/nose)	0
	Hunched and/or fluffed	1
	Ocular discharge, and/or edema	3
	Soft stools (fecal matter around anus)	5
	Pale, white mucus membranes/skin	6
	Bloody diarrhea	9
	Blue mucus membranes/skin (cyanosis)*	12
Respiratory Rate:		
	Normal breathing	0
	Change in breathing (an increase or decrease in respiratory rate from normal baseline)	3
	Abdominal breathing (gasping +/- open mouth breathing)*	12
General Behavior:		
	Normal (based on baseline observations)	0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)	1
	Decreased mobility	2
	Ataxia, wobbly, weak	6
	Inability to stand*	12
Provoked Behavior:		
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))	0
	Subdued; responds to stimulation (moves away briskly)	1
	Subdued even to stimulation (moves away slowly)	3
	Unresponsive to gentle prodding	6
	Does not right when placed gently on side within 5 seconds*	12
Weight loss		
	0-35% with no other clinical signs	0
	≥35% weight loss	12

*** Regardless of score, immediately euthanize (death is imminent)**

Individual or Cumulative Score:

< 6	Normal
6 - 11	Morbid: See VI.D.2 and VI.D.4 in IACUC Policy #10 for guidelines
≥ 12	Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*). Any single criteria of 12* euthanize immediately; consider as 'found dead.'

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	10-23-2015	2nd Resubmission Date:	
Approved/**Returned for Revision**:	11-12-2015	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	11-24-2015	**EXPIRATION DATE:**	12/10/2018
Approved/Returned for Revision:	12/11/2015	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: A STRAIN COMPARISON STUDY ON SURVIVAL, VASCULAR DAMAGE, AND LONG TERM HEALTH EFFECTS IN IRRADIATED MINIPIGS (*Sus scrofa domestica*)

IV. PRINCIPAL INVESTIGATOR: (b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD** Date
Head, Scientific Research Department, AFRRI
Telephone (b)(6)
(b)(6)

- Or -

(b)(6) **PhD, LTC, USA** Date
Head, Scientific Research Department, AFRRI

Telephone (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date
Statistician
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, LTC, VC, USA** Date
Head, Veterinary Sciences Department, AFRRI
Telephone (b)(6) Fax (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date
Safety and Occupational Health Specialist, USUHS
Telephone: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **MA, MS, Lt Col, USAF, BSC**
IACUC Chair (b)(6) Scientific Research Department, AFRRI
Telephone (b)(6)
(b)(6)

PROTOCOL TITLE:
A STRAIN COMPARISON STUDY ON SURVIVAL, VASCULAR DAMAGE, AND LONG TERM HEALTH EFFECTS IN IRRADIATED MINIPIGS (*Sus scrofa domestica*)

PRINCIPAL INVESTIGATOR: [(b)(6)] PhD
Principal investigator [(b)(6)] Scientific Research Department
Armed Forces Radiobiology Research Institute (AFRRI)
Tel: [(b)(6)] Fax [(b)(6)], E-mail: [(b)(6)]

CO-INVESTIGATOR(S): [(b)(6)] DVM, MVSc, PhD
[(b)(6)] Scientific Research Department
Armed Forces Radiobiology Research Institute (AFRRI)
Tel: [(b)(6)] Fax [(b)(6)] E-mail: [(b)(6)]

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER:

I. NON-TECHNICAL SYNOPSIS

More animal models are required to screen candidates for radiation countermeasures. We have extensively characterized the Gottingen minipig as a model of hematopoietic Acute Radiation Syndrome (H-ARS) and Gastrointestinal Acute Radiation Syndrome (GI-ARS). The model has proven to be more sensitive to irradiation than other minipig strains.
The FDA states that animal models must be well-characterized to be used for testing efficacy of drugs under the Animal Rule. Understanding the reason behind the sensitivity of the Gottingen minipig is critical for the development and use of this model, as well as for an informed choice of different miniature strains. We propose here to compare two strains of minipigs, the Gottingen minipig and the Sinclair minipig (supposedly more resistant to radiation damage than the Gottingen minipig), and to investigate potential mechanism of radiation injury that leads to the extensive, severe hemorrhaging and bleeding observed in the Gottingen minipig. In addition, we will further characterize the Gottingen minipig for development of late effects of irradiation in the cardiovascular system, following partial body irradiation (PBI). To provide PBI, a linear accelerator (LINAC) similar to those used in the clinic to target precise tissue and organ areas must be used. Late effects of irradiation in humans may manifest several months after exposure in several organs. In the heart, evidence of coronary artery atherosclerosis leading to myocardial infarct, microvascular damage, fibrosis leading to congestive heart failure, pericardial disease, and cardiomyopathy has been found in patients exposed to radiation therapy, as well as in the H-bomb survivors (Armenian et al, 2015, Stewart et al, 2013; Waler et al, 2013, Yamada et al, 2005). Preliminary data indicate that the Gottingen minipig irradiated at clinically relevant doses (*i.e.* 2 Gy) displays a rapid reduction in HDL cholesterol level after irradiation, which may lead to development of coronary heart diseases and microvascular damage.

II. BACKGROUND

II.1. Background

The FDA states that animal models must be well-characterized to be used for testing efficacy of drugs under the Animal Rule. In parallel to the development of radiation countermeasures to increase survival to acute radiation doses, the issue of late effects of irradiation after exposure to survivable doses becomes relevant and an integral part of the model characterization.
We have extensively characterized the Gottingen minipig as a model of hematopoietic Acute Radiation Syndrome (H-ARS) and Gastrointestinal Acute Radiation Syndrome (GI-ARS). The model has proven to be more sensitive to irradiation than other minipig strains (Mandel et al, 1980; [(b)(4),(b)(6)]. It is characterized by widespread edema and hemorrhages affecting mainly heart, lungs and intestine, as well as by dyslipidemia [(b)(4),(b)(6)]. Damage to the vasculature is expected to be one of the main mediators of the radiation sensitivity of the Gottingen. Development of a PBI minipig model which can maintain the sensitivity of the cardiovascular system to irradiation but is capable to

overcome the acute lethality of the syndrome, will greatly aid our understanding of late effects of radiation. The cardiovascular features of the Gottingen minipig might be exploited to establish a model of late cardiovascular effects that requires relatively short period (months) to develop, as opposed to years. Cardiovascular late effect can manifest as arrhythmia, cardiomyopathy (affecting mainly the left ventricle of the heart, the mitral and aortic valve, and developing into congestive heart failure and infarction), premature coronary artery disease (*i.e.* atherosclerosis), and inflammation (pericarditis and pericardial fibrosis) (Keene & Oeffinger, n.d.).Understanding the reason behind the sensitivity of the Gottingen is critical for the short- and long-term use of this model, as well as for the choice of a different miniature strain.
The minipig model developed at AFRRI has utilized gamma photons from a Cobalt-60 source to irradiate the animals. This setup can be used for total body irradiation, but not for PBI. In order to irradiate only a portion of the animal, a linear accelerator (LINAC) similar to those used in the clinic to target precise tissue and organ areas must be used. AFRRI has an Elekta Linear Accelerator (LINAC) that can be used for partial body irradiation.
For total body irradiation, both Cobalt-60 sources and LINAC can be used. Survival is impacted by the type of radiation used. This study will compare side by side two minipig strains for two radiation doses, and will use Gottingen minipig historical data for two additional doses. We may use either Cobalt-60 or LINAC when comparing strain sensitivity to total body irradiation, for all animals in the protocol. Alternatively, we may deliver some of the doses with Cobalt-60 and some other with LINAC; however all animals in this protocol receiving the same dose will be exposed to the same source, for comparison purposes. Choice of radiation source will depend on availability of the source and in consultation with funding agency.

The insulin-like growth factor 1 (IGF-1) is an important hormone that plays pivotal roles in all phases of human growth and endocrine metabolism (Livingstone, 2013). It is induced following tissue or cellular injury to stimulate repair and homeostasis of tissues (blood vessels, skeletal muscles, heart, brain, intestine), and replication of cells (hematopoietic and intestinal crypt cells). IGF-1 expression changes over the course of the life span in humans, with the highest levels present in the pre-pubertal phase and early adulthood, and the lowest levels at early and late stages in life (Lamberts et al. 1997; Mayo Clinic, n.d). Gender and menopausal state also affect expression of IGF-1 (Roelfsema & Veldhuis, 2015). Low levels of IGF-1 have been associated to increased risk of atherosclerosis and other cardiovascular diseases (Ruidavets et al. 2011).

Alterations in IGF-1 levels (both deficiencies and excess) are associated with several human pathologies (Kim, 2015; Puche, 2012). Cardiovascular mortality, insulin resistance, high blood pressure and dysregulation of vascular tone are associated with both low and high IGF-1 concentration, suggesting the existence of a U-shape relationship between IGF-1 and pathologies, or the onset of IGF-1 resistance at high concentration of IGF-1 (Schutte, 2014). Studies from our lab with the Gottingen minipig suggest that IGF-1 levels are altered as a function of radiation exposure. IGF-1 is induced shortly after irradiation, peaks a few days before the animal becomes moribund, or declines over the course of weeks in surviving animals (Figure 1).

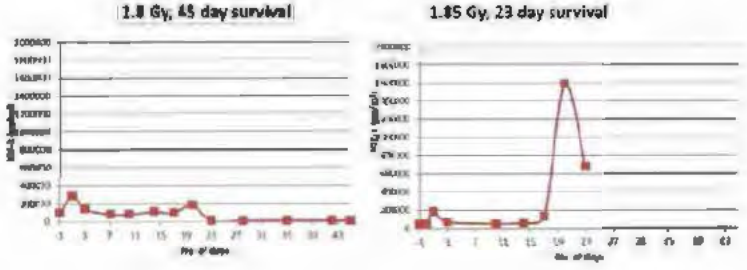
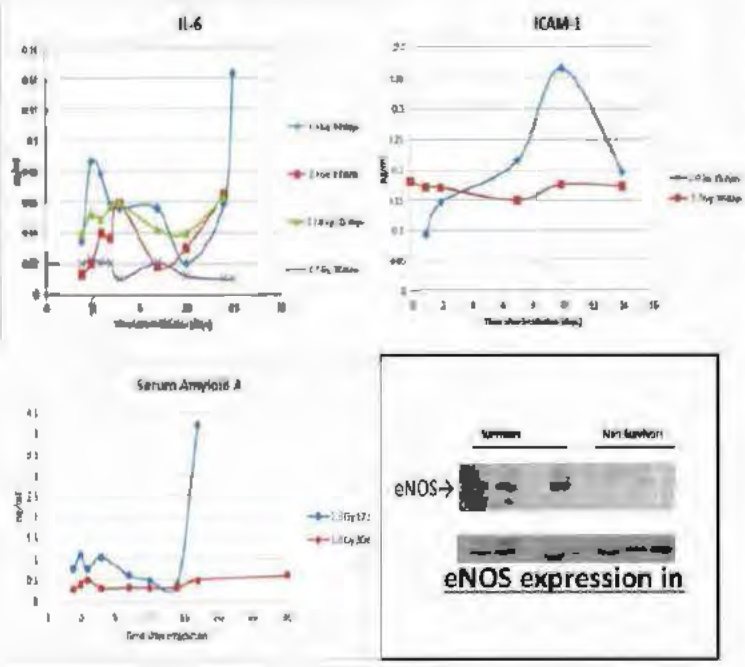


Figure 1: IGF-1 in the plasma of irradiated G. minipigs. The animal on the left survived the entire observation period (45 days), while the animal on the right survived only 23 days.



Our preliminary data suggest that moribund animals undergo a secondary acute inflammatory response, characterized by increase in pro-inflammatory cytokines (IL-6), acute phase proteins (CRP, SAA), inflammation of the endothelium (ICAM-1), and reduction in endothelial Nitric Oxide Synthase (eNOS) (Figure 2), possibly leading to impairment of the vascular tone, hypertension, edema, bleeding, and hemorrhages.

Figure 2: IL-6, ICAM-1, and SAA in plasma of irradiated G. minipigs; eNOS expression in heart tissue of irradiated G. minipigs. Doses and survival time or condition is indicated in each panel.

The cardiovascular protective role of IGF-1 is thought to be mediated by its regulation of C-reactive protein (CRP) (Liu et al. 2014). CRP is an acute-phase reactant protein and systemic marker of inflammation, which displays pro-inflammatory effects on endothelial cells through promotion of adhesion molecules, chemokine expression and vasoconstriction. IGF-1 activates eNOS signaling and improves vascular tone, while CRP promotes uncoupling of eNOS, thus favoring production of superoxide and oxidative stress instead of nitric oxide, and hypertension (Liu et al. 2014). Emerging evidence demonstrates that IGF-1 and CRP levels have an inverse correlation and opposing roles in endothelial cell activation (Liu et al. 2014). Unexpectedly, in the irradiated Gottingen minipig, IGF-1 parallels high levels of the inflammatory markers C-reactive protein and serum amyloid A (SAA) (Figure 3).

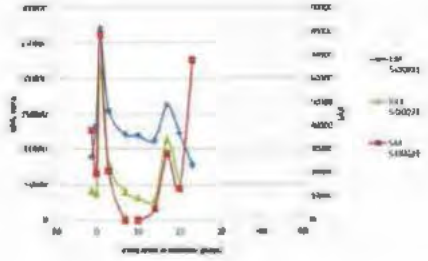


Figure 3: Levels of IGF-1, CRP and SAA from the same plasma sample from one irradiated G. minipig. The animal survived only 23 days.

The roles of IGF-1 and CRP are important in understanding potential mechanisms of sensitivity to radiation, as well as the radiation-induced vascular damage observed in the Gottingen minipig. Surprisingly, results from the serial sample analysis in our lab suggests that the Gottingen minipig may become IGF-1 resistant or that a U-shape relationship exists between IGF-1 and survival, since high

levels of IGF-1 are associated with poor prognosis. Indeed, our preliminary analysis indicates in age matched groups of minipigs suggests that IGF-1 levels are higher in the Gottingen with respect to the Sinclair (Hormel Minnesota), a strain reportedly more resistant to irradiation based on one study with 14 day old Minnesota miniature piglets (Mandel et al. 1980).

The Sinclair minipig was developed by the Hormel Institute at the University of Minnesota in 1949 (Sinclair Bioresources, n.d.). It is the first miniature swine stock developed specifically for research purposes. The Sinclair has a slow growth rate, but is larger than the Gottingen minipig. It has been used in multiple research areas, cardiovascular, musculoskeletal, urogenital, oncology (melanoma), immunology, reproduction toxicology, teratology, dermatology, gastroenterology, respiration, nutrition, pediatrics, *etc.* One lineage of the Sinclair miniature swine develops a malignant spontaneously regressing melanoma (Sinclair Bioresources, n.d.).

Here, we propose to confirm our preliminary results on IGF-1, as well as to evaluate both strains of minipigs in terms of radiation sensitivity and expression of IGF-1. We will determine: i) if different strains of minipigs have the same sensitivity to irradiation; ii) the relationship between IGF-1 levels and sensitivity to irradiation; iii) the relationship between IGF-1, CRP and vascular function/integrity, and iv) if there are late effects detectable within 120 days after exposure to radiation in the Gottingen minipig.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

II.2.2. Date of Search 8/17/2015

II.2.3. Period of Search All available years

II.2.4. Key Words of Search

minipig OR swine AND radiation AND total body
OR
minipig OR swine AND radiation AND partial body
OR
minipig OR swine AND radiation AND late effects
OR
minipig OR swine AND radiation AND Cobalt
OR
minipig OR swine AND radiation AND LINAC
OR
minipig OR swine AND radiation AND C-reactive protein
OR
minipig OR swine AND radiation AND IGF-1

II.2.5. Results of Search

BRD The database was not available during the period 8/17/2015 to 9/2/2105

RePORTER: The results of the research found a few projects dealing with Toll receptors in macrophages and dendritic cell activation, development of medical devices or diagnostic tools, induction of stable B-cell tolerance to transplantation antigens, application of gene based therapy, identification of biomarkers for application other than radiation biodosimetry, and assessment of CD47 function in response to radiation-induced burns.

PubMed: Several publications were found with the key words radiation and minipig; several were from our own laboratory. One article described the hematopoietic radiation toxicity in the Tibet minipig; another studied the effect of fractionated radiotherapy on the parotid gland of the Brazilian minipig. However, no publication described a comparative study between strains; no publication reported assessment of IGF-1 as a potential mechanism of radiation sensitivity; no publication investigated the relationship between radiation, IGF-1 and vascular damage.

III. OBJECTIVE / HYPOTHESIS

Our hypothesis is that (i) the Gottingen and the Sinclair minipigs display different sensitivity to irradiation; (ii) a relationship exists between survival, circulating levels of IGF1, acute phase proteins (*i.e.* CRP, SAA), and vascular function/integrity; and (iii) late effects of irradiation (*i.e.* atherosclerosis, fibrosis) can be observed within 120 days after exposure.

In order to test our hypotheses, we will:

1. Expose Gottingen and Sinclair minipigs (total body irradiation, TBI) to several radiation doses, and compare survival. In addition, we will establish the time course for IGF-1 and acute phase proteins in both strains irradiated at two radiation doses, and perform histological assessment of major organs for vascular integrity, as assessed by presence of edema and hemorrhages, for all animals.
2. Irradiate Gottingen minipigs with partial body irradiation (PBI) sparing 5% of bone marrow (5% PBI), determine survival and time course for IGF-1 and acute phase proteins, and perform histological assessment of heart, lungs, and gastrointestinal tract to assess presence of markers indicative of tissue injury attributable to late effects of irradiation.

   Experimental details are reported in Section V Materials and Methods.

IV. MILITARY RELEVANCE

According to the DoD Chemical and Biological Defense Program 2009 Annual Report to Congress (6): "Although the overall number of nuclear weapons continues to decline because of Russian and U.S. treaty commitments, the United States anticipates an increase in weapon numbers in China, India, and Pakistan. Motivated by economic and strategic interests, Russia and China (or political entities in each) and North Korea continue to supply technologies and components that are dual use and could support weapons of mass destruction and missile programs, especially in the Middle East and South Asia. Iran continues to develop its enrichment program in defiance of United Nations (U.N.) Security Council resolutions and also continues to build a heavy water reactor in Arak, which will be capable of producing plutonium that could be weaponized. While North Korea has halted portions of its nuclear program, it is possible that it has stockpiled several nuclear weapons from plutonium produced at Yongbyon. Non-nuclear radiological dispersal devices (RDD) and radiological exposure devices (RED) pose a significant potential threat especially in the hands of non-state groups. Contamination and recovery issues associated with operating in a radiological environment remain significant concerns for military operations and underscore the need for robust detection, protection, and decontamination systems."

Both early and late health effects of radiation exposure are major concerns for the military. Developing countermeasures to radiation exposure is a top priority for the US Department of Defense. Effective countermeasures would expand the options available to field commanders operating Ionizing Radiation (here defined as IR) threat environments, and improve the morale of personnel at risk of exposure to IR. DoD Defense Technology Objective MD.18 states: "Effective mitigation of health consequences [of IR] will (1) reduce casualty load at medical treatment facilities, (2) sustain a more effective operational force after a radiation exposure event, (3) allow commanders to conduct operations in radiation field environments without undue risk of decremented performance of personnel due to acute tissue injury, and (4) reduce the negative psychological impact on personnel tasked to operate in contaminated environments."

An important barrier to advanced development of radiation countermeasures is the paucity of large, long-lived, non-rodent animal models of radiation injury. The only choices at present are nonhuman primates (NHPs) and canines. The higher sentinent status of NHPs, their expense and danger (occupational health hazards) of working with NHPs, are also disadvantages. Canines are notoriously sensitive to NSAID and

vomiting (Swindle et al. 2012). The addition of miniature swine as a large animal radiation injury model would facilitate development of promising countermeasures for both military and civilian use.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

V.1.1. Experiment 1 Dosimetry

Animals may be required for finalizing dosimetry measurement for Cobalt and LINAC exposure. After the required period of quarantine/acclimation (see Section V.5.1.), animals will be sedated as described in section V.4.1.2.1. Anesthesia / Analgesia / Tranquilization, and transported to the Cobalt and/or LINAC facility, placed on a sling or a table, and measurements such as animal dimension while lying in the sling used for irradiation and CT scans will be taken. For dosimetry measurements, the animals will not be exposed to ionizing radiation. At completion of Experiment 1, animals will either (i) be used for Experiment 2 if final calculations for dosimetry are completed within 7-10 days, or (ii) will be euthanized and blood and tissue samples collected as non-irradiated controls, for model development.

Number of animals requested for Experiment 1 = 8 total (4 Sinclair + 4 Gottingen).

Table 1
Experiment 1: experimental plan

Dose (Gy)	Gottingen	Sinclair	Study Endpoints	Total
Non-irradiated	4	4	Dosimetry measurements	8

V.1.2. Experiment 2 Strain comparison study

After the required period of quarantine/acclimation (see Section V.5.1.), we will irradiate (TBI) Gottingen and Sinclair minipigs using LINAC or Cobalt-60, and we will collect study endpoints according to the Table 2. Animals irradiated at the same dose and exposed to the same radiation quality *(i.e.* either X-rays from LINAC or gamma-photons from Cobalt-60), to enable comparison of survival data between strains.

Table 2
Experiment 2: experimental plan

Doses (Gy)*	Gottingen	Sinclair	Study Endpoints	Total
1.8 (TBI)	4	4	30 d survival, CBC, IGF-1, acute phase proteins, gross- and histo-pathology	8
2.5 (TBI)	4	4	30 d survival, CBC, IGF-1, acute phase proteins, gross- and histo-pathology	8
Dose TBD (hematopoietic range)		4	30d survival, CBC, IGF-1, acute phase proteins, gross- and histo-pathology	4
Dose TBD (hematopoietic range)		4	30d survival, CBC, IGF-1, acute phase proteins, gross- and histo-pathology	4
Total	8	16		24

* Doses other than 1.8 Gy and 2.5 Gy will be determined based on survival data of the Sinclair minipigs

As this is the first time that we irradiate the Sinclair, the dose response of this strain is unknown to us. To compare the two strains, we will irradiate both strains at a dose corresponding to the approximate LD70 for the Gottingen (1.8 Gy), as well as one supralethal dose (2.5 Gy, this dose is above LD100) for side by side comparison between the two strains of pigs. Doses other than 1.8 Gy and 2.5 Gy will be determined based on survival data of the Sinclair minipigs. Blood will be collected according to section V.4.4.2. Biosamples, and used to obtain CBC/differential and plasma samples for further processing. Blood for CBC/differential and plasma will be collected the week before irradiation and the day before irradiation (or 1 hour before irradiation) and, considering Day 0 the day of irradiation, on days 0, +1, +2, +3, +7, +10, +14, +17, +20, +23, +27, +30. Time points may be shifted by 24 - 48 hour, or dropped in case of unforeseen circumstances. Additional blood draws may be taken, per veterinarian request, if needed to assess health of the animal.

Blood will be collected from either a Vascular Access Port (VAP) (b)(4) (b)(6) or an externalized catheter, or from peripheral veins (see list of potential sites under section **V.4.4.2. Biosamples "Collection of blood samples"**; See VAP or externalized catheter placement under section **V.4.3.2. Procedure (s)**). We have perfected the techniques to collect blood samples from VAP as well as peripheral veins for the Gottingen minipig, but we do not have experience with the Sinclair minipig. Optimal modality of blood collection will have to be determined empirically.
Animals will be observed for 30 days. Moribund animals will be euthanized according to pre-established euthanasia criteria (V.4.5); animals completing the survival study will be euthanized at day 30 - 34 after irradiation. Euthanasia will be carried out according to section V.4.6 Euthanasia. Tissues and blood samples will be collected for further analysis (V.4.4.2).

Number of animals requested for Experiment 2 = 24 total (8 Gottingen + 16 Sinclair).

V.1.3. Experiment 3 Long term effect of radiation

After the required period of quarantine/acclimation (see Section V.5.1.), we will expose Gottingen minipigs to X-rays using LINAC, while protecting about 5-10 % of the bone marrow (5-10% PBI). Partial protection of bone marrow is expected to increase survival at doses otherwise lethal, and allow assessment of late effects of radiation. We will irradiate minipigs with 2.0 Gy and 2.5 Gy (Table 3). These doses are 100% lethal under total body irradiation, but are not sufficient to induce the gastro-intestinal Acute Radiation Syndrome (b)(4) (b)(6) Animals irradiated at 2.0 – 2.5 Gy display extensive bone marrow suppression, vascular damage, and increased CRP. Therefore we expect that such doses will not be lethal under 5-10% bone marrow sparing, and will be suitable for studying long term damage associated with radiation-induced bone marrow toxicity.

Table 3
Experiment 3, experimental plan
Distribution of animals per irradiation doses and endpoints

Doses (Gy)	Gottingen	Sinclair	Primary Endpoints	Total
2.0 (5-10% PBI)	4	0	120 d survival, IGF-1, acute phase proteins, atherosclerosis	4
2.5 (5-10% PBI)	4	0	120 d survival, IGF-1, acute phase proteins, atherosclerosis	4
Total	8	0		8

Animals will be observed for 120 days. Blood for CBC/differential and plasma will be collected the week before irradiation and the day before irradiation (or on day 0 before irradiation) and, considering Day 0 the day of irradiation, on days +1, +3, +7, +10, +14, +17, +20, +23, +27, +30 and up to twice a week thereafter. Time points may be shifted by 24-48 hour, or dropped in case of unforeseen circumstances. Additional blood draws may be taken, per veterinarian request, if needed to assess health of the animal. Blood will be collected from either a Vascular Access Port (VAP), or an externalized catheter, or from peripheral veins (see list of potential sites under section **V.4.4.2. Biosamples "Collection of blood samples"**; See VAP or externalized catheter placement under section **V.4.3.2. Procedure (s)**). Moribund animals will be euthanized according to pre-established euthanasia criteria (V.4.5); animals completing the survival study will be euthanized at day 120 - 124 after irradiation. Euthanasia will be carried out

according to section V.4.6 Euthanasia. Tissues and blood samples will be collected for further analysis (V.4.4.2).

Number of animals requested for Experiment 3 = 8 total (8 Gottingen + 0 Sinclair)

V.2. Data Analysis

Experiment 1. Four animals are sufficient to provide medians and means with standard deviations, which will allow calculation of 95% confidence intervals. These miniature swine, produced for research, are of homogeneous size when matched by age. The variation in size is expected to be very small.
Experiment 2 and 3. Experiment 2 is a pilot study, to be followed by a fully powered study if results are as expected and funds are available. There is not enough evidence available on the difference in sensitivity between Sinclair and Gottingen to justify a fully powered study at this point to demonstrate that one strain is more sensitive than the other. Preliminary proportion data at each radiation dose for the two strains can be used to determine sample size needed for fully powered studies.
Four animals, however, will be sufficient to provide medians and means with standard deviations, and provide the possibility to remove one potential outlier in the dataset for each parameter and time point. Differences in means between groups will be assessed using Student's T-test or ANOVA. Strength and direction of association between variables will be estimated using the Pearson Product Moment correlation coefficient, the Spearman coefficient or the Kendall rank correlation, depending on the distribution and level of the variables.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

The purpose of this project is to determine the effect of radiation on survival and expression of hormones and acute phase proteins. There is no computer model or tissue culture that can mimic the effect of radiation on the whole organism and predict lethality. Survival depends on an intricate network of organ cross-talks and cellular microenvironment that cannot be recreated *in vitro.* Consequently, administration of supportive care to cellular components grown *in vitro* will not allow any assessment on survival.

We do not know all of the cell types and tissues involved in radiation injury or how radiation injury causes lethality. The concept of multi-organ failure has been suggested, but how the injured organs affect each other's vital functions is unknown. The present state of the art of tissue culture is still incapable of reproducing the *in vivo* relationships. Computer modeling is similarly limited by lack of full characterization of the biological elements and interactions under study.

These considerations are consistent with the FDA requirement for preclinical research in both small and large mammals before granting approval for drug testing in humans.

V.3.2. Animal Model and Species Justification

Large, long-lived, non-rodent animal models are required for drug studies submitted to the FDA for licensure applications. The close similarity to humans in anatomy and physiology of organs such as the liver, pancreas, kidney and heart has made the pig the primary species of interest as organ donors for xenographic procedures. Minipigs are routinely used for toxicity testing. As such, further and rapid characterization at physiological, molecular and genetic level of the minipig is likely to occur, thus increasing acceptance of this model and facilitating advanced drug development. We have been developing the Gottingen minipig as an additional large animal model for the study of the ARS and advanced development of radiation countermeasures. The current study will help to further characterize the model in terms of natural history of ARS, and to understand the reasons behind inter-individual sensitivity to radiation. Studies on the Sinclair minipig will increase the choice of animal model available to mimic the condition in humans, overall increasing the chances of obtaining a FDA approved drug for the mitigation and treatment of the ARS and eventually decreasing the number of animals required for research. Our previous studies have employed male Gottingen minipigs to develop a probit curve; female minipigs of a corresponding age (approximately 4 months old) are more sensitive to irradiation, based on data from other laboratories. Since we are using our own historical data as one of the terms of comparison, we will continue to use male minipigs for this study

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species *Sus scrofa domestica*

V.3.3.2. Strain / Stock Gottingen miniature swine and Sinclair miniature swine

V.3.3.3. Source / Vendor Marshall BioResources/Marshall Farms Group Ltd, 5800 Lake Bluff Road, North Rose NY 14516; Sinclair Bio Resources, LLC, P.O. Box 658, Columbia, MO 65205.

V.3.3.4. Age 3-5 months

V.3.3.5. Weight Weight appropriate for age (6-14 kg for the Gottingen, and 12-30 kg for the Sinclair)

V.3.3.6. Sex Male

V.3.3.7. Special Considerations NA

V.3.4. Number of Animals Required (by species)

Exp 1: 4 Gottingen, 4 Sinclair
Exp 2: 8 Gottingen, 16 Sinclair
Exp 3: 8 Gottingen
Additionally, we would like to request an additional 10% (4 pigs of either strain as needed) due to unforeseen complications or unexpected deaths.
Total: 20 Gottingen + 20 Sinclair miniature swine + 4 pigs = 44

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement

The study data-points currently mandated by the FDA for approval of radiation countermeasures under the Animal Efficacy Rule is mortality. Hence the primary study data-point in our development of Gottingen minipigs as a model for ARS is survival. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress, using an extensive set of criteria (see animal endpoints, paragraph V.4.5 – Lethargy, weight loss, temperature, respiratory distress, food intake, bleeding *etc.*). Animal care and use procedures will only be performed by trained personnel. We have considerable experience in the care and handling of minipigs. Extensive human interaction every weekday will reduce stress in the animals. Use of slings, topical anesthetics, and anesthetics will minimize distress during blood collections and the animals will be gradually acclimated to the slings for these procedures. VAP design and the blood collection have been refined during multiple iterations of previous studies to minimize discomfort to the animals.

V.3.5.2. Reduction

Blood samples obtained from minipigs before irradiation will serve as controls, thus reducing the number of animals required for this study. Use of historical controls will reduce the number of animals needed for this study.

V.3.5.3. Replacement NONE

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C ___ _0_ (# of animals)

V.4.1.1.1.2. Column D ___ _8_ (# of animals)

V.4.1.1.1.3. Column E ___ _36_ (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1	4 Gottingen, 4 Sinclair		8		8
2	8 Gotingen, 16 Sinclair			24	24
3	8 Gottingen			8	8
	Unexpected complications/losses (either strain)			4	4
Totals:			8	36	44

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

We are planning to use anesthesia/sedation at the time of blood sampling (if necessary), prior to irradiation, surgery (if applicable), and prior to euthanasia. Anesthesia/sedation regimen for these procedures is outlined in Table 4. Atropine will be added for any procedure that requires intubation.

Table 4
*Anesthesia/sedation regimen**

Blood sampling, emergency medical care	i	Midazolam (0.1-0.5 mg/kg s.c.or *i.m.*)
	ii	Acepromazine (0.03-1.1 mg/kg *i.m.)*
	iii	Isoflurane 1-5% (face mask or endotracheal tube)
	iv	Xylazine (2.2 mg/kg) + Telazol (2-6 mg/kg) i.m.
	v	Telazol 2 - 8 mg/kg *i.m.*, with or without isoflurane 1-5% (face mask or endotracheal tube)
	vi	Telazol (4.4 mg/kg *i.m.*) + dexmedtomidine (0.05-0.1 mg/kg *i.m.*) or ketamine (5-20 mg/kg IM)/xylazine (1-2 mg/kg *i.m.*), with or without isoflurane 1-5% (face mask or endotracheal tube)
Irradiation, Surgery, Euthanasia	vii	Telazol (2 - 8 mg/kg *i.m.*), with or without isoflurane 1-5% (face mask or endotracheal tube), Atropine (0.04 mg/kg s.c., i.m.or i.v.) (when intubated)
	viii	Telazol (4.4 mg/kg *i.m.*) + dexmedtomidine (0.05-0.1 mg/kg *i.m.*) or ketamine (10-20 mg/kg IM)/xylazine (1-2 mg/kg *i.m.*), with or without isoflurane 1-5% (face mask or endotracheal tube), Atropine (0.04 mg/kg s.c., i.m.or

	i.v.) (when intubated)
ix	Xylazine (2.2 mg/kg) + Telazol (2-6 mg/kg) i.m. with or without isoflurane 1-5% (face mask or endotracheal tube), Atropine (0.04 mg/kg s.c., i.m.or i.v.) (when intubated)

*Agents will be selected and doses may be refined based on veterinarian's discretion

At the end of the irradiation procedure or surgery, once the animals are back in their cages, dexmedetomidine can be reversed by atipamezole *i.m.* (same volume as dexmedotmidine) to facilitate recovery.

V.4.1.2.2. Pre- and Post-procedural Provisions

Body weights and health check status

Body weights and standard animal health check status (hydration status, hair coat, skin quality, vitals) will be determined at least once prior to irradiation, and on days of blood collection. Data will be recorded on the Animals' Clinical Observation at Blood Collection Form (c) (see Section VIII Enclosures).

Animals will be monitored daily for general indicators of animal health (activity, posture, stool, vomit, respiratory activity, respiratory rate, anorexia and temperature). Monitoring will occur at least once in the morning (am) and once the afternoon (pm) throughout the duration of the study. Monitoring in the critical period of sick animals will be performed with increased frequency, as described in V.5.2.1. Routine Veterinary Medical Care. Daily observations will be recorded on study specific form, Animals' Daily Observations Form (d) (see Section VIII Enclosures).

Supportive care

We will administer prophylactic empirical antibiotic regimen to reduce the risk of infection. Regimen is based on prototype medical orders during a radiation incident and follows guidelines from the Department of Health and Human Services Radiation emergency medical management and the Radiation Injury Treatment Network, RITN (Department of Health and Human Services, Radiation emergency medical management, 2013). For trigger points, will follow guidelines for treatment of afebrile neutropenic patients from chemotherapy and from ablative radiation treatment for hematopoietic stem cell transplantation, as recommended by the American Society of Cancer Oncology, ASCO, National Comprehensive Cancer Network (ASCO, 2011; Flowers, 2013). Furthermore, we will define neutropenia as neutrophil counts <500/microL, and we will use this parameter as trigger point to start/end treatment.

We will start treatment at absolute neutrophil counts (ANC) <500/µL or expected to decrease to <500/µL within the next 48 hours, with or without concurrence of fever (Table 5). We will administer ciprofloxacin plus amoxicillin/clavulanate (p.o.) for a minimum of 3 days. Treatment will continue until bone marrow recovers and ANC remain stable above >500/µL for 2-3 consecutive time points. In case of resistance to ciprofloxacin (temperature higher than 39° Celsius for >3-5 days during fluoroquinolone treatment), we will switch to a different class of antibiotic and we will use gentamicin in combination with amoxicillin/clavulanate (ASCO, 2011). If fever still persists for >3-5 days, we will switch to fluconazole. Oral administration of drugs is preferred over *i.v.* administration because of negative impact of venipuncture on animal survival. Oral administration is also likely to be preferred in case of mass casualties due to limited availability of infrastructure for *i.v.* administration of drugs. We reserve however the option to inject the drugs *i.m.* if oral administration is not feasible. The trigger points to start and end such treatment, as well as the dosage, are described in the Table 5 and Table 6 below -

Table 5
Prophylactic empirical antibiotic regimen: trigger points to start/end treatment

ANC<500/µL or expected to decrease to <500/µL within the next 48 hours	**Start** ciprofloxacin plus amoxicillin/clavulanate (continue for at least 3 days)
ANC stable above >500/µL (2 consecutive CBC reading at 3-4 days distance) and T<39° C	**Stop** treatment
If T > 39° C for >3-5 days while taking ciprofloxacin	**Substitute** ciprofloxacin plus amoxicillin/clavulanate with gentamicin plus amoxicillin/clavulanate. Administer until ANC stable above >500/µL (2 consecutive CBC reading at 3-4 days distance) and T<39° C
T > 39° C for >3-5 days while taking gentamicin	**Substitute** gentamicin plus amoxicillin/clavulanate with fluconazole plus amoxicillin/clavulanate. Administer until ANC stable above >500/µL (2 consecutive CBC reading at 3-4 days distance) and T<39° C

Table 6
Prophylactic empirical antibiotic regimen: drug dosage and modality of administration

Drug	Regimen/route
Ciprofloxacin	2.5 - 7.5 mg/kg, BID, p.o. *
Gentamicin	2-6 mg/kg SID, p.o. *
Amoxicillin/clavulanate	11-13 mg/kg every 12-24 h, p.o. *
Fluconacole (Diflucan)	10-12 mg/kg, SID p.o. *

*or i.m. if PO is not possible, at dosage prescribed by and in consultation with a veterinarian

V.4.1.2.3. Paralytics NA

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched Altweb, AGRICOLA and Pubmed

V.4.1.3.2. Date of Search 9/2/2015

V.4.1.3.3. Period of Search All available

V.4.1.3.4. Key Words of Search swine, total body irradiation, partial body irradiation, ARS, sequelae, pain, distress, death as an endpoint, alternatives

V.4.1.3.5. Results of Search

Alternatives to toxicity testing in live animals have been proposed in the form of cell and tissue culture, which are not feasible for the purpose of this study where the cross-talk among organs and strain sensitivity is being investigated in relationship to survival after acute irradiation. Additionally, bioinformatics or *in silico* approaches applied to life science might be created ad hoc to validate studies; however these approaches cannot be adopted to model the complexity of multi-organ failure in a large animal, because the mechanisms of radiation injury are still for the most part unknown. Alternatives to

irradiation to study the ARS (total body or partial body) do not exist. We will however provide anesthetics and analgesics for pain management at the time of VAP and catheter implantation (See VAP or externalized catheter placement under section V.**4.3.2. Procedure (s)**), as well as during the days that follow surgery, to alleviate the pain produced by the procedures.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification

Irradiation itself is not a painful procedure. Pain and distress are associated with the immune suppression, GI injury and bone marrow suppression post TBI. At equivalent TBI doses, pain and distress after PBI are expected to be less intense, because of the residual, spared, bone marrow which is still capable of producing blood cells. These animals may not have acute effects of irradiation (bleeding, infection *etc.*). Depending upon the irradiation dose, irradiated animals die due to compromised immune responses and opportunistic infections. The percentage of surviving animals is the indicator of the efficacy of a countermeasure. We cannot give systemic anesthetic agents to animals after the irradiation procedures, since they are known to interact with the immune system (see references in Jacobsen, K. O., V. Villa, V. L. Miner, and M. H. Whitnall. 2004. Effects of anesthesia and vehicle injection on circulating blood elements in C3H/HeN male mice. *Contemp Top Lab Anim Sci* 43:8-12.), and would confound the correlation of radiation dose with incidence of moribundity, resulting in a waste of animals. However, we are providing supportive care in terms of antibiotics, and fluids/ nutritional support. Use of supportive care is expected to mitigate the pain associated to the sequelae of irradiation.

V.4.2. Prolonged Restraint

Only short periods of restraint in the sling (<30 minutes) will be necessary for irradiation (in Cobalt facility) procedure, physical examination and blood collections. To minimize discomfort, we have designed a new type of sling that uses softer, elastic material to support the animal, and utilizes a more efficient restraint design that maintains the animal in place while reducing manipulation by technicians and consequently stress to the animals.

During the acclimation period, slings are used for physical examination. At this time, animals are acclimated to the sling for 5-10 minutes. Acclimation to the sling is very rapid and non-stressful; animals are petted and gently spoken to while restrained. It has been our experience that resting on the sling does not agitate the animal at all.

Animals are anesthetized/sedated and transported to the Cobalt Facility holding area before being put into sling for irradiation (<20 minutes). Animals will be kept under continuous observation while in the sling. Slings will be washed and sanitized at the end of each day.

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions Animals will be fasted overnight (approximately 16 – 18 h) prior to VAP implantation surgery. For antibiotic, Cefazolin sodium 20 mg/kg *i.v.* or ampicillin sodium 10-20 mg/kg *i.v.* will be given just prior to the surgery, or intra-operatively, for prophylaxis. At the time of surgery, a temperature microtransponder (Implantable Electronic ID Transponder IPPT-300, Bio Medic Data Systems, Seaford, DE) will be implanted subcutaneously behind the neck, over the shoulder opposite to the site of VAP surgery, to facilitate body temperature measurements.

V.4.3.2. Procedure(s) **VAP implantation**. VAP catheters (SoloPort subcutaneous access port, either PMID or MID model; Instech Solomon, PA) will be surgically implanted in the right external jugular veins under general anesthesia according to the principles of aseptic surgery.

Animals will be lying on their back; vital signs will be taken at regular intervals by vet techs. The catheter tip will be introduced through a 2-3 mm incision in the right jugular vein and advanced to the junction of the external jugular vein and vena cava. Prior to insertion, the VAP catheter will be flushed with locking solution (heparin lock flush solution, 100 IU/ml). Prior to securing the VAP catheter within the vein, 1 preplaced 2-0 or 3-0 vicryl (polyglactin 910) suture (Ethicon, Somerville, NJ) will be used to ligate the vessel just cranial to the insertion point of the catheter tip. A second and third vicryl ligature will be placed around the caudal portion of the vessel containing the VAP catheter and retention bead, with 1 ligature on either side of the bead. The port will be secured within the scapular incision site to the underlying musculature by use of 2-0 or 3-0 poldioxanone (PDS) (Ethicon, Somerville, NJ) at a minimum of 2 of the 5 anchor holes on the port. Catheter function will be confirmed intraoperatively through aspiration of locking solution and withdrawal of a blood sample via the septum. The port reservoir and catheter will be flushed

again with saline and locked with a heparin lock flush solution (100 IU/ml). All subsequent lock solutions will be heparin (100 IU/ml).

During closure of the surgical sites, special attention will be given to eliminating dead space around the port and around the catheter loop on the neck. The muscle and subcutaneous layers will be closed with 2-0 or 3-0 PDS II (polydioxanone suture; Ethicon). Skin incisions will be closed with 2-0 PDS II (Ethicon) in a continuous intradermal pattern. Tissue glue (VetBond, 3M, St Paul, MN) may be used to help seal surgical sites as necessary to help prevent contamination.

Externalized catheter implantation. If needed, an externalized catheter will be placed in the external jugular vein as described above in the VAP catheter placement. However, in this case, the catheter will be tunneled to an exit site on the dorsum of the animal without a VAP. A coil of the catheter will be left underneath the skin and a subcutaneous purse-string suture will be placed around the catheter to secure it at the exit site. Skin will be closed using 2-0 PDS.

V.4.3.3. Post-surgical Provisions

VAP surgery or externalized catheter surgery. Postoperative intramuscular buprenorphine (0.01 to 0.02 mg/kg; Buprenex, Reckitt Benckiser, Richmond, VA) or Carprofen (2.2 mg/kg) will be provided for analgesia before full recovery from anesthesia. Additional post-operative analgesia will be given as Carprofen (2.2 mg/kg *p.o.* BID) for three days starting the day after surgery, or as recommended by an AFRRI veterinarian in consultation with the PI. Alternatively, fentanyl transdermal patch at 5 mcg/kg/hr or buprenorphine sustained release 0.12 – 0.27 mg/kg SC for analgesia can be used as determined by the veterinarian. The fentanyl patch, if used, will be applied to a shaved portion of skin and secured. It takes approximately 12 hrs for the fentanyl patch to take effect. Animals will continue to be monitored until fully recovered from anesthesia; they will be returned to their home cage when ambulatory. Supplemental heat may be provided in the home cage post-operatively (e.g. Bair Hugger warm air blanket, heating pads).

After surgery, 25-50 mg/kg trimethoprim-sulfa *p.o.*(or 5mg/kg *i.m.*), *SID*, will be given for 5 days or more as determined by veterinarian to lessen the risk of post-op infection. Alternatively, enrofloxacin 5-10 mg/kg *i.v./p.o./i.m.* can be given every 24 hrs for 3-5 days or more if needed as determined by the veterinarian. Convenia 5-10 mg/kg *s.c.* may be used as an antibiotic as determined by the veterinarian. The advantage of this antibiotic is that the single injection provides protection against the bacterial infection for several days.
Postsurgical recovery and pain will be monitored twice daily by visual examination of the incisions and overall conditions of the animals. Parameters monitored will be food and water intake, activity, alertness, vocalization, guarding, and response to human contact. Topical antibiotic ointment may be applied at the surgical site. Animals will be allowed to fully recover from the surgery before irradiation is done. The recovery period may last for 2-4 weeks.

V.4.3.4. Location

VSD surgical suite

V.4.3.5. Surgeon

Surgeons will be AFRRI/HJF veterinarians.

V.4.3.6. Multiple Major Survival Operative Procedures NA

V.4.3.6.1. Procedures

V.4.3.6.2 Scientific Justification

V.4.4. Animal Manipulations

V.4.4.1. Injections

All injections will be administered *i.m*, or *s.c.*, or *i.v.*, using sterile needles (19-25 gauge, ¾"-1½") and syringes.

V.4.4.1.1. Pharmaceutical Grade Drugs

Lidocaine (5%), topical
Telazol®
Xylazine
KetamineAtropine sulfate
Cefazolin sodium
Ampicillin sodium
Heparin lock, IV
Saline
Buprenorphine
Carprofen
Fentanyl transdermal patch, topical
Metamucil
Enroflaxacin
Augmentin
Ciprofloxacin
Amoxicillin
Midazolam
Acepromazine
Cetacain topical spray
Atipamezole (Antisedan, Pfizer)
Dexmedetomidine (Dexdomitor, Pfizer)
Convenia (Cefovecin sodium, Pfizer)
Isoflurane
All the drugs listed above are ordered from veterinary product distributors by VSD and they all are pharmaceutical grade drugs.

V.4.4.1.2. Non-Pharmaceutical Grade Drugs NA

V.4.4.2. Biosamples We will collect blood samples throughout the duration of the study, as well as tissue samples at necropsy, for use in our laboratory and for sharing with collaborators. Blood will be used for CBC counts and isolation of plasma/serum for laboratory assays. Tissues will be used for histology as well as molecular laboratory techniques as applicable (*i.e.* Western blot, PCR, ELISA, *etc.*). Tissue samples, to include organs, blood and hair may be taken at the time of euthanasia for tissue blocks and for frozen tissue repository.

Mean blood volume for minipigs is 61-68 ml/kg. (Diehl, 2001) Up to 1% of total blood will be drawn in a single day, and no more than 7% of total blood volume in a week (Walter Reed Army Institute of Research. 1997. Investigators' Handbook. Section I: Handling Techniques and Noninvasive Procedures).

Weight (kg)	Total blood volume (ml)	1% total blood volume (ml)	7% total blood volume (ml)
10	650	6.5	45.5
12	780	7.8	54.6
14	910	9.1	63.7
16	1040	10.4	72.8
18	1170	11.7	81.9
20	1300	13	91
22	1430	14.3	100.1

Collection of blood samples. We will obtain blood samples from externalized catheter (if applicable), or from VAPs (if applicable), or from peripheral veins.

Blood draw from VAP. Before accessing the VAP site, 5% lidocaine ointment USP will be applied topically 15 min prior to sampling, to minimize animal discomfort. Before placement of a non-coring needle, the port injection site will be scrubbed with povidone iodine and 70% isopropyl alcohol until all gross debris is removed (3 to 5 scrubs). To collect a sample, approximately 1 ml fluid will be withdrawn from the catheter and port until blood no longer appears to be mixed with saline. This fluid will be discarded. The blood sample will be collected into a new syringe and placed in appropriate vials for analysis. Next, 3 ml heparin lock flush solution (100 IU/ml) will be infused into the port and catheter as a locking solution. A standard protocol will be followed for obtaining a sample from a problematic catheter. First, external pressure will be applied to the non-coring needle where it entered the port to ensure the tip of the needle is all the way through the septum and into the reservoir. Second, high-pressure turbulent flushing will be attempted with a change in the animal's position. A second person will be needed to extend the neck of the animal by raising it or flexing it away from the side that contains the VAP. Third, the non-coring needle will be replaced; at times the blood may be clotted inside the lumen of the needle. Fourth, if the described series of interventions fail to return the VAP to functioning, the sample will be collected from accessible peripheral vessels using one of the anesthesia regimen as described in section V.4.1.2.1.

Blood draw from externalized catheter. If an externalized catheter is implanted in the external jugular vein (section V.4.3.2) and used for blood collection, the catheter will be flushed with1-5 ml saline and the first 0.5 ml of blood will be discarded. Blood samples will be collected using a new syringe. After blood collection, catheter will be flushed again and locked with 1-3 ml of heparin lock (100 IU/ml).

Blood draw from peripheral veins. Alternatively, we will collect blood from peripheral veins such as auricular, femoral, cephalic, saphenous *etc.* An i.v. catheter may be placed in one of the peripheral veins. Animals will be sedated at the time of blood sampling according to section V.4.1.2.1 Anesthesia/Analgesia/Tranquilization, if necessary. All procedures will be entered into the experimental record. Lidocaine (5%) may be applied topically to the site of venipuncture to minimize discomfort to the animals about 15 minutes before starting the procedure; site will be cleaned with 70% alcohol.

Collection of samples will be performed with the minipig restrained in a sling or on a sedated minipig in the cage; at the same time, we will take vitals. The entire procedure takes less than 30 min. After blood draw, animals will be returned to their cages and checked to ensure that the needle puncture wound is closed and the blood has clotted.

V.4.4.3. Adjuvants NA

V.4.4.4. Monoclonal Antibody (MAb) Production NA

V.4.4.5. Animal Identification Tattoos or ear tags and cage cards/tags

V.4.4.6. Behavioral Studies NA

V.4.4.7. Other Procedures

Temperature transponder. At the time of irradiation, VAP surgery, or catheter implantation, while the animal is anesthetized, a temperature microtransponder (Implantable Electronic ID Transponder IPPT-300, Bio Medic Data Systems, Seaford, DE), will be implanted subcutaneously behind the neck, over the shoulder opposite to the site of VAP surgery, to facilitate body temperature measurements.

Dosimetry. Animals will be sedated, and taken in covered transporting cages to the Cobalt or LINAC facility, where they will be placed on a sling or a table and body measurements will be taken. Sedation will be administered as appropriate.

V.4.4.8. Tissue Sharing Bio-samples (as described in section **V.4.4.2.**) will be collected, to be used for CBC counts, plasma/serum isolation, histo-chemistry/protein/molecular assays among others, and to be shared with collaborators upon request and as experiments permit.

V.4.4.9. Animal By-Products NA

V.4.5. Study Endpoint Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress. The determination of the moribundity must be made in consultation with a veterinarian.
Euthanasia will be carried out either after completion of the observation period (30 day for experiment 2, or 120 day for experiment 3), or when the animal(s) shows at least one of the absolute criteria for moribundity, or a combination of four criteria for non-absolute moribundity. Absolute criteria will be lack of responsiveness (lying down, no effort to get up when people enter the cage, no ability to stand without support), continuous prolonged dyspnea (>30 minutes), body weight loss (loss of greater than 20% of expected weight), and hypothermia (<36°C). Non-absolute criteria will be hyperthermia (>40°C), anorexia (skip 3 consecutive meals), anemia/pallor, petechiae/ecchymosis, vomiting/diarrhea, lethargy, seizures or vestibular signs, and prolonged hemorrhage. Attainment of 4 non-absolute criteria will qualify for immediate euthanasia.

Absolute	
1	Non responsive, assuming the animal has recovered from anesthesia
2	Dyspnea (fast respiration rate, shallow breathing, labored breathing), continuous, prolonged >30 minutes)
3	Loss of 20% of expected weight** (See Appendix)
4	Hypothermia (<36°C) (rectal temperature)
Non-absolute	
1	Hyperthermia (>40°C) (rectal temperature)
2	Anorexia (skip 3 consecutive meals – pig chow)
3	Anemia/pallor, CRT > 2 seconds.
4	Vomiting/diarrhea
5	Lethargy
6	Seizures or vestibular signs (falling, circling or head tilt, paddling)
	Prolonged hemorrhage (hemorrhage = any sign of internal or external bleeding, including presence of petechiae/ecchymosis, bleeding from snout, eye, mouth, anus; presence of blood in the cage; presence of blood in the stool. Hemorrhaging can be also determined by drop in HCT %).

*Attainment of 4 non-absolute criteria will qualify for immediate euthanasia
**Based on Marshall Bio-resources growth chart –weight versus age

Animals will be euthanized according to current American Veterinary Medical Association (AVMA) guidelines.

V.4.6. Euthanasia Animals will be euthanized according to current American Veterinary Medical Association (AVMA) guidelines. Animals will be anesthetized with Telazol® (2-8 mg/kg) *i.m.*, or ketamine (10-20 mg/kg)/xylazine (1-2 mg/kg), *i.m* or 1-5% isoflurane via mask or any one of the combinations described in section V.4.1.2.1. Animals will then be euthanized with Euthasol® (sodium pentobarbital, a minimum dose of 1 ml/4.5 kg, *i.v.* or *i.c.*) or another commercial euthanasia solution. In the rare event that a moribund animal is not responsive and necessitating of immediate euthanasia, euthasol may be injected without prior anesthesia. Animals will be deeply anesthetized with 1-5% isoflurane (via face mask) in addition to the injectable anesthesia during intra-cardiac injection of euthasol. Death will be confirmed by AFRRI veterinarian or veterinary technician via auscultation of the

heart, with cessation of sounds for at least 2 minutes or longer as per current SOP. See item V.4.5 for euthanasia criteria before the end of the study.

V.5. Veterinary Care

V.5.1. Husbandry Considerations Animals will be acclimated for ≥1 week prior to beginning of procedures. Minipigs will be housed in stainless steel cages/runs, in tactile, visual and olfactory contact with adjacent animals. During quarantine/acclimation period, up to day of surgery, if applicable, minipigs may be group-housed. After surgery, they will be singly housed to prevent damage to the VAPs and to allow individual assessment of feed consumption and fecal/urine/blood production. Minipigs will be singly housed after irradiation. Room environment will be maintained as per VSD SOPs.

V.5.1.1. Study Room

Minipigs will be located at AFRRI vivarium for the duration of the study. They will either be in a VSD surgical suite for VAP /catheter implantation if applicable, in transit to and from the Cobalt facility/LINAC in a covered transport cage, in slings for irradiation in the Cobalt facility, or in their housing cage or a VSD prep room for blood collections or euthanasia.

V.5.1.2. Special Husbandry Provisions Filtered tap water will be provided *ad libitum*. Commercial diet will be provided as per vendor's recommendation. Diet may be supplemented with yogurt, metamucil, pumpkin mash, apple sauce, milk formula, and pedialyte may be given, in addition to, or in place of, fresh fruits and vegetables.

Supportive care will be provided after irradiation to all animals, according to Tables 5 and 6.

No treats in the form of marshmallow, peanut butter, crackers will be offered to these animals. Instead, fresh fruits, vegetables, and/or yogurt will be offered 5-7 days a week.

V.5.1.3. Exceptions NA

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care Each animal will be observed at least twice daily for evidence of pain or distress. Body weights will be determined at least once a week and on days of blood collection. Animals will be qualitatively assessed at least twice daily (a.m. and p.m.) for general behavioral status and food consumption by study staff or VSD personnel. Unless the animals are deteriorating, daily observations may not be recorded during weekends, emergency closures, and holidays. Daily observations will be annotated on the Animals' Daily Observation Form (d).

The critical period is defined as day 14-30 post-irradiation. Monitoring of sick animals will be performed with increased frequency during this time to include: an early morning check (before 9 a.m.), a mid-morning check (between 9 a.m. and noon), an afternoon check (between 1 p.m. and 3 p.m.), and a late afternoon check (between 4 p.m. and 6 p.m.). If animal conditions are deteriorating rapidly during the day, an additional evening check (after 8 p.m.) will be done, followed by an early morning check at < 10 hours apart. Rapidly deteriorating conditions are defined as follows: activity score of 2, >1°C change of body temperature per hour, respiratory activity of 1 with continuous dyspnea for more than 10 consecutive minutes, complete anorexia for more than 12 hours, single instance of vomiting/diarrhea, single instance of seizure or vestibular signs, mild hemorrhaging for longer than 6 hours).

V.5.2.2. Emergency Veterinary Medical Care Emergency veterinary medical care is available 24/7 and will be provided based on the restrictions approved in earlier sections of this protocol

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

Physical and occupational enrichment will be done as per the VSD minipig enrichment SOP, except single housing after catheter/VAP implantation if applicable, and irradiation. Dietary enrichment will be done as per section V.5.1.2.

V.5.3.2. Enrichment Restrictions

Minipigs will be single-housed after surgery (if applicable) and after irradiation until completion of the study, to avoid mechanical and infectious complications related to implanted catheters, but they will be in visual, olfactory and auditory contact with other minipigs in the same room. Animals may be group-housed during quarantine.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3, 4, 5, 7 (drug delivery)	(b)(6)
				1,2,3b,4, 5, 6 (VAP), 7 (in vivo BrdU labeling)	
				1, 3, 4, 5	
				1,3,4,5	
				1, 3, 4, 5	

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)					(b)(6)
				1,2,3,4,7	
				1,2,3,4	
				1,2,3,4,5	
				3b,4, minipig husbandry procedures	
				Minipig husbandry procedures	
VSD Back-up technicians					
(b)(6)				All veterinary technical procedures	(b)(6)
				All veterinary technical procedures	
				All veterinary technical procedures	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv

Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY NA

VIII. ENCLOSURES

1) Animals' Daily Observation Form (d).
2) Animals' Clinical Observation at Blood Collection Form (c)
3) Sinclair growth chart
4) Gottingen growth chart

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X. ASSURANCES

Protocol Title: A STRAIN COMPARISON STUDY ON SURVIVAL, VASCULAR DAMAGE, AND LONG TERM HEALTH EFFECTS IN IRRADIATED MINIPIGS (*Sus scrofa domestica*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) _____ _____ 11/24/15_____

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	11-25-2015	2nd Resubmission Date:	
Approved/**Returned for Revision**:	12-14-2015	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	12-22-2015	**EXPIRATION DATE:**	1/3/2019
Approved/Returned for Revision:	1-4-2016	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

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- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: A novel nanoparticle oral formulation of tocotrienols as radiation countermeasure: a pilot study in mice, *Mus musculus*.

IV. PRINCIPAL INVESTIGATOR:

(b)(6) Ph. D Date
Ph. D
Research Biologist, AFRRI-SRD
Office: (b)(6) Fax (b)(6)
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD** Date
Head, Scientific Research Department, AFRRI
Telephone (b)(6)
(b)(6)

- Or –

(b)(6) **PhD, LTC, USA** Date

Head, Scientific Research Department, AFRRI
Telephone: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician
Telephone: (b)(6) Fax (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, LTC, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Safety and Occupational Health Specialist, USUHS
Telephone: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **Ph.D.**

IACUC Chair (b)(6) Scientific Research Department, AFRRI
Telephone (b)(6) Fax: (b)(6)
(b)(6)

PROTOCOL TITLE: A novel nanoparticle oral formulation of tocotrienols as radiation countermeasure: a pilot study in mice, *Mus musculus*.

PRINCIPAL INVESTIGATOR: (b)(6) Ph. D
Research Biologist (AFRRI – SRD)
Office: (b)(6) Fax: (b)(6)
(b)(6)

CO-INVESTIGATOR(S): (b)(6) Ph.D.
Scientist
Office: (b)(6)
(b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER:

I. NON-TECHNICAL SYNOPSIS Radiologic or nuclear events are serious threats to our safety, which can come from sources including devices which spread radiation (including explosive devices), sophisticated nuclear weapons, nuclear power plants, and stolen or mishandled medical sources which are dangerous to health. While the DoD is taking many measures to protect military members and U.S. citizens against these dangerous threats, no drug is currently available to protect people from radiation effects before exposure. Recently, studies by AFRRI scientists have conclusively shown that a single injection of delta- or gamma-tocotrienol (DT3 or GT3) to mice confers a striking survival benefit against irradiation. Both protect mice against injury of the hematopoietic and gastrointestinal systems. Preliminary studies show minimal efficacy by GT3 when administered orally before radiation. (b)(4)
(b)(4)
(b)(4) Some of the advantages of this formulation will include biodegradability, none or very low toxicity, high stability at ambient temperature, significantly high cellular uptake, and feasibility of various routes of administration. The (b)(4) formulation has been tested for stability at various temperatures. Here, we propose to test a promising lipid-based nanoparticle formulation to increase the radioprotective efficacy of tocotrienols following oral (PO) administration.

The current paucity of radiological medical countermeasures warrants serious concern in consideration of credible assessments of potential radiologic or nuclear threat that our military members and first responders are facing. This proposal will evaluate a promising and safe product for use when given orally for protecting against ionizing radiation before sending the U.S. troops in harm's way.

II. BACKGROUND

II.1. Background Threat characterization for radiologic and nuclear events (intentional or accidental) is defined by the nature of the incident [1]. Threat devices or sources include radiologic dispersal devices, radiologic exposure devices, a sophisticated

nuclear weapon, improvised nuclear devices, nuclear power plants, and stolen or mishandled medical radiologic sources [2-5]. AFRRI has been in the forefront in evaluating radiation countermeasures against the deleterious effects of ionizing radiation[(b)(4)]

Tocols as radiation countermeasures: AFRRI investigators and their collaborators have extensively studied tocols including tocopherols, tocotrienols, esters such as tocopherol succinate, and found those to be radioprotective when administered subcutaneously prior to the total body radiation exposure[(b)(4)] Currently no FDA approved countermeasures are available for oral administration. This protocol will allow us to test several formulations of tocols for oral administration with the support of a commercial partner,[(b)(4)] that has interest in advancing tocols as radiation countermeasure.

AFRRI management formed a "tocol" team to initiate studies in collaboration with[(b)(4)] [(b)(4)] has been in consultation with the tocol team to develop[(b)(4)] that might be approved by FDA (use of Generally Regarded as Safe [GRAS] emulsifiers) for human use. These[(b)(4)] [(b)(4)] and will be tested at AFRRI using the current protocol.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched NIHRePORTER, PUBMED, Embase

II.2.2. Date of Search 10-15-2015 to 10-22-2015

II.2.3. Period of Search The search period extended from 2009-present for NIHReporter, 1970 to present for PubMed, 2006 to present for Embase.

II.2.4. Key Words of Search Each individual term: Annatto-derived, [(b)(4)] + (radiation), + (radioprotection), + (radioprotection+mice) + (oral) + [(b)(4)] [(b)(4)]

II.2.5. Results of Search Table 1. Literature search resulted the following hits as tabulated below:

KEY WORDS	PUBMED	NIHRePORTER	Embase
Annatto-derived	1	0	0
[(b)(4)]	0	0	0
[(b)(4)] + radiation	0	0	0
[(b)(4)] + radioprotection+mice	0	0	0
[(b)(4)] + oral	0	0	0
[(b)(4)] + oral + [(b)(4)]	0	0	0

Summary of the Search: The only literature found for 'Annatto-derived' was on effect of[(b)(4)] in postmenopausal osteoporosis. There was no study evaluating radioprotective efficacy of[(b)(4)]

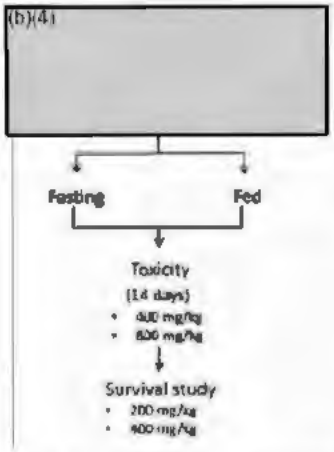
III. OBJECTIVE / HYPOTHESIS The objective of the present protocol is to test the radioprotective efficacy of three oral formulations of (b)(4) (b)(4)

IV. MILITARY RELEVANCE The issue that we will address in this proposal is the urgent need to protect first responders deployed in a radiation field for rescue and recovery military operations, resulting from accidental or intentional nuclear/radiological detonations. Currently there is no drug approved by the FDA to protect first responders deployed in a radiation field for military operations. This is a serious capability shortfall that needs to be addressed. Drugs under various stages of development in small and large animals are found to be toxic at effective doses [3, 4]. Therefore, there is an immediate need for non-toxic radioprotectants useful for military personnel and civilians who may be exposed to a nuclear/radiological scenario [4]. The present proposal directly addresses this need by moving forward with the development of a promising drug delivery approach to increase radioprotective efficacy of oral tocotrienols (T3s).

V. MATERIALS AND METHODS In this study we will test the oral efficacy of (b)(4) (b)(4) using the male CD2F1 mouse model. Drugs will be administered orally to two groups of mice, one with fasting [13] for 5-8 h (fast) and the other without fasting (fed). Fasting of mice is a common procedure performed in association with many different types of experiments mainly in order to reduce variability in investigatory parameters or to facilitate surgical procedures. Since many physiological parameters are regulated by circadian rhythms, fasting initiated at different points in the circadian rhythm has different impacts and produces different results [14, 15]. The oral absorption of tocotrienol is affected by dietary tocopherol content (b)(4) In this study the focus is on absorption of tocols under fed and fasting conditions. Tocopherol is structurally similar to tocotrienols hence competes during absorption. Therefore, we are proposing to evaluate the effect of fasting on radioprotection by tocotrienol. An arm of normal animals will be used as control. Before conducting the efficacy study, we will study the safety of these formulations by conducting a 14-day acute toxicity study [16, 17].

V.1. Experimental Design and General Procedures

General experimental design: In this protocol (b)(4) formulations will be used. All drugs will be administered orally (po). Blank (b)(4) (b)(4) and natural emulsion (NE) will be used as respective negative controls. A schematic diagram of the experimental design is given below:



The experimental design for this protocol is divided in to 2 subsections.

Section v.1.1.: Basic toxicity
Section v.1.2.: Survival studies

Section V.1.1. Basic AcuteToxicity

By the term 'Basic' here means limited toxicology study. The term 'Acute' is used to indicate immediate (short term) toxicity. To evaluate a safe dose of orally administered tocotrienols, we will perform Optimal (non-toxic) Drug Dose for screening (ODDs), which has been used in evaluating several drugs in other IACUC protocols at AFRRI. The purpose of using the ODDs method is that it allows testing in one gender (males in the present proposal), and requires fewer animals when compared to conventional Maximum Tolerated Dose (MTD) study. We will start with four times the dose chosen for survival studies (4X) (e.g. 400 mg/kg) as the starting dose [18-20]. Choice of 4X concentration of the drug for toxicity is from the experience with the (b)(4) screening project, where 4X concentration gave a good window to test efficacy safely. It is also helpful while planning dose optimization studies. Drug will be administered via orogastric gavage (see Section V.4.4.2) once daily for four days to accumulate a steady state concentration of the drug because of its fast clearance after oral delivery. Animals will be divided in two groups, with and without fasting. Animals will be fasted for 5-8 h hours before drug administration (by removing food from those cages. Food will be replaced once the drug has been administered). A maximum of six animals per drug dose will be used to evaluate toxicity, and will be monitored daily in accordance with IACUC policy #10 (Guidelines for establishment of early endpoints in experiments with expected mortality) for acute (1 to 4 h) and chronic (up to 14 days after final dose of the drug is administered) signs of toxicity after administration of drug. Signs of toxicity include decreased activity, squinting eyes, hunching, labored breathing, weight loss or mortality. If the 4X drug dose shows no toxicity in mice, the dose will be escalated to 8X (Table 1). Animals will be euthanized on day 14 and gross necropsy and histopathology will be carried out for any abnormal pathology in all major organs. Maximum tolerated dose (MTD) will be determined from this study.

Serum biochemical and whole blood hematological tests: Whole blood will be collected under anesthesia on day 14 after last dose of drug administration (see section V.4.4.2 for Blood collection) and blood will be transferred to EDTA tubes (200 µl) for

hematological analysis. The rest of the blood will be transferred to serum tubes (300-400 µl) for serum collection for biochemical analysis. Hematological parameters (CBC/differential) and serum biochemistry will be analyzed using an Advia 2120 hematology system from Siemens Corporation, or replacement analyzer, if necessary.

Table 1. Basic Acute toxicity study (14 day) after four doses of T3s (once daily) administered orally.

Drug Dose (mg/kg)	Groups	Diet Condition	# of animals	End point (whole blood)
4X	(b)(4)	Fasting	6	CBC/Differential, Serum Biochemistry
			6	
			6	
Controls			6	
	NE		6	
	Naïve		6	
4X	(b)(4)	Fed	6	CBC/Differential, Serum Biochemistry
	(b)(4) NE		6	
			6	
Controls	(b)(4)		6	
	NE		6	
	Naïve		6	
8X	(b)(4)	Fasting	6	CBC/Differential, Serum Biochemistry
	(b)(4) NE		6	
			6	
8X	(b)(4)	Fed	6	CBC/Differential, Serum Biochemistry
	(b)(4) NE		6	
			5	
		Total # of animals	**120**	

X=Drug dose selected for survival studies

(b)(4)

V.1.2. Efficacy of DG, nano-DGs as radiation countermeasures - survival studies

For survival studies, 16 mice will be used in each group. Mice will be exposed to whole-body γ-radiation. $LD_{70/30}$ dose of radiation will be used such that survival in the control group falls in the range 10% to 50% at 30-days post-irradiation. The exact dose for CD2F1 mice will be obtained from the probit curve (will be determined in Feb 2016 under P(b)(6) . There will be three test article-treated groups and two control groups (Table 2). Drug dose will be determined based on the results obtained from toxicity studies. To see the reproducibility of the results, the experiment will be repeated once, and end point will be 30 day survival. One GT3 in 5% Tween®-80 group (subcutaneous [sc] administration) will be included as positive control.

Table 2. Survival efficacy of oral (b)(4)

Groups	Treatment	Multiplier
1 radiation dose	$LD_{70/30}$	1
3 drug groups	(b)(4) , po	3
3 vehicles (used for drugs)	2 (b)(4) po	4
1 positive control*	GT3 (100 mg/kg, sc, -24 h) and 5%Tween80-saline as its vehicle	2
Drug dose	1/4th of MTD determined by Toxicity study	1

Drug regimen	-3 d, -2 d, -1 d, and -4 h	1
Mice/group	16	16
1 time point	30 day survival	
Fasting/fed	2	2
Iterations	2	2
	Total no. of animals required	**448**

Mice calculation = Rad dose (1) x groups (3+3= 6) x drug regimen (1) x mice (16) x 2 treatments (fast and fed) x iteration (2) =384

*Positive control = 2 groups (GT3 sc, vehicle) x 1drug regimen x 16 mice x 2 iterations = 64

Experimental/Control Group	**Totals**
ODDS safety/toxicity study	120
Radiation survival studies	448
Extra	25
Total # of animals	**593**

Grand total of 593 including 25 additional mice (<5% of total no. of mice required for the protocol) to compensate for incidental loss during the experiment. Based on our previous studies and collection of data, we determine an additional requirement of 25 animals and particularly owing to the de novo nature of studies this becomes even more imperative to account for any unanticipated outcomes. The animals will also serve and support training purposes.

V.2. Data Analysis All data will be reported as means with standards errors or percentages where applicable.
Basic Toxicity (ODDS) – This is a qualitative study so no additional statistics will be conducted.
Preliminary survival study - Based on the power analyses, the sample size (n=16) is adequate to provide 80% power required to detect significant difference between two groups, if any, given 5% type I error and delta/sigma of at least 1.03, where delta is the estimated mean differences between two groups and sigma is the standard deviation. Previously (b)(6) n=24 was used in30-day survival studies to determine if there is 30% improved survival in the drug group compared to vehicle at LD70/30 dose of radiation. Data will be represented as Kaplan-Meier survival curves. Comparison of the survival curves will be made using Log-rank test. A p value of < 0.05 will be considered significant. Experiments will be conducted a maximum of 2 times with 16 mice per group. Due to the *de novo* nature of the study, 25 additional animals have been included to compensate for incidental loss and these animals will be used in the study and also for additional training.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered Although high throughput screening and computer modeling have advanced significantly in recent years in identification of promising drugs, it would be simplistic to assume that cell systems or artificial intelligence modeling can replace the data that can be generated by using an animal model. Irradiation of animals will result in a cascade of changes including death of stem and progenitor cells in bone marrow, alterations in signal transduction pathways,

multiple organ dysfunctions, etc. due to differing radiosensitivities of organs. Currently, there are no alternatives in existence that can predict or compute the complexity of the response to total body irradiation.

V.3.2. Animal Model and Species Justification Recent advances in molecular techniques have allowed significant improvements in understanding the cellular and biochemical processes. These advances are possible only due to whole animal modeling of human ailments. The mouse, in particular, has high sequence homology to humans at a genomic level as well as similar hematological and immunological response to radiation. Mouse models not only confirm primary pathological processes but have also provided a venue for studying basic molecular, cellular, biochemical, and cytological processes. Furthermore, there are extensive data on the mouse for comparison, review and analysis. The practicalities of breeding and housing these small mammals have made the mouse model invaluable. The Principal Investigator's group at AFRRI has significant training and experience in handling mice in radiation countermeasure studies. In this protocol, we will use male CD2F1 mice since this is a historical strain AFRRI has been using for last 30 years. The PI also has used this strain in previous studies. To compare the previous data with this study, PI will use only male CD2F1 mice.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species Mouse. *Mus musculus*

V.3.3.2. Strain / Stock CD2F1

V.3.3.3. Source / Vendor VSD approved vendor (Harlan Inc preferred for CD2F1 mice)

V.3.3.4. Age (Procurement) 7-8 weeks

V.3.3.5. Weight (delivery) 22-24 grams

V.3.3.6. Sex Males have been used historically and will continue to be used in this study.

V.3.3.7. Special Considerations Commercially procured animals must be adventitious disease free (see Appendix 2).

V.3.4. Number of Animals Required (by species) Table

Animal (genus, species and common names)	**Original**
CD2F1	593

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement The PI will take necessary steps for all personnel in the project to be rigorously trained in handling techniques (Tail-tattooing, ear-punch, injections, blood

collection, orogastric gavage and routine cage-side observations) to minimize distress and pain to the animals. Specifically, during capture and restraint of the rodents, care will be taken to ensure that handling does not contribute to distress.

V.3.5.2. Reduction Although basic toxicity and survival will be carried out on all three formulations, additional studies such as dose and time optimization and DRF will be done with the best formulation determined from the survival study. Additional studies will involve various doses of drug as well as various doses of radiation ranging from 100% survival to 100% lethality. Positive controls and negative controls will be used to confirm robustness of experimental conditions. In addition, control groups (such as vehicle) will be shared whenever possible between formulations that are dissolved in similar vehicles. In classical toxicology testing, groups of 10-20 mice of both sexes are routinely used. Optimal (non-toxic) Drug Dose for screening (ODDs), which has been used in evaluating several drugs in other IACUC protocols at AFRRI. In ODDs method testing is done in one gender (males in the present proposal), and the number of animals are 6 per group, a thereby reducing animal numbers significantly.

V.3.5.3. Replacement It is not feasible to use non-animal systems, nor a less-sentient model, in place of the rodent animal models to address the questions in this project.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals 593

V.4.1.1.1.1. Column C ___0__ (# of animals)

V.4.1.1.1.2. Column D ___0__ (# of animals)

V.4.1.1.1.3. Column E ___593__ (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
V.1.1.	ODDS safety/toxicity study			120	120
V.1.2.	Radiation survival studies			448	448
	Extra			25	25
Totals:				593	593

Even though we don't expect any pain or distress during toxicology studies, we would like to keep the animals in category E as we don't have that information.

V.4.1.2. Pain Relief / Prevention Animals might experience pain and distress. Minimizing and alleviating pain in laboratory animals without compromising the methodological integrity of a research project is important both ethically and legally. Mice often exhibit pain and distress with only subtle changes in their behavior [21]. Potential signs associated with pain and distress in mice includes decreased food and

water consumption, weight loss, self-imposed isolation/hiding, rapid breathing, open-mouth breathing, abdominal breathing, decreased movement, abnormal posture, muscle rigidity, twitching, trembling, tremor, etc. as included in Appendix 1 (sample scoring sheet for clinical observations with criteria for rodent euthanasia) in IACUC policy #10 (Guidelines for establishment of early endpoints in experiments with expected mortality).

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization The protocol will not use anesthetics or analgesics during radiation since they will adversely affect the outcome of the experiments or during oral administration of drug. The proposed study is to determine the effects of radiation and countermeasures on the function of the immune and blood forming systems. Use of any analgesics and anesthetics would interfere with the function of the immune system and therefore compromise the interpretation of the results [22]. Topical antibiotics will be applied if wounding occurs via in-cage fighting, under the guidance of the clinical veterinarian in accordance with standard treatment measures.
Anesthesia using standard Isoflurane apparatus under the guidance of the VSD will be carried out in mice for blood collection. All personnel will be extensively trained for efficient administration of the agents as well as to minimize handling stress.
Animals will be placed in the Isoflurane chamber and delivered a metered amount of 3-5% Isoflurane mixed with 100% oxygen (at the flow rate of 500-1000cc/min) until all voluntary motor movement ceases and the animal is recumbent for 10 seconds. Animal's respiration rate will be closely monitored through the Plexiglas chamber during induction of anesthesia. When animals are recumbent, they will be removed from the chamber and their nose/mouth placed in the anesthesia apparatus breathing/gas administration cone for maintenance of anesthesia at 1-3% Isoflurane and 100% O_2.

V.4.1.2.2. Pre- and Post-procedural Provisions Mice will be housed under standard conditions both before and after radiation procedures in accordance with current VSD rodent husbandry Standard Operating Procedures (see section V.5).

V.4.1.2.3. Paralytics N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched AGRICOLA, PubMed. EMBASE

V.4.1.3.2. Date of Search 11/19/2015 to 11/20/2015

V.4.1.3.3. Period of Search AGRICOLA: 1987-2014, EMBASE 1974-present
PubMed ~1970 to present.

V.4.1.3.4. Key Words of Search Radiation and pain, [b](4) toxicity and pain, analgesia, alternative, humane, alternative methods to blood collection by cardiac stick and inferior vena cava.

In order to increase specificity of literature review, several combinations such as radiation plus specific drug, radiation plus specific drug plus mice, alternatives to blood collection, (b)(4) AND toxicity AND pain were evaluated.

V.4.1.3.5. Results of Search

Table 2:Summary of Database Keyword Searches

Keywords	Hits		
	PubMed	AGRICOLA	EMBASE
(b)(4) AND radiation AND pain	0	0	0
(b)(4) AND analgesia AND pain	0	0	0
Radiation AND mouse AND pain	234	0	224
(b)(4) AND toxicity AND pain	0	0	0
Alternatives to blood collection by cardiac stick and inferior vena cava	0	0	0
Pain AND analgesia And mice	1873	52	2236
Radiation AND mouse AND pain AND alternative	5	0	11
Radiation AND humane	12	0	34

There are no alternatives to irradiation. While radiation itself does not cause pain or distress, it induces a number of changes in the body that alters immune response and destroys hematological tissues, resulting in opportunistic infections, and multiorgan dysfunction leading to death. It is anticipated that animals exposed to high lethal radiation doses will become moribund. Unfortunately, literature clearly demonstrates that use of analgesics alters radiation response and therefore leads to increase in animal number for better statistical results or for iteration of entire experiments. Since the purpose here is to compare the efficacy of the three experimental drugs we will be unable to use analgesics and anesthetics because they may interfere with the innate system [17].

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification Because exposure to irradiation is potentially lethal or causes debilitating effects in humans, it is ethically impermissible to test the effectiveness of radiation countermeasures on human volunteers. Irradiation compromises the immune and blood forming systems leading to mortality. The radiation-induced mortality and potential percentage increase of survivors over 30 days using potential radiation countermeasures are study endpoints for this protocol. Administration of drugs and analgesics is not an option since it is well documented that they interact with the immune system which will confuse the interpretation [23-25]. Pain relieving measures are not used because such measures may compromise the experimental integrity of the study. All moribund animals will be euthanized by CO_2 inhalation and confirmatory cervical dislocation (see Section V.4.6 Euthanasia).

V.4.2. Prolonged Restraint The experimental animals will receive radiation at a dose rate 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (according to the latest dosimetry map) using plastic racks. Mice will be inserted into the standard radiation boxes just prior to irradiation (~15 min of exposure time) and taken to the cobalt facility for irradiation. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period. They will be monitored daily following IACUC policy #10 (Guidelines for establishment of early endpoints in

experiments with expected mortality) (at least three times a day during critical period with no more than 10 h interval between late night and morning check and at least twice a day during non-critical period) for 30 days and euthanized at the completion of the observational period.

V.4.3. Surgery N/A

V.4.3.1. Pre-surgical Provisions N/A

V.4.3.2. Procedure(s) N/A

V.4.3.3. Post-surgical Provisions N/A

V.4.3.4. Location N/A

V.4.3.5. Surgeon N/A

V.4.3.6. Multiple Major Survival Operative Procedures N/A

V.4.3.6.1. Procedures N/A

V.4.3.6.2 Scientific Justification N/A

V.4.4. Animal Manipulations

V.4.4.1 Ear punch: Three animals/ shoe box for toxicity studies and four animals/ shoe box for survival studies will be housed. They will be identified either by ear punch or tail tattoo.

Procedure for ear-punch

a. Soak the ear punch (Fine Science Tools, Cat# 24212) in a disinfectant (100% ethanol) before use and between animals.
b. Place the device on the pinna of the ear (external ear) where the skin is thin avoiding the cartilage.

Press firmly to punch a circular hole through the ear

Tattoo procedure: Tattooing will be done either with a machine from Labstamp system following the SOP provided by the company or any other machine certified by VSD.

Irradiation: These will follow the standard AFRRI protocol with mice placed in restrainers shortly prior to exposure, irradiation at the cobalt facility and return to VSD for further monitoring. Briefly, the experimental animals will receive radiation at a dose rate of 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Exact dose of radiation will be determined based on the probit curve. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (the latest dosimetry map) using plastic racks. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period. They will be monitored daily following IACUC policy #10 (Guidelines for establishment of early endpoints in experiments with expected

mortality) (3-4 times a day during critical period with no more than 10 h interval between late night and morning check and at least twice a day during non-critical period) for 30 days and euthanized at the completion of the observational period.

V.4.4.2. Oral gavage: Oral administration will be done by orogastric gavage undertaken using 18-20 gauge gavage needle (animal feeding needles, disposable-sterile, non-toxic and nonpyrogenic, 1 ½" length). Non-disposable, metal ball ended, oral gavage needles of similar gauge and length are included as an alternative to the disposable needles. Drug volume will be 0.1 - 0.2 ml (maximum volume 10 ml / kg). The animal will be restrained in a vertical position to align the spine straight. The bulb will be introduced into the mouth horizontally. Using the needle as lever, the syringe will be moved into a vertical position and the needle dropped down the esophagus into the stomach. The needle contents will be administered when the needle hub touches the animal's mouth. All precautions will be taken to ensure that the placement of the needle is properly positioned and that the animal is not in distress. No forceful feeding will be done to prevent perforation of the esophagus.

Injections: Subcutaneous injections will be given with either a 23 or 25 G needle to the nape of the neck of 0.1 ml (maximum volume 1 ml for 25 to 38 g animals). A disposable sterile needle will be used for all sc injections. One needle will be used per cage containing generally 4 animals. Endotoxin testing for all drugs will be completed prior to any animal testing at AFRRI and a report submitted for IACUC/VSD review and approval. The endotoxin tests are conducted by Charles River Laboratories. At the same time, pH will be tested to ensure that it falls in the range (6-8 for sc administration) stipulated by IACUC. It is expected that the osmolality will not be more than AFRRI IACUC's recommended levels (IACUC Policy #9: Non-pharmaceutical grade experimental drug formulations to be used in experimental animals).

Blood collection: Mice will be anesthetized as described under section V.4.1.2.1. (Anesthesia). Blood draw for hematology is collected either by cardiac stick or from the inferior vena cava (IVC). The animals will be deeply anesthetized in the rodent anesthesia machine; the tail will be pinched for reflexive movement, indicative of insufficient anesthesia. If there is no response, the animal will be moved to the station with individual nose cone for continued anesthesia. An incision will be made on the right side below the abdomen of the animal, closest to the IVC, the vein exposed and blood withdrawn with a 23 - 25 G needle. Similarly, a 23 - 25 G needle will be used to perform cardiac stick while the animal is positioned under nose cone anesthesia. In both cases, the animals will be immediately euthanized on completion of blood draw by cervical dislocation.

V.4.4.1.1. Pharmaceutical Grade Drugs

V.4.4.1.2. Non-Pharmaceutical Grade Drugs Research grade (b)(4) (b)(4) and will be tested at AFRRI using the current protocol (using AFRRI intramural funds for animal studies). The following formulations will be supplied by (b)(4) (b)(4)

(b)(4)

V.4.4.2. Biosamples Blood and tissue

V.4.4.3. Adjuvants N/A

V.4.4.4. Monoclonal Antibody (MAb) Production N/A

V.4.4.5. Animal Identification Generally, cage cards will be used to distinguish drug-treated and control groups. Animals will be randomly distributed in groups. Animals will be identified by ear punch or tattoo at investigator's discretion. Ear punch will be performed as described in V.4.4.1.

V.4.4.6. Behavioral Studies N/A

V.4.4.7. Other Procedures N/A

V.4.4.8. Tissue Sharing
V.4.4.9. Animal By-Products N/A

V.4.5. Study Endpoint The time frame for experiment V.1.1 (basic toxicity) will be 14 days after final drug dose administration. The radiation survival studies will span 30 days after radiation exposure (V.1.2).
It is anticipated that the animals involved in these studies may succumb to death either due to a drug administration or radiation or a combination of the two. To maintain a suitable baseline for humane treatment of the animals while adhering to the study objective, the IACUC recommendations stipulated in IACUC policy #10 (Guidelines for establishment of early endpoints in experiments with expected mortality) with some minor modification (as stated later in the paragraph) will be implemented for judging morbidity and moribundity. The moribund condition is defined as a clinically irreversible condition leading inevitably to death. Signs of morbidity in the mouse include difficulty in breathing, ruffling of hair; drying of lacrimal fluid, fall in body temperature, loss of appetite with/without diarrhea. A modified IACUC policy #10 (Guidelines for establishment of early endpoints in experiments with expected mortality) will be used to record the scoring of clinical signs of pain in animals (attached Appendix 1). PI would like to change the score for the "Changes in breathing" from 3 to 2 and add an in between score of 6 for "Dyspnea / labored breathing" under "Respiratory rate". Description of the score 12 will be "Abdominal breathing/ gasping – open mouth breathing". Previously there was a large jump between a score of 3 (change in breathing) to 12 (abdominal breathing/ gasping – open mouth breathing). From the experience gathered from the studies performed recently, the PI thinks by adding a score of 6 in between 3 and 12 would enhance efficiency of the scoring system giving a fair advantage to the study outcome. PI will seek veterinarian's input for training the staff adequately to learn the suggested scoring system.
a) Body weights will be recorded prior to radiation.

b) Once animals reach a cumulative score of 6, concern cards ("green cards") will be put up, weights will be recorded and monitored with each subsequent scoring change.
c) Animals that exceed 35% body weight loss with any other clinical signs are scored at a 12 and will be euthanized immediately.
To adhere to the policy of preventing animal distress, the PI will facilitate the late PM and early AM checks (not more than 10 h apart) in addition to two times a day checks during the critical lethality period (early morning, late morning, and late afternoon and evening). During non-critical periods, we will continue to check animals except late night. All morbid mice will be monitored carefully and their conditions scored for documentation (Appendix 1, revised scoring sheet).
PI will use a scoring card for each animal in the critical period and scored in each observation. Cumulative Scores below 6 will not be recorded in the scoring sheet. Mice will be euthanized if there is a score of 12 in a single category or a cumulative score of 12 in multiple categories.
No supportive care is proposed, however, moribund animals will be immediately euthanized. VSD veterinarians will be consulted for all matters regarding the animal welfare.

V.4.6. Euthanasia It is anticipated that at high radiation doses, animals will demonstrate varying degrees of morbidity and moribund, which makes it critical to define the criteria for euthanizing the animals without undermining the study objective.
In toxicity studies, if any animal shows discomfort (hunching, decreased activity, inappetence, separation from cage-mates), that animal will be under careful observation on consultation with the attending VSD veterinarian. Mice found moribund (defined in V.4.5.) or meeting the criteria for euthanasia following a modified IACUC policy #10 (Guidelines for establishment of early endpoints in experiments with expected mortality) (score sheet attached) will be euthanized by CO_2 inhalation plus confirmatory cervical dislocation by investigator/technician according to the directives of the VSD-SOP (VS0211: Rodent Euthanasia Guidelines) and in accordance with current AVMA Guidelines on Euthanasia [26, 27]. At the conclusion of the experimental time frame, all animals that survived the procedures of irradiation, drug intervention and controls, will be euthanized by CO_2 inhalation plus confirmatory cervical dislocation and their carcasses disposed of according to VSD regulations. An alternate method of euthanasia by cervical dislocation after exsanguination (by cardiac stick or inferior vena cava) under deep isoflurane anesthesia will be applied in experiment V.1.5 (see Section V.4.4.2 Blood collection).

V.5. Veterinary Care

V.5.1. Husbandry Considerations The AFRRI vivarium follows stringent protocols for the housing and care of all animals. The PI and designated team members will adhere to all the policy and guidelines set forth by standard operating protocols of the VSD. The specific requirements for mice housing should meet the following criteria of opportunity for social interaction, opportunity to carry out normal behavior and the opportunity to rest and withdraw from each other [28]. Mice are social animals and hence will be caged together in groups of 4 or 8. Nestling pads will be provided in each cage during bedding changes. In cage shelters will also be provided as an enrichment strategy at the discretion of the PI depending on experimental conditions. Animals will receive

Harlan Teklad Rodent Diet 8604 and water *ad libitum*. Acidified water (pH 2.5 – 3.0) is routinely provided to AFRRI mice used in radiological experiments. Cages will be regularly changed and cleaned by VSD staff in accordance with Standard Operating Procedure. Animals will have 12 h light/dark cycle. Animals will be habituated to their surroundings and routine procedures prior to experimentation. In the event of procedures that last into the night cycle, care will be taken to minimize exposure to light by using red-lens flashlights.

V.5.1.1. Study Room As assigned by VSD

V.5.1.2. Special Husbandry Provisions N/A

V.5.1.3. Exceptions Mice will be socially (group) housed as described above, except when there is a possibility that during survival studies cage mates may be lost through attrition and for a period of time the sole surviving mouse may remain singly housed until the end of the 30-day study.

V.5.2. Veterinary Medical Care We do not project routine veterinary medical care of animals for injuries/inflammation related to injection site inflammation. In case of minor injuries arising from fighting, topical application of antibiotics is acceptable with consensus from the PI and VSD.

V.5.2.1. Routine Veterinary Medical Care Experimental animals will be observed daily by either the investigator or technician or both as outlined in the **Section V.4.5. Study Endpoint.**

V.5.2.2. Emergency Veterinary Medical Care Moribund animals (gasping, recumbent, non-responsive to mild stimulus) will be euthanized by trained personnel.

V.5.3. Environmental Enrichment: The husbandry staff provides hutches to animals not on active experiments whereas cotton nestlets are provided to animals on active experiments.

V.5.3.1. Enrichment Strategy Nestling pads will be provided in each cage during bedding changes as per AFRRI-VSD SOPs.

V.5.3.2. Enrichment Restrictions

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3, 4b, 5	(b)(6)
				1, 3, 4, 5	
				1, 3, 4b, 5	
				1, 3, 4b, 5	
				1,3b,3c,4,5	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia

Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY All personnel working in the protocol have undergone necessary training in handling experimental animals. The amount of drug used will be small and standard safety precautions will be taken while handling them in the laboratory and in the vivarium. All personnel listed in the present protocol have been trained as general radiation users on a regular basis. All investigators and personnel will use appropriate protective gear while in the vivarium as described by the VSD. All listed personnel are current in safety training and medical surveillance.

VIII. ENCLOSURES none

References:

1. Koerner, J.F., et al., *The medical decision model and decision maker tools for management of radiological and nuclear incidents.* Health Phys, 2014. **106**(6): p. 645-51.
2. Barnett, D.J., et al., *Understanding radiologic and nuclear terrorism as public health threats: preparedness and response perspectives.* J Nucl Med, 2006. **47**(10): p. 1653-61.
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(b)(4),(b)(6)

13. Chaudhry Z.Z. et al., *Streptozotocin is equally diabetogenic whether administered to fed or fasted mice. .* Lab Anim., 2013. **47**(4): p. 257-65.
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16. Wang, L., et al., *Acute and sub-chronic oral toxicity profiles of the aqueous extract of Cortex Dictamni in mice and rats.* J Ethnopharmacol, 2014. **158 Pt A**: p. 207-15.
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22. Colucci, D.G., N.R. Puig, and R. Hernandez-Pando, *Influence of anesthetic drug on immune response: from inflammation to immunosuppression.* OA Anaesthetics, 2013. **21**(3): p. 1-7.
23. Galley, H.F. and N.R. Webster, *Effects of propofol and thiopentone on the immune response.* Anaesthesia, 1997. **52**(9): p. 921-3.
24. Galley, H.F., A.M. Dubbels, and N.R. Webster, *The effect of midazolam and propofol on interleukin-8 from human polymorphonuclear leukocytes.* Anesth Analg, 1998. **86**(6): p. 1289-93.
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X. ASSURANCES

Protocol Title: A novel nanoparticle oral formulation of tocotrienols as radiation countermeasure: a pilot study in mice, Mus musculus.

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM** / **AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

Appendix 1.
Clinical Observations with Criteria for Euthanasia (Rodent)

Criteria:	Description:	Score
Appearance:		
	Normal (smooth coat, clear eyes/nose)	0
	Hunched and/or fluffed	1
	Ocular discharge, and/or edema	3
	Soft stools (fecal matter around anus)	5
	Pale, white mucus membranes/skin	6
	Bloody diarrhea	9
	Blue mucus membranes/skin (cyanosis)*	12
Respiratory Rate:		
	Normal breathing	0
	Change in breathing (an increase or decrease in respiratory rate from normal baseline)	2
	Labored breathing/ Dyspnea	6
	Abdominal breathing (gasping +/- open mouth breathing)/ Diaphragmatic breathing*	12
General Behavior:		
	Normal (based on baseline observations)	0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)	1
	Decreased mobility	2
	Ataxia, wobbly, weak	6
	Inability to stand*	12
Provoked Behavior:		
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))	0
	Subdued; responds to stimulation (moves away briskly)	1
	Subdued even to stimulation (moves away slowly)	3
	Unresponsive to gentle prodding	6
	Does not right when placed gently on side within 5 seconds*	12
Weight loss		
	0-35% with no other clinical signs	0
	≥35% weight loss	12

*** Regardless of score, immediately euthanize (death is imminent)**

Individual or Cumulative Score:

< 6	Normal
6 - 11	Morbid: See VI.D.2 and VI.D.4 in IACUC Policy #10 for guidelines
≥ 12	Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*). Any single criteria of 12* euthanize immediately; consider as 'found dead.'

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	12/11/2015	2nd/3rd Resubmission Date:	2/8; 2/23
Returned for Revision:	12/22/2015	1st Tier Approved:	2/24/2016
1st Resubmission Date:	01/11/2016	EXPIRATION DATE:	2/23/2019
Returned for Revision:	2/2; 2/22/16	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:	2/25/2016	Approved/Returned for Revision:	
Approved/Returned for Revision:	3/2/2016	2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Evaluation of a promising countermeasure, (b)(4) (b)(4) as a radiation mitigator in Rhesus macaque (*Macaca mulatta*)

IV. PRINCIPAL INVESTIGATOR:

(b)(6) Ph.D. Date

(b)(6)

Armed Forces Radiobiology Research Institute (b)(6)

Tel (b)(6) fax (b)(6)

(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD** Date

Head, Scientific Research Department, AFRRI

Telephone: (b)(6) fax (b)(6)

(b)(6)

- Or –

(b)(6) **PhD, LTC, USA** Date

Head, Scientific Research Department, AFRRI

Telephone: (b)(6) fax (b)(6)

(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician, AFRRI

Telephone (b)(6) Fax (b)(6)

(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, LTC (P), VC, USA** Date

Head, Veterinary Sciences Department, AFRRI

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Safety and Occupational Health Specialist, USUHS

Telephone (b)(6)

(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.

(Date)

(b)(6) **PhD, Lt Col, USAF**

IACUC Chair: (b)(6) Scientific Research Department, AFRRI

Telephone (b)(6) Email (b)(6)

PROTOCOL TITLE: Evaluation of a promising countermeasure, [(b)(4)] as a radiation mitigator in Rhesus macaque (*Macaca mulatta)*

PRINCIPAL INVESTIGATOR:

[(b)(6)] Ph.D. Date

[(b)(6)]

Armed Forces Radiobiology Research Institute [(b)(6)]

Tel [(b)(6)] fax [(b)(6)]

[(b)(6)]

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: [(b)(6)]

I. NON-TECHNICAL SYNOPSIS

The threat of a limited radiological attack on American cities (i.e. a "dirty" bomb), or a nuclear incident, requires the development of a radiation countermeasure capable of protecting military personnel and other first responders, who might have to enter the scene and perform their duties for an extended period of time in a contaminated area. This countermeasure should have the capability of mitigating gastrointestinal and hematopoietic syndrome lethality in the general population. At present, there is no effective treatments for the acute radiation gastrointestinal syndrome and only two FDA-approved treatment for hematopoietic syndrome, which requires close patient monitoring by a highly skilled medical team that may not be available during a mass casualty event.

Hematopoietic syndrome usually occurs in humans with a dose between 0.7 and 10 Gy (70 – 1000 rads) though mild symptoms may occur as low as 0.3 Gy (30 rads) and is accompanied by varying degrees of thrombocytopenia that depend on a number of factors including age and health of the individual. Hematopoietic injury that causes death from increased risk of sepsis due to a suppressed immune system and from hemorrhagic events due to depletion of platelets (thrombocytopenia). As the radiation dose escalates, symptoms of a compromised immune and coagulation system persist, along with the addition of damage to the gastrointestinal tract. The impaired integrity of the gastrointestinal epithelium further increases the risk of bleeding, dehydration and sepsis. The increase in radiation dose also involves injury to other late responding tissues such as lung, skin, and kidney making the situation progressively worse and less medically manageable, and ultimately incurable. This study will employ cobalt-60 γ-radiation and utilize the well-defined, nonhuman primate (NHP) model of drug development. The first objective is to estimate radiation dose that will result in 50 - 70% lethality for NHPs after exposure to the AFRRI cobalt-60 γ-radiation source while receiving minimal supportive care defined in our protocol as antibiotics, fluids, and analgesics while excluding the administration of blood products. This would reflect the medical management expected to be available in the field during the first few days after a mass casualty incidence. The second objective is to assess the [(b)(4)] [(b)(4)] efficacy with and without granulocyte colony-stimulating factor (G-CSF) as improved survival following a lethal radiation exposure in NHPs.

II. BACKGROUND

II.1. Background

Military and civil defense organizations have an urgent requirement for medically effective radiation countermeasures. Only two drugs, granulocyte-colony stimulating factor (G-CSF) and pegylated G-CSF, have been cleared by the FDA as a treatment for the hematopoietic acute radiation syndrome (ARS). Another molecule, (b)(4) displays promise as a radiation countermeasure. (b)(4) the countermeasure that is being evaluated in this study, is a (b)(4) (b)(4) has shown multiple potential radioprotective effects in more than one vertebrate animal model. Its therapeutic benefits in preventing ARS appear to be a result of its ability to maintain hematopoietic cell viability [1], prevent apoptosis of irradiated bone marrow cells [2], cause expansion of the primitive progenitor population in combination with other cytokines [2, 3], enhance *in vivo* platelet and erythroid recovery following irradiation [4], reduce expression of endothelial adhesion proteins [5, 6], modulate platelet interactions with vascular endothelium [5, 7-9], prevent formation of petechiae, thrombin and microangiopathies [4, 5, 10-13], reduce fibrinogen and endotoxin levels [5], and enhance bone marrow hematopoietic cell mobilization into peripheral blood [14].

Agonistic activity at the (b)(4) (b)(4) appears to play an important role in modulating the cascade of events resulting in improved survival and reduced morbidity through direct interactions at multiple sites including vascular endothelial cells, bone marrow, and platelets. Administration of (b)(4) at 4, 6 or 24 h after exposure to lethal radiation increased survival and ameliorated radiation induced toxicity in the mouse, rat, and canine, and (b)(4) increased survival in NHP model (see Table 3). The mechanisms by which (b)(4) exerts its protective effects have not been fully characterized, but as summarized above, it has been shown that (b)(4) exerts a broad range of pharmacological effects. Figure 1 illustrates potential pathways by which (b)(4) increases survival in animal models of ARS. Administration of (b)(4) after irradiation acts through its receptor, c-MPL, which is expressed on multiple cell types including primitive hematopoietic progenitors, megakaryocytes, platelets and endothelial cells. Thus apart from restoring platelet production, (b)(4) may prevent radiation-induced vascular damage in a number of sensitive tissues and may enable improved production of endogenous (b)(4) by the irradiated liver.

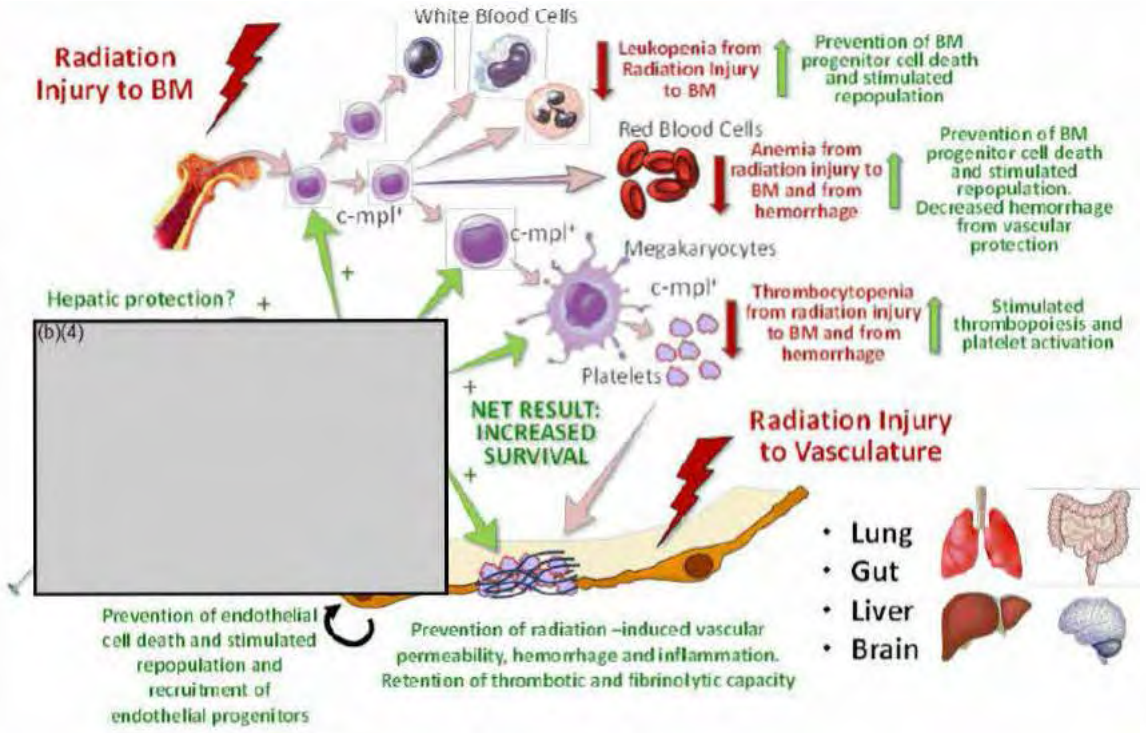


Figure 1: Schematic diagram illustrating the pathways by which (b)(4) may increase survival and reduce morbidity associated with ARS (original creation).

Data showing the impact of (b)(4) on overall survival and in reducing morbidity are described in the sections that follow.

Toxicity and pharmacokinetic studies: A single intravenous (iv) dose of (b)(4) (30 to 300 µg/kg) resulted in an increased platelet count in rats that reached a maximum after 6 days and returned to baseline after 12 days. The pharmacokinetics of (b)(4) were evaluated in studies conducted in rats, rabbits, and canines after single-dose iv administration, and in studies conducted in rats or canines after multiple-dose (once-daily or twice-daily) iv or subcutaneous (sc) administration. The half-life ($t_{1/2}$) of (b)(4) after single or repeat-dose iv administration was between 8 to 31 h in rats, ~26 to ~36 h in rabbits, and 18 to 56 h in canines. The long $t_{1/2}$ of (b)(4) in canines corresponded to a low clearance (CL). In all species, the volume of distribution was low, indicating that (b)(4) resides primarily in the vascular compartment.

The toxicity of (b)(4) was evaluated after a single iv dose (3 mg/kg) in rats, daily iv doses (0.3 mg/kg/day) for 1 week in rats and canines, weekly iv or sc doses (0.3 mg/kg/wk) for 4 weeks in rats and canines, and weekly iv doses (0.3 mg/kg/wk) for 13 and 26 weeks in rats and 13 and 39 weeks in canines. The main findings were increased megakaryocytes in spleen and bone marrow, platelet counts, and mean platelet volume, which were dose-related in rats and canines, increased spleen weights in rats, and increased intravascular platelets in the lung vasculature of canines (13- and 39-week study). These findings were considered to be expected pharmacologic responses. Bone marrow fibrosis and hyperostosis (new bone formation) observed in rats (only in the 1-week and 4-week studies), but not canines, were considered secondary to exaggerated pharmacologic responses. All findings were reversible. In addition, there were no

antibodies to (b)(4) detected in rats or canines given weekly iv doses for up to 26 and 39 weeks, respectively, or weekly sc doses for 4 weeks.

(b)(4) was negative for genotoxicity. In iv and sc studies evaluating immunogenic potential in mice (once weekly doses) and rats (twice weekly doses), no antibodies against the (b)(4) were detected with no cross reactivity with endogenous (b)(4) Similarly, antibodies were not detected in the 4-week iv and sc (weekly doses) toxicity studies in rats and canines.

Mouse Survival Data: (b)(4) was administered by sc injection to mice 6 and 24 h after exposure to an $LD_{50/30}$ or $LD_{70/30}$ total body dose of γ-radiation from a ^{137}Cs source. Each treatment group had 10 males and 10 females (total of 20 animals). Survival was monitored for 30 d. When (b)(4) was administered 6 h after an $LD_{50/30}$ or $LD_{70/30}$ dose of radiation, mice were completely protected (100% survival) against lethal radiation (Figure 2). When administered 24 h after an $LD_{50/30}$ dose of radiation, 90% survival was observed, compared with 75% in the vehicle treated control group. When (b)(4) was administered 24 h after an $LD_{70/30}$ dose of radiation, 75% survival was observed, compared with 55% survival in the vehicle treated control group.

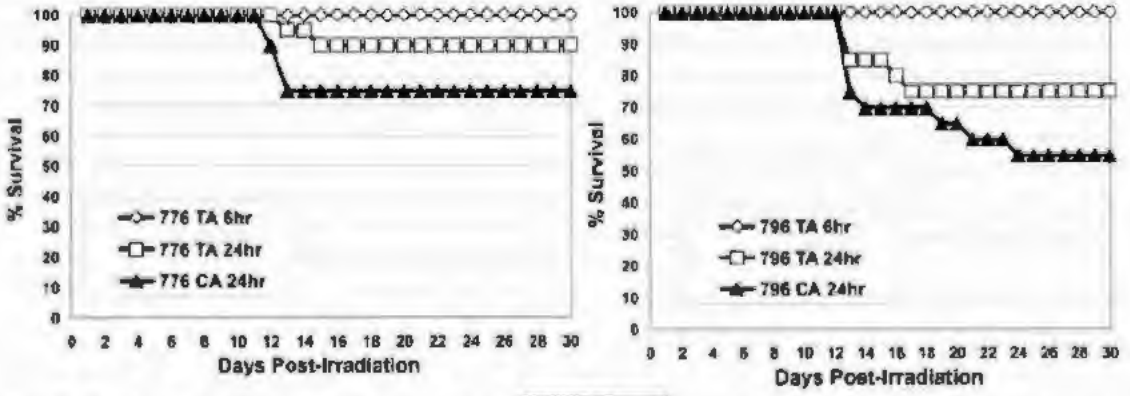
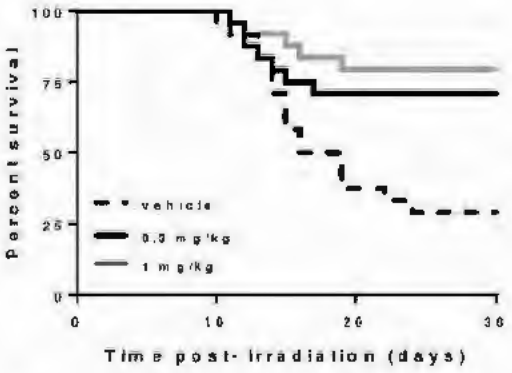


Figure 2. Survival of mice (n = 20) treated with (b)(4) or vehicle (CA) following two doses levels of lethal total body exposure to γ-radiation (776 cGy – Left Figure and or 796 cGy – Right Figure).

Using the same manufacturing batch that will be used in the proposed NHP studies, an additional acute radiation study was recently conducted at AFRRI in a different strain of mice (12-14 week old CD2F1 male mice) that were exposed to ^{60}Co TBI (9.3 Gy, $LD_{90/30}$, 6 Gy/min). Mice were administered a single injection (300 or 1,000 µg/kg) of (b)(4) sc, prior to irradiation, 4 and 24 h post-irradiation. Post-irradiation survival was monitored for 30 d. A statistically significant survival benefit was observed in all groups that received (b)(4) (at 24 h: 71% survival for 300 µg/kg (b)(4) 79% survival for 1,000 µg/kg (b)(4) versus 29% survival of saline group, (Log rank test $p = 0.0013$).



P value	0.0013

Figure 3: Survival of mice (n = 24) treated with (b)(4) at two doses levels (0.3 and 1 mg) or vehicle of lethal total body exposure to radiation (9.3 Gy – ^{60}Co TBI)

The positive and rapid onset of activity observed for (b)(4) drug substances in this initial screening in mice warrants further evaluation in the large animal model. These results are consistent with those obtained with recombinant human (b)(4) in the NHP model.

Rat Pilot Radiation Study: (b)(4) was administered sc to female rats (n = 8) at 6 or 24 h after exposure to an $LD_{70/30}$ TBI dose of γ-radiation (Gammacell 3000 irradiator). Survival was substantially increased in the animals that received 3,000 µg/kg of (b)(4) compared with animals that received vehicle or 30 µg/kg of (b)(4) (Figure 4). Survival of animals receiving (b)(4) at 6 and 24 h post-irradiation are similar but both are significantly higher than that of rats receiving (b)(4) at 48 h post-irradiation.

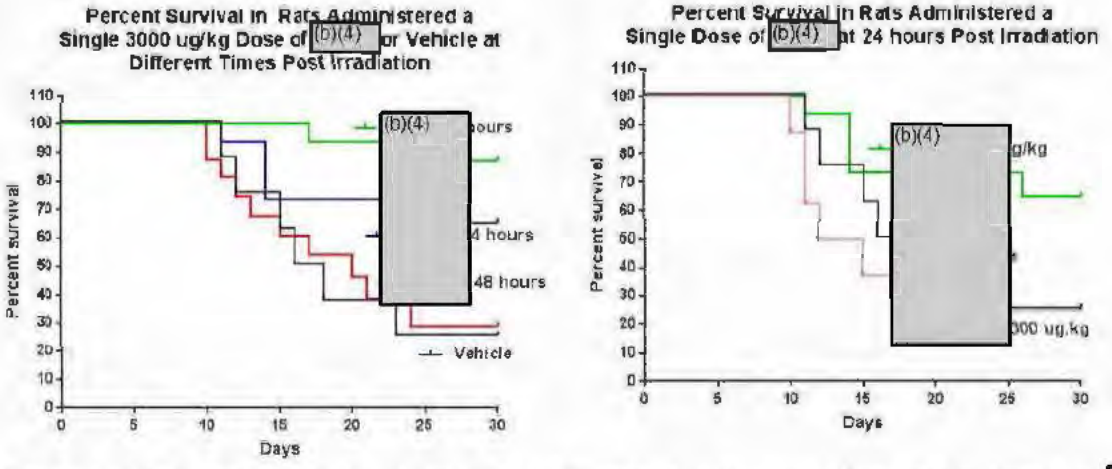


Figure 4. Pilot survival study in rats (n = 8) treated with escalating single doses of (b)(4) administered at 6, 24 or 48 h or vehicle following lethal total body exposure to γ-radiation. In the left panel only the 24 h control data has been plotted for clarity. All controls for other time points (6 hand 48 h) had similar survivors.

Canine Survival Data: A pilot radiomitigation study was conducted in canines with (b)(4) These are initial results as dose had not been optimized for canine and they are

known to be less sensitive to the platelet elevating effects of (b)(4) compared to other species. Results are presented in Table 1 and Figure 5.

Table 1. Effects following a single 10 mg/kg dose of (b)(4) or vehicle administered to canine 24 h after total body radiation exposure.

Endpoint	Vehicle	(b)(4) Treated
Mortality	9/12	7/12
Mean survival time (days)	17	36
Diarrhea	4/12	2/12
Blood transfusions	2.25/animal	1.25/animal
Severe neutropenia duration (days)	8.3	5.4
Neutropenia nadir	0.02 x 10^9/L	0.09 x 10^9/L
Days of febrile neutropenia	2.92	1.08
Mild to severe hematopoietic bone marrow hypocellularity	10/12	6/12
Mild to moderate necrohemorrhagic bronchioalveolar or minimal subacute bronchioalveolar inflammation	10/12	6/12

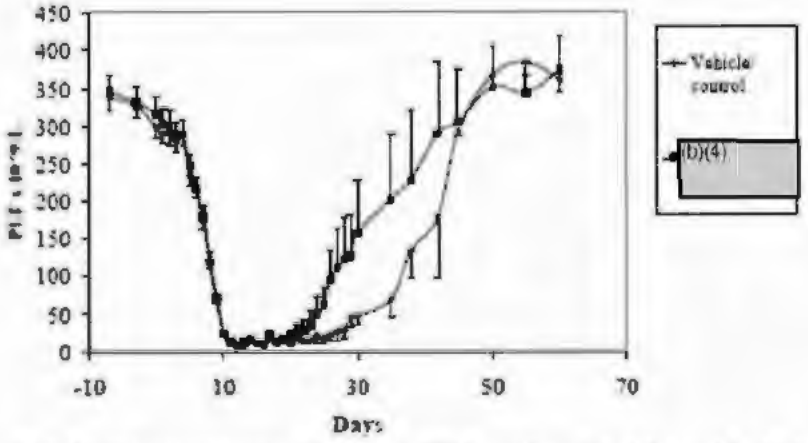


Figure 5. Time course of platelet concentrations following TBI in canines administered a single sc dose of saline vehicle or 10 mg/kg (b)(4) 24 h after irradiation (n=12, 2.7 Gy).

NHP Data with (b)(4) NHPs were exposed to 7 Gy of γ-radiation and then administered vehicle, (b)(4) (b)(4) 10 µg/kg) sc at 0.5 and 24 h post-radiation. Blood transfusions were administered to all treatment groups except (b)(4) As shown in Table 2 and Figure 6 (b)(4) treatment significantly promoted hematopoiesis recovery, improved the quality of life and additionally simplified supportive care in ARS rhesus monkeys [15].

Table 2. Survival and selected clinical observations following TBI to NHP.

Group	No. of Animals	No. of Survival Animals	Average No. Of Days to Mortality	No. of Animals with Fever	Transfusion Volume (ml)	Highest Body Temp (°C)
IR = Vehicle	6	2	19.4±1.7	6	135 ± 31.5**	40.4
(b)(4)	6	5	17.0	2	120 ± 37.9**	40.7
	6	6	-	1	0	39.8

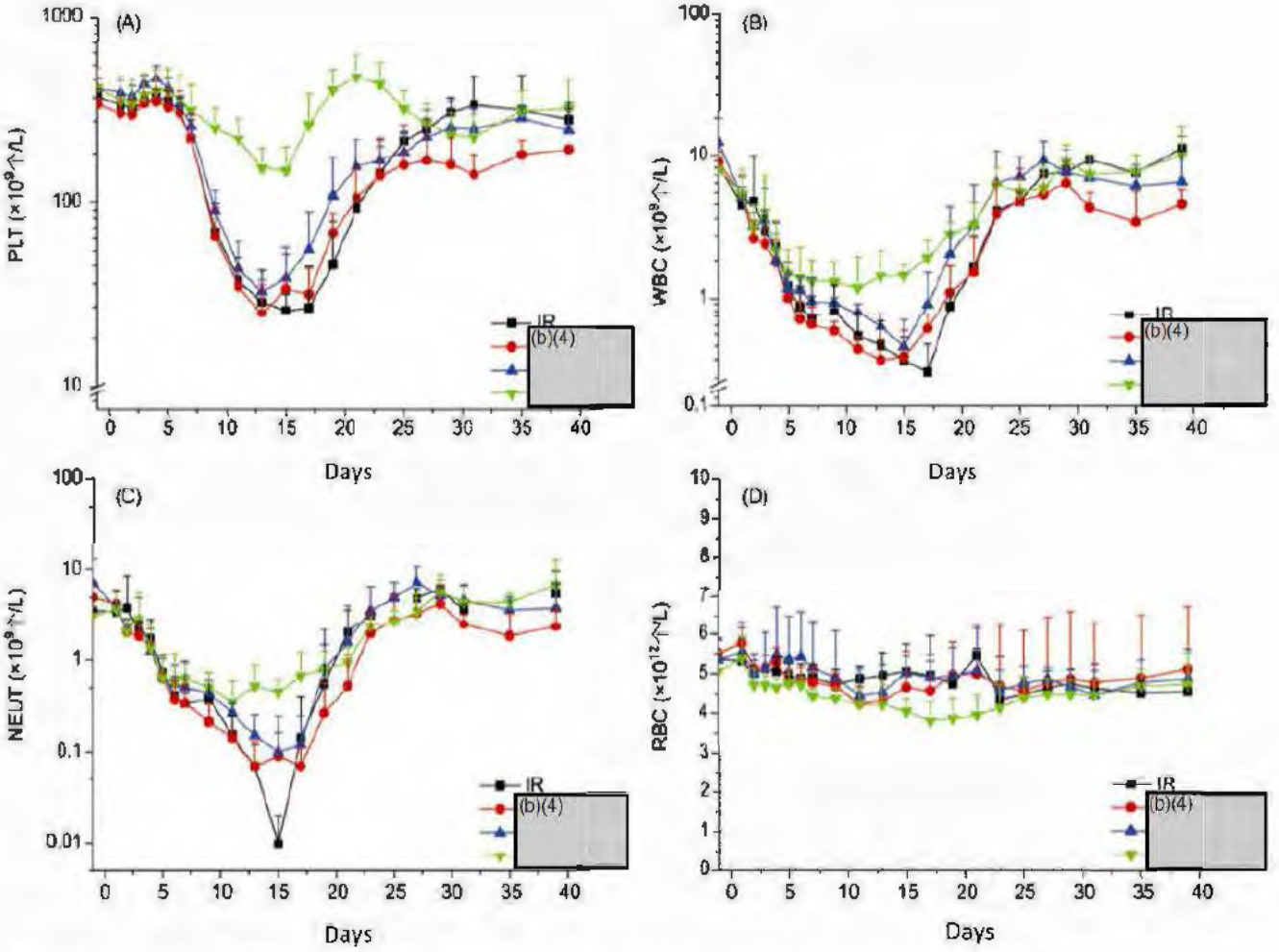


Figure 6. Platelet, WBC, neutrophil and RBC counts vs. time in days post-irradiation (7 Gy) to Rhesus macaques followed by either (b)(4) (10 µg/kg) sc at 0.5 and 24 h post-radiation.

Differences in potency resulting in platelet count elevation amongst species

The (b)(4) dose required to achieve maximal platelet elevation in humans, mice, rats, canines and rhesus macaques is shown in Figure 7. The dose required to achieve a 2-3-fold elevation in humans is ~100-fold lower compared to mice, ~1,000-fold lower compared to rats and >10,000-fold lower compared to canines and NHPs. The maximum platelet elevation was greater than 3-fold for all species except canine is the least in which the maximum platelet elevation was ~1.7 fold, suggesting that the canine is the least responsive species. Figure 8 shows the platelet elevation in NHPs vs. time following a single dose of (b)(4) at 0.5, 2, 10, 20 or 40 mg/kg. The species differences in potency has been described for *other* (b)(4) in the literature and is attributed to differences in receptor affinity (b)(4) Despite the differences in dose, there is clear evidence that comparable maximum platelet response are observed in some species with (b)(4)

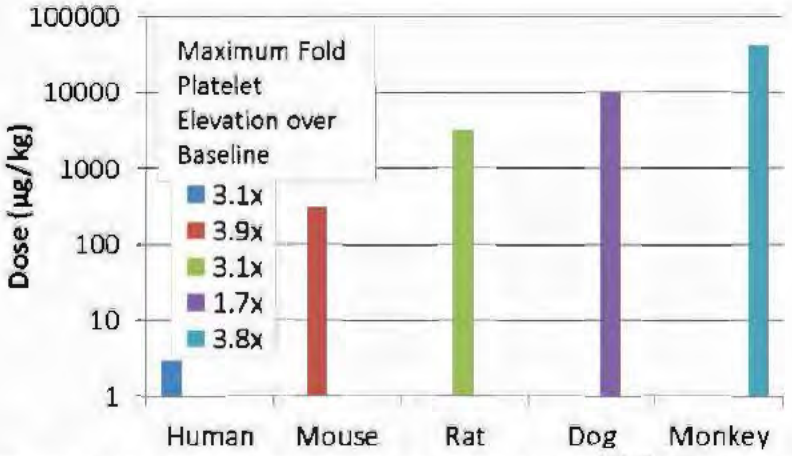


Figure 7. Cross species comparison of the dose of (b)(4) required to achieve the maximum platelet elevation compared to baseline.

(b)(4),(b)(6)

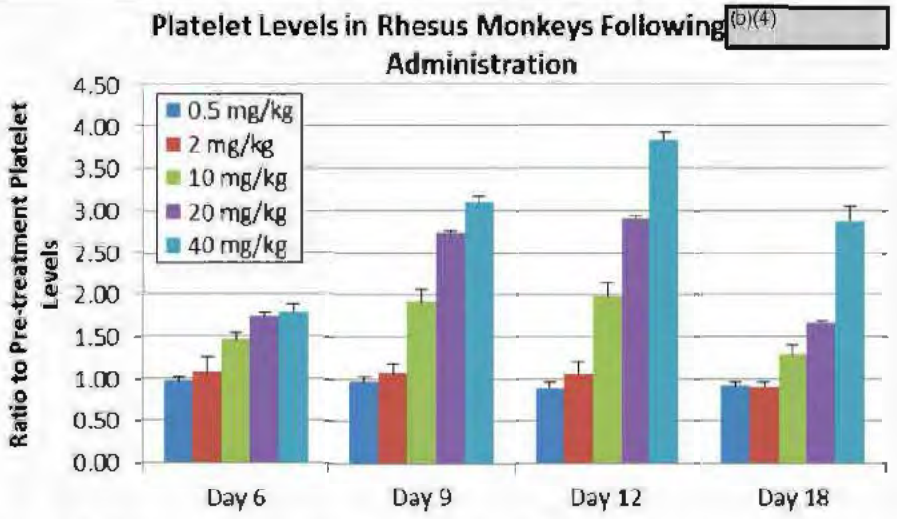


Figure 8. Ratio of the platelet concentration to the pre-treatment platelet concentration following a single dose of (b)(4) to Rhesus monkeys.

(b)(4),(b)(6)

Clinical studies: (b)(4) has been investigated in two human studies. The safety, tolerability, pharmacokinetics, and pharmacodynamics of single ascending iv doses of 0.375, 0.75, 1.5, 2.25, and 3.0 µg/kg (b)(4) administered to 40 healthy men were

evaluated in the first clinical study (b)(6) and it was found to be safe and well tolerated. In the (b)(6) study conducted in subjects with cancer treated with platinum-based chemotherapy, results of analysis of platelet counts in subjects who received (b)(4) at the 2.25 and 3.0 µg/kg dose levels indicated a reduction in chemotherapy-induced decline in platelets and a faster recovery relative to placebo, suggesting potential efficacy for (b)(4) in the prevention of chemotherapy-induced thrombocytopenia.

Justification of dose for NHP study

A 30 mg/kg dose of (b)(4) is proposed for this NHP whole body irradiation study and it is based on different activities between (b)(4) This dose is justified based on platelet elevation in unirradiated animals and survival data following whole body irradiation (see above). In these studies, doses that produce a minimum of 3 fold increases in platelet's concentrations in unirradiated animals appear to consistently produce a survival benefit in irradiated animals (Table 3). Where survival benefit has been demonstrated (b)(4) administration, there is also an estimate of ~3 fold change in platelet concentration. A 30 mg/kg dose of (b)(4) in Rhesus macaques is expected to increase platelet concentrations 3.3 fold of the baseline (Figure 8). The difference in the magnitude of the dose for (b)(4) (0.02 mg/kg) compared to the proposed dose of 30 mg/kg for (b)(4) in NHPs is a result of (b)(4) (b)(4) (b)(4) Because of its sequence homology to endogenous (b)(4) (b)(4) Species differences in potency have been described for other (b)(4) (b)(4) Nevertheless, despite the substantial species differences in dose required for platelet elevation, there is enhanced survival in mice and rats treated with (b)(4) at these doses.

Taken together these data (specifically data from NHP studies) support the hypothesis that administration of (b)(4) at a dose of 30 mg/kg will provide a survival benefit in this study. The platelet response to (b)(4) at 30 mg/kg in non-irradiated NHPs will be verified in an initial study before comparing the NHP response to (b)(4) after irradiation and before studies to assess a survival benefit. The (b)(4) response may be diminished in irradiated animals compared to non-irradiated NHPs due to the depletion of hematopoietic progenitors caused by radiation exposure.

Table 3: Summary of survival results for (b)(4) and vehicle treated animals at different time points post irradiation in animal models of ARS and corresponding elevations of platelets at the doses indicated in unirradiated animals.

Species	Strain	n	Radiation Dose (Gy)	Drug	Dose (mg/kg)	Drug Administration Time Relative to Irradiation (Hours)	Maximum Platelet Elevation in Non-Irradiated Animals (fold increase)	Survival Treatment	Survival Vehicle	Ref#
Mouse	C57BL/6	20	7.76	(b)(4)	0.3	6	3.9	100%	75%	a
Mouse	C57BL/6	20	7.76		0.3	24	3.9	90%	75%	a
Mouse	C57BL/6	20	7.96		0.3	6	3.9	100%	55%	a

				(b)(4)						
Mouse	C57BL/6	20	7.96		0.3	24	3.9	75%	55%	a
Mouse	CD2F1	24	9.25		0.3	-24	3.9	83%	38%	h
Mouse	CD2F1	24	9.25		0.3	4	3.9	88%	29%	h
Mouse	CD2F1	24	9.25		0.3	24	3.9	55%	55%	h
Mouse	CD2F1	24	9.3		0.3	24	3.9	71%	29%	h
Mouse	CD2F1	24	9.3		1	24	4.3	79%	29%	c
Rat	SD	8	7.14		0.3	6	1.7	25%	25%	d
Rat	SD	16	7.14		3	6	3.2	88%	25%	d
Rat	SD	8	7.14		0.3	24	1.7	25%	25%	d
Rat	SD	16	7.14		3	24	3.2	69%	25%	d
Rat	SD	8	7.14		0.3	48	1.7	50%	25%	d
Rat	SD	16	7.14		3	48	3.2	38%	25%	d
Dog	Beagle	12	2.7		10	24	1.7	58%	25%	e
NHP	Rhesus	6	7		0.02	0.5 and 24	3	100%	33%	f,g

It is important to note that platelet elevation (fold increase) data (PD) have come from independent experiments with unirradiated animals (as mentioned in Table 3, column 8 heading) and survival data are from irradiated animal experiments (study source referred).

(b)(4),(b)(6)

.II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

PubMed: United States National Library of Medicine
FEDRIP: Federal Research in Progress Database
DTIC: Defense Technical Information Center

II.2.2. Date of Search: 10/22/2015 to 10/30/2015

II.2.3. Period of Search

PubMed: 1965 - 2015
FEDRIP: 1992 - 2015
DTIC: 1986-2015

II.2.4. Key Words of Search Please see table below.

Literature Search for Duplication				
#	Keywords of Search	Literature Sources and Periods of search		
		DTIC: 1986 - 2015	**PubMed: 1965 - 2015**	**FEDRIP: 1992 - 2015**
		Results of Search		
1	Thrombopoietin	88	4253	23
2	(b)(4)	3	94	1
3		71	9	0
4		3	99	0
5		0	2	0
6	Thrombopoietin and radiation	45	173	3
7	Thrombopoietin and acute radiation syndrome	30	10	0
8	(b)(4) and radiation	1	5	1
9	(b)(4) and primates	0	79	0
10	(b)(4) and radiation and primates	0	3	0
11	(b)(4) and acute radiation syndrome	1	0	0
12	(b)(4) and radiation countermeasure	0	1	0
13	(b)(4) and thrombocytopenia	0	61	1
14	(b)(4) and radiation and hematopoietic injury	1	1	0
15	(b)(4) and GI syndrome	1	0	0
16	(b)(4) and G-CSF and radiation	1	1	1
17	(b)(4) and radioprotection	0	0	0
18	(b)(4) and hematopoietic cells	2	25	1
19	(b)(4) and primates	2	2	0
20	and radiation	22	1	0
21	and radiation and primates	0	0	0
22	and acute radiation syndrome	1	0	0
23	(b)(4) and radiation countermeasure	4	0	0
24	(b)(4) and thrombocytopenia	0	1	0
25	and radiation and hematopoietic injury	0	0	0

26	(b)(4) and GI syndrome	2	0	0
27	and G-CSF and radiation	0	0	0
28	and radioprotection	0	0	0
29	and hematopoietic cells	0	0	0
30	and primates	0	87	0
31	and radiation	0	6	0
32	and radiation and primates	0	4	0
33	and acute radiation syndrome	0	1	0
34	(b)(4) and radiation countermeasure	0	0	0
35	(b)(4) and thrombocytopenia	0	52	0
36	and radiation and hematopoietic injury	0	0	0
37	(b)(4) and GI syndrome	0	0	0
38	and G-CSF and radiation	0	0	0
39	and radioprotection	0	0	0
40	and hematopoietic cells	3	36	0
41	(b)(4) and radiation	0	0	0
42	and primates	0	2	0
43	and radiation and primates	0	0	0
44	(b)(4) and acute radiation syndrome	0	0	0
45	(b)(4) and radiation countermeasure	0	0	0
46	(b)(4) and hematopoietic cells	0	2	0

II.2.5. Results of Search

Summary of Results

Several studies have been conducted using other (b)(4) mainly (b)(4) but mostly for chronic immune thrombocytopenia or thrombocytopenia induced by liver disease or chemotherapy. Administrations of (b)(4) (b)(4) resulted in adverse effects in clinical trials, however were successful at increasing platelet concentrations. (b)(4) (b)(4) have been successful in the clinic for treating idiopathic thrombocytopenia purpura and are FDA approved for these indications.

The safety and pharmacokinetics (PK)/pharmacodynamics (PD) of (b)(4) in humans has been evaluated in healthy human volunteers. Platelet levels in patients treated with (b)(4) showed dose-related elevation as compared to results with placebo. The pharmacokinetic profile was characterized for doses of 2.5 and 3.0 µg/kg, although the dose relationship could not be fully elucidated.The two highest doses of (b)(4) appeared to increase burst-forming units-erythroid and colony-forming unit counts, indicating some effects on progenitor lineages. (b)(4) was well tolerated, with

no evidence of antibody formation in this single-dose study. A non-clinical safety assessment of (b)(4) was completed in rats, dogs and rabbit model.

III. OBJECTIVE / HYPOTHESIS

The first objective is to define a dose level that is determined to be close to the $LD_{50\ 70}$ for NHPs after exposure to the cobalt-60 γ-radiation while receiving minimal supportive care including antibiotics, fluids and analgesics. The second objective is to assess the (b)(4) efficacy with and without G-CSF as improved survival following a lethal radiation exposure in NHPs. This study is to investigate the beneficial effects of radiation countermeasures such as (b)(4) (either alone or in combination with G-CSF) as measured by significant increase in 60-day post-irradiation survival when the drug administered post-irradiation (as a radiomitigator) in an NHP model with minimal supportive care (antibiotics, fluid, analgesics). This study also includes studying hematopoietic recovery including neutrophil and platelet profiles, mean survival time (MST), incidence of febrile neutropenia, thrombocytopenia, infection, and incidence/severity of diarrhea in NHPs.

To achieve the above objective, we need to determine the radiation doses close to estimated $LD_{50\text{-}70}$ value of cobalt 60 γ-radiation source for NHPs under minimal supportive care. Though experiments have been performed in the past for evaluation of various radiation countermeasures without any supportive care using historic values from other institutions, the level of lethality for cobalt-60 source at our institute has not been determined under minimal supportive care as mentioned above. This objective is necessary before proceeding to an efficacy study with the proposed radiation countermeasure. In addition, to use an appropriate drug dose in the efficacy study, PD of the drug need to be evaluated. Hence, we will be investigating PD of the countermeasure in both non-irradiated and irradiated NHPs before proceeding to the efficacy study.

We hypothesize that (b)(4) (alone or in combination with any additional countermeasure used in this study) will be an effective radiation countermeasure in NHPs and administration of this agent post-irradiation will enhance survival over and above the protection provided by the vehicle control group with minimal supportive care.

IV. MILITARY RELEVANCE

The search for treatments to counter potentially lethal radiation injury has been underway for the past several decades, resulting in multiple classes of radiation countermeasures. However, to date only G-CSF and pegylated G-CSF have been approved by the US FDA for the treatment of neutropenia associated with acute radiation syndrome (ARS). Though G-CSF has already been procured, along with GM-CSF, for the strategic national stockpile for use in a radiological emergency [18], the adverse consequences of G-CSF (exacerbation of long-term stem cell damage through excessive differentiation stimulation, exacerbates delayed lung damage in animal model of ARS) which need to be taken into consideration. Thus there is a need for developing additional radiation countermeasures to address thrombocytopenia associated with ARS. Follow-on studies may be needed to address the potential interaction with or synergism with concomitant G-CSF administration.

The problem has become more acute in recent times due to the unpredictable nature of terrorist attacks. Recognizing this urgent need, the U.S. Department of Defense has assigned top priority to the "development of medical countermeasures to radiation exposure" against both early and late arising health effects. The current proposed studies are conducted with interagency collaboration and funding support from (b)(4) These concerns imply the urgent need to develop an appropriate countermeasure for radiation injuries potentially sustained by troops during combat operations and enable commanders to make judicious decisions in troop deployment. There are several drugs at different stages of development. This study will perform essential research and development of promising radiation countermeasures for potential injuries sustained by military personnel who have been exposed to ionizing radiation.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

We propose to carry out the following experiments to estimate the LD_{50-70} value of cobalt-60 γ-radiation in NHPs receiving minimal supportive care (antibiotics, fluid, and analgesics) and to (b)(4) (alone or in combination with G-CSF).

V.1.1. Experiment 1: Determination of estimated radiation dose that will result in 50 - 70% lethality in a minimal supportive care (antibiotics, fluid, and analgesics) NHP model

A recent publication suggests that a lethality curve of NHPs exposed to various qualities of radiation (with and without supportive care) has only been determined to a limited extent [19]. To the best of our knowledge, there is no published report detailing either a full lethality curve or $LD_{50/60}$ value of NHPs receiving cobalt-60 γ–radiation and minimal supportive care (antibiotics, fluid, and analgesics).

Based on extensive discussion with the statistician, we propose to use two different doses of radiation (7.0 Gy and 7.4Gy) with 10 NHPs in each group. It is important to note that we have recently conducted an experiment using 7.2 Gy in 16 NHPs receiving exactly the same supportive care proposed in the current protocol resulting in 43% mortality within 60 days post-irradiation. Data of this study along with data resulting from two additional doses of radiation proposed in this protocol will be used to estimated radiation dose that will result in 50 - 70% lethality.

Table 4. Blood sample collection for LD_{50-70} estimation study

Time in relation to irradiation	Blood			
	CBC 0.5 ml		Biochemistry 0.5 ml	Total blood ml
-7 d	+		+	1.0
-4 d	+		+	1.0
-1 d	+			
0 d (Irradiation)				
1 d	+		+	1.0

4 d	+		+	1.0
7 d	+			0.5
10 d	+		+	1.0
13 d	+			0.5
19 d	+		+	1.0
25 d	+			0.5
31 d	+		+	1.0
38 d	+			0.5
45 d	+		+	1.0
52 d	+			0.5
60 d	+		+	1.0

We will be well within the acceptable published blood collection volumes [20] that are followed at AFRRI. According to these guidelines 1% blood (2.8 ml per day for 4 kg NHP – the minimum body weight of NHPs in this experiment) can be withdrawn every 24 h. Up to 10% of circulating blood volume can be drawn in a single day repeated every 3-4 weeks. For the entire span of the study, the total blood volume drawn will be well below the recommended maximum limits as outlined in section V.4.4.2 Biosamples.

V.1.2. Experiment 2: Determination of pharmacodynamics of (b)(4) in sham irradiated and irradiated NHPs

One 30 mg/kg dose (20 mg/ml in 0.9% saline solution administered at a dose volume of 1.5 ml/kg) of (b)(4) will be tested in four NHPs for PD. The (b)(4) will be administered sc (dorsal scapular region – midline, injection details under section V.4.4.1. Injections) 24 h after sham irradiation to factor in relevant stress in the hematopoietic parameters. Blood samples for PD (CBC – platelet counts) will be collected as shown in the table given below and as outlined in section V.4.4.2 Biosamples. Each animal will be observed twice daily by Veterinary Science Department/research staff for evidence of pain or distress. Hematology and body temperature will be monitored throughout the study. Body temperature via implanted microchips, blood pressure via arm cuf/Doppler, body weights and pulse will be taken at the time of each blood collection.

Table 5. Blood sample collection for PD study with sham irradiation

Time in relation to drug injection	CBC (0.5 ml)	Total vol (ml)
- 7 d	+	0.5
- 4 d	+	0.5
- 1 d	+	0.5
0 d (Sham irradiation, 0 Gy)		
1 d	+	0.5
3 d	+	0.5
6 d	+	0.5
9 d	+	0.5
12 d	+	0.5
18 d	+	0.5
24 d	+	0.5
30 d	+	0.5

For the entire span of the study, the total blood drawn will be well below the recommended maximum limits [20].

After a 30 d or more wash-out period, the same four animals will be used to determine PD of (b)(4) in an irradiated model. NHPs will be irradiated with 7.2 Gy and one dose of (b)(4) (30 mg/kg) will be administered sc 24 h after irradiation. Blood samples for PD (CBC – platelet count) will be collected as shown in Table 6 given below.

Table 6. Blood sample collection for PD study with irradiation

Time in relation to irradiation	CBC (0.5 ml)	Total vol (ml)
- 7 d	+	0.5
- 4 d	+	0.5
- 1 d	+	0.5
0 d (Irradiation)		
1 d	+	0.5
3 d	+	0.5
6 d	+	0.5
9 d	+	0.5
12 d	+	0.5
18 d	+	0.5
24 d	+	0.5
30 d	+	0.5

For the entire span of the study, the total blood volume drawn will be well below the recommended maximum limits [20] as outlined in section V.4.4.2 Biosamples.

V.1.3. Experiment 3: Investigate the radiomitigative efficacy of (b)(4) in a pilot study in NHPs

The radiomitigative potential of (b)(4) will be evaluated in irradiated NHPs. Based on the results from Experiment 1, we will be targeting a radiation exposure dose that will contribute to a lethality rate between 50 – 70% (not above 8 Gy). Once the value is determined, PI will provide a memorandum to the IACUC notifying them of the radiation dose that will be used for the drug efficacy studies (Experiment 3 and Experiment 4). We will be irradiating 16 animals (8 animals for (b)(4) using 30 mg/kg and 8 animals for control using an equal volume of normal saline) first with both groups injected sc 24 h after irradiation as outlined in section V.4.4.1. Injections. This will be repeated once (for a total of two iterations using a total of 32 animals) to assess reproducibility. Here our interest is to develop a radiation countermeasure that is effective when administered 24 h after radiation exposure, the assumed time frame required to make radiation countermeasures available to victims in the event of mass casualty scenario. Blood samples will be collected as presented in Table 7 as outlined in section V.4.4.2 Biosamples. This is the pilot phase of the multiyear interagency agreement between (b)(4) and AFRRI. We will be irradiating 16 animals (8 for control and 8 for (b)(4) first.

Table 7. Blood sample collection for (b)(4) pilot efficacy study

Time in relation to irradiation	Blood		
	CBC 0.5 ml	Biochemistry 0.5 ml	Total blood ml

-7 d	+		+	1.0
-4 d	+		+	1.0
-1 d	+		+	1.0
0 d (Irradiation)				
1 d (drug administration)	+		+	1.0
4 d	+		+	1.0
7 d	+			0.5
10 d	+		+	1.0
13 d	+			0.5
19 d	+		+	1.0
25 d	+			0.5
31 d	+		+	1.0
38 d	+			0.5
45 d	+		+	1.0
52 d	+			0.5
60 d	+		+	1.0

For the entire span of the study, the total blood volume drawn will be well below the recommended maximum limits [20].

V.1.4. Experiment 4: Investigate the radiomitigative efficacy of (b)(4) with and without G-CSF (standard of care) in NHPs

After evaluating the efficacy of (b)(4) in a pilot study (experiment 3), (b)(4) will be evaluated with and without G-CSF, the current standard of care, using a larger sample size. There will be four groups (Vehicle control (normal saline), (b)(4) G-CSF, and (b)(4) + G-CSF) with 20 NHPs in each group. (b)(4) will be administered sc (30 mg/kg) 24 h after irradiation (injection details under section V.4.4.1. Injections).

The US FDA recently approved G-CSF (Neupogen/filgrastim) for the treatment of radiation-induced myelosuppression (hematopoietic acute radiation syndrome) in patients following a radiological/nuclear incident [21]. The route, dose, and schedule of G-CSF administration in this study is based on the currently licensed indication and the recent publication demonstrating its efficacy in irradiated NHPs [22, 23]. G-CSF (10 μg/kg/d) will be administered sc at 24 h after irradiation and then daily until the ANC ≥1,000/μl for 3 consecutive days or if at any time the ANC is ≥10,000/μl. If at any point the ANC falls <500/μl again, daily injections will be reinitiated the same day and continued until the ANC is ≥1,000/ μl for 3 consecutive days.

Table 8. Blood sample collection for (b)(4) efficacy study in combination with G-CSF, standard of care

Time in relation to irradiation	Blood			
	CBC 0.5 ml		Biochemistry 0.5 ml	Total blood ml
-7 d	+		+	1.0

-4 d	+		+	1.0
-1 d	+		+	1.0
0 d (Irradiation)				
1 d (drug administration)	+			0.5
4 d	+		+	1.0
7 d	+			0.5
10 d	+		+	1.0
13 d	+			0.5
19 d	+		+	1.0
25 d	+			0.5
31 d	+		+	1.0
38 d	+			0.5
45 d	+		+	1.0
52 d	+			0.5
60 d	+		+	1.0

For the entire span of the study, the total blood volume drawn will be well below the recommended maximum limits [20] as outlined in section V.4.4.2 Biosamples.

Experiment 4 will be conducted as proposed if the survival benefit of the drug in experiment 3 is at least 25%, even if the benefit does not reach statistical significance. If the survival benefit is < 25% we will contact IACUC and discuss the matter to take decision about experiment 4. The proposed sample size is based on an expected survival advantage of 45% (which is conservative, but we expect that a recalculation of sample size based on the results of experiment 3 may allow a smaller sample size in expt 4). We have no way to estimate the survival advantage before doing the experiment as this is the first experiment with combination of these drugs. NHPs will be irradiated in a minimum of, but not limited to, 4 cohorts, based on existing situation.

V.2. Data Analysis

For experiment 1, the $LD_{50\text{-}70}$ will be estimated. The proposed sample size (10 per group at 7.0 and 7.4 Gy, to be analyzed with existing data on 16 animals at 7.2 Gy) will yield a 95% confidence interval for the $LD_{50/60}$ with an expected width of 0.4 Gy, assuming mortality is less than 50% at 7.0 Gy and between 50-70% at 7.4 Gy. The likelihood of a confidence interval wider than 0.6 Gy is about 5%. Estimates are based on simulating 500 random data sets with the above probabilities and sample sizes and estimating a probit model for each.

For experiment 2, statistical analyses will be primarily descriptive. Data may be compared over time using paired *t*-tests or repeated measures ANOVA as appropriate. A sample size of 4 animals will have 80% power to detect a difference of 2 standard deviations between time points or conditions if the within-subject correlation is 0.6, based on a paired *t*-test with 5%, two-sided significance level.

For experiment 3, survival will be described using Kaplan-Meier curves and compared between groups using the log rank test. If 60 day survival is 50% in the control group and 99% in the treated group, a sample size of 16 for each of the two groups will

have 80% power to detect a significant difference based on a log rank test with 5%, two-sided significance level.

For experiments 4, survival will be described using Kaplan-Meier curves and compared between groups using the log rank test. If survival is 50% in the control group and 95% in the comparison group, a sample size of 20 for each of the two groups will have 80% power to detect significant differences based on a log rank test with 5% two-sided significance level. The expected survival will be higher for the group receiving the (b)(4) and G-CSF combination compared to group administered only (b)(4)

For additional parameters (blood components and blood biochemistry), data mean values with standard errors (when applicable) will be reported. Independent samples *t*-tests or analysis of variance (ANOVA) will be used to detect if there are significant differences between experimental groups. All statistical tests will be two-sided, with a 5% significance level. Statistical software SPSS version 22 (IBM, Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) will be used for analyses. A sample size of 10 per group (experiment 1) will have 80% power to detect differences of 1.3 standard deviations between groups, and a sample size of 16-20 NHPs per group (experiments 3 and 4) will have 80% power to detect differences of approximately 1 standard deviation between groups, based on a *t*-test for independent samples with 5%, two-sided significance level.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

Both computer models and tissue culture have been considered as alternatives to animals. These alternatives lack the knowledge base and complexity needed to evaluate the effects of radiation and radiation countermeasures on the immune system. The phenomena under study involve complex information-processing networks comprising of a large number of cell types and biological signal transduction molecules. Responses to irradiation and radioprotective agents involve interactions between the central nervous system, the peripheral nervous system including the enteric nervous system, endocrine glands, the immune and inflammatory systems, hematopoietic system, GI system, etc. We do not know all of the cell types and tissues involved and many of the signal transduction molecules have not been discovered yet. Even if all of the tissues, cell types and signaling molecules were known, the present state of tissue culture would be incapable of reproducing *in vivo* relationships. One would have to reproduce much of an entire mammalian organism in culture to study these phenomena *in vitro*. As for computer models, the most powerful supercomputers available in the near future would be incapable of analyzing interactions between so many elements in the network. Thus these drugs necessitate investigation in whole animal models to see overall outcome.

V.3.2. Animal Model and Species Justification

Demonstrating efficacy of a countermeasure in a large animal species (LAS) is a critical requirement of the FDA Animal Rule. Rhesus macaque has 95+% DNA sequence identity with humans. NHPs are necessary for the pre-clinical development of a drug candidate intended for use in humans, because drug metabolism and physiology are so similar between NHPs and humans. Rhesus macaques are the model of choice for investigations of toxicity, pharmacokinetics, biomarkers, radiation injury, and

countermeasures, because the large database available from the existing literature. The FDA has accepted rhesus macaques as the appropriate animal model for pivotal efficacy testing of radiation countermeasures under the Animal Efficacy Rule, when efficacy testing cannot be performed in humans. Our ultimate goal is to generate data suitable for submission to US FDA to support the approval of the radiation countermeasure. The pig model (specifically Gottingen minipig) is currently under initial stages of development for studying radiation injury and this model is not defined or mature enough to evaluate radiation countermeasures nor has the minipig model reached a stage of acceptance in the scientific community (b)(4) Therefore, this study utilizes the rhesus macaque to guide future development of this class of radiation countermeasures. There are several reports of using NHPs in radiation research and countermeasure development [23, (b)(4) 29].

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species
Macaca mulatta (Rhesus macaques)

V.3.3.2. Strain / Stock

Chinese origin

V.3.3.3. Source / Vendor

Animals will be procured from vendors registered with the USDA or DoD.

V.3.3.4. Age: 3 - 7 years

V.3.3.5. Weight: 4 - 6 Kg

V.3.3.6. Sex: Male

V.3.3.7. Special Considerations

Animals should test antibody negative for Herpes B virus (aka *Macacine herpesvirus*), Simian T-cell leukemia virus type 1 (STLV-1), Simian Immunodeficiency virus (SIV) and Simian Retrovirus (SRV) Types 1, 2, 3, and 5. Animals shall also test negative by means of virus isolation or polymerase chain reaction (PCR) for SRV Type 2. NHPs will either be vaccinated for measles or, in the case of previously measles-vaccinated NHPs, tested for the presence of measles antibodies. NHPs shall come from the vendor colony negative for *Salmonella*, *Shigella* and *Yersinia*. Vendor must provide the health reports indicating the pathogen free status of the NHPs for the agents listed in this section. The animals will be pole and collar trained by the vendor before their use in above experiment.

V.3.4. Number of Animals Required (by species): 136

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement

For efficacy studies, the endpoint currently mandated by the FDA for approval of radiation countermeasures is mortality or major morbidity. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress, using an extensive set of criteria (listed under section V.4.5). The actual irradiation procedure does not cause pain or distress. Animal care and use procedures will only be performed by trained and certified personnel.

V.3.5.2. Reduction

As we move forward with experiments, plans will be reevaluated at every stage of the experiments, in light of incoming data in an attempt to reduce the numbers of experiments and groups, if possible. If deemed not necessary based on incoming data, some investigations may not be carried out to optimize the use of experimental animals.

V.3.5.3. Replacement

There are no *in vitro* techniques available to demonstrate that this drug will counter the effects of whole-body irradiation in humans. Efficacy and safety have already been demonstrated in rodents. The preferred large animal model for preclinical evaluation of radiation countermeasures intended for use in humans is NHP primates. This study represents the next step necessary to develop this drug for FDA approval. According to FDA scientists and the radiobiology community, the large database of radiation studies in *Macaca mulatta* makes this the most useful large animal model to evaluate interactions of radiation injury and drugs intended for use in humans.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals 136

V.4.1.1.1.1. Column C 0 (# of animals)

V.4.1.1.1.2.Column D 0 (# of animals)

V.4.1.1.1.3. Column E 136 (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
Experiment 1	$LD_{50\text{-}70}$ estimation			20	20
Experiment 2	PD of (b)(4)			4	4
Experiment 3	Pilot efficacy study with (b)(4)			32	32
Experiment 4	Efficacy study with (b)(4) in combination with G-CSF			80	80
Totals:				136	136

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization

As mentioned under section 'V.4.4.7. Other Procedures' animals will be sedated with ketamine (5 -15 mg/kg, im, 22 - 25 G needle length of ¾-1") or Telazol (2 - 5 mg/kg, im, 22 - 25 G needle length of ¾-1") to place in restraint box for transporting to cobalt facility.

Microchips: These are inserted under ketamine anesthesia after animals clear the quarantine period. The chips are used to monitor body temperature and are pre-loaded in a specially designed needle/syringe device that is inserted sc between the shoulder blades. Once the chip is placed, the needle is withdrawn and the skin is pinched between 2 fingers for about 10 - 20 seconds to ensure that the chip itself (about the size of a grain of rice) does not dislodge out through the punctured site. If a chip fails, a

second chip will be inserted. If necessary, a drop of surgical glue will be placed at the punctured site to ensure that the chip stays in place.

***Supportive Care*:** Antibiotics will be administered prophylactically and initiated on day 3 and will continue through day 30. The primary antibiotic will be enrofloxacin (Baytril Bayer HealthCare LLC, Shawnee Mission, KS). The dose will be 10 +/- 0.5 mg/kg administered sc daily (QD). If the body temperature is > 103 ^{o}F, gentamicin sulfate (GentaMax, Phoenix Scientific, Inc.) (5 mg/kg, sc or iv, QD) will be administered in combination with Baytril, and this treatment will continue for 24 h. If high fever persists, ceftriaxone (Rocephin, Roche Laboratories Inc., Nutley, NJ) (50 mg/kg, im, every 24 h) will be administered after gentamicin sulfate is discontinued. An alternative antibiotic to be used under such situation will be clindamycin, 12.5 mg/kg, administered intramuscularly (im) every 8 h. Based on veterinarian suggestion, Ceftiofur sodium free acid (EXCEDE) sc at 5 mg/kg (lasting 2 days) or sc at 20 mg/kg (lasting 7 days) will be used. For this purpose, a bacterial sensitivity culture may be arranged by Veterinary Science Department.

***Fluid support*:** Administration of iv fluid to animals in restraint chair is not possible. Additionally, frequent parenteral route administration to animals with low platelet counts increases the risk of uncontrolled bleeding and every time animals need to be put in restraint chair causing significant stress. Without anesthetizing animals, iv administration of iv fluid is not practicable and frequent sedation will also cause stress. Thus, we will provide water/electrolytes in bottles and fruits and vegetables with high water content (such as watermelon, oranges, cantaloupes etc.) for the dehydrated NHPs.

***Analgesics and non-steroidal anti-inflammatory drugs*:** Buprenorphine SR (0.12 – 0.2 mg/kg, sc, lasting for 3 days) will be administered when there is sign of pain. Rimadyl (Carprofen, 15 mg tab, BID, po or injectable 50 mg/ml; 1 - 5 mg/kg/dose, BID, sc) can be administered as an alternative to buprenorphine when evidence of pain is still present.

V.4.1.2.2. Pre- and Post-procedural Provisions

Animals will be observed for signs of pain and distress by either the veterinary or research staff at least twice daily. After specialized procedures, NHPs will be monitored at least twice daily for signs of complications that may have occurred by the investigative staff. Study staff will adhere to IACUC Policy #10, Guidelines for Establishment of Early Endpoints in Experiments with Expected Mortality during the critical period and with additional checks (maximum 10 h between two checks).

V.4.1.2.3. Paralytics: No paralytic agent will be used in this study.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched

IVIS: International Veterinary Information Service
PubMed: United States National Library of Medicine
Web of Science

V.4.1.3.2. Date of Search: 10/22/2015 - 1/4/2016

V.4.1.3.3. Period of Search

IVIS: 1998 - 2016

PubMed: 1965 - 2016
Web of Science: 1970 - 2016

V.4.1.3.4. Key Words of Search: See table below

V.4.1.3.5. Results of Search

#	Keywords of Search	Literature Sources and Periods of search		
		IVIS: 1998 - 2016	**PubMed: 1965 - 2016**	**Web Of Science: 1970 - 2016**
		Results of Search		
1	Pain and radiation	341	11618	5967
2	Ionizing radiation and pain	22	539	268
3	Ionizing radiation and pain and supportive care	0	1	0
4	Ionizing radiation and pain and sepsis	0	1	0
5	Ionizing radiation and pain and bone marrow depletion	0	0	0
6	Ionizing radiation and pain and intestinal damage	0	0	0
7	Alternative and radiation	213	13382	32031
8	Alternative and radiation and pain	118	463	522
9	Thrombopoietin and pain	0	21	9
10	Alternative and thrombopoietin and pain	0	1	0
11	Thrombopoietin and pain and radiation	0	0	0
12	Alternative and Thrombopoietin and pain and radiation	0	0	0
13	(b)(4) and pain	0	0	0
14	Alternative and (b)(4) and pain	0	0	0
15	(b)(4) and side effects	0	2	0
16	and toxicity	0	1	1
17	and pain and radiation	0	0	0

Summary of Results: No citations for pain were associated with exposure to ionizing radiation, the use of analgesia in conjunction with sub-lethal doses of ionizing radiation, or the use of analgesia in conjunction with (b)(4) in all of the searched databases. The use of (b)(4) was not associated with any reports of pain. GLP studies conducted in rats and canines show (b)(4) to be well tolerated and low toxicity even when administered at a high dose (300 mg/kg given *iv*) (b)(4),(b)(6)

(b)(4),(b)(6)

(b)(4),(b)(6)

(b)(4) The non-clinical safety studies of (b)(4) as well as the results from safety pharmacology, genetic and reproductive toxicology studies demonstrate that (b)(4) is safe and well tolerated with large safety margins for a clinical dose. No alternatives were cited as being a replacement for irradiation.

The literature search did not indicate specific alternatives to pain, other than, what is described below.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

Irradiated animals finally succumb due to a compromised immune response and microbial infections. In the event that there is pain and distress to animals, we will use antibiotics, analgesics, and antipyretic agent, food supplements, and oral electrolyte supplements in this study as minimal symptomatic supportive care to relieve pain and discomfort to the animals. Since we are not sure about the extent of pain by irradiation and to what we are able to relieve the pain, we decided to put all irradiated animals under category E.

V.4.2. Prolonged Restraint

Animals to be irradiated will be placed in restraint devices for irradiation in the cobalt facility. Animals will be sedated for this purpose as outlined in section V.4.1.2.1. Anesthesia/Analgesia/Tranquilization.

For blood collection, NHPs will be placed in a primate restraint chair. NHPs will not be left in the restraint chair for more than 1 hour.

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure(s): N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: N/A

V.4.3.6.2 Scientific Justification: N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections

Injections of test drug, vehicle (normal saline), and G-CSF will be performed with a sterile 21 - 25 gauge needle length of ¾-1". The site for injection will be prepared as a surgical site before the injection: hair will be clipped using # 40 surgical blade and the site will be scrubbed at least 3 times using either betadine or chlorhexidine and 70%

alcohol. Injections will be performed by appropriately trained staff listed in this protocol under section VI. Injections for ketamine, Telazol, Buprenorphine, Rimadyl, and antibiotics will be administered im with 22 - 25 gauge needle length of ¾-1". The injection volume for this will be 0.1 - 1.0 ml.

Sodium pentobarbital sodium will be used as outlined in section V.4.6 Euthanasia.

V.4.4.1.1. Pharmaceutical Grade Drugs

Pharmaceutical grade drugs (G-CSF, antibiotics, analgesics, anesthetics, and fluids) will be procured from the USUHS pharmacy or VSD pharmacy at the discretion of the veterinary staff.

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

(b)(4) is a white to off-white powder with a molecular weight of 3,295 Daltons for the parent compound without polyethylene glycol (PEG) and approximately 43,295 Daltons with 2 PEG chains (MW ~20,000 Daltons each). (b)(4) is composed of 2 identical 14 chain amino acid peptide chains linked by a lysyl residue and linked on each N-terminal to a PEG chain. GLP toxicology studies were performed with (b)(4) (b)(4) which were accepted on the basis of the manufacturer's certificate of analysis and released for use in clinical studies.

(b)(4) comes from a batch of GMP grade drug substance that requires requalification for GMP use and has been stored at -20 °C under sterile conditions and has been tested for endotoxins. The batch of (b)(4) that will be used in this study comes from the same batch that showed significant survival benefits in the mouse studies conducted at AFRRI (Protocol (b)(6) Biopotency will be confirmed in the NHP PD study just prior to efficacy study. Appropriate safety precautions including osmomolarity and pH of final solutions will be in the range stipulated by IACUC Policy #9, non-Pharmaceutical Grade Experimental Drug Formulations to be Used in Experimental Animals. All procedures will be performed under sterile conditions.

V.4.4.2. Biosamples

Blood withdrawals will be carried out according to the schedules presented above and on necropsy. Blood will be collected from a peripheral vessel (saphenous or cephalic veins) rather than a femoral vein with a 21 - 25 gauge sterile needle length of ¾-1"attached to a 2 - 3 ml syringe, to reduce the chances of bleeding in irradiated animals. The femoral vein will be used if necessary for blood collection prior to euthanasia.

For aseptic collection for bacterial samples, the area surrounding the saphenous vein (dorsal region of the lower limb) will be swabbed three times with Providone-iodine. After each application of the Providone-iodine, the area will be wiped off with alcohol. Samples collected for other tests will only utilize alcohol swabs. The blood sample will be drawn as described above. While withdrawing the needle, pressure will be applied at the same time with a sterile bandage to reduce bleeding from the venipuncture site. While making regular routine observation of animals, the venipuncture site will be checked for bleeding. In the event the site becomes infected, topical triple antibiotics (bacitracin, neomycin, and polymyxin) or similar agent will be applied as needed.

Partial necropsies will be performed on all animals following euthanasia by pathology necropsy technicians and/or trained non-pathology personnel. The following tissues will be collected; brain, heart, lung, kidney, spleen, duodenum, jejunum, ileum, large intestine, bladder, and sternum will be collected.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAb) Production: N/A

V.4.4.5. Animal Identification

Animals arrive tattooed and such tattoos will be used for identification. Additionally, cage tags or ID's written on cages with permanent markers are used for identification.

V.4.4.6. Behavioral Studies: No behavioral studies will be performed in this protocol.

V.4.4.7. Other Procedures

Transport plan: For whole body irradiation, the sedated animals will be placed in positioning aide devices and will be transported inside a cart to the High Level Gamma Radiation (HLGR) facility. HEPA filtered transport carts will be used for transportation of animals through common hallways/areas. At no time will the animals be removed from the restraint boxes while outside of the VSD vivarium. At least two personnel will accompany the animals. Additional ketamine may be administered as needed for chemical restraint (animals are not awake during irradiation), and to provide animal handling support. After irradiation, the NHPs in their restraint boxes will be removed from the irradiation platform, returned to the transport cart and returned to the vivarium.

V.4.4.8. Tissue Sharing: None

V.4.4.9. Animal By-Products

No animal by-products will be used in the proposed study.

V.4.5. Study Endpoint

The primary endpoint used in the efficacy study will be mortality. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress. Necropsy and histopathological analysis will be performed on all euthanized animals. The following parameters will provide the guidelines for moribundity.

1. Weight loss: Significant weight loss (10%) from the baseline (NHPs will be weighed whenever the animal is placed on the restraint chair attached to the scale for blood collection),

2. Inappetance: Complete anorexia for 2 days with deteriorating conditions based on clinical examination,

3. Weakness/inability to obtain feed or water: Inability or extreme reluctance to stand which persists for 1 h,

4. Minimal or absence of response to stimuli (animal does not move when prodded),

5. Core body temperature: Below 96.6º F following a period of febrile neutropenia (such as >103º F and <500 neutrophils/ml),

6. Severe anemia: <13% hematocrit due to acute blood loss or <40 g/L hemoglobin (decision in consultation with AFRRI veterinarians),

7. Severe thrombocytopenia (<10,000 platelets/μl)

8. Other signs of severe organ system dysfunction with a poor prognosis as determined by a veterinarian:

8.1 Respiratory: any dyspnea or severe cyanosis.

8.2 Gastrointestinal: sustained vomiting or diarrhea, obstruction, intussusceptions; peritonitis (transient vomiting and diarrhea are expected results of whole-body gamma irradiation, therefore, as criteria for euthanasia, these symptoms should be severe and life-threatening per se, i.e., hemorrhagic diarrhea to the point of causing anemia as listed above or severe uncompensated dehydration).

8.3 Urogenital: renal failure based on clinical chemistry parameters (BUN, creatinine) and urinalysis (urine specific gravity).

8.4 Nervous: sustained CNS depression, seizures, paralysis of one or more extremities.

8.5 Integumentary: non-healing wounds, repeated self-trauma, severe skin infections, indicating severe organ system dysfunction with a poor prognosis.

When an animal reaches a state of moribundity (i.e. point of no return) the animal will be euthanized. Any single parameter listed under this section will not lead to euthanasia of the animal, unless in conjunction with the recommendation to euthanize by an AFRRI veterinarian. Moribundity status of the animal will be determined by a team effort between institutional veterinarian, principal investigator, research staff, veterinary technicians and husbandry staff based on the combination of criterions described above.

Surviving animals at the end of the study (60 d post-irradiation) will be euthanized for gross necropsy and tissue collection as described under section V.4.6. Histopathology of various organs will be conducted to study the effects of radiation and drug.

V.4.6. Euthanasia

Animals will be humanely euthanized using the most current American Veterinary Medical Association (2013 AVMA) guidelines. These animals will be given sodium pentobarbital sodium iv (saphenous or cephalic), needle size 20-25 gauge ¾-1" length, (100 mg/kg, 1 - 5 ml). Prior to pentobarbital sodium administration, the animal will be sedated using ketamine (5 - 15 mg/kg, im). Intra-cardiac administration will be performed if unable to administer pentobarbital sodium through peripheral veins. The animal will be deeply anesthetized by Isoflurane (1 - 5%) with O_2 at 1 - 4 liters per minute via mask before giving intra-cardiac injection. The animals will be euthanized only under the guidance of a staff veterinarian or a trained technician in consultation with the veterinarian if the animal becomes moribund. After pentobarbital sodium administration, the animals will be examined (heart auscultation and pulse) to confirm death.

V.5. Veterinary Care

V.5.1. Husbandry Considerations

All non-human primates will be housed individually in cages with at least 4.3 square feet of floor space per NHP in accordance with *The Guide for the Care and Use of Laboratory Animals*. In addition, every cage will be equipped with built in perches. Excreta and food waste will be removed from inside each indoor primary enclosure, once per day, to prevent the primates from becoming soiled, and to reduce disease hazards, insects, pests, and odors. Care will be exercised when washing the primary enclosure so that the animals remain dry at all times. Primary enclosures will be sanitized every two weeks. This period can be extended to four weeks for animals in the critical period. The secondary enclosure (room) will be sanitized once per month. Foraging boards as well as other enrichment devices will follow the same schedule as the primary enclosures of the primate.

Special care will be given to animals that have been irradiated as their natural immunity will be suppressed (more prone to infections). Irradiation boxes will be sanitized after every use or more often as needed.

V.5.1.1. Study Room: Study rooms will be assigned by VSD.

V.5.1.2. Special Husbandry Provisions: None

V.5.1.3. Exceptions: None

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care

All animals will be quarantined for 45 days prior to any experiment. For this study, NHPs will arrive at AFRRI after completing quarantine at supplier's facility. As per VSD SOP VS0202, Animal Health Rounds, veterinary staff will monitor all animals at least twice daily during morning and afternoon rounds. Any abnormalities will be reported immediately to the on-call veterinarian and PI/his staff. In addition, animals will be observed daily by this protocol's research staff. Any sick animal will be observed at least twice a day (early morning and late afternoon/night) by the research staff. When animals become ill or debilitated, a veterinarian will be contacted immediately to assess the animal and provide adequate/emergency care. If the animal becomes moribund, it will be euthanized as described in section V.4.6. Euthanasia.

V.5.2.2. Emergency Veterinary Medical Care

The on-call veterinary officer will be available via telephone 24 hours a day. The on-call roster is located outside the VSD conference room (Room # (b)(6) on the bulletin board and at the security watch desk. These procedures are in accordance with VSD SOPs.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

In this study, all primates will receive regular enrichment IAW VSD SOP VS0226, Nonhuman Primate Environmental Enrichment. Enrichment provided will include sanitizable toys, contact with husbandry and research staff, and visualization of conspecifics. To facilitate the consumption of food post-irradiation, the primates may be provided with an array of drinking and food products that will heighten their interest. Such items will include kool-aid type drinks, jello and yogurt (as examples of soft type

foods), and bananas, sweet potatoes, and celery with peanut butter (as examples of harder type foods). This brief listing is not meant to exclude any drink or food product that will aid in the consumption of diet designated for the animals depending on clinical observations; monthly enrichment can be modified per the veterinarian.

V.5.3.2. Enrichment Restrictions

None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				7 (off-hour observations)	(b)(6)
				1,3a,3b,3d,4,5,7 (off-hour observation)	
				1,3a, 3b, 3d,4,5,7 (off-hour observation)	
				1,7 (off-hour observations)	
				1, 3b, 5	
				7 (off-hour observations)	
				7 (off-hour observations)	
				7 (off-hour observations)	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage, 3d-iv) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-inferior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

Hands-on training for additional personnel will be provided by study staff designated as trainers by VSD or by VSD technicians using the animals and procedures described in the approved protocol. This will include pole and collar handling of NHPs as well as blood collection and injections. The IACUC will be provided with updated records as training is accomplished.

VII. BIOHAZARDS/SAFETY

There is no plan to use any potential biohazard. Standard laboratory safety precautions will be observed throughout this study.

VIII. ENCLOSURES

Form 310
List of references

X. ASSURANCES

Protocol Title: Evaluation of a promising countermeasure (b)(4) as a radiation mitigator in Rhesus macaque (*Macaca mulatta)*

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I (**AM**) / **AM NOT** (*circle one*) conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress (**WILL**) / **WILL NOT** (*circle one*) be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) _____ _____ _____

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

References

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DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	12/21/2015	2nd Resubmission Date:	2/19/2016
Approved/**Returned for Revision**:	1/15/2016	**Approved**/Approval Withheld by IACUC:	2/22/2016
1st Resubmission Date:	1/20/2016	**EXPIRATION DATE:**	2/21/2019
Approved/**Returned for Revision**:	2/19/2016	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:	2/23/2016	**Approved**/Returned for Revision:	3/2/2016
Approved/**Returned for Revision:**	2/24/2016	2nd Resubmission Date:	
1st Resubmission Date:	2/25/2016	Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and subsection, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. **NAME OF FACILITY:** Armed Forces Radiobiology Research Institute

II. **PROTOCOL NUMBER:** (b)(6)

III. **PROTOCOL TITLE:** Development of a lethality curve with supportive care in the Rhesus macaque (*Macaca mulatta*)

IV. **PRINCIPAL INVESTIGATOR:**

(b)(6) Ph.D. Date

(b)(6)

Armed Forces Radiobiology Research Institute (b)(6)

Tel (b)(6) fax (b)(6)

(b)(6)

V. **DEPARTMENT HEAD:** This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD** Date

Head, Scientific Research Department, AFRRI
Telephone: (b)(6)
(b)(6)
- Or –

(b)(6) **PhD, LTC, USA** Date
Head, Scientific Research Department, AFRRI
Telephone: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) **PhD** Date
Statistician, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by enesthetics or analgesics.

(b)(6) **DVM, DACLAM, LTC (P), VC, USA** Date
Head, Veterinary Sciences Department, AFRRI
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis).*

(b)(6) Date
Safety and Occupational Health Specialist, USUHS
Telephone: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____
(Date)

(b)(6) **PhD, Lt Col, USAF**
IACUC Chair (b)(6) Scientific Research Department, AFRRI
Telephone: (b)(6)

PROTOCOL TITLE: Development of a lethality curve with supportive care in the Rhesus macaque (*Macaca mulatta)*

PRINCIPAL INVESTIGATOR:

(b)(6) Ph.D. Date
(b)(6)
Armed Forces Radiobiology Research Institute (b)(6)
Tel (b)(6) fax (b)(6)
(b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS

Due to increased terrorist activity and the dissemination of nuclear materials, the chances of military or civilian personnel being exposed to radiation from dirty bombs or improvised nuclear devices have risen dramatically in recent years. There are currently no FDA-approved drugs for use by the military or civilian populations for the prevention or treatment of whole body radiation-induced injury. At present, there is no effective treatment for acute radiation gastrointestinal syndrome and only one FDA approved treatment for hematopoietic syndrome, which can be used only after radiation exposure and requires close patient monitoring by a highly skilled medical team.

In an effort to identify suitable radiation countermeasures (agents given before or after radiation exposure that can prevent/treat injuries), many strategies have been investigated during the past several years. In order to develop future countermeasures, we need to develop the lethality curve for cobalt-60 γ-radiation with supportive care. This study will develop the lethality curve of γ-radiation before investigating any radiation countermeasure for efficacy.

II. BACKGROUND

II.1. Background

Although the search for radiation countermeasures for acute radiation syndrome (ARS) was initiated more than half a century ago, only one radiation countermeasure, granulocyte colony-stimulating factor (G-CSF), has been approved by the United States Food and Drug Administration (US FDA) for the ARS (b)(4) There is a need to develop additional radiation countermeasures for use by our military and civilians.

To achieve the goal of having suitable radiation countermeasures approved by the US FDA, we need to investigate radiation countermeasures against various subsyndromes of acute radiation syndrome such as hematopoietic and gastrointestinal subsyndromes. For this purpose, we need to determine the lethality curve of cobalt-60 γ-radiation for NHPs with supportive care. In the past, experiments have been performed for evaluation of different radiation countermeasures without any supportive care using historic or values from other institutions. Thus, we need to determine the lethality curve of our cobalt γ-radiation in a NHP model before proceeding to an efficacy study with any radiation countermeasure.

2. Literature Search for Duplication

II.2.1. Literature Sources Searched

PubMed: United States National Library of Medicine
FEDRIP: Federal Research in Progress Database
DTIC: Defense Technical Information Center

II.2.2. Date of Search 10/23/2015 to 10/30/2015

II.2.3. Period of Search

DTIC: 1986 - 2015
PubMed: 1965 - 2015
FEDRIP: 1992 - 2015

II.2.4. Key Words of Search See table for single and multiple keyword combinations.

II.2.5. Results of Search

Keywords	**PubMed** 1965 - present	**FEDRIP** 1992 - present	**DTIC** 1986 - present
	Results of Search		
Primates and radiation	341353	38	2110
Primates and hematopoietic	153235	33	598
Primates and hematopoietic and radiation	4510	3	436
Primates and hematopoietic and radiation and neutropenia	178	2	90
Primates and stem cells	136567	73	1540
Primates and stem cells and radiation	5055	2	1030
Primates and stem cells and hematopoietic and radiation	1772	1113	323
Primates and stem cells and hematopoietic and radiation and neutropenia	68	119	76
Radiation and supportive care	671	11	46
Primates and gastrointestinal	287	14	29
Primates and gastrointestinal and radiation	14	0	7
None of these searched references address the questions to be tested in this protocol.			

Summary of Results

No publications addressing the questions of this protocol were found. Above literature searches rule out any possibility of duplication.

III. OBJECTIVE / HYPOTHESIS

The long term objective of the present study is to investigate the beneficial effects of future radiation countermeasures as evaluated by significant increase in 60-day post-irradiation survival when the drug is administered post-irradiation (as a radiomitigator) in conjunction with supportive care (antibiotics, fluid, analgesics, blood products). The secondary objectives include studying hematopoietic recovery including neutrophil and platelet profiles, mean survival time (MST), incidence of febrile neutropenia, infection, and incidence/severity of diarrhea in NHPs.

To achieve these objectives, we need to determine the lethality curve of cobalt-60 γ-radiation for NHPs. In the past, experiments have been performed for evaluation of different radiation countermeasures without any supportive care using historic or values from other institutions. Lethality curve for cobalt-60 γ-radiation at our institute has not been determined, particularly with supportive care as mentioned above. Thus, we need to determine lethality curve of our institute cobalt-60 γ-radiation in a NHP model before proceeding to an efficacy study with the proposed radiation countermeasure. This lethality curve will be helpful in the future for investigating efficacy of radiation countermeasures against specific lethal doses of radiation.

IV. MILITARY RELEVANCE

Radiation-associated casualties, whether caused by acute injuries that occur soon after exposure or those resulting from chronic effects, or combined injury, have the potential to impact military operations in various ways. (a) Radiation-associated incapacitating events (such as nausea, vomiting, diarrhea) are some of the early hazards on exposure to lethal radiation. (b) Non-lethal low doses of radiation may not affect military operation immediately but can cause late life pathologies (cancer, pulmonary complications). (c) Lack of readily available radiation countermeasures may cause fear and panic among exposed individuals, and can severely impact the readiness for rescue operations. (d) The impact of chemical and biological weapons can be significantly enhanced by simultaneous exposure to radiological exposure. A non-lethal dose of radiation may become lethal upon simultaneous exposure to a chemical or biological agent or thermal burn.

The above concerns reinforce the urgent need to develop an appropriate modality to sustain the war-fighting capabilities of our military. Developing effective therapies for sepsis following lethal doses of ionizing radiation remains a challenge. The proposed study presents a novel approach to solve the problem of ARS. With the deployment of a radiation countermeasure, forces exposed to moderate to high doses of ionizing radiation, combined injury of radiation and blast/burn will demonstrate enhanced survivability, expanding the range of operable threat environments. The options available to our military will increase, and the morale of personnel at risk of radiation and combined injury exposures will significantly improve. Determination of lethality curve for AFRRI cobalt-60 γ-radiation with supportive care is a first step in this direction.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

We propose to carry-out the following experiments to develop the lethality curve for cobalt-60 γ-radiation in NHPs with supportive care. After determining lethality curve, various LD values will be used to investigate the radiomitigative efficacy of identified radiation countermeasures in the future.

V.1.1. Experiment 1 . Establish lethality curve for AFRRI cobalt-60 γ-radiation in NHPs with supportive care

A recent publication extensively reviewing supportive care for NHPs exposed to radiation suggests that supportive care studies such as the one proposed in this protocol have been executed only to a limited extent [4]. In the publication cited above,

the $LD_{50/60}$ value and probit curve for supportive care has been provided using a 6 MV linear accelerator (LINAC). This study suggests a dose modifying factor of 1.13 for animals provided supportive care compared to animals receiving no supportive care. The radiation source and type of radiation exposure in the published report differ from the AFRRI cobalt-60 pool type facility. To the best of our knowledge, there is no published report for with cobalt-60 γ–radiation and supportive care. Though such study was initiated at AFRRI earlier, it was abandoned and thus, the proposed study still needs to be conducted.

Based on extensive discussion and modeling by the statistician of the sponsor of this study (b)(4) (see data analysis section and also annexure with the protocol for details), we propose to use 6 radiation doses (6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 Gy) with 6 NHPs in each group (total 36 NHPs). Animals will be irradiated in groups of no more than 6 at a time, 1 or more weeks apart or at the discretion of the VSD & Cobalt schedules. Animals will be provided supportive care as described below and monitored for blood profile as stated in table 1. There will be 26 blood donor animals.

Table 1. Blood sample collection for lethality curve determination study

Time of blood draw in relation to irradiation	Blood		
	CBC 0.5 ml	Biochemistry 0.5 ml	Total blood ml
-7 d	+	+	1.0
-4 d	+	+	1.0
-1	+	+	1.0
Day 1 (24 h)	+		0.5
Day 2	+		0.5
Day 4	+		0.5
Day 6	+		0.5
Day 8	+		0.5
Day 10	+	+	1.0
Day 12	+		0.5
Day 14	+		0.5
Day 16	+		0.5
Day 18	+		0.5
Day 20	+	+	1.0
Day 22	+		0.5
Day 24	+		0.5
Day 26	+		0.5
Day 28	+	+	1.0
Day 30	+		0.5
Day 34	+		0.5
Day 38	+	+	1.0
Day 42	+		0.5
Day 50	+	+	1.0
Day 60	+	+	1.0

We will be well within the acceptable published blood collection volumes [5] that are followed at AFRRI. According to these guidelines 1% blood (2.8 ml per day for 4 kg NHP – the minimum body weight of this experiment) can be withdrawn every 24 h. Up

to 10% of circulating blood volume can be drawn in a single day repeated every 3-4 weeks. For the entire span of the study, the total blood drawn will be well below the recommended maximum limits [5] as outlined in section V.4.4.2 Biosamples.

Donor blood draw: A maximum of 12.5% of blood will be collected once every 30 days if needed in consultation with VSD veterinarians. Donor animals will get a CBC done prior to donation to confirm they do not have anemia.

Donor animals will be used for collecting blood for transfusion to recipients. The donor animals will not receive any agents before or after blood collection unless some health issue comes up for that individual donor during the course of this study.

A 4 kg recipient will need 40 ml of blood based on an average transfusion volume of 10 ml/kg (ranging 7 - 14 ml/kg). According to good practice guide [5], NHPs can donate 15% of total blood volume every four weeks. In this protocol, we have projected to take 12.5% upon veterinarian recommendation. A 6 kg donor animal, with a 336 ml blood volume (56 ml/kg), can donate 42 ml per transfusion. Based on radiation doses used in this study, we expect, on average, two transfusions for each recipient within a 4 wk period, thus, for each recipient we will need 2 donors. Following this logic, for 12 recipients we will need 24 donors.

We have projected 26 donors so that if two animals, for any reason are not able to donate the blood, our experiment will not suffer. If needy animals are not supplied with necessary blood, it will be a serious health issue and the study will be wasted.

We will be conducting this experiment in a manner that limits each donor to one donation during the experimental time frame. These donor animals will be used again as donors for future evaluations of countermeasure efficacy using a model of supportive care.

V.2. Data Analysis

A probit model will be used to establish the lethality curve for AFRRI cobalt-60 γ-radiation in NHPs with supportive care. A series of simulations (see attachment at the end of this protocol) indicates that a design with six different doses of radiation (6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 Gy) and 6 animals in each group will be sufficient to estimate the LD_{30}, LD_{50} and LD_{70} to within 0.5 Gy of the true value 90% of the time. The simulations show that this design has greater precision than similar designs with more doses or a wider range of doses, and only marginally less precision than a design using 48 NHPs instead of 36.

For additional parameters (blood components and blood biochemistry), data mean values with standard errors (when applicable) will be reported. Independent samples *t*-tests or analysis of variance (ANOVA) will be used to detect if there are significant differences between experimental groups. All statistical tests will be two-sided, with a 5% significance level. Statistical software SPSS version 22 (IBM, Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) will be used for analyses.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

Both computer models and tissue culture have been considered as alternatives to animals. These alternatives lack the knowledge base and complexity needed to evaluate the effects of radiation and radiation countermeasures on the immune system. The phenomena under study involve complex information-processing networks comprising large number of cell types and biological signal transduction molecules. Responses to irradiation and radioprotective agents involve interactions between the central nervous system, the peripheral nervous system including the enteric nervous system, endocrine glands, the immune and inflammatory systems, hematopoietic system, the GI system, etc. We do not know all of the cell types and tissues involved and many of the signal transduction molecules have not been discovered yet. Even if all of the tissues, cell types and signaling molecules were known, the present state of tissue culture would be incapable of reproducing *in vivo* relationships. One would have to reproduce much of an entire mammalian organism in culture to study these phenomena *in vitro*. As for computer models, the most powerful supercomputers available in the near future would be incapable of analyzing interactions between so many elements in the network. Thus, these drugs merit investigation in whole animal models to see the overall outcome.

V.3.2. Animal Model and Species Justification

Demonstrating efficacy of a countermeasure in a large animal species (LAS) is a critical requirement of the FDA Animal Rule. Rhesus macaque has 95+% DNA sequence identity with humans. NHPs are necessary for the pre-clinical development of a drug candidate intended for use in humans, because the drug metabolism and physiology are so similar between NHPs and humans. Rhesus macaques are the model of choice for investigations of toxicity, pharmacokinetics, biomarkers, radiation injury, and countermeasures, because of the large database available from the existing literature. The FDA has accepted Rhesus macaques as the appropriate animal model for pivotal efficacy testing of radiation countermeasures under the Animal Efficacy Rule, where efficacy testing cannot be performed in humans. Our ultimate goal is to generate data which will be submitted to US FDA and our data should be acceptable to them for approval of the radiation countermeasure. The use of pig (specifically Gottingen minipig) is currently under the initial stage of development for studying radiation injury. This model is not well defined and mature enough to evaluate radiation countermeasures. The minipig model has not reached that stage of acceptance yet in the scientific research community (b)(4) Therefore, this study in the rhesus macaque is being undertaken to guide future development of this class of radiation countermeasures. There are several reports for use of NHPs in radiation research and countermeasure development [9-12].

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species

Rhesus macaques (*Macaca mulatta*)

V.3.3.2. Strain / Stock

Chinese or Indian origin

V.3.3.3. Source / Vendor

Animals will be procured from vendors registered with the USDA or DoD.

V.3.3.4. Age: 3 to 7 years

V.3.3.5. Weight: 4 to 12 kg

V.3.3.6. Sex: Male and Female

V.3.3.7. Special Considerations

Animals should test antibody negative for Herpes B virus (aka *Macacine herpesvirus 1*), Simian T-cell leukemia virus type 1 (STLV-1), Simian Immunodeficiency virus (SIV) and Simian Retrovirus (SRV) Types 1, 2, 3, and 5. Animals shall also test negative by means of virus isolation or polymerase chain reaction (PCR) for SRV Type 2. NHPs will either be vaccinated for measles or, in the case of previously measles-vaccinated NHPs, tested for the presence of measles antibodies. NHPs shall come from the vendor colony negative for *Salmonella, Shigella* and *Yersinia*. Vendor must provide the health reports indicating the pathogen free status of the NHPs for the agents listed in this section. The animals will be pole and collar trained by the vendor before their use in above experiment.

V.3.4. Number of Animals Required (by species): 62 Rhesus macaque (*Macaca mulatta)*

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement

For efficacy studies, the endpoint currently mandated by the FDA for approval of radiation countermeasures is mortality. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress, using an extensive set of criteria (listed under section V.4.5, Study Endpoint). The actual irradiation procedure does not cause pain or distress. Animal care and use procedures will only be performed by trained and certified personnel.

V.3.5.2. Reduction

As we move forward with experiments, plans will be reevaluated at every stage of the experiments, in light of incoming data in an attempt to reduce the numbers of experiments and groups, if possible. If deemed not necessary based on incoming data, some investigations may not be carried out to optimize the use of experimental animals. The simulations show that our experimental design has greater precision than similar designs with more doses or a wider range of doses, and only marginally less precision than a design using 48 NHPs instead of 36.The donor animals will be reused in future study for PK/PD of any drug or donor in experiment evaluating countermeasure for efficacy study with any drug.

V.3.5.3. Replacement

There are no *in vitro* techniques available to demonstrate that any drug will counter the effects of whole-body irradiation in humans. The preferred large animal model for preclinical evaluation of radiation countermeasures intended for use in humans is NHPs. This study represents the next step necessary to develop a drug for FDA approval. According to US FDA scientists and the radiobiology community, the large database of radiation studies in *Macaca mulatta* makes this the most useful large animal model to evaluate interactions of radiation injury and drugs intended for use in humans.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals:

V.4.1.1.1.1. Column C 0 (# of animals)

V.4.1.1.1.2. Column D 26 (# of animals)

V.4.1.1.1.3. Column E 36 (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
Expt 1	Lethality curve - six radiation doses with 6 NHPs each i.e. 36 recipients			36	36
Expt 1	Donors - 26		26		26
Totals:			26	36	62

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization

As mentioned under section 'V.4.4.7. Other Procedures, animals will be sedated with ketamine (5 -15 mg/kg, im, 22 - 25 G needle length of ¾-1") or Telazol (2 - 5 mg/kg, im, 22 - 25 G needle length of ¾-1") to place in restraint box for transporting to cobalt facility.

Monitoring of Sedated NHPs: During procedures which require short duration of anesthesia, but which the animal is un-restrained (such as blood tranfusions), anesthetic monitoring will be achieved through use of anesthetic monitors which will include the minimum of SpO2 % and heart rate measurement. Visual assessment of respiratory rate will be conducted during the procedure. Other measurements such as ECG, EtCO2, and blood pressure will be used as available and circumstances allow. The animal will be sedated with an im injectable agent as described in V.4.1.2.1 and checked for appropriate sedation by signs of prostration, limb flaccidity, and lack of palpebral reflex. Once these signs are achieved, the animal is removed from the cage and transported to a warmed table in the treatment room and maintained on 1 - 5% (more commonly 2 - 3%) isoflurane delivered through face mask with 1 L/min of 100% oxygen. The dose of isoflurane will be subjectively determined by the technician or veterinarian based on any signs of alertness or increased heart & respiratory rate (above approximately 150-160 bpm and 40 rpm, respectively).

After sedating animal for transport to the cobalt facility, the animal is checked for appropriate sedation by signs of prostration, limb flaccidity, and lack of palpebral reflex. Once these signs are achieved, the animal is removed from the cage and placed in the restraint box and subsequently the HEPA transport cart. The transport cart has a Plexiglas window to observe the NHP during transport and staging prior irradiation. Signs of increasing alertness such as yawning, extremity movement, and blinking will necessitate an anesthetic "bump" which may be administered within the box. This dose will be subjectively determined by technician or veterinarian based on extent of alertness and will be equivalent to approximately ¼ dose to the full dose originally

given to the NHP. Once transported from cobalt back to the home cage, NHPs are placed in their cages and monitored visually until fully awake, alert, and ambulatory.

Analgesics and non-steroidal anti-inflammatory drugs: Buprenorphine SR (0.12 – 0.02 mg/kg, sc, 22 - 25 G needle length of ¾-1", lasting for 3 days) will be administered when there is signs of pain. Rimadyl (Carprofen 15 mg/tab, BID, po or injectable 50 mg/ml; 1 – 5 mg/kg/dose, BID, sc, 22 - 25 G needle length of ¾-1") can be administered as an alternative to buprenorphine when evidence of pain is still present.

V.4.1.2.2. Pre- and Post-procedural Provisions

Animals will be observed for signs of pain and distress by either the veterinary or research staff at least twice daily. After specialized procedures, NHPs will be monitored at least twice daily for signs of complications. Study staff will adhere to IACUC Policy #10, Guidelines for Establishment of Early Endpoints in Experiments with Expected Mortality during the critical period and with additional checks (maximum 10 h between two checks).

V.4.1.2.3. Paralytics No paralytic agent will be used in this study.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched

IVIS: International Veterinary Information Service
PubMed: United States National Library of Medicine
WOS: Web of Science

V.4.1.3.2. Date of Search 10/22/2015 - 10/30/2015

V.4.1.3.3. Period of Search

IVIS: 1998 - 2015
PubMed: 1965 - 2015
Web of Science: 1970 - 2015

V.4.1.3.4. Key Words of Search: See table below

V.4.1.3.5. Results of Search

Keywords	IVIS 1998 - 2015	PubMed 1965 - 2015	Web Of Science 1970 - 2015
	Results of Search		
Pain and radiation	341	11618	5967
Ionizing radiation and pain	22	539	268
Ionizing radiation and alternatives	2	175	939
Stem cells and pain	164	911	1097
Stem cells and side effects	115	1250	3440
Stem cells and pain and radiation	45	46	100
Pain and primate and radiation	7	6	4

The literature search did not indicate specific alternatives to pain, other than, what is described below.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: N/A

Irradiated animals experience symptoms of Acute Radiation Syndrome (ARS) due to a compromised immune response and potential microbial infections. In the event that there is pain and distress to animals, we will administer therapies as described previously to provide symptomatic supportive care to relieve pain and discomfort to the animals. Since we are not sure about the extent of pain by irradiation and the potential for pain or distress to persist despite intervention, we decided to put all irradiated animals under category E.

V.4.2. Prolonged Restraint

Animals to be irradiated will be placed in restraint devices for irradiation in the cobalt facility. Animals will be sedated for this purpose as outlined in section V.4.1.2.1. Anesthesia/Analgesia/Tranquilization.

For blood collection, NHPs will be placed in a primate restraint chair. NHPs will not be left in the restraint chair for more than 1 hour.

Animals are acclimated to restraint chair before any procedure. This training starts with vendor before NHPs are sent to our quarantine facility at Poolesville and continued during the acclimatization period at AFRRI. NHPs do not need training for the irradiation procedure's radiation box, as they are sedated throughout the procedure to minimize movement and stress of the animal.

Simple blood draw takes significantly less time than an hour, however, while restrained we take vital sign measurements, do a thorough evaluation of the animals overall health, clean any wounds and provide any other treatment the NHPs may need. Most of the time, it will be less than one hour. Only when a veterinarian examination is requested, NHPs may be in the chair for a maximum of an hour.

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure(s): N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: N/A

V.4.3.6.2 Scientific Justification: N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections

Injections will be performed by appropriately trained staff listed in this protocol under section VI, Study Personnel Qualifications and Training. Injections will be performed by appropriately trained staff listed in this protocol under section VI. Injections for ketamine, Telazol, Buprenorphine, and Rimadyl will be administered as

outlined in section V.4.1.2.1 Anesthesia/Analgesia/Tranquilization. Antibiotics will be administered iv, im or sc as outlined in section V.4.4.7 Other Procedures using a 22 - 25 gauge needle length of ¾-1". The injection volume for this will be 0.1 - 1.0 ml. Lactated Ringers Solution and Anti-emetics will be administered as outlined in section V.4.4.7 Other Procedures using a 22 - 25 gauge needle length of ¾-1". Sodium pentobarbital sodium will be used as outlined in section V.4.6 Euthanasia.

V.4.4.1.1. Pharmaceutical Grade Drugs

Pharmaceutical grade drugs (antibiotics, analgesics, anesthetics, and fluids) will be procured from the USUHS pharmacy or VSD pharmacy at the discretion of the veterinary staff.

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

V.4.4.2. Biosamples

Blood withdrawals will be carried out according to the schedules presented above and on necropsy. Blood will be collected from a peripheral vessel (saphenous or cephalic veins) rather than a femoral vein with a 21 - 25 gauge sterile needle length of ¾-1"attached to a 2 - 3 ml syringe, to reduce the chances of bleeding in irradiated animals. The femoral vein will be used if necessary for blood collection prior to euthanasia.

For aseptic collection for bacterial samples, the area surrounding the saphenous vein (dorsal region of the lower limb) will be swabbed three times with Providone-iodine. After each application of the Providone-iodine, the area will be wiped off with alcohol. Samples collected for other tests will only utilize alcohol swabs. The blood sample will be drawn as described above. While withdrawing the needle, pressure will be applied at the same time with a sterile bandage to reduce bleeding from the venipuncture site. While making regular routine observation of animals, the venipuncture site will be checked for bleeding. In the event the site becomes infected, topical triple antibiotics (bacitracin, neomycin, and polymyxin) or similar agent will be applied as needed.

Partial necropsies will be performed on all animals following euthanasia by pathology necropsy technicians and/or trained non-pathology personnel. The following tissues will be collected; brain, heart, lung, kidney, spleen, duodenum, jejunum, ileum, large intestine, bladder, and sternum will be collected. An outside laboratory (Histoserv) will process the tissue and mount on slides for the VSD pathologist to read. The VSD pathologist will review the gross necropsy observations.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAb) Production: N/A

V.4.4.5. Animal Identification

Animals arrive tattooed and such tattoos will be used for identification. Additionally, cage tags or ID's written on cages with permanent markers are used for identification.

V.4.4.6. Behavioral Studies: No behavioral studies will be performed in this protocol.

V.4.4.7. Other Procedures

As mentioned under section 'V.4.1.2.1. Anesthesia/Analgesia/Tranquilization', animals will be sedated with ketamine or Telazol to place in restraint box for transporting to cobalt facility.

Transport plan: For whole body irradiation, the sedated animals will be placed in positioning aide devices and will be transported inside a cart to the High Level Gamma Radiation (HLGR) facility. HEPA filtered transport carts will be used for transportation of animals through common hallways/areas. At no time will the animals be removed from the restraint boxes while outside of the VSD vivarium. At least two personnel will accompany the animals. Additional ketamine may be administered as needed for chemical restraint (animals are not awake during irradiation), and to provide animal handling support. After irradiation, the NHPs in their restraint boxes will be removed from the irradiation platform, returned to the transport cart and returned to the vivarium.

Microchips: These are inserted under ketamine anesthesia after animals clear the quarantine period. The chips are used to monitor body temperature and are pre-loaded in a specially designed needle/syringe device that is inserted subcutaneously between the shoulder blades. Once the chip is placed, the needle is withdrawn and the skin is pinched between 2 fingers for about 10 - 20 seconds to ensure that the chip itself (about the size of a grain of rice) does not dislodge out through the punctured site. If a chip fails, a second chip will be inserted. If necessary, a drop of surgical glue will be placed at the punctured site to ensure that the chip stays in place.

Supportive Care: Antibiotics will be initiated if the absolute neutrophil count (ANC) is< 500 cells/µl and will continue until the NHP ANC reaches >500 cells/µl. The primary antibiotic will be enrofloxacin (Baytril® Bayer HealthCare LLC, Shawnee Mission, KS). The dose will be 5 +/- 0.25 mg/kg administered intramuscularly (im) or subcutaneously (sc) twice a day (BID); or 10 +/- 0.5 mg/kg administered im or iv once daily (QD). If the body temperature is > 103 ºF, ceftiofur (5 mg/kg, sc - lasts 2 days or 20 mg/kg – lasts 7 days) or gentamicin sulfate (GentaMax®, Phoenix Scientific, Inc.) (5 mg/kg, im or iv, QD) will be administered in combination with Baytril, and this treatment will continue for 24 h. If high fever persists, ceftriaxone (Rocephin®, Roche Laboratories Inc., Nutley, NJ) (50 mg/kg, im, every 24 h) will be administered after gentamicin sulfate is discontinued or if microbial resistance is demonstrated to enrofloxacin or gentamicin. If microbial resistance is demonstrated to enrofloxacin, gentamicin and ceftriaxone, an alternative antibiotic to be used under such situation will be clindamycin, 12.5 mg/kg, administered im every 8 h. For this purpose, a bacterial sensitivity culture may be arranged by Veterinary Science Department.

Additional supportive care details: Additional supportive care therapies may be given upon the descretion of an AFRRI veterinarian.

Table. Summary of plan for medical management and medication use

Drug class	Allowed medication or supportive care agents	Indication and/or criterion for administration

Parenteral fluids	Equal volume of Lactated Ringer's Solution (LRS) and LRS with 5% Dextrose; each at 5 ± 2.5 mL/kg body weight via slow IV push; twice daily depending on extent of dehydration; or LRS at 40 ± 5 mL/kg body weight by IV drip over 2 hrs. Fluid administration rates and amounts may be changed at the discretion of AFRRI veterinarian. Pedialyte or Gastrolyte orally 15 ± 5 mL/kg of body weight Bottles containing diluted fruit juice or oral rehydration solutions (Prang™, Bio-Serv)	**Dehydration:** • Mild to moderate dehydration: Ringer's lactate and Ringer's lactate with 5% dextrose via slow IV push. • Severe dehydration: Ringer's lactate by IV drip. • Pedialyte® or Gastrolyte® or similar commercial solutions containing sodium, potassium, and glucose may also be used if IV access is difficult or precluded. **Fever (temperature ≥103 °F)**
Anti-diarrheal	Loperamide hydrochloride (Imodium), Initial dose 0.04 mg/kg QD Diphenoxylate HCl/atropine sulfate (Lomotil), 0.1 mg/kg, (2.5 mg/tablet) will be administered BID, PO. The 2.5 mg tablet first will be dissolved in 5 mL of tap water. Then, 1 mL of the Lomotil solution (0.5 mg/mL) will be added to 5–10 mL tap water, with OralLRS solution or liquid nutrition for 3 days.	**Diarrhea:** • At the first sign of soft to loose stools: Imodium will be administered up to twice daily and should continue for 3 days. It may be stopped earlier than 3 days if the diarrhea resolves. • Severe (diarrhea or if Imodium has been administered for 3 days): Lomotil, up to BID, PO for 3 days. It may be stopped earlier than 3 days if the diarrhea resolves. If the diarrhea does not resolve, the Imodium treatment will be repeated.
Anti-ulcer	Sucralfate (Carafate) 1 g/day, (0.5 g BID) daily from days 5 – 30, PO	For treatment of possible ulcers of the stomach or proximal small intestine
Anti-emetics	Ondansetron (1-2 mg/kg), IM, IV or PO 25-90 minutes prior to irradiation and 30-45 minutes following irradiation to suppress emesis or Granisetron (0.25 mg/kg), PO or IV	• Administered pre-and post-radiation to suppress emesis

Blood products	Whole blood or packed red blood cells will be obtained from a commercial source or obtained from donor animals and blood product irradiated @ 2500 cGy with a source in (b)(4) prior to transfusion Whole blood at 7 – 14 mL/kg or Packed red blood cells (RBC). Note: Repeat blood collection from donor animals will be defined on the total volume collected each time, recovery period and other criteria as described by Diehl et al 2001[5]. A maximum of 12.5% of blood will be collected once every 30 days if needed. Donor animals will get a CBC done prior to donation to confirm they do not have anemia – per consultation with veterinarian.	Whole blood will be transfused if: • PLT counts are <20,000/µL and hemoglobin (Hgb) <7 g/dL or hematocrit (Hct) <20%; • An animal experiences a 5-unit decrease in Hct in a 24-hour period that results in an Hct of ≤ 25%; • An animal that has previously received a transfusion demonstrates continued evidence of bleeding, such as bloody stools and/or any decrease in Hct relative to the most recent result obtained; or • If there is obvious evidence of uncontrolled hemorrhage. • An animal experiences a 7-10 unit decrease in Hct in a 24-hour period; or • An animal's Hct is ≤ 25% for 2 consecutive days and PLT counts are <3,000/µL. Packed RBCs will be administered in preference to whole blood when PLT counts are satisfactory (≥100,000/µL with no clinical bleeding) but the Hgb or Hct remain low (Hgb <7 g/dL or Hct <20 g/dL). • PLT counts are satisfactory (≥100,000/µL with no clinical bleeding) but the Hgb or Hct remain low (Hgb <7 g/dL or Hc <20 g/dL).
Nutritional Support	BIO-SERV Rhesus Liquidiets To facilitate the consumption of food post-irradiation, the primates may be provided with an array of drinking and food products that will heighten their interest. Such items will include kool-aid type drinks, jello and yogurt (as examples of soft type foods), and bananas, sweet potatoes, and celery with peanut butter (as examples of harder type foods). This brief listing is not meant to exclude any drink or food product that will aid in the consumption of diet designated for the animals.	**Weight Loss (Anorexia):** Nutritional support may be administered via oral gastric tube when body weight is <85% of baseline and will be continued as long as the body weigh remains <85% of baseline, and the animal is not eating. In the case where an animal loses its ability to regain weight following irradiation despite actively eating and drinking, nutritional supplementation via oral gastric tube may be foregone in order to avoid daily anesthesia.

V.4.4.8. Tissue Sharing: Brain tissue will be shared with (b)(6) (b)(6) at part of his genome project.

V.4.4.9. Animal By-Products:

Whole blood anti-coagulated with 10% citrate, dextrose phosphate with adenine obtained from healthy animals will be used. The blood will be irradiated prior to use.

V.4.5. Study Endpoint

The primary endpoint used in this study will be mortality. This study is to determine the lethality of ^{60}Co-gamma-radiation using probit curve with various doses of radiation. Such lethality curve is required to know the exact dose for radiation for various levels of lethality (e.g. LD_{30}, LD_{50}, LD_{70}, LD_{90} etc.). Based on lethality estimation in this study, different doses of radiation will be used for evaluating efficacy of radiation countermeasures in future studies. Such lethality information is required for evaluating efficacy against hematopoietic syndrome (LD_{30} or LD_{50}) or gastrointestinal syndrome (LD_{70} or LD_{90}). To develop radiation countermeasures for US Food and Drug Administration approval, one needs to demonstrate that drug significantly increases survival in animals compared to placebo. To demonstrate increase in survivors, end point needs to be mortality. In this study, we are establishing animal model with full supportive care for a future radiation countermeasure study.

Two previous publications have been provided below which have used mortality as an endpoint for studying efficacy of two radiation countermeasures for US Food and Drug Administration approval. Neupogen [9] and Neulasta [13] received US Food and Drug Administration approval based on studies using mortality as an endpoint.

Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress. Necropsy and histopathological analysis will be performed on all euthanized animals. The following parameters will provide the guidelines for moribundity.

1. Weight loss: Significant weight loss (10%) from the baseline (NHPs will be weighed whenever the animal is placed on the restraint chair attached to the scale for blood collection),

2. Inappetance: Complete anorexia for 2 days with deteriorating conditions based on clinical examination,

3. Weakness/inability to obtain feed or water: Inability or extreme reluctance to stand which persists for 1 h,

4. Minimal or absence of response to stimuli (animal does not move when prodded),

5. Core body temperature: Below 96.6º F following a period of febrile neutropenia (such as >103º F and <500 neutrophils/ml),

6. Severe anemia: <13% hematocrit due to acute blood loss or <40 g/L hemoglobin (decision in consultation with AFRRI veterinarians),

7. Severe thrombocytopenia (<10,000 platelets/μl)

8. Other signs of severe organ system dysfunction with a poor prognosis as determined by a veterinarian:

8.1 Respiratory: any dyspnea or severe cyanosis.

8.2 Gastrointestinal: sustained vomiting or diarrhea, obstruction, intussusceptions; peritonitis (transient vomiting and diarrhea are expected results of whole-body gamma irradiation, therefore, as criteria for euthanasia, these symptoms should

be severe and life-threatening per se, i.e., hemorrhagic diarrhea to the point of causing anemia as listed above or severe uncompensated dehydration).

8.3 Urogenital: renal failure based on clinical chemistry parameters (BUN, creatinine) and urinalysis (urine specific gravity).

8.4 Nervous: sustained CNS depression, seizures, paralysis of one or more extremities.

8.5 Integumentary: non-healing wounds, repeated self-trauma, severe skin infections, indicating severe organ system dysfunction with a poor prognosis.

When an animal reaches a state of moribundity (i.e. point of no return) the animal will be euthanized. Any single parameter listed under this section will not lead to euthanasia of the animal, unless in conjunction with the recommendation to euthanize by an AFRRI veterinarian. Moribundity status of the animal will be determined by a team effort between institutional veterinarian, principal investigator, research staff, veterinary technicians and husbandry staff based on the combination of criterions described above.

This full supportive care study includes the administration of anesthetics and analgesics. If the animal reaches a point where it is appears to be in excessive pain, despite the administration of analgesics and other therapeutics, a decision to euthanize will be made in consultation with institute veterinarian.

Surviving animals at the end of the study (60 d post-irradiation) will be euthanized for necropsy and tissue collection as described under section V.4.4.2 Biosamples.

V.4.6. Euthanasia

Animals will be humanely euthanized using the most current American Veterinary Medical Association (2013 AVMA) guidelines. These animals will be given pentobarbital sodium iv (saphenous or cephalic), needle size 20-25 gauge ¾-1" length, (100 mg/kg, 1 - 5 ml). Prior to pentobarbital sodium administration, animal will be sedated using ketamine (5 - 15 mg/kg, im). Intra-cardiac administration will be performed if unable to administer pentobarbital sodium through peripheral veins. The animal will be deeply anesthetized by Isoflurane (1 - 5%) with O_2 at 1 - 4 liters per minute via mask before giving intra-cardiac injection. The animals will be euthanized only under the guidance of a staff veterinarian or a trained technician in consultation with the veterinarian. After pentobarbital sodium administration, the animals will be examined (heart auscultation and pulse) to confirm death.

V.5. Veterinary Care

Animal procedures performed at AFRRI will be in compliance with the *Animal Welfare Act* and the *Guide for the Care and Use of Laboratory Animals.* The AFRRI facility is accredited by Association for Assessment and Accreditation of Laboratory of Animal Care (AAALAC)-International.

V.5.1. Husbandry Considerations

All non-human primates will be housed individually in cages with at least 4.3 square feet of floor space per NHP in accordance with The *Guide for the Care and Use of Laboratory Animals*. In addition, every cage will be equipped with built in perches. Excreta and food waste will be removed from inside each indoor primary enclosure,

once per day, to prevent the primates from becoming soiled, and to reduce disease hazards, insects, pests, and odors. Care will be exercised when washing the primary enclosure so that the animals remain dry at all times. Primary enclosures will be sanitized every two weeks, or longer if it is necessary to decrease stress to the animals during the critical period (displaying clinical signs and symptoms related to acute radiation syndrome). The secondary enclosure (room) will be sanitized once per month. Foraging boards as well as other enrichment devices will follow the same schedule as the primary enclosures of the primate.

Special care will be given to animals that have been irradiated as their natural immunity will be suppressed (more prone to infections). Irradiation boxes will be sanitized after every use or more often as needed.

V.5.1.1. Study Room: Study rooms will be assigned by VSD.

V.5.1.2. Special Husbandry Provisions: None

V.5.1.3. Exceptions: None

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care

All animals will be quarantined for 45 days prior to any experiment. For this study, NHPs will arrive at AFRRI after completing quarantine at supplier's facility. As per Standard Operating Procedures, investigator staff will monitor all animals at least twice daily during morning and afternoon rounds. Any abnormalities will be reported immediately to the on-call veterinarian and PI or PI's staff. In addition, animals will be observed daily by this protocol's research staff. Any sick animal will be observed at least twice a day (early morning and late afternoon/night) by the research staff. When animals become ill or debilitated, a veterinarian will be contacted immediately to assess the animal and provide adequate/emergency care. If the animal becomes moribund, it will be euthanized as outlined in section V.4.6 Euthanasia.

V.5.2.2. Emergency Veterinary Medical Care

The on-call veterinary officer will be available via telephone 24 hours a day. The on-call roster is located outside the VSD conference room (Room (b)(6) on the bulletin board and at the security watch desk. These procedures are in accordance with VSD SOPs.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

In this study, all primates will receive regular enrichment IAW VSD SOP VS0226, Nonhuman Primate Environmental Enrichment. Enrichment provided will include sanitizable toys, contact with husbandry and research staff, and visualization of conspecifics. To facilitate the consumption of food post-irradiation, the primates may be provided with an array of drinking and food products that will heighten their interest. Such items will include kool-aid type drinks, jello and yogurt (as examples of soft type foods), and bananas, sweet potatoes, and celery with peanut butter (as examples of harder type foods). This brief listing is not meant to exclude any drink or food product that will aid in the consumption of diet designated for the animals depending on clinical observations; monthly enrichment can be modified per the veterinarian.

V.5.3.2. Enrichment Restrictions

None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				7 (off-hour observation)	(b)(6)
				1,3a,3b,3d,4,5,7 (off-hour observation)	
				1,3a,3b,3d,4,5,7 (off-hour observation)	
				1,7 (off-hour observations)	
				1, 3b, 5	
				7 (off-hour observations)	
				7 (off-hour observations)	
				7 (off-hour observations)	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-inferior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

Hands-on training for additional personnel will be provided by study staff designated as trainers by VSD or by VSD technicians using the animals and procedures described in the approved protocol. This will include pole and collar handling of NHPs as well as blood collection and donation, gavage, and injections. The IACUC will be provided with updated records as training is accomplished.

VII. BIOHAZARDS/SAFETY

There is no plan to use any potential biohazard. Standard laboratory safety precautions will be observed throughout this study.

VIII. ENCLOSURES

Form 310
List of references

X. ASSURANCES

Protocol Title: Development of a lethality curve with supportive care in the Rhesus macaque (*Macaca mulatta)*

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): (**I AM**) **AM NOT** (*circle one*) conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress (**WILL**) **WILL NOT** (*circle one*) be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

References

[4] Farese AM, Cohen MV, Katz BP, Smith CP, Jackson W, 3rd, Cohen DM, et al. A nonhuman primate model of the hematopoietic acute radiation syndrome plus medical management. Health Phys. 2012;103:367-82.

[5] Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, et al. A good practice guide to the administration of substances and removal of blood, including routes and volumes. J Appl Toxicol. 2001;21:15-23.

[9] Farese AM, Cohen MV, Katz BP, Smith CP, Gibbs A, Cohen DM, et al. Filgrastim improves survival in lethally irradiated nonhuman primates. Radiat Res. 2013;179:89-100.

[11] Farese AM, Cohen MV, Stead RB, Jackson W, 3rd, Macvittie TJ. Pegfilgrastim administered in an abbreviated schedule, significantly improved neutrophil recovery

after high-dose radiation-induced myelosuppression in rhesus macaques. Radiat Res. 2012;178:403-13.

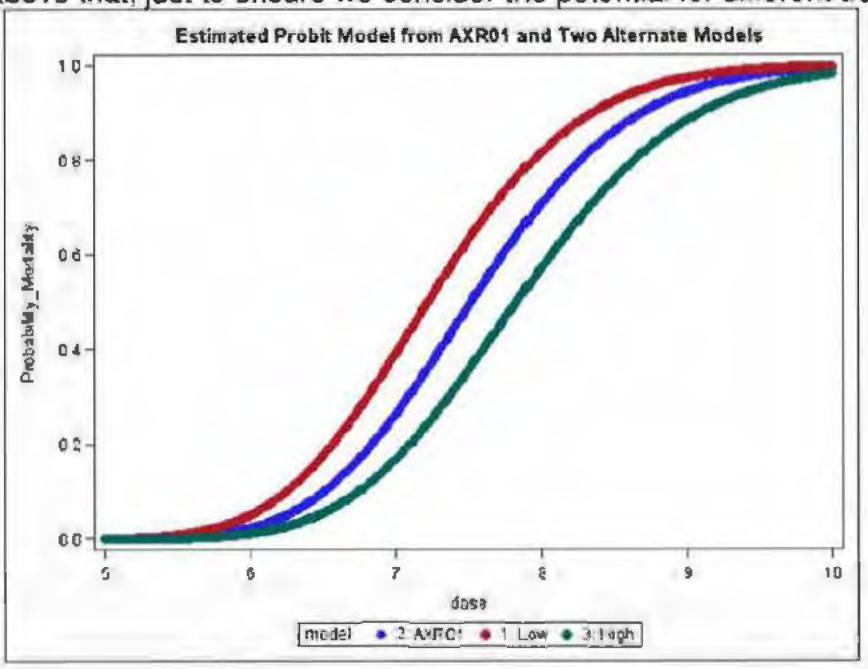
[12] Farese AM, MacVittie TJ, Roskos L, Stead RB. Hematopoietic recovery following autologous bone marrow transplantation in a nonhuman primate: effect of variation in treatment schedule with PEG-rHuMGDF. Stem Cells. 2003;21:79-89.

[13] Hankey KG, Farese AM, Blaauw EC, Gibbs AM, Smith CP, Katz BP, et al. Pegfilgrastim Improves Survival of Lethally Irradiated Nonhuman Primates. Radiat Res. 2015;183:643-55.

Dear Team,
After our meeting on 10/2/2015, we considered some refinements to the simulations to evaluate the impact of sample size and dosing schedule on the estimation of LD30, LD50, and LD70. Based on the feedback, I estimated the middle 90% of estimates for LD30, LD50, and LD70 in each scenario. Designs with narrower ranges of estimates in my simulations will tend to provide more precise estimates.
I evaluated 4 dosing regimens and 2 total sample sizes (for a total of 8 designs). This evaluation was done for each of three models: a model consistent with AXR01 data, a model with lower lethality curve, and one with higher, to ensure that the results will be robust across a range of possible true curves:

- 4 dosing regimens
  - 8 doses spread uniformly from 6 to 9.5 (6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5)
  - 8 doses spread uniformly from 6.5 to 9 (6.5, 6.86, 7.21,7.57, 7.93, 8.29, 8.64, 9)
  - 6 doses spread uniformly from 6 to 9.5 (6, 6.7, 7.4, 8.1, 8.8, 9.5)
  - 6 doses spread uniformly from 6.5 to 9 (6.5, 7, 7.5, 8, 8.5, 9)
- 36 total animals versus 48 total

Bottom Line: The dosing regimen highlighted above provided the most precise estimates of LD30, LD50, and LD70, regardless of which the three models was true. The advantage of this dosing regimen was relatively modest, but it is the clear winner (because there is no trade-off that I know of). The advantage of using 48 total animals versus 36 was also relatively modest; the sample size decision requires a judgement as it is a trade-off of increased efficiency versus the total cost.
I considered three possible true mortality curves (as shown below) – where the blue curve represents the model from the AXR01 data, and the other curves represent shifts below and above that, just to ensure we consider the potential for different true models



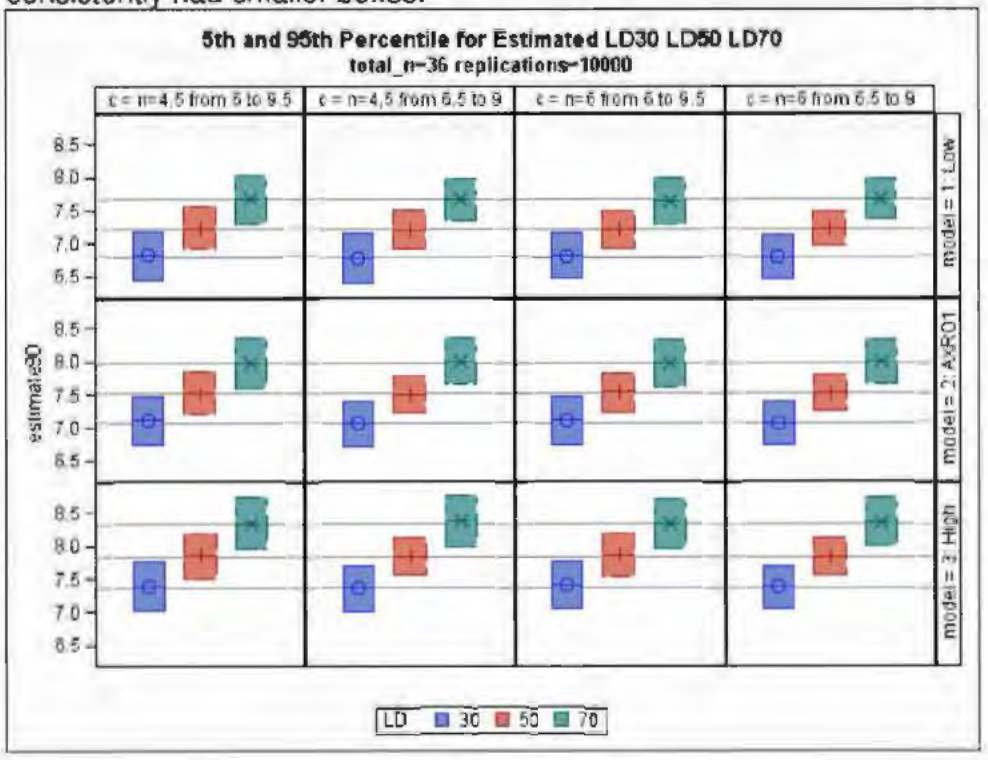
For each of these models, I evaluated 8 different designs. I varied doses (6 or 8), the range of the doses (6 to 9.5 or 6.5 to 9), and the total number of animals studied (36 or 48).

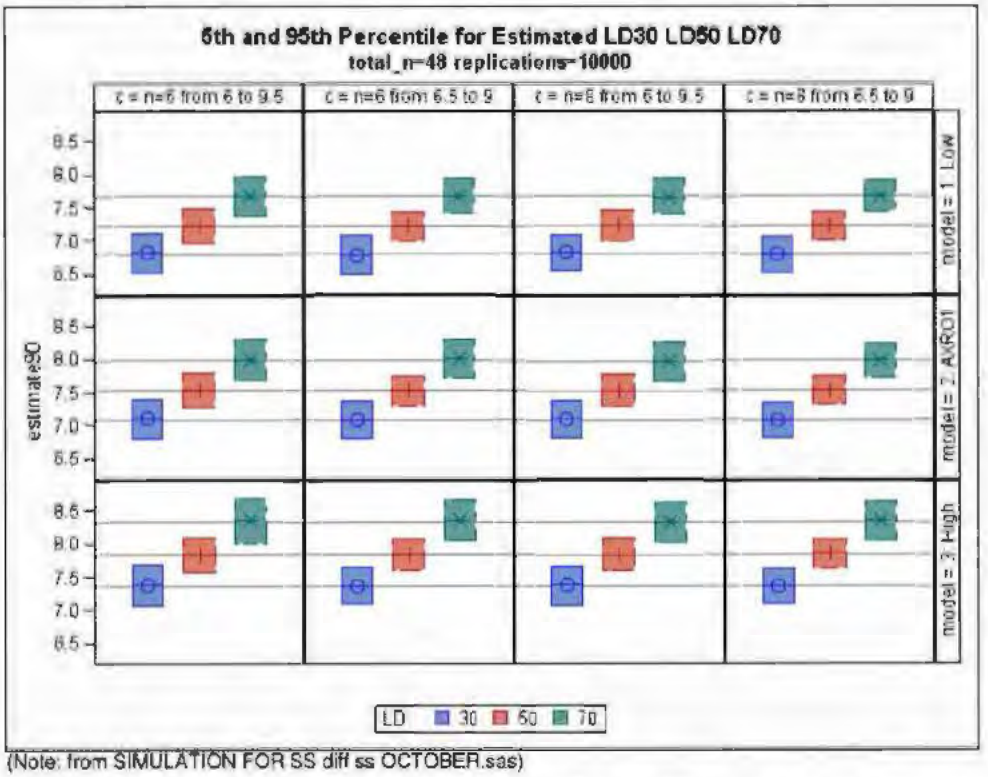
Dosing Regimen		Total N=36	Total N=48
# Doses	Doses Spread Uniformly in this Interval	Animals Per Dose	Animals Per Dose
8	6 to 9.5	Alternate Between 4 & 5*	6
8	6.5 to 9	Alternate Between 4 & 5	6
6	6 to 9.5	6	8
6	6.5 to 9	6	8

* Thus, for example, the doses were 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5; At Dose=6, there were 4 animals, and at Dose=6.5, there were 5, and Dose=7, there were 4 animals, and so on. This design had 36 animals.

To determine the most efficient dosing regimen I looked across the four columns for each of the 6 rows in the plots below (i.e., 3 rows per total sample size). Each row has the same total sample size and same model, so if any dosing regimen consistently provides increased precision, then it would be the best choice. In every comparison the boxes in the right most row which corresponds to 6 doses from 6.5 to 9 were narrower than the boxes in the other three columns. The differences were modest, but consistent. This dosing regimen corresponds to the highlighted one above. One caveat is that differences in precision between the dosing regimens is not dramatic, so that if there is some other important consideration that favors a different dosing regimen, then it should be considered. Discussion about the total sample size choice follows after the plots.

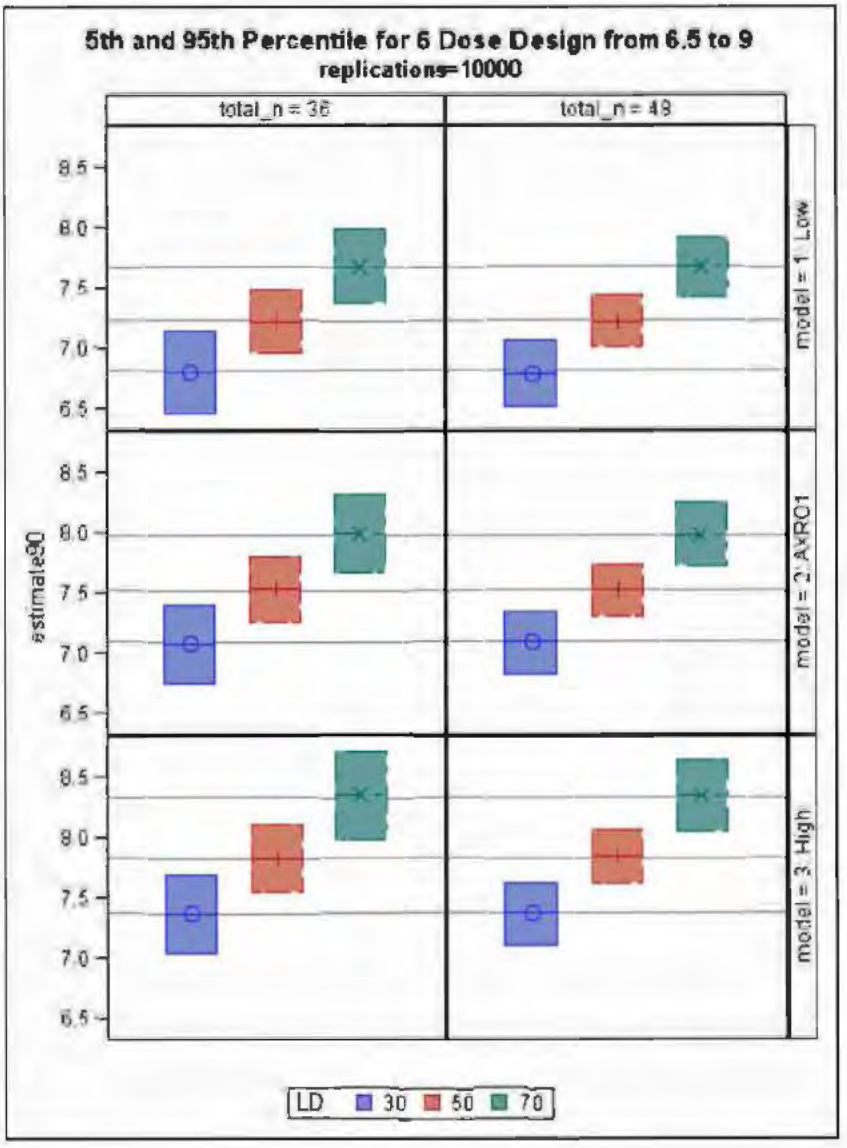
Results comparing 8 designs under 3 different true mortality models. The gray lines represent the true LD30, LD50, and LD70 under each true model. The fourth column consistently had smaller boxes.





(Note: from SIMULATION FOR SS diff ss OCTOBER.sas)

The choice between total N of 36 versus 48 is a different sort of comparison. By definition, the 48 will give more precise estimates, so it's a trade-off between the increased efficiency versus the additional cost associated with studying more animals. I assume that the above selected dosing regimen with n=36 versus n=48 (i.e., 6 doses between 6.5 and 9 each with 6 animals, versus the same doses with 8 animals) would be used. We look at the same box plots as on the previous page, but narrowing in on the relevant panels. Each row illustrates the results under each of the three models. We see the boxes associated with the larger sample size are clearly smaller, although differences are somewhat modest. Nonetheless, given that the usefulness of future designs depend on the curve being estimated reasonably well, this gain in efficiency may be important.


5th and 95th Percentile for 6 Dose Design from 6.5 to 9
replications=10000
total_n = 36
total_n = 48
model = 1: Low
model = 2: AxRO1
model = 3: High
estimate90
8.5
8.0
7.5
7.0
6.5
LD
30
50
70

May 20, 2016

To: IACUC Chair

Subject: This memorandum is for informational purpose for protocol # (b)(6)

We recently conducted a full supportive care (blood transfusions and antibiotic treatments as necessary) NHP experiment in two batches with 6 NHPs each; each NHP received a different dose of radiation (6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 Gy). In the first batch, all study animals received a combination of enrofloxacin and ceftiofur as antibiotic treatments as directed by veterinarian and died by SD 22. In the second batch, enrofloxacin was used as the preferred antibiotic and ceftiofurwas used only as directed by the veterinarian; all but one of our study animals died by SD 15.

Batch 1			Batch 2		
Rad dose (Gy)	Number of transfusions	Euthanasia (SD)	Rad dose (Gy)	Number of transfusions	Euthanasia (SD)
6.5	2	22	6.5	2	12
7.0	2	22	7.0	3	Still alive
7.5	1	15	7.5	0	15
8.0	1	14	8.0	2	14
8.5	3	15	8.5	3	14
9.0	0	10	9.0	2	15

Histopathology results of tissue indicate a large number of cocci bacteria present in the samples from batch 1 (most likely *Streptococcus sp.; Staphylococcus sp.*). Similarly, there were bacterial cocci colonies noted in 4 of the 5 euthanized animals from batch 2. In none of our past studies has such prominent bacterial presence in histopathological findings been noted. Furthermore, batch two animals presented spikes in neutrophil counts at SD-1, perhaps in response to infection.

These results have led us to believe that the increased mortality in these two batches may be due in part to "dormant" antibiotic resistant bacteria within the first batch, which then "bloomed" as a result of radiation-induced immunosuppression; the infection then spread to batch 2, prior to irradiation further compromising their overall health, leading to rapid deterioration of all animals within a short time frame (3 days). Bacterial cultures from the lone survivor of the second batch (b)(4) are under investigation for remaining bacterial presence, if any, and sensitivity. Additionally, a direct correlation could not be demonstrated between mortality and vendor/breeder of multiple studies (4+) done over the course of several years.

A study of this nature is complex with a potential for multiple contributing factors to mortality. Several strategies have been adopted to ensure data integrity of future batches; we will employ more vigorous culture and sensitivity methods to ensure judicious use of antibiotics and take any additional precautions or recommendations by AFRRI veterinarians.

Sincerely,

(b)(6)

Principal Investigator

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	1/20/2016	2nd Resubmission Date:	
Approved/**Returned for Revision**:	2/8/2016	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	2/17/2016	**EXPIRATION DATE:**	2/25/2019
Approved/Returned for Revision:	2/26/2016	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and *subsection*, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Screening potential drugs for radioprotection after total body irradiation using laboratory mouse (*Mus musculus*)

IV. PRINCIPAL INVESTIGATOR:

(b)(6) **Ph. D** Date

Research Biologist, AFRRI-SRD
Office: (b)(6) Fax (b)(6)
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD** Date

Head, Scientific Research Department, AFRRI
Telephone: (b)(6)

(b)(6)

- Or –

(b)(6) **PhD, Lt Col, USAF** Date
Deputy Head, Scientific Research Department, AFRRI
Telephone: (b)(6)
(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date
Statistician
Telephone (b)(6) Fax (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, COL, VC, USA** Date
Head, Veterinary Sciences Department, AFRRI
Telephone (b)(6) Fax (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date
Safety and Occupational Health Specialist, USUHS
Telephone: (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **PhD, Lt Col, USAF** Date
(b)(6) Scientific Research Department, AFRRI
Telephone (b)(6)
(b)(6)

PROTOCOL TITLE: Screening potential drugs for radioprotection after total body irradiation using the laboratory mouse (*Mus musculus*)

PRINCIPAL INVESTIGATOR: (b)(6) Ph. D
Research Biologist (AFRRI – SRD)
Office: (b)(6) Fax: (b)(6)
(b)(6)

CO-INVESTIGATOR(S): (b)(6) Ph.D.
Scientist
Office: (b)(6)
(b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER:

I. NON-TECHNICAL SYNOPSIS One of the major missions of Armed Forces Radiobiology Research Institute (AFRRI) is to develop non-toxic, safe, effective and approved radioprotectors for use in victims of an accidental or deliberate radiation exposure. AFRRI is involved in testing various classes of drugs as radiation countermeasure agents under (b)(4) interagency agreement. These drugs can stimulate immunity, remove toxic free radicals and/or activate biological systems to counteract the damage caused by ionizing radiation. In the present protocol, we propose to test new drugs and new formulations for their efficacy in radiation protection under the Intramural Screening Program recently established at AFRRI. In order to establish an optimum dose for radiation countermeasure studies, basic toxicity of various drug formulations will be carried out in mice. The toxicity studies will be followed by basic survival experiments in drug-treated mice using Cobalt-60 gamma radiation at AFRRI. Study endpoint will be thirty days post-irradiation survival. Once a drug shows efficacy either as a prophylactic or therapeutic agent, additional studies which include drug dose optimization, effect of time of drug administration on 30-day survival, and a dose reduction factor to determine the effectiveness of the drugs will be performed.

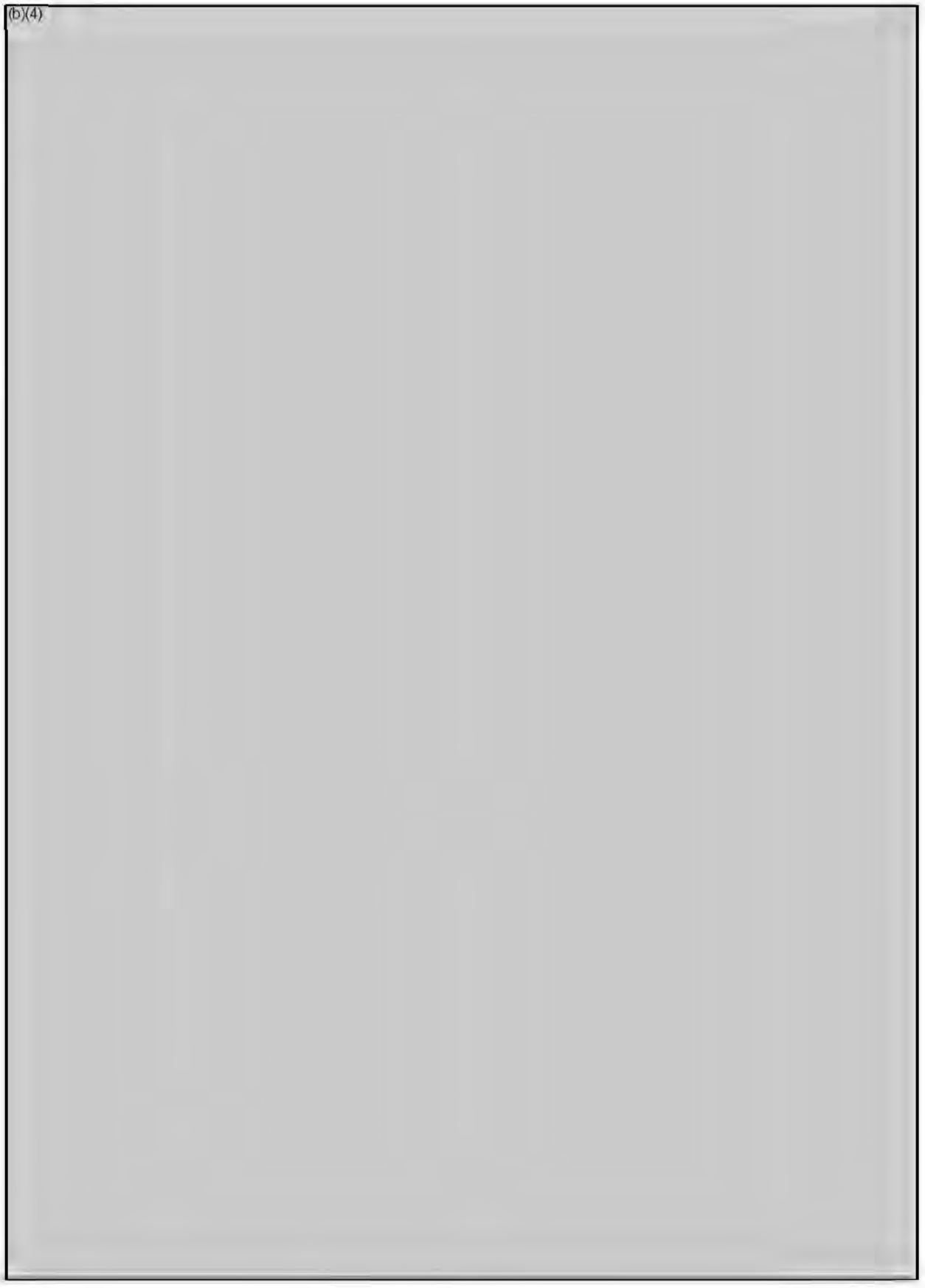
II. BACKGROUND

II.1. Background Radiation exposure can occur in a variety of scenarios [1], including nuclear weapons, accidental exposure in power plants, in nuclear submarines and via terrorist activities using radiological dispersal devices (RDDs) as well as during extended space travel. Acute effects of high-dose radiation include hematopoietic cell loss, immune suppression, mucosal damage (gastrointestinal and oral), and potential injuries to other sites such as lung, kidney and central nervous system (CNS) [2]. As of today, no radiation countermeasure is available for human use except recently approved drugs, Neupogen® and Neulasta®. Since these are of limited use, there is an urgent need to develop safer and more effective radioprotectors for national stockpile to protect emergency first responders and civilians from harmful radiation damage. This is a critical issue for combat commanders in the planning and execution of military operations [2].

Over the years investigators have identified several classes of compounds which act as radioprotectants. These include thiols [3], cytokines [4], steroids (b)(4) prostaglandin analogs, antioxidants (b)(4) and nutraceuticals (b)(4) Early searches for radioprotectors have been dominated by the study of sulphydryl compounds such as amifostine [10]. Despite amifostine being considered a gold standard radioprotector because of its effectiveness in various species in minimizing the deleterious effects of ionizing radiation, it has not been advanced for use during a nuclear event because of its significant performance –degrading characteristics [10,] as well as hypoglycemic (b)(4) response [12]. However, in clinical settings, its cytoprotective characteristics (protection of normal tissue without similar protection of tumor tissue) have been effectively used. It has been approved for clinical use in conjunction with cisplatin and for patients undergoing radiotherapy for head and neck cancer [10]. (b)(4) (b)(4) as a prophylactic radiation countermeasure (b)(4) (b)(4) (b)(4) to evaluate the safety, tolerability and pharmacokinetics of the drug in healthy volunteers (b)(4) (b)(4) (b)(4) Although there are a few compounds in different stages of development, until now only Neupogen® and Neulasta® are approved by FDA for limited use for treating ARS. Therefore, there is an urgent need to develop small molecules/agents/compounds for national stockpile that are less toxic, and efficacious.

AFRRI is involved in testing various classes of drugs as radiation countermeasure agents under (b)(4) interagency agreement. These chemicals/drugs can stimulate immunity, remove toxic free radicals and/or activate biological systems to counteract the damage caused by ionizing radiation. One of AFRRI's missions is to develop non-toxic radioprotectors and therapeutic agents which can be available to our emergency responders and uniformed personnel before deployment to a radiation exposed field. In order to fulfill AFRRI's mission, an Intramural Screening Program (ISP) has recently been established at AFRRI as a core program. Drugs/compounds are brought to the Intramural Screening Program committee (ISPC) by (b)(4) (b)(4) Drugs are reviewed by the ISPC and get approved or disapproved for screening based on available literature/data. We will follow AFRRI's standard protocols used for screening radiation countermeasure drugs under (b)(4) interagency agreement. In order to establish an optimum dose for radiation countermeasure studies, the basic toxicity of various drug formulations will be carried out in mice. Drugs will be tested for radioprotective efficacy (prophylactic and therapeutic) after total body irradiation (Cobalt-60). Mice will be monitored for 30-days as study end point. Further studies (drug dose optimization, time optimization and dose reduction factor determination) will be performed with potential drugs which pass initial efficacy study.

Classification of drugs (for the study):
The new drugs included in the current protocol are subject to legal requirements of confidentiality and material transfer agreement (moderated by the (b)(4) (b)(4)


(b)(4)

(b)(4)

(b)(4)

Positive controls used in the survival study:

1.Gamma-tocotrienol (GT3): Gamma-tocotrienol (GT3), Delta-tocotrienol and (DT3), and tocopherol succinate (TS) are tocols demonstrating radioprotective efficacy against high doses of radiation. These tocols are optimally effective when administered 24 h before irradiation as their radioprotective efficacy can be negated through the administration of antibodies to G-CSF, an essential cytokine playing an important role in protection from ARS. AFRRI researchers have conducted multiple murine studies with CD2F1 mice (male) with several isomers of vitamin E. One of these isomers GT3, has demonstrated strong radioprotection in mice when administered subcutaneously 24 hours prior to total body irradiation (TBI).
GT3 demonstrates almost 100% radioprotective efficacy in mice against the $LD_{90/30}$ dose of radiation (b)(4) Drug reduction factor (DRF) reported for GT3 (200 mg/kg) was1.29 (b)(4) In several mouse studies done at AFRRI, GT3 has shown consistent results of 100% radioprotection at 200 mg/kg body weight.

2. Androstenediol (5-AED): Androstenediol is a direct metabolite of the most abundant steroid produced by the human adrenal cortex, dehydroepiandrosterone (DHEA). It is less androgenic and has been found to stimulate the immune system. Its potential use as a radiation countermeasure was introduced at AFRRI with possible mechanism mainly due to stimulation of production of white blood cells and platelets (b)(4)
5-AED has been used as a positive control (80 – 100% survival when given 24 h prior to irradiation at 30 mg/ kg subcutaneously) in IACUC protocol P# (b)(6)

(b)(6)

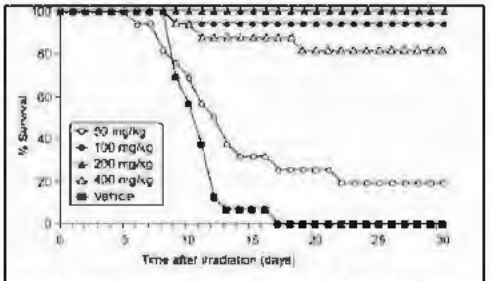


Figure 2. Radioprotection provided by GT3 at 11 Gy. Thirty-day survival of mice (n=16 per group) treated 24 h before receiving 11 Gy of cobalt-60 gamma radiation, with a single SC injection of vehicle (5% Tween 80) or GT3 at doses of 50 to 400 mg/kg body weight. Mice that received a GT3 dose of 100, 200, and 400 mg/kg exhibited a significant increase from vehicle control group ($p<0001$. Chi-square=69.74, df=4).

Representative survival plot has been shown here.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched
NIHRePORTER, PUBMED , Embase
II.2.2. Date of Search 11-22-2015 to 12-12-2015
II.2.3. Period of Search The search period extended from 2009-present for NIHReporter, 1970 to present for PubMed, 2006 to present for Embase.

II.2.4. Key Words of Search Each individual term + (radiation), + (radioprotection), + (radioprotection+mice)

II.2.5. Results of Search Table 1. Literature search resulted the following hits as tabulated below:

KEY WORDS	PUBMED	NIHRePORTER	Embase
(b)(4)	5	1	7
(b)(4) + radiation	5	1	5
(b)(4) + radioprotection	2	1	1
(b)(4) +radioprotection+mice	2	1	1
(b)(4)	1644	36	957
(b)(4) + radiation	46	1	33
(b)(4) + radioprotection	2	0	1
(b)(4) + radioprotection+ mice	1	0	0
(b)(4)	6546	116	8140
(b)(4) + radiation	117	6	120
(b)(4) + radioprotection	8	0	4
(b)(4) + radioprotection+ mice	2	0	0

Summary of the Search: Literature for (b)(4) is mostly from the same research group which is providing AFRRI the drug for testing and is specific to radioprotection. However, the proposed study with (b)(4) is not a duplication since the published work was done in (b)(4) (b)(4) and is used to treat urea cycle disorders. It is also a (b)(4) leading respectively to research into its use as an anti-cancer agent and in protein misfolding diseases such as cystic fibrosis. As a result of multiple applications for this class of compounds many literature articles are found in the searches. This study with (b)(4) is not a duplication as the study in the literature has been done in (b)(4) (b)(4) All references available for (b)(4) are either as a dietary supplement or a biomarker.

III. OBJECTIVE / HYPOTHESIS The overall objective of this proposal is to evaluate the prophylactic and therapeutic efficacy of countermeasure agents (b)(4) (b)(4) in mice using a standardized screening protocol. We hypothesize that by screening these compounds, we will be able to identify a countermeasure which can be taken to the next step of evaluation for further development as a radioprotectant and/ or mitigator. Our goal is to develop a drug which is effective in prolonging survival in mice when given either before or after whole body radiation.

IV. MILITARY RELEVANCE The current lack of availability of radioprotectors and therapeutic agents that are non-toxic, effective for use in victims of an accidental or terrorist nuclear event scenario is a major problem in preparing for such an eventuality. Therefore, there is a desperate need to develop effective countermeasures to the pathological impact of ionizing radiation for use by emergency response personnel, military personnel in harm's way prior to deployment, and the general population at risk.

Exposure to whole body irradiation of humans above a dose of about 1 Gy causes depletion of bone marrow progenitor cells, leading to neutropenia and increased susceptibility to infection. Exposure at doses above 2 Gy causes mortality in humans [2]. Our uniformed emergency personnel could be exposed to these levels of ionizing radiation in scenarios involving nuclear detonations or the use of other radiological weapons by international terrorist. One of AFRRI's missions is to develop non-toxic radioprotectors and therapeutic agents which can be made available to our emergency responders and uniformed personnel before deployment to a radiation exposed field. In order to fulfill AFRRI's mission, an Intramural Screening Program (ISP) has recently been established at AFRRI as one of its core activities. In the proposed study we will screen compounds (b)(4)

V. MATERIALS AND METHODS In this study we will test the efficacy of new drugs using either CD2F1 or C57BL/6 mouse model. Reasons for choosing these strains of mice are (i) at AFRRI CD2F1 mice have been used for screening for countermeasures. (ii) In the literature many researchers use C57BL/6 mice in their studies. (iii) In the case where results of the preliminary study are being validated at AFRRI. (iv) efficacy of drugs on varied radio-sensitive strains [27] can be addressed by testing on CD2F1 (LD70/30 = 9.25 Gy) and C57BL/6 (LD70/30 = 8.2 Gy). Drugs will be administered either subcutaneously or by orogastric gavage (see **Section V.4.4**). In case of drugs by orogastric administration, animals will be divided in two groups, with and without fasting. Animals will be fasted for 5-8 h hours before drug administration. Before conducting the efficacy study, we will study the safety of these drugs by conducting a 14-day acute toxicity study.

V.1. Experimental Design

General experimental design: The experimental design for this protocol is divided in to 5 subsections and each subsection covers a specific area:

Section v.1.1.: Basic acute toxicity
Section v.1.2.: Survival studies to test efficacy as an ARS countermeasure
Section v.1.3.: Drug optimization – survival studies
Section v.1.4.: Dose reduction factor - survival studies
Section v.1.5.: Hematological studies - sublethal dose of radiation

Section V.1.1. Basic Acute Toxicity

We will be using the modified toxicity protocol Optimal (non-toxic) Drug Dose for screening (ODDS) which has been used in our previous protocols (b)(6) (b)(6) at AFRRI to determine maximum tolerated dose (MTD). The purpose of using the ODDS method is that it allows testing in one gender and requires fewer animals when compared to the FDA protocol. If the initial drug dose (based on recommendation from the sponsor or literature) doesn't show any signs of basic toxicity (using the clinical signs discussed in modified Policy#10, Appendix 1), then the drug dose will be escalated 2 – 3 fold. However, if toxicity is observed, then the drug dose will be reduced by 2 – 3 fold or as suggested based on the discussion with the company [28]. For either scenario (escalation or reduction 2 – 3 fold), the IACUC will be notified by memorandum. For survival studies we will use 1/4th of the observed non-toxic dose. A maximum of six animals per drug dose will be used to evaluate toxicity, and will be monitored daily for

acute (1 to 4 h and up to 14 days) signs of toxicity after administration of drug. Signs of acute toxicity include decreased activity, squinting eyes, hunching, labored breathing or mortality (see modified Policy#10, Appendix 1). Animals will be euthanized on day 14 and gross necropsy will be carried out by the research staff for any abnormal pathology in all major organs. Research staff will also collect tissues by Pluck method [28].

Serum biochemical and whole blood hematological tests: Whole blood will be collected under anesthesia on day 14 after drug administration (see **section V.4.4.2** for Blood collection) and blood will be transferred to EDTA tubes for hematological analysis. The rest of the blood will be transferred to serum tubes for serum collection for biochemical analysis. Hematological parameters (CBC/differential) will be analyzed using an Advia 2120 hematology system from Siemens Corporation, or replacement analyzer, if necessary. Serum biochemistry will be done using either Vitros 350 (manufactured by Ortho) or Daytona Plus (manufactured by Randox) or any other suitable analyzer.

General Experimental Design: Total no. of animals required

Drug	Groups	Route of Injection	Diet Condition	# of animals	End point (whole blood)
Drug 1	Naïve	SC	N/A	6	CBC/Differential, Serum Biochemistry
	Vehicle			6	
	Drug dose 1			6	
	Drug dose 2			6	
	Drug dose 3			6	
	Drug dose 4			6	
Drug 1	Naïve	PO	Fasting	6	CBC/Differential, Serum Biochemistry
	Vehicle			6	
	Drug dose 1			6	
	Drug dose 2			6	
	Drug dose 3			6	
	Drug dose 4			6	
Drug 1	Naïve	PO	Fed	6	CBC/Differential, Serum Biochemistry
	Vehicle			6	
	Drug dose 1			6	
	Drug dose 2			6	
	Drug dose 3			6	
	Drug dose 4			6	
Drug 2	Naïve	SC	N/A	6	CBC/Differential, Serum Biochemistry
	Vehicle			6	
	Drug dose 1			6	
	Drug dose 2			6	
	Drug dose 3			6	
	Drug dose 4			6	
Drug 2	Naïve	PO	Fasting	6	CBC/Differential, Serum Biochemistry
	Vehicle			6	
	Drug dose 1			6	
	Drug dose 2			6	
	Drug dose 3			6	
	Drug dose 4			6	
Drug 2	Naïve	PO	Fed	6	CBC/Differential, Serum Biochemistry
	Vehicle			6	
	Drug dose 1			6	
	Drug dose 2			6	
	Drug dose 3			6	
	Drug dose 4			6	
Drug 3	Naïve	SC	N/A	6	CBC/Differential, Serum Biochemistry
	Vehicle			6	

	Drug dose 1			6	
	Drug dose 2			6	
	Drug dose 3			6	
	Drug dose 4			6	
Drug 3	Naïve	PO	Fasting	6	CBC/Differential, Serum Biochemistry
	Vehicle			6	
	Drug dose 1			6	
	Drug dose 2			6	
	Drug dose 3			6	
	Drug dose 4			6	
Drug 3	Naïve	PO	Fed	6	CBC/Differential, Serum Biochemistry
	Vehicle			6	
	Drug dose 1			6	
	Drug dose 2			6	
	Drug dose 3			6	
	Drug dose 4			6	
			Total # of animals	**324**	

V.1.2. Efficacy of drugs as radiation countermeasures - survival studies (General design)

1) We will use the LD70/30 – LD90/30 radiation dose, a dose that results in 70% - 90% mortality within 30 days following exposure for all drugs we wish to test to preclude additional amendments.

2) There are three major arms to the survival study: one prophylaxis (24 h pre-TBI), and two mitigation (post-irradiation) arms, namely 4 h and 24 h post-TBI.

3) Survival of ≥30% over vehicle is considered the 'pass criteria'.

The efficacy of each drug in enhancing survival of lethally irradiated CD2F1 or C57BL/6 mice will be tested in both the prophylactic and therapeutic regimen (-24 h, +4 h or +24 h after exposure [0 h]) as described below. This regimen could be changed based on the discussions with the company and preliminary data available for a specific drug. A memo will be submitted to IACUC before the study begins.

Each study will consist of 1 positive control (GT3/ 5-AED), 1 negative control (5% tween80®-saline/ PEG400) and 3 groups (Drug, vehicle and Naïve if the vehicle is other than PBS or saline) at 3 regimens (-24 h, +4 and +24 h)

General Experimental Design of Survival Study for 1 drug:

Groups	Route of injection	Regimen	Diet Condition	# of animals
5% tween80®-saline/ PEG400	SC	-24 h	N/A	10
GT3/ 5-AED				10
Naïve	PO	-24 h	Fasting	24
Vehicle				24
Drug				24
Naïve	PO	-24 h	Fed	24
Vehicle				24
Drug				24

Naïve	PO	+4 h	Fasting	24
Vehicle				24
Drug				24
Naïve	PO	+4 h	Fed	24
Vehicle				24
Drug				24
Naïve	PO	+24 h	Fasting	24
Vehicle				24
Drug				24
Naïve	PO	+24 h	Fed	24
Vehicle				24
Drug				24
Naïve	SC	-24 h	Fed	24
Vehicle				24
Drug				24
Naïve	SC	+4 h	Fed	24
Vehicle				24
Drug				24
Naïve	SC	+24 h	Fed	24
Vehicle				24
Drug				24
			# of animals for 1 drug	***668***
			Total # of animals for 3 drugs	**2004**

Specific experimental designs for 3 drugs described below:

Experimental design for (b)(4)

Based on recommendation from (b)(4),(b)(6) (b)(4) (**Section II, Background**), basic acute toxicity study for (b)(4) will be conducted at 1800 mg/kg administered orally by gavage to fed and fasting (fasted for 5-8 h prior to drug administration) mice. If the drug dose of 1800 mg/kg is found to be toxic, then lower dose (1500 mg/kg) will be tested for toxicity. Maximum tolerated dose (MTD) will be determined from this study.

Toxicity study

Drug	Groups	Route of injection	Diet Condition	# of animals	End point (whole blood)
(b)(4)	Naïve	PO	Fasting	6	CBC/Differential, Serum Biochemistry
	Water			6	
	(b)(4) at 1800 mg/kg			6	
	Naïve	PO	Fed	6	CBC/Differential, Serum Biochemistry
	Water			6	
	(b)(4) at 1800 mg/kg			6	
			Total # of animals	36	

30-day survival study will be carried out at 1/4th of the MTD of (b)(4) (determined in the toxicity study) administered orally by gavage 1 h prior to TBI (Radiation dose LD70/30 determined by the probit curve which will be generated in February 2016, at the rate of 0.6 Gy/min at AFRRI). Survival study will involve two groups of animals – fasting (for 5-8 h prior to administration) and fed mice. (b)(4) will be administered 1 h prior to TBI. Animals will be monitored for 30 days post-TBI. Endpoint of the study is as described in Section **V.4.5. Study Endpoint.**

Survival study

Groups	Administration time point(s)	Route of injection	Diet Condition	# of animals
5% tween80®-saline/ PEG400	-24 h	SC	Fed	10
GT3/ 5-AED	-24 h			10
water	-1 h	PO	Fasting	24
(b)(4)				24
water	-1 h	PO	Fed	24
(b)(4)				24
			Total # of animals	**116**

CD2F1 mice will be used for the **toxicity and survival** study of (b)(4) **The animals for these studies are included in the total animals requested in this protocol.**

Experimental design for (b)(4) : Basic acute toxicity study will be conducted at 100 mg/ kg administered subcutaneously in the nape of the neck of mice. Drug dose determined from the literature information of MTD (b)(4) (**Section II, Background**) . If the dose of PB at 100 mg/kg (sc) is found to be toxic, lower dose (50 mg/kg) will be tested for toxicity. Maximum tolerated dose (MTD) will be determined from this study.

Toxicity study

Drug	Groups	Route of injection	Diet Condition	# of animals	End point (whole blood)

(b)(4)	Naïve	SC	N/A	6	CBC/Differential, Serum Biochemistry
	Saline			6	
	(b)(4) at 100 mg/kg			6	
			Total # of animals	**18**	

Survival study will be conducted at 1/4th of the MTD of (b)(4) administered subcutaneously 24 h pre-TBI, 4 h post-TBI. (Radiation dose LD70/30 determined by the probit curve which will be generated in February 2016, at the rate of 0.6 Gy/min). Animals will be monitored for 30 days post-TBI. Endpoint of the study is as described in Section **V.4.5. Study Endpoint.**

Survival study

Groups	Administration time point(s)	Route of injection	Diet Condition	# of animals
5% tween80®-saline/ PEG400	-24 h	SC	Fed	10
GT3/ 5-AED	-24 h			10
Saline	-24 h	SC	Fed	24
(b)(4)				24
Saline	+4 h	SC	Fed	24
(b)(4)				24
			Total # of animals	**116**

CD2F1 mice will be used for the **toxicity and survival study** of (b)(4) **The animals for these studies are included in the total animals requested in this protocol.**

Experimental design for (b)(4)

Toxicity study Drug	Groups	Route of injection	Diet Condition	# of animals	End point (whole blood)
(b)(4)	Naïve	SC	N/A	6	CBC/Differential, Serum Biochemistry
	Saline			6	
	(b)(4)			6	
			Total # of animals	18	

Survival study

Groups	Administration time point(s)	Route of injection	Diet Condition	# of animals
5% tween80®-saline/ PEG400	-24 h			10
GT3/ 5-AED	-24 h	SC	Fed	10
Vehicle	-24 h	SC	Fed	24
(b)(4)				24
Vehicle	+4 h	SC	Fed	24
(b)(4)				24
Vehicle	-24 h	SC	Fed	10
			Total # of animals	**126**

Radiation dose, drug dose, strain of mice (CD2F1 or C57BL/6) for toxicity and survival studies of (b)(4) will be decided later. **The animals for these studies are included in the total animals requested in this protocol.**

Estimated numbers of animals for toxicity studies:

Drug	# of mice for 2 iterations
(b)(4)	36 x 2 = 72

(b)(4)	18 x 2 = 36
	18 x 2 = 36
Total	**144**

Estimated numbers of animals for survival studies:

Drug	# of mice for 2 iterations
(b)(4)	116 x 2 = 232
	116 x 2 = 232
	126 x 2 = 252
Total	**716**

Section V.1.3. Optimization studies: Optimization studies will be initiated only if any of the drugs tested meets the AFRRI criteria of ≥30% survival than vehicle in the preliminary radiation survival screen. These studies will be conducted in the mouse strain (CD2F1 or C57BL/6) which was used for the survival studies. Assuming that three drugs meet the AFRRI criteria, we request animals to carry out optimization studies and DRF for three drugs.

Drug dose optimization study: 6 groups (Five drug doses determined based of the Survival study + 1 vehicle) x 1 radiation dose x 2 regimen (pre- and post-exposure) x 1 route (*sc* or *po*) x 3 drugs (chosen based on the efficacy (≥30% survival compared to the vehicle group) in the survival study) x 24 animals per group

Subtotal = **864 mice (CD2F1 or C57BL/6)**

Time optimization study: 4 different time points (determined based on Survival study) x 2 groups (1 optimum dose [based on the Drug dose optimization study] + 1 vehicle) x 1 radiation dose (LD70/30) x 1 route (*sc* or *po*) x 3 drugs x 24 animals per group

Subtotal = **576 mice (CD2F1 or C57BL/6)**

Section V.1.4. Dose reduction factor study (DRF):

The dose reduction factor (DRF) studies will use the optimum drug dose and times to evaluate the effectiveness of the drug against a range of radiation doses at the rate of 0.6 Gy/min in CD2F1 / C57BL/6 mice.

6 irradiated vehicle groups (8.5, 9, 9.5, 10, 10.5 and 11 Gy, 0.6 Gy/min)* + 6 irradiated drug groups (8.5, 9, 9.5, 10, 10.5, and 11 Gy)* x 24 mice per group x 3 drugs.

Subtotal = **864 mice (CD2F1 or C57BL/6)**

*These radiation doses are projected based on an earlier probit study and are considered guidance. The appropriate radiation doses will be determined using the new dosimetry map (Feb 2016) and the IACUC will be notified by memorandum.

Section V.1.5. Hematology, clinical chemistry, and bone marrow studies .These studies are anticipated only for those candidates that demonstrate 30% increase in survival.

Data from these studies will form the basis of understanding the mode of action of the drug,
3 groups (Naïve + vehicle + drug) x 7 time-points (0, 1,4,7,10,14 and 30 d post-TBI) x 10 mice per group x 1 route of administration x 2 drugs

Subtotal = 420 mice (CD2F1 or C57BL/6)

2 experimental groups (vehicle-irradiated, Drug-irradiated) x 7 time-points (0, 1,4,7,10,14 and 30 d post-TBI) x 10 mice per group x 1 route of administration x 2 radiation doses x 2 drugs

Subtotal = 560 mice (CD2F1 or C57BL/6)

Grand total no. of mice required in this protocol: Toxicity (324) + Survival (2004) + Optimization (1440) + DRF (864) + Hematology (980) = 5612

V.2. Data Analysis All data will be reported as means with standards errors or percentages where applicable.
Basic Toxicity (ODDS) – This is a qualitative study so no additional statistics will be conducted.
Preliminary survival study - Based on the power analyses, the sample size (n=24) is adequate to provide 80% power required to detect significant difference between two groups, if any. Previously (b)(6) n=24 was used in 30-day survival studies to determine if there is 30% improved survival in the drug group compared to vehicle. Data will be represented as Kaplan-Meier survival curves. Comparison of the survival curves will be made using Log-rank test will be used to compare survival curves. A p value of < 0.05 will be considered significant. Experiments will be repeated once with 24 mice per group, thus providing adequate statistical power for any conclusions in the survival studies. Experiment V.1.4. (Dose Reduction Factor study) probit analysis and estimation of DRF will be done using IBM SPSS Statistics 22 software or any other certified software. In the experiment V.1.5, t-test, Fisher's test or ANOVA will be used to evaluate the outcome and comparisons.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered Although high throughput screening and computer modeling have advanced significantly in recent years in identification of promising drugs, it would be simplistic to assume that cell systems or artificial intelligence modeling can replace the data that can be generated by using an animal model. Irradiation of animals will result in a cascade of changes including death of stem and progenitor cells in bone marrow, alterations in signal transduction pathways, multiple organ dysfunctions etc. due to differing radiosensitivities of organs. Currently, there are no alternatives in existence that can predict or compute the complexity of the response to total body irradiation. The US FDA recognizes the ethical considerations involved in testing the efficacy of radioprotective drugs in humans. Therefore FDA requires preclinical drug assessments (safety and efficacy) to be performed using both small and large animal models [29] prior to granting approval for safety testing in humans.

V.3.2. Animal Model and Species Justification Recent advances in molecular techniques have allowed significant improvements in understanding the cellular and biochemical processes. These advances are possible only due to whole animal modeling of human ailments. The mouse, in particular, has high sequence homology to humans at a genomic level as well as similar hematological and immunological response to radiation. Mouse models not only confirm primary pathological processes but have also provided a venue for studying basic molecular, cellular, biochemical, and cytological processes. Furthermore, there are extensive data on the mouse for comparison, review and analysis. The practicalities of breeding and housing these small mammals have made the mouse model invaluable. The Principal Investigator's group at AFRRI has significant training and experience in handling mice in radiation countermeasure studies. In this protocol, we will use CD2F1 or C57BL/6 mice. The PI also has used these strains in previous studies.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species Mouse, *Mus musculus*

V.3.3.2. Strain / Stock CD2F1, C57BL/6

V.3.3.3. Source / Vendor Harlan Inc., Jackson laboratories or any other approved vendors

V.3.3.4. Age (Procurement) 7-8 weeks

V.3.3.5. Weight (delivery) 20-24 grams

V.3.3.6. Sex Males have been used historically and will continue to be used in this study.

V.3.3.7. Special Considerations
Commercially procured animals must be adventitious disease free (see Appendix 2).

V.3.4. Number of Animals Required (by species) Table

Animal (genus, species and common names)	**Study**	**Original**
CD2F1	(b)(4) (Toxicity and survival)	304
CD2F1	(b)(4) (Toxicity and survival)	268
CD2F1 or C57Bl/6	(b)(4) (Toxicity and survival)	288
CD2F1 or C57Bl/6	Future 3 studies	4752
	Total # of animals	**5612**

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement
PI will take necessary steps for all personnel in the project to be rigorously trained in handling techniques (Tail-tattooing, ear-punch, injections, blood collection, orogastric

gavage and routine cage-side observations) to minimize distress and pain to the animals. Specifically, during capture and restraint of the rodents, care will be taken to ensure that handling does not contribute to distress.

V.3.5.2. Reduction

The protocol will use a tiered approach to reduce the number of animals for testing 3 drugs/ year. Although basic toxicity and survival will be carried out on all the drugs, additional studies will depend on the initial results from survival study. Positive controls and negative controls are used to confirm robustness of experimental conditions and drugs that don't meet our minimal criteria of 30% increased survival over the vehicle control will be eliminated. Based on previous experience, we have proposed that only 3 drugs may be advanced for optimization and dose reduction factor studies (Section V.1.3 and V.1.4). Further, control groups (such as vehicle) will be shared whenever possible between drugs that are dissolved in similar vehicles. In classical toxicology testing, groups of 10-20 mice and both sexes were routinely used; in using the ODDS method (current protocol), we have reduced the number of animals to 6 per group, and use only males, thereby reducing animal numbers significantly. Four drug doses have been proposed for toxicity screening; however, in some cases a smaller number might be selected based on literature search and recommendation by the manufacturers. Similarly, two routes of drug administration have been contemplated in the experimental design; but depending on the existing data and company suggestions, a single and optimal route may be chosen. Wherever possible we have reduced the number of repeats planned for the experiments shown above.

V.3.5.3. Replacement

It is not feasible to use non-animal systems, nor a less-sentient model, in place of the rodent animal models to address the questions in this project.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals 4144

V.4.1.1.1.1. Column C ___0__ (# of animals)

V.4.1.1.1.2. Column D ___0__ (# of animals)

V.4.1.1.1.3. Column E ___5612__ (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
V.1.1.	ODDS safety/toxicity study			324	324
V.1.2.	Radiation survival studies			2004	2004
V.1.3.	Dose and Time optimization			1440	1440
V.1.4.	DRF			864	864
V.1.5.	Hematology			980	980

Exp. #	Experimental/Control Group	C	D	E	Totals
Totals:				5612	5612

V.4.1.2. Pain Relief / Prevention Animals can experience pain and distress. Minimizing and alleviating pain in laboratory animals without compromising the methodological integrity of a research project is important both ethically and legally. Mice often exhibit pain and distress with only subtle changes in their behavior [30]. Potential signs associated with pain and distress in mice includes decreased food and water consumption, weight loss, self-imposed isolation/hiding rapid breathing, open-mouth breathing, abdominal breathing, decreased movement, abnormal posture, muscle rigidity, twitching, trembling, tremor, etc. as included in Appendix 1 (sample scoring sheet for clinical observations with criteria for rodent euthanasia) in modified IACUC policy#10 (Guidelines for establishment of early endpoints in experiments with expected mortality, Appendix 1).

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization
The protocol will not use anesthetics or analgesics during radiation since they will adversely affect the outcome of the experiments or during oral administration of drug. The proposed study is to determine the effects of radiation and countermeasures on the function of the immune and blood forming systems. Use of any analgesics and anesthetics would interfere with the function of the immune system and therefore compromise the interpretation of the results [31]. Topical antibiotics will be applied if wounding occurs via in-cage fighting, under the guidance of the clinical veterinarian in accordance with standard treatment measures.
Anesthesia using standard Isoflurane apparatus under the guidance of the VSD will be carried out in mice for blood collection. All personnel will be extensively trained for efficient administration of the agents as well as to minimize handling stress.
Animals will be placed in the Isoflurane chamber and delivered a metered amount of 3-5% Isoflurane mixed with 100% oxygen (oxygen at the flow rate of 500-1000cc/min) until all voluntary motor movement ceases and the animal is recumbent for approximately 10 seconds. Animal's respiration rate will be closely monitored through the Plexiglas chamber during induction of anesthesia. When animals are recumbent, they will be removed from the chamber and their nose/mouth placed in the anesthesia apparatus breathing/gas administration cone for maintenance of anesthesia at 1-3% Isoflurane and 100% O_2.

V.4.1.2.2. Pre- and Post-procedural Provisions Mice will be housed under standard conditions both before and after radiation procedures in accordance with current VSD rodent husbandry Standard Operating Procedures (see section V.5).

V.4.1.2.3. Paralytics N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched AGRICOLA, PubMed, EMBASE

V.4.1.3.2. Date of Search 12-07-2015 – 12-14-2015

V.4.1.3.3. Period of Search AGRICOLA: 1987-2014, EMBASE 1974-present
PubMed ~1970 to present.

V.4.1.3.4. Key Words of Search Radiation and pain, (b)(4) toxicity and pain, (b)(4) toxicity and pain, (b)(4) and toxicity and pain, analgesia, alternative, humane, alternative methods to blood collection by cardiac stick and inferior vena cava. In order to increase specificity of literature review, several combinations such as radiation plus specific drug, radiation plus specific drug plus mice, alternatives to blood collection.

V.4.1.3.5. Results of Search

Table 2:Summary of Database Keyword Searches

Keywords	Hits		
	PubMed	AGRICOLA	EMBASE
(b)(4) AND radiation AND pain	0	0	0
AND analgesia AND pain	0	0	0
(b)(4) AND radiation AND pain	0	0	0
AND analgesia AND pain	1	0	0
(b)(4) AND radiation AND pain	0	0	3
AND analgesia AND pain	2	0	4
Radiation AND mouse AND pain	234	0	224
(b)(4) AND toxicity AND pain	0	0	0
(b)(4) AND toxicity AND pain	0	0	0
(b)(4) AND toxicity AND pain	4	0	14
Alternatives to blood collection by cardiac stick and inferior vena cava	0	0	0
Pain AND analgesia And mice	1873	52	2236
Radiation AND mouse AND pain AND alternative	5	0	11
Radiation AND humane	12	0	34

There are no alternatives to irradiation. While radiation itself does not cause pain or distress, it induces a number of changes in the body that alters immune response and destroys hematological tissues, resulting in opportunistic infections, and multi-organ dysfunction leading to death. It is anticipated that animals exposed to high lethal radiation doses will become moribund. Unfortunately, literature clearly demonstrates that use of analgesics alters radiation response and therefore leads to increase in animal number for better statistical results or for iteration of entire experiments. Since the purpose here is to compare the efficacy of the three experimental drugs we will be unable to use analgesics and anesthetics because they may interfere with the innate system [31].

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification
Because exposure to irradiation is potentially lethal or causes debilitating effects in humans, it is ethically impermissible to test the effectiveness of radiation countermeasures on human volunteers. Irradiation compromises the immune and blood forming systems leading to mortality. The radiation-induced mortality and potential percentage increase of survivors over 30 days using potential radiation countermeasures are study endpoints for this protocol. Administration of drugs and analgesics is not an option since it is well documented that they interact with the immune system which will confuse the interpretation [32-34]. Pain relieving measures

are not used because such measures may compromise the experimental integrity of the study. All moribund animals will be euthanized by CO_2 inhalation and confirmatory cervical dislocation (see **Section V.4.6 Euthanasia**).

V.4.2. Prolonged Restraint

The experimental animals will receive radiation at a dose rate 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (according to the latest dosimetry map) using plastic racks. Mice will be inserted into the standard radiation boxes just prior to irradiation (~15 min of exposure time) and taken to the cobalt facility for irradiation. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period. They will be monitored daily following modified IACUC policy#10 (Guidelines for establishment of early endpoints in experiments with expected mortality) (at least three times a day during critical period with no more than 10 h interval between late night and morning check and at least twice a day during non-critical period) for 30 days and euthanized at the completion of the observational period.

V.4.3. Surgery N/A

V.4.3.1. Pre-surgical Provisions N/A

V.4.3.2. Procedure(s) N/A

V.4.3.3. Post-surgical Provisions N/A

V.4.3.4. Location N/A

V.4.3.5. Surgeon N/A

V.4.3.6. Multiple Major Survival Operative Procedures N/A

V.4.3.6.1. Procedures N/A

V.4.3.6.2 Scientific Justification N/A

V.4.4. Animal Manipulations

V.4.4.1 Ear punch:

Three animals/ shoe box for toxicity studies and four animals/ shoe box for survival studies will be housed. They will be identified either by ear punch or tail tattoo.

Procedure for ear-punch

a. Soak the ear punch (Fine Science Tools, Cat# 24212) in a disinfectant (70% ethanol) before use and between animals.

b. Restrain the mouse manually. Place the device on the pinna of the ear (external ear) where the skin is thin avoiding the cartilage. Press firmly to punch a circular hole through the ear

Tattoo procedure: Tattooing will be done either with a machine from Labstamp system following the SOP provided by the company or any other machine certified by VSD. An alphabet followed by two digits will be used as a numbering scheme (e.g. A11).

Irradiation:
These will follow the standard AFRRI protocol with mice placed in restrainers shortly prior to exposure, irradiation at the cobalt facility and return to VSD for further monitoring. Briefly, the experimental animals will receive radiation at a dose rate of 0.6 Gy per/min in the AFRRI Cobalt 60 gamma radiation facility. Exact dose of radiation will be determined based on the probit curve. Animals will be irradiated in Lucite boxes (8 animals/box) and arranged in an array (the latest dosimetry map) using plastic racks. Animals will be restrained for no more than 60 min and returned to cages at the end of the irradiation period. They will be monitored daily following modified IACUC policy#10 (Guidelines for establishment of early endpoints in experiments with expected mortality, Appendix 1) (3-4 times a day during critical period with no more than 10 h interval between late night and morning check and at least twice a day during non-critical period) for 30 days and euthanized at the completion of the observational period.

V.4.4.2. Oral gavage:
Oral administration will be done by orogastric gavage undertaken using 20-24 gauge gavage needle (animal feeding needles, disposable-sterile, non-toxic and nonpyrogenic, 1" length curved gavage needle). Non-disposable, metal ball ended, oral gavage needles of similar gauge and length are included as an alternative to the disposable needles. Drug volume will be 0.1 - 0.2 ml (maximum volume 10 – 50 ml / kg). Drug dosage will vary from one drug to another based on discussions with the company which is providing the drug. The animal will be restrained in a vertical position to align the spine straight. The bulb will be introduced into the mouth horizontally. Using the needle as lever, the syringe will be moved into a vertical position and the needle dropped down the esophagus into the stomach. The needle contents will be administered when the needle hub touches the animal's mouth. All precautions will be taken to ensure that the placement of the needle is properly positioned and that the animal is not in distress. No forceful feeding will be done to prevent perforation of the esophagus.

Injections:
Subcutaneous injections will be given with a 23 - 25 G, 5/8" needle to the nape of the neck of 0.1 ml (maximum volume 1 ml for 25 to 38 g animals). A disposable sterile needle will be used for all sc injections. One needle will be used per cage containing generally 4 animals. Endotoxin testing for all drugs will be completed prior to any animal testing at AFRRI and a report submitted for IACUC/VSD review and approval. The endotoxin tests are conducted by Charles River Laboratories. At the same time, pH will be tested to ensure that it falls in the range (6-8 for sc administration) stipulated by IACUC. It is expected that the osmolality will not be more than AFRRI IACUC's

recommended levels (IACUC Policy#9: Non-pharmaceutical grade experimental drug formulations to be used in experimental animals).

Blood collection:

Mice will be anesthetized as described under section V.4.1.2.1. (Anesthesia). Blood draw for hematology is collected either by cardiac stick or from the inferior vena cava (IVC). The animals will be deeply anesthetized in the rodent anesthesia machine; the tail and the toe will be pinched for reflexive movement, indicative of insufficient anesthesia. If there is no response, the animal will be moved to the station with individual nose cone for continued anesthesia. 70% ethanol will be sprayed on the abdominal area of the animal. An incision will be made on the right side below the abdomen of the animal, closest to the IVC, the vein exposed and blood withdrawn with a 23 - 25 G, 5/8" needle. Similarly, a 23 - 25 G, 5/8" needle will be used to perform cardiac stick while the animal is positioned under nose cone anesthesia. In both cases, the animals will be immediately euthanized on completion of blood draw by cervical dislocation.

V.4.4.1.1. Pharmaceutical Grade Drugs

V.4.4.1.2. Non-Pharmaceutical Grade Drugs Research grade (b)(4)

(b)(4)

V.4.4.2. Biosamples Blood and tissue

V.4.4.3. Adjuvants N/A

V.4.4.4. Monoclonal Antibody (MAb) Production N/A

V.4.4.5. Animal Identification Generally, cage cards will be used to distinguish drug-treated and control groups. Animals will be randomly distributed in groups. Animals will be identified by ear punch or tattoo at investigator's discretion. Ear punch will be performed as described in V.4.4.1.

V.4.4.6. Behavioral Studies N/A

V.4.4.7. Other Procedures N/A

V.4.4.8. Tissue Sharing N/A

V.4.4.9. Animal By-Products N/A

V.4.5. Study Endpoint

The time frame for experiment V.1.1 (basic toxicity) will be 14 days after final drug dose administration. The radiation survival studies will span 30 days after radiation exposure (V.1.2).

It is anticipated that the animals involved in these studies may succumb to death either due to a drug administration or radiation or a combination of the two. To maintain a

suitable baseline for humane treatment of the animals while adhering to the study objective, the IACUC recommendations stipulated in IACUC policy#10 (Guidelines for establishment of early endpoints in experiments with expected mortality) with some minor modification (as stated later in the paragraph) will be implemented for judging morbidity and moribundity. The moribund condition is defined as a clinically irreversible condition leading inevitably to death. Signs of morbidity in the mouse include difficulty in breathing, ruffling of hair; drying of lacrimal fluid, fall in body temperature, loss of appetite with/without diarrhea. A modified IACUC policy#10 (Guidelines for establishment of early endpoints in experiments with expected mortality) will be used to record the scoring of clinical signs of pain in animals (attached Appendix 1). PI would like to change the score for the "Changes in breathing" from 3 to 2 and add an in between score of 6 for "Dyspnea / labored breathing" under "Respiratory rate". Description of the score 12 will be "Abdominal breathing/ gasping – open mouth breathing". Previously there was a large jump between a score of 3 (change in breathing) to 12 (abdominal breathing/ gasping – open mouth breathing). From the experience gathered from the studies performed recently, the PI thinks by adding a score of 6 in between 3 and 12 would enhance efficiency of the scoring system giving a fair advantage to the study outcome. PI will seek veterinarian's input for training the staff adequately to learn the suggested scoring system.

a) Body weights will be recorded prior to radiation.

b) Once animals reach a score of 6, concern cards ("green cards") will be put up, weights will be recorded and monitored with each subsequent scoring change.

c) Animals that exceed 35% body weight loss with any other clinical signs are scored at a 12 and will be euthanized immediately.

To adhere to the policy of preventing animal distress, the PI will facilitate the late PM and early AM checks (not more than 10 h apart) in addition to two times a day checks during the critical lethality period (early morning, late morning, and late afternoon and evening). During non-critical periods, we will continue to check animals except late night. All morbid mice will be monitored carefully and their conditions scored for documentation (Appendix 1, revised scoring sheet).

PI will use a scoring card for each animal in the critical period and scored in each observation. Scores below 6 will not be recorded in the scoring sheet. Mice will be euthanized if there is a score of 12 in a single category or a cumulative score of 12 in multiple categories.

No supportive care is proposed, however, moribund animals will be immediately euthanized. VSD veterinarians will be consulted for all matters regarding the animal welfare.

V.4.6. Euthanasia

It is anticipated that at high radiation doses, animals will demonstrate varying degrees of morbidity and moribund, which makes it critical to define the criteria for euthanizing the animals without undermining the study objective.

In toxicity studies, if any animal shows discomfort (hunching, decreased activity, inappetence, separation from cage-mates), that animal will be under careful observation on consultation with the attending VSD veterinarian. Mice found moribund (defined in V.4.5.) or meeting the criteria for euthanasia following a modified IACUC policy#10 (Guidelines for establishment of early endpoints in experiments with expected mortality) (score sheet attached) will be euthanized by CO_2 inhalation plus confirmatory cervical

dislocation by investigator/technician according to the directives of the VSD-SOP (VS0211: Rodent Euthanasia Guidelines) and in accordance with current AVMA Guidelines on Euthanasia [35, 36]. At the conclusion of the experimental time frame, all animals that survived the procedures of irradiation, drug intervention and controls, will be euthanized by CO_2 inhalation plus confirmatory cervical dislocation and their carcasses disposed of according to VSD regulations. An alternate method of euthanasia by cervical dislocation after exsanguination (by cardiac stick or inferior vena cava) under deep isoflurane anesthesia will be applied in experiment V.1.5 (see Section V.4.4.2 Blood collection).

V.5. Veterinary Care

V.5.1. Husbandry Considerations The AFRRI vivarium follows stringent protocols for the housing and care of all animals. The PI and designated team members will adhere to all the policy and guidelines set forth by standard operating protocols of the VSD. The specific requirements for mice housing should meet the following criteria of opportunity for social interaction, opportunity to carry out normal behavior and the opportunity to rest and withdraw from each other [37]. Mice are social animals and hence will be caged together in groups. Nestling pads will be provided in each cage during bedding changes. In cage shelters will also be provided as an enrichment strategy at the discretion of the PI depending on experimental conditions. Animals will receive Harlan Teklad Rodent Diet 8604 and water *ad libitum*. Acidified water (pH 2.5 – 3.0) is routinely provided to AFRRI mice used in radiological experiments. Cages will be regularly changed and cleaned by VSD staff in accordance with Standard Operating Procedure. Animals will have 12 h light/dark cycle. Animals will be habituated to their surroundings and routine procedures prior to experimentation. In the event of procedures that last into the night cycle, care will be taken to minimize exposure to light by using red-lens flashlights.

V.5.1.1. Study Room As assigned by VSD

V.5.1.2. Special Husbandry Provisions N/A

V.5.1.3. Exceptions Mice will be socially (group) housed as described above, except when there is a possibility that during survival studies cage mates may be lost through attrition and for a period of time the sole surviving mouse may remain singly housed until the end of the 30-day study.

V.5.2. Veterinary Medical Care We do not project routine veterinary medical care of animals for injuries/inflammation related to injection site inflammation. In case of minor injuries arising from fighting, topical application of antibiotics is acceptable with consensus from the PI and VSD.

V.5.2.1. Routine Veterinary Medical Care

Experimental animals will be observed daily by either the investigator or technician or both as outlined in the **Section V.4.5. Study Endpoint.**

V.5.2.2. Emergency Veterinary Medical Care Moribund animals as per modified IACUC policy#10 (Guidelines for establishment of early endpoints in experiments with expected mortality, Appendix 1) (gasping, recumbent, non-responsive to mild stimulus) will be euthanized by trained personnel.

V.5.3. Environmental Enrichment:
The husbandry staff provides hutches and nestlets to animals not on active experiments whereas cotton nestlets are provided to animals on active experiments.

V.5.3.1. Enrichment Strategy Nestling pads will be provided in each cage during bedding changes as per AFRRI-VSD SOPs.

V.5.3.2. Enrichment Restrictions N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3, 4, 5	(b)(6)
				1, 3, 4, 5	
				1, 3, 4, 5	
				1, 3, 4, 5	
				1,3b,3c,4, 5	
				1, 3, 4	
				1, 3	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY All personnel working in the protocol have undergone necessary training in handling experimental animals. The amount of drug used will be small and standard safety precautions will be taken while handling them in the laboratory and in the vivarium. All personnel listed in the present protocol have been trained as general radiation users on a regular basis. All investigators and personnel will use appropriate protective gear while in the vivarium as described by the VSD. All listed personnel are current in safety training and medical surveillance.

VIII. ENCLOSURES none

References:

1. Mettler, F.A., Jr. and G.L. Voelz, *Major radiation exposure--what to expect and how to respond.* N Engl J Med, 2002. **346**(20): p. 1554-61.
2. Pellmar, T.C. and S. Rockwell, *Priority list of research areas for radiological nuclear threat countermeasures.* Radiat Res, 2005. **163**(1): p. 115-23.
3. Davidson, D.G., MM. Sweeny, TR. , *Biological Characteristics of Some Improved Radioprotectors.* . Radiation Sensitizers, their use in the clinical managemtn of cancer, ed. L.W. Brady. 1980, Masson, NY.
4. Waddick, K.G., et al., *Comparative analysis of the in vivo radioprotective effects of recombinant granulocyte colony-stimulating factor (G-CSF), recombinant granulocyte-macrophage CSF, and their combination.* Blood, 1991. **77**(11): p. 2364-71.

(b)(4),(b)(6)

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(b)(4)

12. Glover, D., et al., *Hypocalcemia and inhibition of parathyroid hormone secretion after administration of WR-2721 (a radioprotective and chemoprotective agent).* N Engl J Med, 1983. **309**(19): p. 1137-41.

(b)(4),(b)(6)

(b)(4)

27. Roderick, T.H., *The Response of Twenty-Seven Inbred Strains of Mice to Daily Doses of Whole-Body X-Irradiation.* Radiat Res, 1963. **20**: p. 631-9.

28. Parkinson, C.M., et al., *Diagnostic necropsy and selected tissue and sample collection in rats and mice.* J Vis Exp, 2011(**54**).

29. FDA, A.R. *21 CFR 314.610 and 21 CFR 601.91.* 2002.

30. Jacobsen, K.R., et al., *Postsurgical food and water consumption, fecal corticosterone metabolites, and behavior assessment as noninvasive measures of pain in vasectomized BALB/c mice.* J Am Assoc Lab Anim Sci, 2012. **51**(1): p. 69-75.

31. Colucci, D.G., N.R. Puig, and R. Hernandez-Pando, *Influence of anesthetic drug on immune response: from inflammation to immunosuppression.* OA Anaesthetics, 2013. **21**(3): p. 1-7.

32. Galley, H.F. and N.R. Webster, *Effects of propofol and thiopentone on the immune response.* Anaesthesia, 1997. **52**(9): p. 921-3.

33. Galley, H.F., A.M. Dubbels, and N.R. Webster, *The effect of midazolam and propofol on interleukin-8 from human polymorphonuclear leukocytes.* Anesth Analg, 1998. **86**(6): p. 1289-93.

34. Beilin, B., et al., *Effects of anesthesia based on large versus small doses of fentanyl on natural killer cell cytotoxicity in the perioperative period.* Anesth Analg, 1996. **82**(3): p. 492-7.

35. Association, A.V.M. *AMVA Guidelines on Euthanasia.* http://www.avma.org/issues/animal_welfare/euthanasia.pdf, 2007.

36. Association, A.V.M., *AMVA Guidelines on Euthanasia*, in http://wwwavmaorg/issues/animal_welfare/euthanasiapdf; . 2013.
37. AWIC, http://awic.nal.usda.gov/nal_display/index. 2010.

X. ASSURANCES

Protocol Title:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

Appendix 1.
Clinical Observations with Criteria for Euthanasia (Rodent)

Criteria:	Description:	Score
Appearance:		
	Normal (smooth coat, clear eyes/nose)	0
	Hunched and/or fluffed	1
	Ocular discharge, and/or edema	3
	Soft stools (fecal matter around anus)	5
	Pale, white mucus membranes/skin	6
	Bloody diarrhea	9
	Blue mucus membranes/skin (cyanosis)*	12
Respiratory Rate:		
	Normal breathing	0
	Change in breathing (an increase or decrease in respiratory rate from normal baseline)	2
	Labored breathing/ Dyspnea	6
	Abdominal breathing (gasping +/- open mouth breathing)/ Diaphragmatic breathing*	12
General Behavior:		
	Normal (based on baseline observations)	0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)	1
	Decreased mobility	2
	Ataxia, wobbly, weak	6
	Inability to stand*	12
Provoked Behavior:		
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))	0
	Subdued; responds to stimulation (moves away briskly)	1
	Subdued even to stimulation (moves away slowly)	3
	Unresponsive to gentle prodding	6
	Does not right when placed gently on side within 5 seconds*	12
Weight loss		
	0-35% with no other clinical signs	0
	≥35% weight loss	12

*** Regardless of score, immediately euthanize (death is imminent)**

Individual or Cumulative Score:

< 6	Normal
6 - 11	Morbid: See VI.D.2 and VI.D.4 in IACUC Policy #10 for guidelines
≥ 12	Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*). Any single criteria of 12* euthanize immediately; consider as 'found dead.'

+

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	2/17/2016	2nd Resubmission Date:	
Approved/**Returned for Revision:**	3/3/2016	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	3/8/2016	**EXPIRATION DATE:**	3/7/2019
Approved/Returned for Revision:	3/8/2016	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and *subsection*, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE:

Study of Biomarkers and Acute Radiation Sickness Prognosis/Outcome Factors after Mixed-Field (Neutron and Gamma) Radiation in a Laboratory Mouse (*Mus musculus*) Total-body Irradiation Model for Use in an FDA Approved Point-of-Care Biodosimetry System

IV. PRINCIPAL INVESTIGATOR:

(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) Date

(b)(6)

- Or –

(b)(6) Date

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) Date

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) Date

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6)

PROTOCOL TITLE:
Study of Biomarkers and Acute Radiation Sickness Prognosis/Outcome Factors after Mixed-Field (Neutron and Gamma) Radiation in a Laboratory Mouse (*Mus musculus*) Total-body Irradiation Model for Use in an FDA Approved Point-of-Care Biodosimetry System.

PRINCIPAL INVESTIGATOR:
(b)(6)

CO-INVESTIGATOR(S):
(b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS

The vast majority of studies on the acute radiation syndrome or sickness (ARS) and biodosimetry have been performed using photon irradiation (gamma and x-rays). Risks for acute mortality from radiation exposure are well known to be influenced by physics-based parameters, such as a radiation quality, low or high linear energy transfer (LET), dose rate, total-body vs. partial body exposure, etc. (Hall and Giarcia, 2011). After an enhanced radiation weapon (ERW) or nuclear device detonation, initial radiation at the time of the nuclear reaction consists of gamma-rays and neutrons (mixed field) produced within the first minute after detonation and its penetration capability, leading to increased damage requiring quality specific biodosimetry as neutrons have different mechanisms of injury to cells and tissues compared to photons, with a higher relative biological effectiveness (RBE).

The present work will evaluate biomarkers for radiation dose-assessment, ARS prognosis, and gender/age effects following mixed-field (gamma-rays and neutrons) using AFRRI TRIGA (Training, Research, Isotope, General Atomic) reactor in a mouse (B6D2F1/J) total-body irradiation (TBI) model. Biomarker measurements will be performed using the Meso Scale Diagnostics (MSD) high-throughput (HT) MULTI-ARRAY plate-format platform and results compared with earlier (b)(4) (b)(4) funded gamma radiation studies.

The proposed efforts supplement an ongoing project to deliver an FDA approved biodosimetry device by potentially expanding the use of its capability for a broader spectrum radiation exposure (mixed neutrons and gamma-rays). This effort is entirely novel. AFRRI is one of a limited number of facilities with the capability to study mixed-field exposures. The proposed studies on radiation biomarker responses to mixed-field irradiation for use with the Meso Scale Diagnostics biodosimetry system is completely new ground. We expect that this project will contribute to bridge a gap that exists in the current capabilities to identify and then rapidly as well as effectively to assess radiation exposure early after a radiation exposure and especially after a mass-casualty radiological incident. In particular, these efforts contribute to validating an early test to distinguish individuals exposed and injured by radiation in order to assist physicians to choose the appropriate medical treatments and hence reducing the adverse acute effects or long-

term risks associated with radiation exposure. The advancement in this type of research might also provide a powerful tool for the accurate assessment of an individual's radiation risk response early after an incident, especially after a mass-casualty radiological incident.

II. BACKGROUND

II.1. Background

A radiological terrorist attack or nuclear accident creates a risk for mass casualties (DiCarlo et al. 2011; Waselenko et al. 2004). The detonation of a nuclear weapon or a nuclear accident represents possible events with significant exposure to mixed-field (neutron + gamma) radiation. The mechanisms of injury of these low linear energy transfer (LET) radiations (pure gamma) are different from those of high-LET radiation such as neutrons or mixed-field, and these differences may affect the radiation dose assessment and countermeasure efficacy. Acute mortality from radiation exposure is well known to be influenced by physics-based parameters such as radiation quality (neutrons or gamma-rays) and dose rate. An enhanced radiation weapon, if detonated, would emit a large portion of neutron radiation with increased radius of exposure and penetration capability, leading to increased damage requiring quality-specific biodosimetry. Currently, a dose of 2 Gy of acute whole-body exposure is thought of as the threshold for medical intervention (Fliedner et al. 2001; Koenig et al. 2005; (b)(4) (b)(4) Moyer 2015). However, absolute reliance on this threshold ignores the impact of radiation quality and is of concern because neutrons are a significant component of the initial radiation released by a fission nuclear device. Hence, there is a current need to accurately determine exposure levels where mixed field combinations of neutrons and gammas are a threat. We have successfully completed four standard gamma only biodosimetry projects funded by (b)(4) (b)(4) (b)(4) the final goal of which is to deliver an FDA-approved hand-held, field deployable point-of-care biodosimetry device, which determines quickly (in few minutes) the radiation dose absorbed by evaluating blood protein biomarker levels after a large-scale radiological or nuclear event (IACUC protocol (b)(6),(b)(4)

Previously established animal (*Mus musculus*, *Macaca mulatta*) total-body irradiation (γ-exposure at a single dose-rate of 0.6 Gy/min) models have evaluated a panel of radiation-responsive proteins that, together with peripheral blood cell counts, to create a multiparametric dose-predictive algorithm with a threshold for detection of ~1 Gy from 1 to 7 d after exposure. The acute radiation syndrome (ARS) severity score systems were also created based on multiple biodosimetric endpoints. The mouse ARS severity score system was created using data collected in individual animals that were identified by IPTT-200, Bio Medic Data System (BMDS) implantable programmable transponders/microchips. Individual tracking of animals via implanted microchips allowed for assessment of criteria based on individuals rather than by group averages. The mouse ARS severity score system created at AFRRI includes the Observational Grading System (currently an IACUC policy #10 [Guidelines for establishment of early

endpoints in experiments with expected mortality]) developed at the AFRRI VSD, survival rate, weight changes, temperature, peripheral blood cell counts and radiation-responsive protein expression profile (Figures 1-3; (b)(6),(b)(4)

(b)(6),(b)(4)

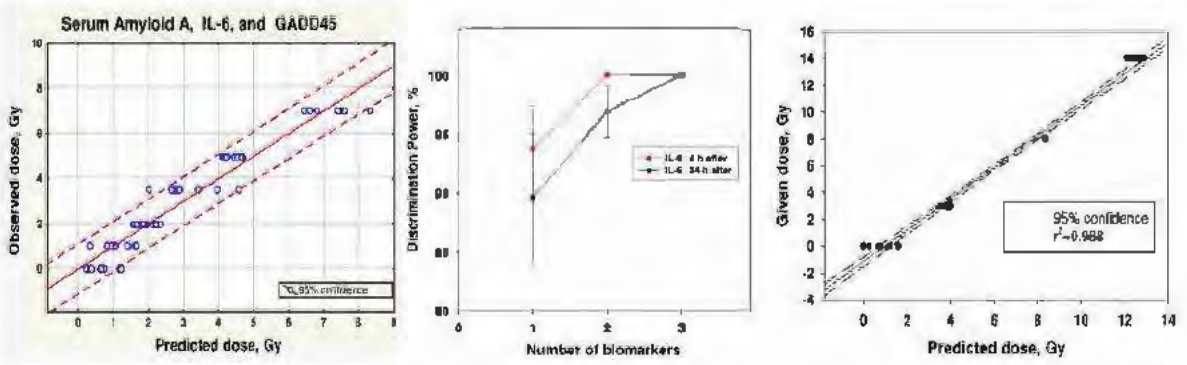

Figure 1. Multiparameter dose-assesment calibration curve (left plot), enhanced discrimination power at radiation dose of 1 Gy (middle plot) and biodosimetry evaluation in "blinded" study (right plot) in mouse TBI model (^{60}Co γ-rays at dose rate of 0.6 Gy/min).

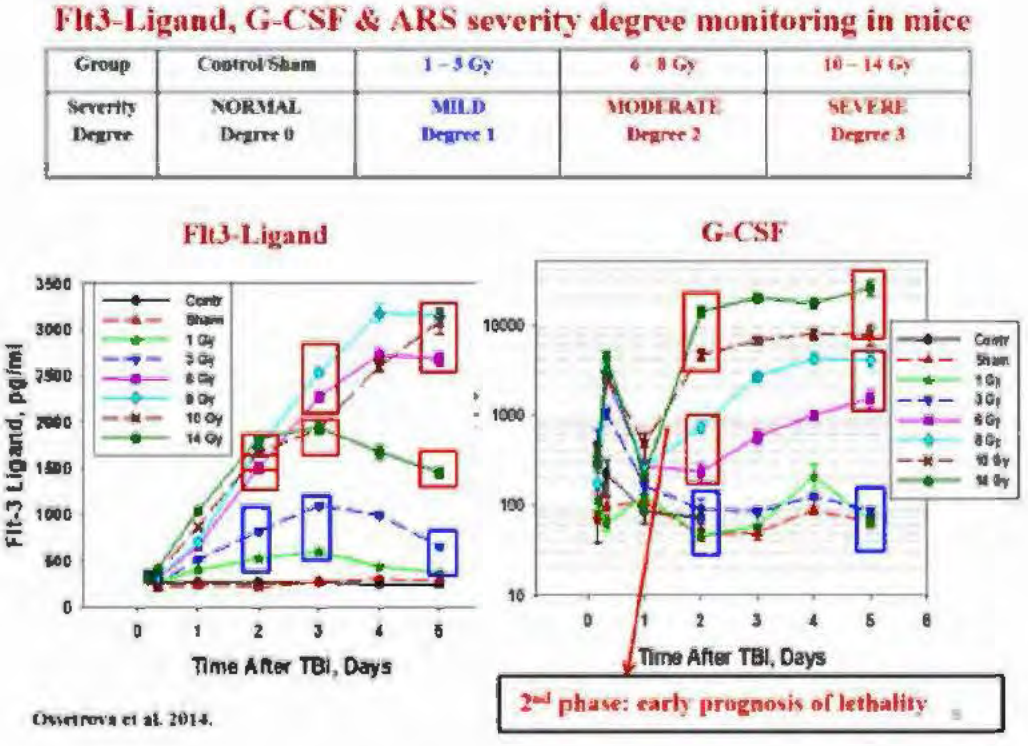

Figure 2. Flt3-Ligand, G-CSF and ARS severity degree monitoring in mouse TBI model (^{60}Co γ-rays at dose rate of 0.6 Gy/min).

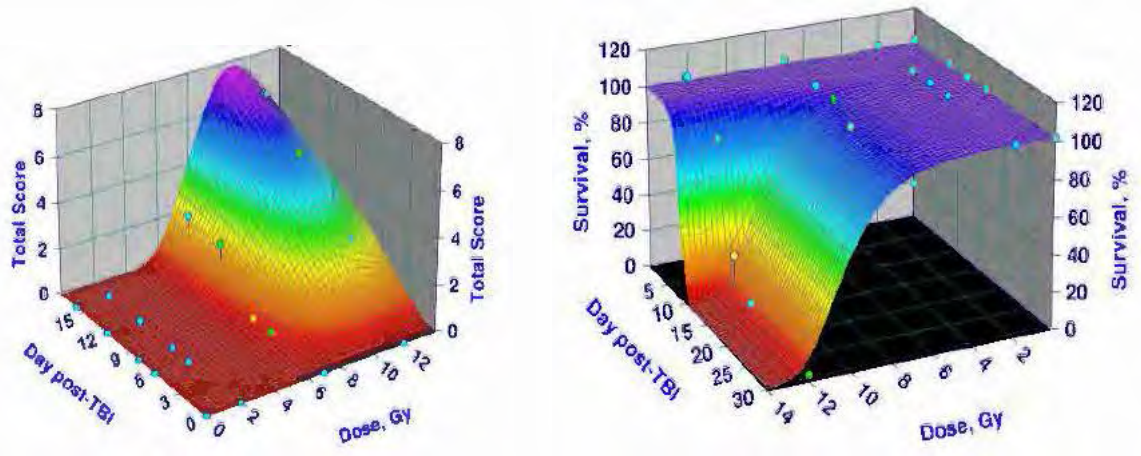

Figure 3. The radition-dose and time-dependent total mean ARS interventional score (left plot) and survival rate (right plot) in mouse TBI model (^{60}Co γ-rays at dose rate of 0.6 Gy/min) that might be used as an early predictive values for ARS prognosis or outcome.

The ARS response category assessment tool was created in order to quantify severity responses for ARS sub-syndromes and determine the criteria and bioindicators for early prognosis of lethality and point of euthanasia. The ARS severity degrees or response categories (RC), defined under the radiation-dose controlled conditions and animal recovery prognosis, were as follows: RC0 (Degree 0) for 0 Gy (control/sham); RC1 (Degree 1) for the dose-range of 1-3 Gy and animal recovery was certain with a low risk of critical phase (mild radiation damage); RC2 (Degree 2) was for the dose-range of 6-8 Gy and animal recovery was likely with a high risk of critical phase (moderate, but reversible damage); RC3 (Degree 3) was for the dose-range of 10-14 Gy and animal recovery was most unlikely (severe, irreversible damage) (Figures 2-3).

Since those studies were performed using only a pure gamma-ray exposure (^{60}Co), the needed dose-assessment modifications for mixed neutron and gamma exposures, a very militarily relevant scenario, remain unknown. There is a current need for biodosimetry and countermeasure drugs to treat ionizing radiation injury, for use in the field by military personnel and emergency responders. Radiation damages blood-forming tissues, resulting in mortality due to infection and hemorrhage (hematopoietic syndrome). (b)(6) (b)(6) assumes that in realistic scenarios, access to medical facilities will be unlikely for some time after a nuclear detonation. Neutrons and photons interact with cells in different ways: photons interact with orbital electrons, and neutrons interact with nuclei. Neutrons have a higher LET compared to photons, and neutron-induced DNA damage is less repairable (an effect not explained solely by frequency of double strand breaks, DSB). Neutrons and photons have different effects depending on the specific cells and tissues being considered. Neutron efficacy in inducing damage is higher than that of photons, i.e., the RBE (dose necessary to cause a degree of injury compared to the photon dose) is greater than one. Tissues and cell types differ in their rate of

accumulation of radiation-induced injury and in their repair capacity. The neutron RBE for GI injury (6 day mortality in mice) is about 3.5, while that for hematopoietic injury (30 day death) is about 2.4 (b)(6),(b)(4) After a nuclear detonation, personnel will be exposed to mixed radiation fields with different neutron to gamma ratios for detonations of concern (from weapons likely to be used by terrorists) (NATO Handbook 1994). In current studies, we have chosen a (33% n + 67% γ) and (67% n + 33% γ) to be representative of the possible fields.

In this protocol, a murine TBI model with a minimal supportive care will be used in order to simulate a catastrophic event involving large number of casualties. In this scenario, there will be limited medical and physical resources. Early and rapid dose assessment is required in radiation disasters that involve a large number of victims and a finite amount of medical resources available to responders and healthcare providers. The goal of this project is to develop biodosimetric assays to permit early and rapid radiation exposure assessment applicable for a forward field application.

Current project approach and aims:

Studies supplement an ongoing efforts to deliver an FDA approved proteomic biodosimetry capability by its expanding to a mixed-field (MF) radiation exposure.

- **Aim 1**: Perform mixed-field (MF, neutron + gamma) radiation validation studies and develop biodosimetry algorithm using hematological and proteomic profiles.
- **Aim 2**: Demonstrate accurate radiological detection, sensitivity and specificity of biomarker combinations to discriminate study groups into tertiles of doses: 0 -1.5 Gy, 1.5 - 3 Gy, 3 - 6 Gy.
- **Aim 3**: Characterize the response category severity score system for ARS prognosis / outcome based on biodosimetry endpoints and survival study results.
- **Aim 4**: Evaluate potential confounding effects of dose-rate and gender on use of proteomic biomarkers for biodosimetry and ARS prognosis / outcome.
- **Aim 5**: Compare MF study results with ones earlier obtained in pure-gamma studies (^{60}Co γ-rays).

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

PubMed: 1985 - 2016 (http://www.ncbi.nlm.nih.gov/pubmed/advanced)
Federal Research in Progress (FEDRIP) Database
FEDRIP: 1995 – 2016 (http://www.ntis.gov/products/databases/federal-research-in-progress/)
NIH Reporter: 1995 – 2016 (https://projectreporter.nih.gov/reporter.cfm)

II.2.2. Date of Search

1/6/2016 – 1/12/2016

II.2.3. Period of Search

PubMed: 1985 - 2016
FEDRIP: 1995 – 2016

NIH Reporter: 1995 – 2016

II.2.4. Key Words of Search

1). Biomarkers AND mixed-field radiation
2). Biological AND dosimetry AND neutrons
3). Biological dosimetry AND gamma- rays
4). Biological dosimetry AND mixed-field radiation
5). Mixed-field radiation AND biomarkers AND mice and whole-body
6). Multiple protein biomarkers AND biodosimetry AND radiation AND dose assessment
7). Multiple protein biomarkers AND biodosimetry AND mixed-field radiation
8). Acute radiation syndrome AND mice
9). Acute radiation syndrome AND mice AND whole-body
10). Acute radiation syndrome AND mice AND whole-body AND mixed-field radiation

II.2.5. Results of Search

There was no duplicative research found for the proposed study evaluating the combined blood based proteomic and hematological approach on biodosimetry endpoints (dose-response relationships for radiation dose assessment, dose-dependent discrimination index of radiation exposure, the response category severity score of the acute radiation syndrome using murine TBI model mixed-field radiation model. The only literature identified (b)(6) and regarding studies done with pure photons (^{60}Co γ-rays and x-rays). Other relevant papers were found reported: (1) measurement of multiple radiation-induced protein expression profiles (using the Luminex system) and chromosome aberrations associated with long-term health risks exposure of astronauts to a mixed-field of space radiation (i.e., protons, high charge and energy ions, neutrons, alpha particles); (2) measurements of dicentrics in peripheral blood lymphocytes and electron paramagnetic resonance (EPR) spectroscopy in tooth enamel for biodosimetry evaluated in pure photon and mixed-field (neutrons and gamma) irradiations; (3) AFRRI papers related to screening of radiation countermeasure against mixed-field (neutrons and gamma) irradiations. None of these studies duplicated the studies proposed in this protocol.

III. OBJECTIVE / HYPOTHESIS

General Hypothesis:

Multiple blood protein and hematological biomarkers along with hematological surrogates will provide enhanced diagnostically useful indices to discriminate between irradiated and non-irradiated (worried well) individuals. A panel of protein biomarkers, each with different radiation responses, coupled with peripheral blood cell counts or hematology surrogates will provide accurate assessment as well as an enhanced discrimination index of radiation exposure.

The proposed mixed-field radiation studies will complement the completed pure-photon mouse TBI studies (IACUC protocol number (b)(6) in terms of (1) develop dose-response relationships for radiation dose assessment; (2) investigate the effect of exposure to different doses of radiation on survival of mice in order to determine associations among protein expression profile, hematology parameters, body weight, and symptoms/signs related to the radiation dose and ARS prediction/outcome.

In addition, studies will evaluate potential confounding effects of dose-rate, age and gender on use of proteomic and hematological biomarkers.

IV. MILITARY RELEVANCE

Radiological terrorist attack or accidental mass-casualty exposures are highly possible. Military personnel responding to such emergencies can be exposed to radiation. Recognizing unpredictable mass casualties, U.S. Department of Defense has given top priority, as stated in the "Defense Technology Objectives (DTO)" to the development of rapid biodosimetry and medical countermeasures to radiation exposure" against both early- and late-arising health effects. In the case of mass casualties, it is difficult to screen out the severely exposed patient from the less exposed or not exposed individuals due to lack of immediate and convenient dose measurement technology.

Proposed studies targeted needs to support radiation diagnostic device (simple, rapid, high-throughput and point-of-care) development efforts based on a multiparametric biomarker-based biodosimetry diagnostic approach to facilitate treatment triage in a mass-casualty situation, and is also essential to the optimal use of scarce therapeutic resources. This strategy addresses the need for developing a high-throughput multiparametric "***Field Radiological Biodosimetry***" system and promotes effective command decisions and force structure planning to ensure mission success. This system should be compatible with military field laboratories, homeland security applications, as well as with radiation therapy centers to assess radiation exposure based on blood protein biomarkers capability. In addition, assessment of a population's exposure to other radiation threats, such as nuclear accidents and terrorism mass casualty scenarios addresses the need for a "***Clinical Radiological Biodosimetry***" system to provide physicians with the ability to triage radiation victims, make appropriate treatment decisions, and reduce uncertainties associated with the variability of individual response to radiation exposure.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

B6D2F1/J female and male 14-16 wk old mice will be used in experiments. Total-body irradiations (TBI) of mice will be performed at different radiation doses and dose-rates in the AFRRI facilities: TRIGA (Training, Research, Isotope, General Atomic) Mark-F nuclear research reactor is capable of delivering a mixed-LET field of fission neutrons and γ-rays and ^{60}Co (low-LET, pure γ-rays) with reliable and accurate physical dosimetry. Dosimetry for ^{60}Co irradiation was performed using an alanine/electron paramagnetic resonance system, with calibration factors traceable to the National Institute of Standards and Technology and confirmed by an additional check against the national standard ^{60}Co source of the UK National Physics Laboratory. Dosimetry for the reactor-produced radiation fields was performed with paired ionization chambers, as described in AFRRI Technical Report (b)(4) TBI will be given as a single exposure. Mice will be irradiated as outlined in section V.4.4.1.4. Irradiation procedure.

Statistical justification for the number of animals in each experiment is provided in section V.2. Data Analysis. In Experiments 1-4 and 6, blood from mice will be collected by intracardiac puncture or inferior vena cava at sampling time-points 1, 2, 4, and 7d after exposure. Blood for cell counts and differentials (CBC/diff) will be analyzed within several hours after biosampling using a clinical hematology analyzer (Bayer Advia 120, Bayer, Tarrytown, NY) at the AFRRI Veterinary Sciences Department (VSD) facility or sent out to Bioreliance or another approved lab as a backup. The remaining blood will be processed for protein profile (18 biomarkers) measurements in plasma using a Meso Scale Diagnostics (MSD) high-throughput (HT) MULTI-ARRAY plate-format platform as previously described (b)(6),(b)(4) Collected data will be analyzed to create the mixed-field biodosimetry algorithm and ARS prediction outcome. Results will be compared with ones earlier obtained in ^{60}Co γ-rays studies (IACUC protocol number (b)(6),(b)(4)

Summary Table 1 is for all experiments (1-6), detail Tables (2-11) are for each experiment.

Table 1. Summary Table for all experiments (1-6).

Exp. #	Experiment Title / Table #	Number of females	Number of males	Totals
1	***TRIGA mixed-field dose-response study***: (67% n + 33% γ) @ 0.6 Gy/min; TBI doses: 1.5, 3, and 6 Gy / Table 2	172	0	172
2	***TRIGA mixed field dose-response study:*** (33% n + 67% γ) @ 0.6 Gy/min; TBI doses: 1.5, 3, and 6 Gy / Table 3	172	0	172
3	***Dose-rate effect study***: ^{60}Co γ-rays @ 0.6 and 1.9 Gy/min; TBI doses: 6 and 12 Gy / Tables 4-5	202	0	202
4	***Dose-rate effect study***: TRIGA mixed-field (67% n + 33% γ) @ 0.6 and 1.9 Gy/min; TBI doses: 3 and 6 Gy /Tables 6-7	220	0	220
5	***Survival study:*** TRIGA mixed-field (67% n + 33% γ) @ 0.6 Gy/min; TBI doses: 4, 5, 6, 7 Gy / Table 8	136	0	136
6	***Gender comparison study:*** ^{60}Co γ-rays @ 0.6 Gy/min; TBI doses: 3, 6 and 12 Gy / Tables 9-10	130	130	260
	TRIGA mixed-field (67% n + 33% γ) @ 0.6 Gy/min; TBI doses: 1.5, 3 and 6 Gy / Table 11	0	140	140
Totals:		**1032**	**270**	**1302**

V.1.1. Experiment 1

Perform the dose-response radiation mixed-field (MF) (67% n + 33% γ) study (1.5, 3, and 6 Gy at dose-rate of 0.6 Gy/min) using female mice.

Table 2. Mixed-field (n + γ) radiation dose-response study design in female mice.

Groups	TRIGA (67% n + 33% γ) @ 0.6 Gy/min Number of female mice (14-16 wks) Sampling time-points after TBI				Total
	1d	2d	4d	7d	
Control	8	8	8	8	32
Sham	8	8	8	8	32
1.5 Gy	8	8	8	8	32
3 Gy	8	8	8	10	34
6 Gy	8	8	12	14	42
Totals	40	40	44	48	**172**

V.1.2. Experiment 2

Perform the dose-response radiation MF (33% n + 67% γ) study (1.5, 3, and 6 Gy at 0.6 Gy/min) using female mice.

Table 3. Mixed-field (n + γ) radiation dose-response study design in female mice.

Groups	TRIGA (33% n + 67% γ) @ 0.6 Gy/min Number of female mice (14-16 wks) Sampling time-points after TBI				**T**otal
	1d	2d	4d	7d	
Control	8	8	8	8	32
Sham	8	8	8	8	32
1.5 Gy	8	8	8	8	32
3 Gy	8	8	8	10	34
6 Gy	8	8	12	14	42
Totals	40	40	44	48	**172**

V.1.3. Experiment 3

Perform low-LET dose-rate effect study with ^{60}Co γ-rays (6 and 12 Gy and dose-rates of 0.6 and 1.9 Gy/min) using female mice.

Table 4. Low-LET (^{60}Co γ-rays) dose-rate effect (0.6 Gy/min) experimental study design in female mice.

Groups	Co-60 γ-rays @ 0.6 Gy/min Number of female mice (14-16 wks)	Total

	Sampling time-points after TBI				
	1d	2d	4d	7d	
Sham	8	8	8	8	32
6 Gy	8	8	8	8	32
12Gy	8	8	8	10	34
Totals	24	24	24	26	**98**

Table 5. Low-LET (^{60}Co γ-rays) dose-rate effect (1.9 Gy/min) experimental study design in female mice.

Co-60 γ-rays @ 1.9 Gy/min					
Groups	Number of female mice (14-16 wks)				Total
	Sampling time-points after TBI				
	1d	2d	4d	7d	
Sham	8	8	8	8	32
6 Gy	8	8	8	10	34
12Gy	8	8	10	12	38
Totals	24	24	26	30	**104**

V.1.4. Experiment 4

Perform high-LET dose-rate effect study with MF (67% n + 33% γ) doses of 3 and 6 Gy and dose-rate of 0.6 and 1.9 Gy/min) using female mice.

Table 6. High-LET (mixed-field, n + γ) dose-rate effect (0.6 Gy/min) study design in female mice.

TRIGA (67% n + 33% γ) @ 0.6 Gy/min					
Groups	Number of female mice (14-16 wks)				Total
	Sampling time-points after TBI				
	1d	2d	4d	**7d**	
Sham	8	8	8	8	32
3 Gy	8	8	8	10	34
6 Gy	8	8	12	14	42
Totals	24	24	28	32	**108**

Table 7. High-LET (mixed-field, n + γ) dose-rate effect (1.9 Gy/min) study design in female mice.

TRIGA (67% n + 33% γ) @ 1.9 Gy/min

Groups	Number of female mice (14-16 wks)				Total
	Sampling time-points after TBI				
	1d	2d	4d	7d	
Sham	8	8	8	8	32
3 Gy	8	8	10	12	38
6 Gy	8	8	12	14	42
Totals	24	24	30	34	**112**

V.1.5. Experiment 5

Perform the 30-d survival study after radiation with MF (67% n + 33% γ) to doses of 4, 5, 6, 7 Gy at dose-rate of 0.6 Gy/min) using 14 – 16-wk female old mice to investigate the effect of exposure to different doses of MF in order to find associations between protein expression profile, hematology parameters, body weight, symptoms and signs related to the radiation dose and the ARS severity and create the ARS severity score system. Compare results with ones collected earlier in ^{60}Co γ-rays studies.

Table 8. Mixed-field (n + γ) survival study design in female mice.

	TRIGA (67% n + 33% γ) @ 0.6 Gy/min					
	Number of female mice (14-16 wks)					Total
Groups	Total-body irradiation doses, Gy					
	Sham	4Gy	5 Gy	6 Gy	7 Gy	
# mice	20	20	24	28	32	124
				10% extra		12
Totals	20	20	24	28	32	**136**

Animal survival will be monitored daily for 30 days after irradiation. Based on review by the AFRRI statistician, we plan to use 20-32 mice per treatment group for measurement of animal survival endpoints. Total of 136 mice (124 plus 10% extra) will be used. The extra 10% of mice is needed in order to eliminate the animal body weight outliers. Mice will be individually identified by tattoos performed on their tails as outlined in section V.4.4.1.3 Tattoo Procedures. Observations will be recorded using the Bio Medic Data System (BMDS) electronic data recording system. Mice will be weighed on a Sartoruis ED5201 scale (BMDS data acquisition system) and the weight will be recorded to electronic data files. Clinical observations will be conducted twice daily in noncritical and up to four times per day (including late-night checks) in critical period of ARS (severe pancytopenia). Clinical signs and certain criteria selected to define the morbidity or moribundity in irradiated mice will be scored following the IACUC Policy #10 developed at AFRRI VSD (Appendix 1). The mutiparametric ARS Severity score system following by high-LET (mixed-field, n + γ) irradiations will be created similar to one created earlier in low-LET (^{60}Co γ-rays) studies (b)(6),(b)(4)

V.1.6. Experiment 6

Task 7. Perform the gender-comparison radiation studies with ^{60}Co γ-rays (3, 6, 12 Gy) and mixed-field (67% n + 33% γ) (1.5, 3, and 6 Gy) at 0.6 Gy/min.

Table 9. Low-LET (^{60}Co γ-rays) gender-comparison study design in female mice.

	Co-60 γ-rays @ 0.6 Gy/min				
	Number of female mice (14-16 wks)				Total
Groups	Sampling time-points after TBI				
	1d	2d	4d	7d	
Sham	8	8	8	8	32
3 Gy	8	8	8	8	32
6 Gy	8	8	8	8	32
12 Gy	8	8	8	10	34
Subtotals	32	32	32	34	130
				TOTAL	**130**

Table 10. Low-LET (^{60}Co γ-rays) gender-comparison study design in male mice.

	Co-60 γ-rays @ 0.6 Gy/min				
	Number of male mice (14-16 wks)				Total
Groups	Sampling time-points after TBI				
	1d	2d	4d	7d	
Sham	8	8	8	8	32
3 Gy	8	8	8	8	32
6 Gy	8	8	8	8	32
12 Gy	8	8	8	10	34
Subtotals	32	32	32	34	130
				TOTAL	**130**

Table 11. High-LET (mixed-field) gender-comparison study design in male mice.

	TRIGA (67% n + 33% γ) @ 0.6 Gy/min				
	Number of male mice (14-16 wks)				Total
Groups	Sampling time-points after TBI				
	1d	2d	4d	7d	
Sham	8	8	8	8	32
1.5 Gy	8	8	8	8	32
3 Gy	8	8	8	10	34
6 Gy	8	8	12	14	42

Subtotals	32	32	36	40		140
					TOTAL	**140**

V.2. Data Analysis

Number of animals n=8-14 per group (dose, and sampling time-point) is necessary for experiments 1 -4 and 6. It has been determined by AFRRI statistician from similar studies to provide power >90% for two-tailed Student's t-test to get two standard deviations (2 STD) or 95% confidence interval in distinguishing animal groups and less than 10% shifts in biomarker values (reproducibility). Values of $P < 0.05$ will be considered statistically significant. An increase in the number of animals per group up to n=10-14 for TBI doses of ≥3 Gy (TRIGA) and ≥ 6 Gy (^{60}Co) at sampling time-points 4 and 7d is necessary in order to provide the sufficient statistical accuracy because at this dose and later time points there is expected to be potential early morbidity (from 30% to 70% in the 30-d monitoring period) by developing hematopoietic syndrome as well as a broadening of the distribution of radiation responsive biomarkers. Control mice will not be placed into boxes nor transported to the radiation facilities. Comparison of results for sham groups and control groups evaluated any effect of stress induced by handling of mice as it was performed in earlier ^{60}Co studies (b)(6),(b)(4) In 30-day survival study (Experiment 5), we plan to use 20-32 mice per treatment group for measurement of animal survival endpoints. Number of mice will be increased from 20 to 32 due to statistical justification provided below.

The 20 mice per group could have 80% power to detect a significant difference between two groups, given type I error of 5% and a treatment group survival rate of at least 73%, if the control group is 25%. Similar statements would apply, provided the treatment groups display survival of at least 86%, 83%, or 78%, if the respective control groups would be 40%, 35%, and 30%, respectively. Note: power analysis may suggest smaller groups are adequate. However, radiobiologists at AFRRI and elsewhere observe considerable variability from experiment to experiment in mouse survival studies that is not reflected in the power analysis. In fact, some institutions have gone to a group size of 30 in these types of experiments for this reason.

Statistical software, PC SAS, will be used for statistical data analysis (SAS Institute Inc., 2000). Multivariate analysis of variance (MANOVA) Wilks' Lambda statistics will be used when comparing more than two groups and two-sided Student's *t* test will be used when comparing two groups to determine significant differences among observational time-points in radiation biomarkers studies and ARS severity degree categories as previously described (b)(6),(b)(4)

Multiple linear regression analysis will be used to develop dose-response relationships for combination of hematological and protein biomarkers for radiation dose assessment at the 95% confidence level. Multivariate discriminant function analysis of PC SAS will be used to demonstrate accurate radiological detection of the proposed protein and hematological biomarkers from biological samples into tertiles of doses 0-1.5 Gy, 1.5-3 Gy, and 3-6 Gy and also to demonstrate which combinations of proposed protein and hematological biomarkers will better reflect the subgroup differences. Receiver Operating Characteristic curve (or ROC curve) will be used to demonstrate the sensitivity and specificity of the proposed protein and hematological biomarkers to

reflect subgroup (dose and sampling time-point) differences. Results will be shown as a ROC plot of the true positive rate against the false positive rate for the different possible cut-points of a diagnostic test. Kaplan-Meier survival curves will be constructed to determine the survival time probability to estimate the LD30/30, LD50/30, and LD70/30. The Table Curve 3D statistical software will be used to create the dose- and time-dependent fitting equations for ARS scores as prognostic indexes of ARS outcome. Biomarker results and ARS severity score system received in high-LET (mixed-field) experiments will be compared with ones received in irradiations performed with low-LET (^{60}Co γ-rays)[(b)(6),(b)(4)]

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

Currently, there are no alternatives in existence that can predict or compute the complexity of the response to total body irradiation. Computer modeling and cell cultures are insufficient to determine whether or not a given radiation exposure will result in a characteristic radio-response of molecular changes, protein expression of blood or other tissues. An integrated, functional, computer model of a mammal has not yet been achieved for the purpose of examining intricate physiological interactions among cells, tissues, and organs that occur after exposure to ionizing radiation. The resiliency of organs, such as the hematopoietic system or gastrointestinal system, to ionizing radiation depends on a complex network of interactive signaling systems to sense the magnitude of tissue damage, and to initiate repair, recovery, and other defense processes. The physiology of these interacting networks is altered by radiation in ways that cannot yet be modeled. Alterations of expression of the proteins we propose to measure are influenced by the same complex network of regulatory controls that cannot be duplicated outside of use of living animals.

V.3.2. Animal Model and Species Justification

The mouse model was chosen for this *in vivo* validation study for the following reasons: (1) it is well-defined immunological animal model for this type of research; (2) qualitative similarities exist between human and mouse proliferative tissue including bone marrow (Thompson 1962); (3) molecular responses of mouse and human peripheral blood to gamma radiation are expected to be similar; (4) the ease of use of a mouse model system ensures reliable data collection; (5) median lethal doses (LD50/30) for radiation-induced death for this strains of mice is known (Hendry 1995;[(b)(4)] [(b)(4)] (6) model provides experimental and statistical validity; (7) AFRRI staff has an extensive experience with mice, including B6D2F1 mice, in radiation injury and countermeasure research. In addition, B6D2F1 mice were used in previous studies (^{60}Co γ-rays, protocol number[(b)(6)]

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species

Mice (*Mus musculus)*

V.3.3.2. Strain / Stock
B6D2F1/J

V.3.3.3. Source / Vendor
B6D2F1/J mice: Areas AX-27 or AX-28, The Jackson Laboratory, 600 Main Street, Bar Harbor, Main 04609-1500, Tel: 800-422-MICE or 207-288-5845, Fax: 207-288-6150, www.jax.org <http://www.jax.org/> or any other approved vendors.

V.3.3.4. Age
Age at irradiation from 14 to 16 wks; 11-12 wk of age at purchase, 14-16 wk of age at use.

V.3.3.5. Weight
Approximately 24 to 26 g (20-22 g upon arrival, 23 g or more at the time of experimental manipulation).

V.3.3.6. Sex
Males and females.

V.3.3.7. Special Considerations
Commercially procured animals must be adventitious disease free, including: *Pseudomonas aeruginosa* and *Pasteurella*. All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus, *Mycoplasma pulmonis*, and *Clostridium piliforme.*

V.3.4. Number of Animals Required (by species)
Total number of mice: 1302.

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement
In this protocol, some radiation doses (≥ 6 Gy) are sublethal, minimizing discomfort due to ARS. Project personnel will take necessary steps to be highly trained in handling techniques (irradiation procedure, tail-tattooing, blood collection, and routine cage-side observations) to minimize distress and pain to the animals. In a survival study, in order to minimize discomfort when animals become morbid, investigators and animal care staff will examine the mice at least twice daily, including the early morning and late afternoon. Number of observations will be increased as mice irradiated to sublethal doses will be in a critical period (pancytopenia). Moribund animals will be considered to have arrived at the study endpoint and will be euthanized as included in Appendix 1

(sample scoring sheet for clinical observations with criteria for rodent euthanasia), an IACUC Policy #10 (Guidelines for establishment of early endpoints in experiments with expected mortality).
Analgesics were considered as a way of minimizing discomfort during the onset of opportunistic infections, which will occur during the survival experiments. However, analgesics and anesthetics are well-known to alter the functional properties of the immune system and resistance to infection (Galley et al. 1997; Elena et al. 1997; Beilin et al. 1996), which would defeat the purpose of the study and results will not be comparable with ones obtained in earlier ^{60}Co studies (IACUC protocol number (b)(6) (b)(6)

V.3.5.2. Reduction
Control groups will be shared between experiments wherever possible. The maximum number of assays will be performed on each tissue sample, minimizing the number of required animals. Tissues from these animals are available for sharing with other investigators. A statistician has reviewed this protocol to ensure that the minimum number of animals will be used to gain statistically significant results. We will collect multiple data from individual animals, providing information on biomarkers and microbiological and hematological endpoints.

V.3.5.3. Replacement
None. It is not feasible to use non-animal systems to address the questions in this project.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals: 1302

V.4.1.1.1.1. Column C 372 (# of animals)

V.4.1.1.1.2. Column D 0 (# of animals)

V.4.1.1.1.3. Column E 930 *(# of animals)*

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1	TRIGA dose-response: (67% n + 33% γ) @ 0.6 Gy/min	64	0	108	172
2	TRIGA dose-response: (33% n + 67% γ) @ 0.6 Gy/min	64	0	108	172
3	Dose-rate effect: ^{60}Co γ-rays @ 0.6 and 1.9 Gy/min	64	0	138	202
4	Dose-rate effect: TRIGA (67% n + 33% γ) @ 0.6 and 1.9 Gy/min	64	0	156	220
5	Survival study: TRIGA (67% n + 33% γ) @ 0.6 Gy/min	20	0	116	136

Exp. #	Experimental/Control Group	C	D	E	Totals
6	Gender comparison: ^{60}Co γ-rays and TRIGA (67% n + 33% γ) @ 0.6 Gy/min	96	0	304	400
Totals:		**372**	0	**930**	**1302**

E. Justification if animals have been added to "E" category:
There are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. Radiation itself does not cause pain or distress. A number of animals in these experiments will receive either sublethal or near-lethal doses. These dose levels will lead to the clinical syndromes following irradiation, which may lead to distress/pain and moribundity. Moribund animals will be considered to have arrived at the study endpoint and will be euthanized as included in Appendix 1 (sample scoring sheet for clinical observations with criteria for rodent euthanasia), an IACUC Policy#10 (Guidelines for establishment of early endpoints in experiments with expected mortality).

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization
In these studies, experiments will be conducted with a minimal supportive care in order to simulate a catastrophic event involving large number of casualties. In this scenario, there will be limited medical and physical resources. Systemic antibiotics, anesthetics or analgesics will not be used as in previous radiation only (not combined injury) studies (IACUC protocol number (b)(6) If wounding occurs via in-cage fighting, topical antibiotics will be applied under the guidance of the veterinarian in accordance with standard treatment measures.

Anesthesia using standard isoflurane rodent anesthesia machine under the guidance of the VSD will be carried out in mice for blood collection. Animals will be placed in the induction chamber and delivered a metered amount of 3-5% isoflurane mixed with 100% oxygen (at the flow rate of 500-1000 cc/min) until all voluntary motor movement ceases and the animal is recumbent for 10 seconds. Animal's respiration rate will be closely monitored through the Plexiglas chamber during induction of anesthesia. The toe pinch response will be used to determine the depth of anesthesia. The tail will be pinched for reflexive movement, indicative of insufficient anesthesia. If there is no response, the animal will be moved to the station with individual nose cone for maintenance of anesthesia at 1-3% isoflurane and 100% oxygen at 500-1000 cc/min to perform blood collection.

V.4.1.2.2. Pre- and Post-procedural Provisions
Mice will be housed and monitored in accordance with VSD standard operating procedures both before and after irradiation procedures (see section V.5. Veterinary Care). For special husbandry considerations post-procedural, see section V.5.1.2.
Project personnel will examine the mice at least twice daily and number of observations will be increased in a critical period of ARS. Moribund animals will be euthanized if criteria will be met as included in Appendix 1 (sample scoring sheet for clinical observations with criteria for rodent euthanasia). Euthanasia will be conducted as outlined in section V.4.6. Euthanasia.

V.4.1.2.3. Paralytics
NA

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched
AGRICOLA and PubMed.

V.4.1.3.2. Date of Search
1/12/2016

V.4.1.3.3. Period of Search
AGRICOLA: 2009 - 2016
PubMed: 1995-2016

V.4.1.3.4. Key Words of Search
Ionizing radiation AND pain, neutron AND pain, mixed-field AND pain, alternative, humane, alternative methods to blood collection.

V.4.1.3.5. Results of Search
There are no alternatives to irradiation. Irradiation itself is not a painful process, but it induces various changes in the body, and kills hematopoietic cells. In irradiated animals, the immune response is compromised, and opportunistic infections and multi-organ dysfunction/failure may occur. Results of literature search clearly demonstrate that use of analgesics alters radiation response and therefore leads to increase in animal number for better statistical results or for iteration of entire experiments.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification
Animals irradiated to sub- or lethal doses become immune compromised and might die due to opportunistic infections and /or multi-organ dysfunction/failure. We anticipate that animals exposed to sub- or lethal radiation doses will become moribund. As discussed above, in order to compare results with earlier studies (IACUC protocol number (b)(6) (b)(6) we will not give anesthetic and/or analgesic agents to animals since they are known to interact with the immune system, and would confound the interpretation, resulting in a waste of animals (see references in section V.3.5.1). All moribund animals will be euthanized as outlined in section V.4.6 Euthanasia. As mentioned in section V.4.1.2.1. Anesthesia / Analgesia / Tranquilization, systemic antibiotics, anesthetics or analgesics will not be used. The topical antibiotics will be applied under the guidance of the veterinarian in accordance with standard treatment measures if wounding occurs via in-cage fighting of mice.

V.4.2. Prolonged Restraint
No prolonged restrain.

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions
NA

V.4.3.2. Procedure(s)

Collection of blood and tissues.
Mice will be deeply anesthetized in the anesthesia machine. The toe pinch response will be used to determine the depth of anesthesia. The tail will be pinched for reflexive movement, indicative of insufficient anesthesia. If there is no response, the animal will be moved to the station with individual nose cone for continued anesthesia. Blood from mice anesthetized by isoflurane inhalation will be drawn by either cardiac stick or from the inferior vena cava using a heparinized 23-25 gauge sterile 5/8" needle attached to 1-3-ml syringe. Typically 0.7-0.9 ml of blood is collected; sometimes more than 1 ml in non-irradiated (sham/control) mice, less blood is available after radiation exposure. After blood collection, animals will be euthanized as outlined in section V.4.6. Euthanasia. Tissues will be made available to other investigators (see also in V.4.4.2. Biosamples).

V.4.3.3. Post-surgical Provisions
NA

V.4.3.4. Location
NA

V.4.3.5. Surgeon
NA

V.4.3.6. Multiple Major Survival Operative Procedures

V.4.3.6.1. Procedures
NA

V.4.3.6.2 Scientific Justification
NA

V.4.4. Animal Manipulations

V.4.4.1. Injections
NA

V.4.4.1.1. Pharmaceutical Grade Drugs
NA

V.4.4.1.2. Non-Pharmaceutical Grade Drugs
NA

V.4.4.1.3. Tattoo procedure:

Tattooing of mice in survival study will be done either with a Labstamp machine Labstamp system following the SOP provided by the company or any other machine certified by VSD.

V.4.4.1.4. Irradiation procedure:
Total-body irradiations (TBI) of mice will be performed at different radiation doses and dose-rates in the AFRRI facilities: TRIGA reactor (mixed-field, n + γ) and ^{60}Co (pure γ). TBI will be given as a single exposure. Animal irradiations will be arranged in an array according to the latest dosimetry map provided by the Radiation Sciences Department. For irradiation of mice at the ^{60}Co facility, animals will be placed into well-ventilated Plexiglas boxes and irradiated bilaterally. For irradiation of mice in the reactor, animals will be inserted into aluminum holders placed onto a carousel that rotates the mice within the exposure beam to ensure uniform exposures to all subjects. Sham-irradiated mice will undergo the same procedures as the irradiated mice (transport to radiation facility, placement into Plexiglas box or aluminum cylinder), minus the radiation exposure. In the reactor, non-neutron components of the exposure are controlled by shielding around the carousel. All irradiations will be performed before noon in order to be consistent with studies earlier performed (IACUC protocol (b)(6) It is important because the radiation sensitivity and survival rate varied on time of irradiation (morning, midday, or afternoon) as reported by Plett and colleagues (Plett et al. 2012). Mice will be restrained during the irradiation procedure. They will be inserted into the standard radiation boxes or aluminum holders just prior to irradiation (~15 min of exposure time) and taken to the radiation facilities for irradiation. The restraint time should be less than 60 minutes. After irradiation, mice will be returned to VSD and placed to cages with a fresh bedding/food/water for further monitoring. Mice will be identified by cage cards using VSD SOP Animal Identification and Accountability.

All personnel involved in reactor irradiation experiments will remain current with respect to radiation safety training and will continue to receive annual radiation worker training. They will also undergo all required specialized training provided by Health Physics Department (HPD), and Radiation Sciences Department (RSD), including AFRRI reactor exposure room training given by the reactor facility staff. Appropriate personal dosimeters will be worn at all times when working in reactor area. This includes the standard personal thermoluminescence dosimeter (TLD) along with a self-reading pocket chamber dosimeter to serve as a backup.

All reactor-irradiated mice, their tissues, and their bedding will be considered radioactive unless declared otherwise as a result of HPD surveys. Source accountability and ALARA principles will be adhered to at all times, and the SWIMS procedure will be followed in case of any spills of potentially radioactive materials.

V.4.4.2. Biosamples
Blood (as in the project Statement of Work) and tissues (bone marrow harvested from femurs and legs, spleen, lung, liver, sternums, GI, thymus, and kidney will be shared with other investigators upon request). Biosamples will be collected as outlined in section V.4.3.2. Procedure(s) Collection of blood and tissues. After blood collection, animals will be euthanized as outlined in section V.4.6. Euthanasia.

V.4.4.3. Adjuvants
NA

V.4.4.4. Monoclonal Antibody (MAb) Production
NA

V.4.4.5. Animal Identification
Mice will be housed in groups of 4-6 per cage. They will be identified by cage cards using GLP SOP Rodent Cage Cards. Each card states the investigator's name, protocol number, experiment number, cage number, start date, end date, species, item number (animal lot), birthdate, gender, strain, number of animals in the cage, vendor, arrival date, treatments, and dates of death, including the initials of the staff person who found and removed the dead animals. In addition, mice in Experiment 5 (survival study) will be identified by tattoo for individual tracking (see section V.4.4.1.3).

V.4.4.6. Behavioral Studies
NA

V.4.4.7. Other Procedures
NA

V.4.4.8. Tissue Sharing
Tissues will be made available to other investigators at the time of scheduled euthanasia.

V.4.4.9. Animal By-Products
NA

V.4.5. Study Endpoint
The endpoint of the studies will be euthanasia at the predetermined terminal blood collections (Experiments 1-4 and 6) and based on assessment of moribundity (Experiment 5). Clinical observations for pain and distress in mice will be monitored by project personnel at least twice daily and the number of observations will be increased in a critical period of ARS (pancytopenia). This monitoring schedule includes weekends and holidays. To minimize animal pain or distress, moribund animals will be scored according to sample scoring sheet for clinical observations with criteria for rodent euthanasia (Appendix 1) and euthanized as outlined in section V.4.6. Euthanasia. Mice that survive the 30-d monitoring period after irradiation will be euthanized. Sham mice from survival study may be transferred to another approved protocol.

V.4.6. Euthanasia
In the dose-response study (Experiments 1-4 and 6), for terminal blood sample collections, mice first will be deeply anesthetized with isoflurane and then euthanized by cervical dislocation that will be performed by protocol personnel. In the survival study (Experiment 5), irradiated mice that survived monitoring period (30 days) will be euthanized. Moribund animals that met IACUC Policy #10 euthanasia criteria will be euthanized by VSD staff or protocol personnel. Euthanasia of mice will be performed by

CO_2 inhalation followed by cervical dislocation and their carcasses will be disposed according to VSD SOPs.

V.5. Veterinary Care

V.5.1. Husbandry Considerations

All animals will be housed and maintained in accordance with the "Guide for the Care and Use of Laboratory Animals" and AFRRI VSD Standing Operating Procedures. Mice will be placed in groups of 4 – 6 per cage during experiments. Animals will receive Harlan Teklad Rodent Diet (w) 8604 and water *ad libitum*. Acidified water (pH 2.5 – 3.0) is routinely provided to AFRRI mice used in radiological experiments. Cages will be regularly changed and cleaned by VSD staff and project personnel (see V.5.1.2. Special Husbandry Provisions for mice irradiated with reactor) in accordance with Standard Operating Procedure. Animals will have 12 h light/dark cycle. In the event of procedures that last into the night cycle, care will be taken to minimize exposure to light by using red-lens flashlights.

V.5.1.1. Study Room

As assigned by VSD, study room number will be (b)(6) within the vivarium.

V.5.1.2. Special Husbandry Provisions

Reactor-irradiated animals will be monitored for radiation levels by the Radiation Safety staff. The activated animals will be marked with radiation activity indicator cards that will be removed after natural activity of the isotopes results in levels of radiation not distinguishable from background (estimated to be ~24 hours, judging from past experience of other investigators at AFRRI).

The room (b)(6) housing the irradiated mice will be designated a "radiation room" by RSD and HPD, labeled as such, and access will be restricted to properly trained staff until declared clear by RSD/HPD radiological surveys. Standard radiological room operating procedures will require removal of all PPE before exiting the room and surveying all personnel, equipment, and materials for radioactivity with a Geiger-Müller counter before allowing them to be removed from the room. Following standard operating procedures in sample collections, gloves will be worn at all times when handling the irradiated mice. Finger ring dosimeters will be worn by all personnel handling irradiated mice until they have been declared non-radioactive by HPD. Before mice have been declared non-radioactive by HPD, carcasses and bedding will be disposed by project personnel in the Rad Waste Facility area designated for such material. Irradiated mice remains will be disposed of in the Radiation Safety Storage area designated for such material until the mice can be surveyed for radiation hazard level before final disposal.

V.5.1.3. Exceptions

Mice will be socially (group) housed, except for the possibility that during survival study cagemates may be lost through attrition and for a period of time the sole surviving mouse may remain singly housed until the end of the 30-day study. When this occurs, singly housed animals will be able to view con-specifics in neighboring cages housed on the same rack in the same room.

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care

The animals will be observed daily by protocol personnel. In the critical phase, mice will be monitored three times daily by investigative staff in conjunction with VSD personnel with no more than 10 hours between the late night and early morning check as per IACUC Policy #10. As observations in the Experiment #5 (survival study) also include the weighting of mice, late night, weekend, and holiday checks will be done by investigative staff. Any moribund mice will be euthanized as soon as possible. If needed, a VSD veterinarian will be contacted for decision to euthanize.

V.5.2.2. Emergency Veterinary Medical Care

Emergency veterinary medical care is available 24 hours a day, seven days a week by VSD veterinarians and technicians and will include immediate euthanasia of any moribund mice. The on-call roster is located outside the VSD conference room (b)(6) (b)(6) on the bulletin board and at the security watch desk. These procedures are in accordance with VSD SOP (Duty Roster and Responsibilities of the "On-Call" Veterinary Officer and Veterinary Technician).

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

The animals will be housed in standard/conventional rodent caging. Mice will be group-housed, 4 - 6 mice per cage, with cotton bedding nestlets as per AFRRI VSD SOPs. Plastic tubes, hutches etc. will be provided once mice have been declared non-radioactive by SHD.

V.5.3.2. Enrichment Restrictions

Plastic tubes, hutches etc. will be not be provided to use immediately after irradiation to avoid contaminating the enrichment devices, but will be introduced once SHD has declared the mice non-radioactive.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3, 4a, 4b, 6 (micro-chips between shoulder blades), 7 (loading into radiation boxes)	(b)(6)
				1, 3, 4a, 4b, 5, 7 (loading into radiation boxes)	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv

Code 4= Blood collection under anesthesia (4a-cardiac, 4b-inferior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY

See V.5.1.2. Special Husbandry Provisions. See also attached AFRRI Form 310.
The primary radiation hazard to workers arises from potential exposure to tissues made radioactive as a result of neutron activation of various elements within those tissues. Activation products of primary concern include Na-24, Cl-38, and P-32, because of their relative abundance in living cells (see AFRRI Form 310).
Those isotopes have a short half-life time (few hours) and were reported to be neutron-activated at the very low residual radioactivity (<0.5 uCi) (Hall DE, 2009. Modeling and Validation of Dosimetry Measurement Assumptions Within The Armed Forces Radiobiology Research Institute TRIGA Mark-F Reactor and associated Exposure Facilities using Monte Carlo Techniques.
All protocol personnel will have a VSD safety training, general radiation users training and additional ones related to irradiations with reactor. Protocols for clean-up of small spills (alcohol, acids, bases, solvents, biological samples, etc.) are maintained in the Biodosimetry Research Group SOP Protocols Binder located in laboratory (b)(6) The reporting of small spills generally includes notification of protocol PI and HPD staff.

VIII. ENCLOSURES

1. AFRRI Form 310

X. ASSURANCES

Protocol Title:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. **Responsibility:** I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. **Scientific Review:** This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. **Painful Procedure(s):** I **AM** / **AM NOT** (*circle one*) conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL** / **WILL NOT** (*circle one*) be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) Ph.D.

Principal Investigator (Printed Name) Principal Investigator (Signature) (Date)

References:

(b)(4)

Beilin, B., et al., Effects of anesthesia based on large versus small doses of fentanyl on natural killer cell cytotoxicity in the perioperative period. Anesth Analg, 1996. 82: p. 492-7.

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Elena G, Puig NR, Bay ML, Urízar L, Barragán J, Comba J, Amerio N. Inhalatory anesthetic (halothane) associated changes in the immune response in mice. International Journal of Immunopharmacology, 1997. 19: p. 699-707.

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Hall EJ and Giarcia AJ, 2011. Radiobiology for the Radiologist, 7th Edition.

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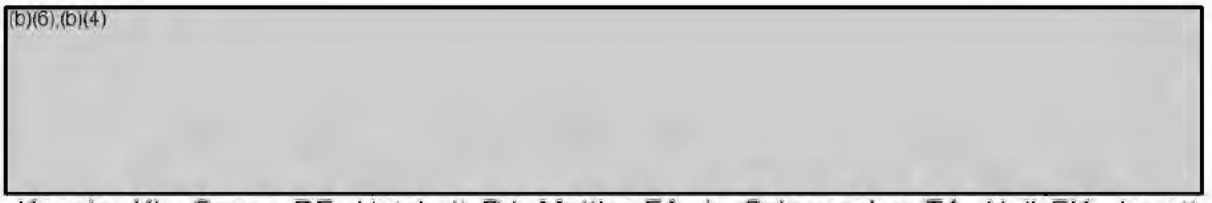
Galley, H.F. and N.R. Webster, Effects of propofol and thiopentone on the immune response [letter; comment]. Anaesthesia, 1997. 52: p. 921-3.

(b)(4)



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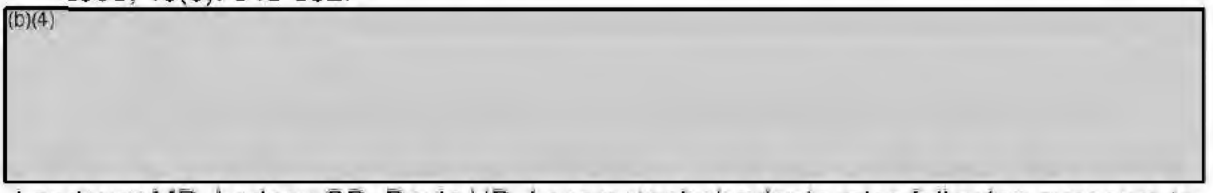

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(b)(6),(b)(4)

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(b)(4)

Appendix 1
Sample Score Sheet for
Clinical Observations with Criteria for Rodent Euthanasia

DATE:	Time:	Animal ID#	Score
Appearance:			
	Normal (smooth coat, clear eyes/nose)		0
	Hunched and/or fluffed		1
	Ocular discharge, and/or edema		3
	Soft stools (fecal matter around anus)		5
	Pale, white mucus membranes/skin**		6
	Bloody diarrhea		9
	Blue mucus membranes/skin (cyanosis)*		12
Respiratory Rate:			
	Normal breathing		0
	Increased breathing (double normal rate, rapid, shallow)		6
	Abdominal breathing (gasping +/- open mouth breathing)*		12
General Behavior:			
	Normal (based on baseline observations)		0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)		1
	Decreased mobility		2
	Ataxia, wobbly, weak**		6
	Inability to stand*		12
Provoked Behavior:			
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))		0
	Subdued; responds to stimulation (moves away briskly)		1
	Subdued even to stimulation (moves away slowly)		3
	Unresponsive to gentle prodding **		6
	Does not right when placed gently on side within 5 seconds*		12
Weight Loss: (Only to be used if the total summed score is ≥ 6 in the other four categories. Said weight should not be taken daily, but rather every few days.):			
	Normal < 20%		0
	20-25%		3
	26-30%**		6
	31-34%**		9
	≥35%*		12
TOTAL			_____

**** Regardless of total score, notify appropriate veterinary staff personnel immediately.**

*** Regardless of score, immediately euthanize (death is imminent)**

Total Score:

< 6	Normal
6 - 11	Morbid: See VI.D.2 and VI.D.4 in IACUC Policy #10 for guidelines
≥ 12	Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*). Any single criteria of 12* euthanize immediately; consider as 'found dead.'

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (December 2015)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

***** DELETE THIS PAGE PRIOR TO PROTOCOL SUBMISSION *****

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	3/11/2016	2nd Resubmission Date:	
Approved/**Returned for Revision**:	3/30/2016	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	4/21/2016	**EXPIRATION DATE:**	4/25/2019
Approved/Returned for Revision:	4/26/2016	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

NOTES to PI:

- To DISPLAY this Word document's red-text instructions, single-click Word's Show/Hide button (shown, right), located in Word's top-of-page panel (click Word's "Home" tab if Show/Hide isn't displayed).
- To HIDE the red-text instructions, single-click the Show/Hide button again.

- For each section and *subsection*, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: Evaluate the radioprotective and mitigative effects and mechanisms of γ-tocotrienol (GT3), δ-tocotrienol (DT3) and (b)(4) as novel radiation countermeasures in *Mus musculus*

IV. PRINCIPAL INVESTIGATOR:

(b)(6)
Radiation Countermeasures, SRD, AFRRI
Telephone: (b)(6)
(b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD** Date
Scientific Advisor; Head, Scientific Research Department, AFRRI
Telephone: (b)(6)
(b)(6)

- Or –

(b)(6) **PhD, Lt Col, USAF** Date

(b)(6) Scientific Research Department, AFRRI

Telephone: (b)(6)

(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician, AFRRI

Telephone: (b)(6) Fax: (b)(6)

(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, COL, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI

Telephone: (b)(6) Fax (b)(6)

(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Safety and Occupational Health Specialist, USUHS

Telephone: (b)(6)

(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.

(Date)

(b)(6) **PhD, Lt Col, USAF**

IACUC Chair: (b)(6) Scientific Research Department, AFRRI

Telephone (b)(6)

(b)(6)

PROTOCOL TITLE: Evaluate the radioprotective and mitigative effects and mechanisms of γ-tocotrienol (GT3), δ-tocotrienol (DT3) and (b)(4) as novel radiation countermeasures in *Mus musculus*

PRINCIPAL INVESTIGATOR: (b)(6)
CO-INVESTIGATOR(S):
AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER: (b)(6)

I. NON-TECHNICAL SYNOPSIS

Nuclear attacks and terrorism are serious security threats. Exposure to ionizing radiation (IR) causes rapid hematopoietic, gastrointestinal (GI), skin, and vascular system damage, and can result in acute radiation syndrome (ARS). This multiple organ failure and mortality can result in human and animal death. There is an urgent need in developing effective and non-toxic countermeasures to safeguard military personnel and civilians against the effects of IR exposure. We and others have reported that the vitamin E isomers γ-tocotrienol (GT3) and δ-tocotrienol (DT3) have significant radioprotective effects. Currently, (b)(4) (b)(4) (b)(4) Side by side comparison of the toxicity and survival efficacies of (b)(4) DT3 and GT3 in mice after total-body gamma-radiation will be evaluated in this study. This application is complementary to that effort and will compare mechanisms of efficacy of the three countermeasure candidates. Successful understanding of the effects and mechanisms of tocotrienols on radioprotection and mitigation would increase survival of personnel who are victims of radiation exposure scenarios.

II. BACKGROUND

II.1. Background

Today, nuclear proliferation, terrorist activity, and the distribution of nuclear and radioactive materials make incidents involving radiation injuries of increasing concern. Exposure to IR causes damage to DNA, protein, and lipids in mammalian cells, with subsequent cell cycle checkpoint arrest, apoptosis, and necrosis. Hematopoietic and gastrointestinal (GI) systems are the most radiation sensitive organs. In humans, IR exposures doses above 1 Gy can pose a risk of destruction of the bone marrow (BM) and damage to the hematopoietic system, leading to decreases in blood cell and platelet counts, long-term compromised immune function and increased susceptibility to infection and internal hemorrhage. The acute generalized GI syndrome appears after high-dose (≥ 10 Gy) total-body irradiation (TBI) (*1*, [redacted]) Radiation induces loss of (b)(4),(b)(6) intestinal crypts, damages crypt stem cells, and breakdown of the GI mucosal barrier, which produce symptoms including nausea, vomiting, diarrhea and abdominal cramping (*3*). The number of crypts that survive following radiation damage determines how intact the intestinal mucosa is and, hence, how well an animal can survive the damage. Injury after prompt IR of hematopoietic and GI tissue is due to DNA damage and apoptosis in their cells occurring over a period of hours to days (*4, 5*). The mechanisms of the IR-induced multiple organ cellular and molecule damage and mortality are extremely complex and effective medical countermeasures are lacking(*6*).

Vitamin E is an essential nutrient that includes eight distinct analogs: α-, β-, γ-, and δ-tocopherols; and α-, β-, γ-, and δ-tocotrienols which differ in the number and position of the methyl groups on the chroman ring and differ in the side chain attached to the chroman ring which could be associated with their function. Most previous studies have been conducted with tocopherols, the most commonly used vitamin E supplement and the most abundant vitamin E isoform in human and animal tissue. During the last few decades, tocotrienol research has gained substantial momentum. Tocotrienols have shown neuroprotective, anticancer, anti-oxidative stress and cholesterol-decreasing effects that are often not exhibited by tocopherols (*7, 8*). However, the mechanisms are not understood. Some studies suggested that tocotrienols' anticancer properties were through apoptotic mechanisms and regulation of PI3K/Akt, NFkB, and MAPK signaling (*9, 10*). Further, many research reports revealed that tocotrienols induced apoptosis preferentially in cancer cells but not in normal cells (*11, 12*).

(b)(4)

Pure DT3 and GT3 (desmethyl tocotrienols) are both derived from three most common natural plant sources: palm, rice bran, and annatto seeds (*7, 17*). (b)(4)

(b)(4)

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched

NIH Reporter, PubMed

II.2.2. Date of Search

December 17 to December 29, 2015

II.2.3. Period of Search

All available years. NIH Reporter and PubMed: includes MEDLINE (1966–present) and OLDMEDLINE (1950–1965)

II.2.4. Key Words of Search

Tocotrienol

γ-tocotrienol (GT3) or δ-tocotrienol (DT3) or (b)(4)

GT3 or DT3 or (b)(4) and radiation

GT3 or DT3 or and hematopoietic cells and radiation damage

GT3 or DT3 or and gastrointestinal tissue and radiation damage

GT3 or DT3 or and radiation countermeasures

GT3 or DT3 or and stress signaling

II.2.5. Results of Search

Articles in all tocotrienols studies = 1217

Articles in GT3 /or DT3 /or (b)(4) studies = 354/178/1

Articles in GT3 and radiation studies = 33

Articles in DT3 and radiation studies = 11
Articles in DG and radiation studies = 0
Articles in GT3 and radiation and hematopoietic cells studies = 10
Articles in DT3 and radiation and hematopoietic cells studies = 3
Articles in GT3 and radiation and gastrointestinal cells studies =4
Articles in DT3 and radiation and gastrointestinal cells studies = 1
Articles comparing effects of GT3 and DT3 on radiation injury = 0
Articles comparing effects of GT3, DT3 and (b)(4) on radiation injury = 0

The proposed study has no overlapping with published data.

III. OBJECTIVE / HYPOTHESIS:

An approved collaborative study (under the purview of the TT) was commenced in FY15; side by side comparison of the survival efficacies of (b)(4) DT3 and GT3 in mice after TBI will be evaluated (IACUC protocol number (b)(6) This application is complementary to that effort. In the present study, we propose to evaluate and compare the effects of GT3/DT3/(b)(4) on protection (given before irradiation) and mitigation (given post-irradiation) of γ-irradiated hematopoietic and gastrointestinal systems using a mouse model. The proposed study will explore the following hypotheses: (1) (b)(4) may have greater radioprotective and/or mitigative effects than highly purified single T3 candidates alone; (2) radioprotective and mitigative effects of T3s may be through different signal pathways in irradiated mice.

IV. MILITARY RELEVANCE Military personnel are potentially exposed to ionizing radiation in various scenarios. However, no safe and non-toxic radioprotective agent is available for military use to date. Developing countermeasures to radiation exposure is a top priority for the U.S. Department of Defense. We and others have reported that the vitamin E isomers γ-tocotrienol (GT3) and δ-tocotrienol (DT3) have significant radioprotective effects. Currently (b)(4)
(b)(4)

Thus, we propose to conduct a study to further explore, evaluate and compare mechanisms and efficacy of the three countermeasure candidates (GT3, DT3 and (b)(4) on survival of the mouse hematopoietic and gastrointestinal system after different doses of TBI.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

Mice: Animals will be randomly grouped (criteria-weight within 10% etc.) for different treatments prior to each experiment and re-housed (6-8 to animals per cage).

Drug preparation and administration in mice model: The candidate T3s, highly purified DT3, highly purified GT3, and (b)(4) will be administered at doses of 100 mg/kg as a single subcutaneous (sc) dose to mice 24 h before or 6 h or 24 h after exposure to whole-body gamma radiation as described below. The drugs will be solubilized in saline with 5% Tween-80 that also served as the vehicle control. SC injection will be performed as outlined in section V.4.4.1 Injections. The optimal doses of these T3s were obtained from the aforementioned survival study by AFRRI Tocol Team.

Irradiation: Mice will be placed in ventilated Plexiglas containers and exposed bilaterally to gamma-radiation from the AFRRI cobalt-60 source at doses of 0.0 (sham-irradiation), 7, 8, 9, 10 or 11 Gy at a dose-rate of 0.6 Gy/min. Sham-irradiated mice will be treated exactly the same way as the gamma-irradiated animals, except the cobalt-60 sources will not be raised from shielding water pool.

V.1.1. Experiment 1 Compare the radioprotective and mitigative effects of (b)(4) DT3, and GT3 (b)(4) DT3/GT3) on the mouse hematopoietic system

a) CD2F1 mice (6 animals + 2 phantoms/group) will be placed in ventilated Plexiglas and exposed bilaterally to γ-radiation from the AFRRI cobalt-60 source at doses of 0.0 (sham-irradiation), 7, 8 or 9 Gy (b)(4) DT3/GT3 (100 mg/kg) or vehicle control will be administrated as a single subcutaneous (sc) dose to mice 24 h before or 6 h or 24 h after irradiation. After irradiation, mice will be returned to their specific cages.

b) After TBI, animals will be anesthetized prior to blood collection and then euthanized for tissue collection at indicated time points. For all assays, blood cells, bone marrow (BM), thymus, spleen, and jejunum tissue will be collected from the sham-irradiated and γ-irradiated mice 4 h and 24 h (for groups with drug given 24 h before and/or 6 h after IR), and 3, 7, 14, 21, and 28 days after radiation. Mouse blood will be analyzed in a Siemens ADVIA® 2120 Hematology System. Mouse BM cells will be collected from femurs and humeri. Total myeloid cell numbers and viability from individual mouse BM will be counted after irradiation. Clonogenicity of mouse BM cells will be quantitated. Death and apoptotic markers and cell surface phenotypes will be determined in all collected tissues using BD FACS Calibur flow cytometry (BD Biosciences, San Jose, CA).

c) BM pathological changes in mice treated with (b)(4) DT3/GT3 or vehicle at 1, 3, 7, and/or 14 days post-irradiation will be evaluated by VSD pathologist. Mouse BM structure and cellularity will be examined in HE stained mouse sternum longitudinal sections. BM cellularity especially megakaryocyte restoration will be measured in microscopic fields for each sectioned sternum.

d) Effects of (b)(4) DT3/GT3 on cytokines and chemokines production in mouse serum and cells lyses will be measured by ELISA, cytokine array, and immunoblotting assays.

V.1.2. Experiment 2 Compare the radioprotective effects of (b)(4) DT3/GT3 on the mouse GI tract.

a) CD2F1 mice (6 animal + 2 phantoms/group) will be placed in ventilated Plexiglas and exposed bilaterally to γ-radiation from the AFRRI cobalt-60 source at doses of 0.0 (sham-irradiation), 10 or 11 Gy (b)(4) DT3/GT3 (100 mg/kg) or vehicle will be administrated 24 h before irradiation. After irradiation, animals will be anesthetized prior to blood collection and then euthanized for jejunum tissue collection from γ-irradiated mice 24 h, 3.5 days, 10 days and 14 days after irradiation. Blood and jejunum samples from sham-irradiation will be shared with experiment 1.

b) H&E stained mouse intestinal tissue slides will be examined for intestinal mucosal surface area, crypt cell apoptosis, intestinal crypt colony generation, and villi at 3.5 days after TBI. Surviving crypts will be counted in transverse cross sections.

c) Cytokine release from jejunum mucosa will be measured using ELISA, and compared in different treatment groups. Effects of (b)(4) DT3/GT3 on radiation-induced inflammatory cytokines production in mouse jejunum mucosa will be evaluated.

d) Radiation-induced intestinal bacterial translocation in liver, heart blood and spleen and radiation-induced sepsis will be quantified by bacterial translocation assay. Radiation-induced bacterial translocation starts around day 7 and peaks around 2 weeks after TBI. Analysis of bacterial translocation will be performed on day 10 after exposure to 10 or 11 Gy TBI. The samples collected from euthanized mouse heart blood, liver, and spleen will be cultured for bacteria growth. The single colonies of isolated microorganisms will be observed for their morphological characteristics. Gram stain characteristics will be determined by Gram staining. They can be subcultured and the pure culture will be analyzed by a Vitek 2 Compact automated system to determine the type of bacteria.

V.1.3. Total number of animals: 1900 male CD2F1 mice

Note: The Sham–irradiated mice will be shared between Experiment 1 and Experiment 2.

Sham –irradiated section for Experiment 1 and 2 = 4 groups (vehicle (AO) and DT3, GT3 (b)(4) × 6 animals/group × 7 time points (4 hr and 1, 3, 7, 14, 21, and 28 days) after drug treatment =168 animals

Irradiated section for Experiment 1 = 4 groups (vehicle (AO) and DT3, GT3 (b)(4) × 6 animals/group × 3 drug dosing time (24 h before, 6 h and 24 h post) × 7 sample collection time points (4 hr and 1, 3, 7, 14, 21, and 28 days) after irradiation × 3 radiation doses (7, 8 and 9 Gy) = 1512 animals

Irradiated section for Experiment 2 = 4 groups (vehicle (AO) and DT3, GT3 (b)(4) × 6 animals/group × 2 radiation doses (10 and 11 Gy) × 4 sample collection time points (1, 3.5, 10 and 14 days) after irradiation = 192 animals

Sum of 168+ 1512+192+ 28 (for unforeseen losses or complications) animals = **1900 animals**

V.2. Data Analysis

Statistical software PC SAS will be used for statistical data analysis. Six animals per group will be used to provide statistical power >80% for two-tailed student's t-test. Means and standard deviation (SD) with a significance level at 5% set for each test. We will report the mean ± SD or percentage when applicable. In order to evaluate the hypothesis set forth and establish a comparison of significance of deviation from the hypothesis, Fisher Exact Test can be used. The test can provide the significance and deviation of efficacy of the measures used. The test can provide estimation of changes

as a series of plots of declining magnitude against a sample used to detect difference in the group population.

When inferential statistics is used such as t-test, Fisher's tests, Analysis of Variance (ANOVA) etc, to evaluate the outcome and comparisons, sample size (n) needs to be determined to provide sufficient statistical capability for performance. Based on the power analyses the sample size n=6 for each experiment is sufficient to deliver more than 80%, power to detect a significant difference between two groups if any, given type I error of 5% and delta/sigma has at least 1.03, where the delta is the estimated mean differences between the two groups and sigma is the standard deviation. ANOVA with mixed-effect model will be used when testing one dependent variable and Multivariate analysis of variance (MANOVA) will be used when testing more than one dependent variable to detect if there is significant difference among the groups.
For comparison and effects of survival of γ-irradiated hematopoietic and gastrointestinal systems using a mouse model and data derived from approved study (IACUC protocol # (b)(6) will be used.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered

Radiation-induced adjustment in cellular tissue homeostasis triggered by various molecular responses related to inter- and intracellular signaling causes both acute and late effects depending upon the interactions in different organs and tissues in animal models. The morphological changes in tissues can only be seen in tissues obtained from the animals. The hematological and cytokine and chemokine measurement can only be performed from blood collected from the animals. Therefore the observation of changes in hematopoietic and GI systems after exposure to radiation will be more meaningful in an *in vivo* model for the proposed study.

V.3.2. Animal Model and Species Justification

The murine model is the most well-defined animal model for research. The mouse was chosen over other animal models because of extensive published data that is readily available for comparison, review and analysis. The mouse model has significant hematological and immunological similarities to higher animals and humans. AFRRI's staff has extensive experience with mice, including male CD2F1 in radiation injury and countermeasure research.

V.3.3. Laboratory Animals

V.3.3.1. Genus / Species Mice (*Mus musculus*)
V.3.3.2. Strain / Stock CD2F1
V.3.3.3. Source / Vendor Envigo (Indianapolis, IN)
V.3.3.4. Age 12-14 weeks
V.3.3.5. Weight 24–30 g
V.3.3.6. Sex Male
V.3.3.7. Special Considerations N/A

V.3.4. Number of Animals Required (by species)

Mice: 1900

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement

All animal users will be properly trained to ensure minimum stress during animal handling

V.3.5.2. Reduction

We shall make every effort to minimize the use of non-necessary animals. Control groups will be shared between experimental groups. At every stage of the research, plans will be reevaluated in light of incoming data in an attempt to reduce the numbers of experiments and groups.

V.3.5.3. Replacement

It is not feasible to use non-animal systems in place of animal models to address the research questions in this protocol. (See section V.3.1 above.)

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C 0 (# of animals)

V.4.1.1.1.2. Column D Sham-irradiated controls 168 for exp-1 and 2 (# of animals). These mice will have drug or vehicle sc. Injection and/or blood collected via cardiac puncture.

V.4.1.1.1.3. Column E All irradiated mice 1732 (# of animals). These mice will be irradiated and will not receive any intervention for pain relief. However, they will be euthanized if found moribund according to AFRRI-IACUC policy # 10.

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1	*IR-induced hematopoietic syndrome*		*168*	*1512*	*1680*
2	*IR-induced GI syndrome*			*220*	*220*
Totals:			*168*	1732	1900

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

For blood sample collection, animals will be anesthetized with isoflurane 1-5% in 100% oxygen using the anesthesia machine in the VSD rodent procedure room. Animal respiratory rates will be closely monitored through the Plexiglas chamber during induction of anesthesia. When animals are recumbent and not response to toe/tail pinch, they will be removed from the chamber and their nose/mouth placed in the anesthesia apparatus breathing/gas administration cone for maintenance of anesthesia at 3% Isoflurane and 100% O_2(with the O_2 flow rate at 500 ml/min) during cardiac puncture for blood collection.

V.4.1.2.2. Pre- and Post-procedural Provisions N/A

V.4.1.2.3. Paralytics No paralytic agent will be administered.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched

AGRICOLA

PubMed

V.4.1.3.2. Date of Search December 20-29, 2015

V.4.1.3.3. Period of Search

AGRICOLA – 1976–present

PubMed: includes MEDLINE (1966–present) and OLDMEDLINE (1950–1965)

V.4.1.3.4. Key Words of Search
Irradiation or Pain and Distress or Alternatives or Animal Model; Mouse and Irradiation or Pain or Alternatives; GT3/DT3[b)(4)] or Treatment or Irradiation or Mouse; Blood collection or mouse or cardiac puncture or alternative.

V.4.1.3.5. Results of Search
Irradiation with ionizing radiation is not a painful process but it induces various changes in the body and kills hematopoietic cells and gastrointestinal epithelium. In irradiated animals, the immune response is compromised, and opportunistic infections may ensue. There is no less painful or distressful alternative found that can be used to develop protective and mitigating agents for irradiation. No references were found suggesting any effect of GT3/DT3[b)(4)] or AO (vehicle-control) on pain or distress. There are alternatives for blood collection by cardiac puncture in a mouse, such as tail vein and facial vein blood draw, etc. However, none of these alternatives can provide more than 0.1-0.2 ml of blood but we need 1 ml blood/mouse for experiments. We will use cardiac puncture for blood collection and we will do it while the animal is anesthetized. Therefore, it will not cause more than momentary pain or distress.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification
There is no alternative *in vitro* system and no less painful/distressful *in vivo* system available to test new therapies against acute irradiation syndrome. Thus, we have to use this mouse model to test the protective and mitigative/therapeutic effects of GT3/DT3[b)(4)] on mouse hematopoietic and gastrointestinal systems after different doses of gamma-irradiation.

Furthermore, we will need to collect about 1 ml of blood sample to run all the tests for our experiments in the proposed study. However, there is no other alternative available to obtain 1 ml of blood from a mouse other than cardiac puncture. Therefore, we will be using cardiac puncture for blood collection. The animal will be properly anesthetized during blood collection, so there should not be any pain or distress.

V.4.2. Prolonged Restraint N/A

V.4.3. Surgery
V.4.3.1. Pre-surgical Provisions N/A
V.4.3.2. Procedure(s) N/A
V.4.3.3. Post-surgical Provisions N/A
V.4.3.4. Location N/A
V.4.3.5. Surgeon N/A
V.4.3.6. Multiple Major Survival Operative Procedures N/A
V.4.3.6.1. Procedures N/A
V.4.3.6.2 Scientific Justification N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections

Animal will be manually restrained and subcutaneous (sc) injections will be given with either a 23-25G x ¾ inch to the nape of the neck of 0.1 ml (maximum volume 1 ml for 25 – 38 g animals). Injections will be carried out in VSD animal housing room.

V.4.4.1.1. Pharmaceutical Grade Drugs N/A

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

a) Research grade DT3, GT3, and (b)(4)

(b)(4)

b) The drugs will be solubilized in saline with 5% Tween-80 that also served as the vehicle control.

These experimental formulations were developed by a commercial formulation lab on behalf of (b)(4) and have been tested at AFRRI.

V.4.4.2. Biosamples

Animals will be anesthetized with isoflurane 1-5% in 100% oxygen before blood collection. Blood (0.7-1 ml/mouse) will be drawn by cardiac puncture using 23-25G x ¾ inch needle in anesthetized mice. After blood collection, animals will be euthanized as outlined in section V.4.6 Euthanasia and tissue samples will then be taken. Blood, bone marrow, spleen, and Jejunum tissue will be collected from the sham-irradiated and gamma-irradiated mice.

V.4.4.3. Adjuvants N/A

V.4.4.4. Monoclonal Antibody (MAb) Production N/A

V.4.4.5. Animal Identification

Generally, cage cards will be used to distinguish drug-treated and control groups. Some experiments might necessitate tattoo by personnel to identify individual animals in a cage.

V.4.4.6. Behavioral Studies N/A

V.4.4.7. Other Procedures N/A

V.4.4.8. Tissue Sharing

Tissues will be shared upon availability and request from other Investigators.

V.4.4.9. Animal By-Products N/A

V.4.5. Study Endpoint

In the proposed study, the animals will be kept for maximum of 30 days in Experiment 1 (7, 8 and 9 Gy) and maximum of 14 days in experiment 2 (10 and 11 Gy). We do anticipate the animals to become morbid and moribund in this study. IACUC Policy #10, Guidelines for Establishment of Early Endpoints in Experiments with Expected Mortality will be implemented for judging morbidity and moribundity. Signs of morbidity in the mouse include difficulty in breathing, ruffling of hair, drying of lacrimal fluid, fall in body temperature, loss of appetite with/without diarrhea. No supportive care is proposed, but the morbid mice will be examined at least three times daily, early morning and late afternoon by the research staff (including weekends and holidays) in addition to the

regular VSD health checks. The timing between the late evening and early morning check will not exceed ten hours during the critical period.

The moribund condition is defined as a clinically irreversible condition leading inevitably to death. Animals involved in experiments that may lead to moribundity or death will be monitored daily by personnel experienced in recognizing signs of moribundity (illness, injury, or abnormal behavior) for at least the following: abnormal posture, rough hair coat, head tucked into abdomen, exudates around eyes and/or nose, skin lesions, abnormal breathing, difficulty with ambulation, cyanosis, decreased food or water intake, or self-mutilation (*19*).

Animals will be immediately euthanized when they display abnormal breathing, are recumbent, or unable to respond to mild external stimulus by the research staff and scored as dead on removal. VSD veterinarians will be consulted for all matters regarding animal welfare.

V.4.6. Euthanasia

Animals in experiments involving blood collection will be anesthetized with 1-5% isoflurane before blood collection. After blood collection, cervical dislocation as a secondary method to confirm animal death will be performed while the animal is still anesthetized.

For the non-blood collection groups, before tissue collection or mice found moribund will be euthanized by CO_2 inhalation plus confirmatory cervical dislocation by investigator/technician according to the directives of the VSD-SOP and in accordance with current AVMA Guidelines on Euthanasia. At the conclusion of the experimental time frame, all animals that survived the procedures of irradiation, drug intervention and controls, will be sacrificed by CO_2 inhalation plus confirmatory cervical dislocation and their carcasses disposed of according to VSD regulations.

V.5. Veterinary Care

V.5.1. Husbandry Considerations

Mice are social animals and will be housed together in groups. Nesting material/igloos/tunnels will be provided in each cage. Animals will receive Harlan Teklad Rodent Diet (w) 8604 and water *ad libitum*. Acidified water (pH 2.5–3.0) will be provided. Cages will be regularly changed and cleaned by VSD staff. Animal rooms will be kept on a 12 h/12 h light/dark cycle.

V.5.1.1. Study Room As assigned by VSD

V.5.1.2. Special Husbandry Provisions N/A

V.5.1.3. Exceptions N/A

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care

Routine veterinary care will be provided as per VSD SOPs. This includes am and pm health checks on all the animals by veterinary technicians on weekdays. On holidays and weekends, am checks are done by animal care takers and pm checks by veterinary technicians. In the course of the study, as animals approach morbidity, the research staff observations will be increased to a minimum of three times a day, early morning and late afternoon (includes weekends and holidays). The timing between the late evening and early morning check will not exceed ten hours during the critical period.

There is no supportive therapy contemplated since this would compromise the experimental results (described earlier in the end point and pain category sections).

V.5.2.2. Emergency Veterinary Medical Care
On weekends and holidays, morning rounds will be done by VSD animal care takers and evening rounds will be done by VSD veterinary technicians. Animal care takers and veterinary technicians can contact the on call veterinarian if needed. Our (PI) staff will provide afterhours coverage (outside of the routine veterinary coverage provided by VSD staff) on as needed basis as determined by the VSD veterinarians. Our staff will contact the on call veterinarian if needed.

V.5.3. Environmental Enrichment
V.5.3.1. Enrichment Strategy
Nestling pads, tunnels and/or igloos will be provided in each cage during bedding changes.
V.5.3.2. Enrichment Restrictions N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3b, 4a, 5, anesthesia	(b)(6)
				1, 3b, 4a, and 5, anesthesia	

Procedure and manipulation codes:
Code 1= Animal handling and euthanasia
Code 2= Surgery (aseptic technique) pre- and post-operative care
Code 3= Injections (3a-ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv
Code 4= Blood collection under anesthesia (4a-cardiac, 4b-inferior vena cava)
Code 5= Tissue collection (after euthanasia) - doesn't need to be specific
Code 6 = Implantation (provide details)
Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY
All investigators and personnel will use appropriate protective gear while in the vivarium, as described by the VSD. All listed personnel are current regarding safety training and medical surveillance. All listed personal have completed the Animal Care and Use in Research and Education training (AALAS learning library) courses. Recently they took three refresher courses: Common Compliance Issues, 8th Edition of the Guide for the Care and Use of Laboratory Animals, Pain Recognition and Alleviation in Laboratory Animals and passed the exams.

VIII. ENCLOSURES
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X. ASSURANCES

Protocol Title: Evaluate the radioprotective and mitigative effects and mechanisms of γ-tocotrienol (GT3), δ-tocotrienol (DT3) and (b)(4) as novel radiation countermeasures in *Mus musculus*

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** (*circle one*) conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** (*circle one*) be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date)

DOD ANIMAL CARE AND USE STANDARDIZED PROTOCOL FORMAT WITH EMBEDDED INSTRUCTIONS (April 2015)

Reference DOD Instruction 3216.01 & AFRRI Instruction 3216.2H

****

Information requested in the following animal use protocol template reflects the requirements of the Animal Welfare Act Regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD instructions.

****

This document is intended to be an aid in the preparation of DOD-sponsored animal use protocols. It is a companion document to an identical protocol template that does not have embedded instructions. Either template may be used to format DOD-sponsored animal research proposals; however, reference to the instructions during protocol preparation is highly recommended. If using the template with instructions, the instructions should be deleted prior to submitting the finished protocol for IACUC review. Please use Arial, regular, size 12, black font to prepare the protocol. With the exception of section headings, all paragraphs require a response. Paragraphs that do not apply should be marked N/A. There is no space limitation for responses to the information requested.

Approved standard operating procedures (SOPs) promulgated by the AFRRI Veterinary Sciences Department (VSD) or similar VSD-validated documents that describe specific procedures may be referenced by SOP number and title in lieu of repeating the details in the protocol. However, it is essential to include general descriptions of all animal manipulations to be carried out under the IACUC-approved protocol and to provide details where no approved references are cited. To do otherwise would constitute a breach of statutory regulations. Manipulations not covered in the originally approved protocol can be initiated only after IACUC approval of a formal amendment adding the new procedures. Additionally, Principal Investigators (PIs), or other delegated research personnel, should maintain accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates with their approved protocol. Protocol PIs will be required to provide this and other information during annual protocol reviews.

Any supporting information that may be of use to the IACUC during its review may be attached to the completed protocol. Adding subparagraphs within the body of the protocol template is permissible, *provided that the numbering sequence of paragraphs in the original standard format is maintained.* In other words, all of the labeled paragraphs and subparagraphs in this template must be retained, with additional information being presented as sub-numbered paragraphs containing information that supports or complements what is requested.

INTELLECTUAL PROPERTY STATEMENT

Research protocols drafted by Government scientists include intellectual property (e.g., concepts, ideas, experimental approaches, etc.), some of which is innovative or original and therefore considered proprietary to the investigators and/or the sponsoring agency. All Government and non-Government personnel handling this protocol shall exercise EXTREME CARE, to ensure that the information contained herein is NOT DUPLICATED OR DISCLOSED, in whole or part, for any purpose other than to evaluate the protocol, without the written permission of the principal investigator or the sponsoring agency.

***** DELETE THIS PAGE PRIOR TO PROTOCOL SUBMISSION *****

DEPARTMENT OF DEFENSE SPONSORED ANIMAL RESEARCH PROPOSAL SIGNATURE COORDINATION SHEET

FOR IACUC USE ONLY			
PROTOCOL NUMBER: (b)(6)			
Submission Date:	3/18/2016	2nd Resubmission Date:	
Approved/**Returned for Revision**:	4/7/2016	Approved/Approval Withheld by IACUC:	
1st Resubmission Date:	4/11/2016	**EXPIRATION DATE:**	4/14/2019
Approved/Returned for Revision:	4/15/2016	Previous Protocol Number *(if related)*	
SECOND TIER REVIEW *(if required)*			
Submission Date:		Approved/Returned for Revision:	
Approved/Returned for Revision:		2nd Resubmission Date:	
1st Resubmission Date:		Approved/Approval Withheld:	

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- For each section *and subsection*, enter your text into the underlined data field ( ) only. Do not make entries into the red-text instruction text. Single-click your mouse cursor in the data field, then begin typing.

I. NAME OF FACILITY: Armed Forces Radiobiology Research Institute

II. PROTOCOL NUMBER: (b)(6)

III. PROTOCOL TITLE: DEVELOPMENT OF WELL-CHARACTERIZED MINIPIG (*Sus scrofa domestica*) MODELS OF GI- AND H-ARS

IV. PRINCIPAL INVESTIGATOR: (b)(6)

V. DEPARTMENT HEAD: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) **PhD** Date

Head, Scientific Research Department, AFRRI

Telephone: (b)(6)

(b)(6)

- Or –

(b)(6)

IACUC Chair (b)(6) Scientific Research Department, AFRRI

Telephone (b)(6)

(b)(6)

VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensures that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study

(b)(6) **PhD** Date

Statistician
Telephone: (b)(6) Fax: (b)(6)
(b)(6)

VII. ATTENDING VETERINARIAN: In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) **DVM, DACLAM, COL, VC, USA** Date

Head, Veterinary Sciences Department, AFRRI
Telephone: (b)(6) Fax (b)(6)
(b)(6)

VIII. SAFETY OFFICER: This animal use protocol received appropriate review for safety and biohazards. *(Attach copy of completed AFRRI Form 310, EHS Research Protocol Hazard Analysis)*

(b)(6) Date

Safety and Occupational Health Specialist, USUHS
Telephone (b)(6)
(b)(6)

IX. IACUC APPROVAL: This protocol was reviewed and approved by the Institute Animal Care and Use Committee on _____.
(Date)

(b)(6) **MA, MS, Lt Col, USAF, BSC**

IACUC Chair (b)(6) Scientific Research Department, AFRRI
Telephone (b)(6)
(b)(6)

PROTOCOL TITLE:
DEVELOPMENT OF WELL-CHARACTERIZED MINIPIG (*Sus scrofa domestica*) MODELS OF GI- AND H-ARS

PRINCIPAL INVESTIGATOR: (b)(6) PhD
Principal investigator (b)(6) Scientific Research Department
Armed Forces Radiobiology Research Institute (AFRRI)
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CO-INVESTIGATOR(S): (b)(6) DVM, MS, PhD
(b)(6) Scientific Research Department
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Tel: (b)(6) Fax (b)(6) E-mail (b)(6)

AFRRI SCIENTIFIC RESEARCH PROTOCOL NUMBER:

I. NON-TECHNICAL SYNOPSIS We are continuing the development of well-characterized minipig models for the gastrointestinal (GI) and hematopoietic (H) Acute Radiation Syndrome (ARS), as surrogate models for the human ARS and for ultimate use by Biomedical Advanced Research and Development Authority (BARDA). The ultimate goal is to evaluate the efficacy of medical countermeasures to mitigate the human H-ARS and GI-ARS, and to support submission of pre-clinical applications to the Food and Drug Administration. This study will fulfill the purpose of providing a well-characterized model for radiation countermeasure testing, as required by the FDA Animal Rule.

II. BACKGROUND

II.1. Background We are continuing the development of well-characterized minipig models for the GI- and H -ARS, as surrogate models for the human ARS and for ultimate use by BARDA. The ultimate goal is to evaluate the efficacy of medical countermeasures to mitigate the human H-ARS and GI-ARS, and to support submission of pre-clinical applications to the Food and Drug Administration. The purpose of this proposal is continue to characterize the natural history of minipigs irradiated at H-ARS and GI-ARS doses, under conditions reproducible at other centers within the BARDA minipig consortium, and to provide probit curves for the testing of radiation medical countermeasures (MCM) under GLP-like conditions, within AFRRI capabilities.

For the H-ARS, we will use a unilateral sequential exposure modality (γ irradiation Cobalt-60 source). The unilateral sequential exposure mimics the rotating plane on which animals are placed for irradiation when a punctiform source of Cobalt-60 is employed, in contrast to AFRRI set up where several rods of radioactive materials are lifted to produce a large field in proximity of the object to be targeted.

Preliminary data using the unilateral sequential exposure suggest that this geometry provides lethality profiles closer to those observed at other sites within the minipig consortium; however data need to be confirmed. In a single experiment done at AFRRI comparing unilateral sequential versus bilateral simultaneous irradiation, under (b)(4) funds, a total of 16 animals were irradiated with 1.85 Gy, using Cobalt 60. Of these, eight were exposed to unilateral sequential irradiation, and eight were exposed to bilateral simultaneous irradiation. Of the animals irradiated bilaterally, two out of eight survived (25%). Of the animals irradiated unilaterally, five out of eight survived (62.5%). Higher counts (thousands per microliter) were seen with unilateral animals for white blood cells (WBC) (3.73 vs. 2.12) and absolute neutrophil counts (ANC) (2.37 vs. 1.66) at day 3 (p 0.001), and for platelets at day 3 (497 vs. 364) and 7 (261 vs. 165), (p 0.001), when comparing complete blood counts (CBC) between animals subjected to different irradiation modalities. Other blood counts did not show major differences. Gross pathology showed hemorrhages in multiple organs (i.e. intestine, heart, lungs) for both groups euthanized due to morbidity before the end of the study. However, petechiae/ecchymoses disappeared around day 23 for unilateral irradiation, but persisted till day 30 (end of study) for bilateral (even though platelet counts were comparable between groups).

Since the gastrointestinal syndrome is 100% lethal after total body irradiation (TBI) in the absence of treatment, for the GI-ARS we will use a linear accelerator (LINAC Elekta) to irradiate only the GI and the bottom half of the animal while sparing thoracic cavities, upper limbs and head, in alignment with dosimetry protocols used at other centers using minipigs and sponsored by (b)(4) We have previously shown that doses of 2 to 5 Gy are associated with an accelerated hematopoietic syndrome, characterized by villus blunting and fusion, the beginning of sepsis, and a mild transient reduction in plasma citrulline concentration (b)(4),(b)(5) At higher doses, the Göttingen minipig exposed to γ radiation of 5 to 12 Gy demonstrates a dose-dependent occurrence of all parameters classically associated with acute GI-ARS. Our results suggested that the Göttingen minipig is a suitable model for studying GI-ARS after total body irradiation (b)(4),(b)(5) However the use of supportive care and/or partial body shielding to extend survival beyond 10 days for characterizing overlapping H-ARS, as expected in humans, is strongly recommended. Unlike total body irradiation, partial body irradiation (PBI) exposure is expected to allow survival at gastrointestinal doses that are otherwise lethal because of concomitant underlying hematological-acute radiation syndrome, gastrointestinal and pulmonary complications. Furthermore, non-uniform or partial body exposure leading to some bone marrow shielding is likely to be the most common modality of exposure in case of a radiation accident. This approach will provide a specific and reproducible GI and hematopoietic injury. The natural history of GI and bone marrow damage will be characterized in terms of general indicators of animal health, as well as by apoptotic and mitotic activity in GI tissues, structure and function of the GI, hematological depression and recovery of the bone marrow.

The Göttingen minipig has demonstrated marked sensitivity to the development of coagulation anomalies and vascular leakage after exposure to irradiation, leading to bleeding, multi-organ dysfunction, and death. Animals that succumb to lethal doses of radiation, starting at hematopoietic doses, are

characterized by widespread hemorrhages, edema, deposition of fibrin, and blood clots. The effect of radiation on blood coagulation is an area that has been largely neglected, although much evidence of induction of coagulation disorders is found among individuals exposed to irradiation from clinical, accidental and malicious exposures. The molecular mechanisms behind species variations in sensitivity to radiation are unknown. Krigsfeld et al (2014a) report that disseminated intravascular coagulation (DIC) may play an integral role in death at the LD50 dose of either gamma or solar particle event (SPE)-like proton radiation. In pigs and ferrets, DIC was characterized by impaired whole blood clotting as measured by thromboelastometry alterations, decreased platelet counts, elevated d-dimer concentrations in the blood, hemorrhaging, and deposition of fibrin in tissues (Krigsfeld, 2014a; Krigsfeld, 2014b). Inflammation, hemostasis and hemodynamics are intimately linked, with each process propagating the other, creating a vicious cycle of thrombogenesis, inflammation and hypo-perfusion, leading to coagulopathies and multi-organ dysfunction. Coagulopathy may derive from thrombocytopenia and reduced levels or absence of clotting factors, or through stimulation of pro-coagulant activities, dysregulation of the thrombomodulin pathway, and inflammation (through increase in levels of fibrinogen, and CRP-induced complement activation). As part of animal model development, we propose to evaluate the dose dependent occurrence of radiation-induced coagulopathies in the Göttingen minipig. This study will fulfill the purpose of providing a well-characterized model for radiation countermeasure testing, as required by the FDA Animal Rule.

II.2. Literature Search for Duplication

II.2.1. Literature Sources Searched RePORTER; BRD; PubMed

II.2.2. Date of Search 1/6/2016

II.2.3. Period of Search 2000 - 2016

II.2.4. Key Words of Search

minipig OR swine AND radiation
OR
minipig OR swine AND acute radiation
OR
minipig OR swine AND radiation AND coagulation
OR
minipig OR swine AND radiation AND Cobalt
OR
minipig OR swine AND radiation AND LINAC

OR

minipig OR swine AND radiation AND intestine

II.2.5. Results of Search

BRD The database is no longer available.

RePORTER: The search with swine and radiation gave 117 results. Among these results, the term "radiation" was used mainly within the context of medical devices (i.e. X-rays, MRI, laser) or for local therapy targeting the eyes, local tumors, or for surgical applications. The project most relevant to this proposal consisted in engineering transgenic pigs to enable the study of adult stem cells following diseases/injuries, including cancer, and regenerative responses resulting from radiation/chemotherapeutic or viral/ bacterial damage. When the term coagulation or intestine were added to the search, the results consisted of projects evaluating interventional techniques (intravascular imaging, MRI, spectra CT, laparoscopy, etc), immune response to specific coagulation factors, blood flow regulation in glaucoma, and the research on genetically engineered transgenic pigs. There were no studies addressing the consequences of acute radiation injury on coagulation parameters or intestinal function.

PubMed: The search with minipig OR swine and radiation gave over 5000 results. Most "hits" were not pertinent to acute radiation, as it is in the case of this proposal. The most relevant publications using acute radiation as search terms were our own (see section on "References" at the end of the protocol), and a few publications on the radiation-induced gastrointestinal syndrome in the Göttingen minipig and its application in clinical research (Shim et al, Rad Res, 2014; Wang et al, PLoS ONE, 2013). In our previous studies we have not investigated intestinal function or the role of coagulation in the etiology of the ARS. The work by Shim et al (2014) uses the approach of ileo-cutaneous anastomosis to gain a "window" over the intestine of irradiated animals which allow the investigators to follow morphological changes overtime following abdominal irradiation. No assays addressing intestinal function were done in that study. The work by Wang et al (2013) suggests that radiation can reduce the energy metabolism, as measured by levels of adenosine-triphosphate (ATP), adenosine-diphosphate (ADP), adenosine-monophosphate (AMP), total adenine nucleotides (TAN), and presence of aberrant mitochondria in Tibetan minipigs.
Of the 178 "hits" found when searching for swine, radiation and coagulation, only one result was relevant to coagulopathies resulting from total body, acute irradiation as in the case of this study. Radiation-induced coagulopathies were assessed using the Yucatan minipig irradiated acutely with total body using solar particle event (SPE)-like proton (Krigsfeld et al, Life Sci Space Res, 2014), or following skin thermal injury after laser irradiation. Other studies were done using laser pulses, ultrasounds, UV, X-rays and neutron scattering. All studies found completely differ from our proposal, in the quality of radiation and endpoints evaluated.

III. OBJECTIVE / HYPOTHESIS Our objectives are:

(i) To establish the dose dependent natural history of animals exposed at H-ARS doses to unilateral sequential (Cobalt-60) irradiation, and to follow the development of radiation-induced coagulopathies.
(ii) To establish the relationship between radiation-induced intestinal bleeding and function of the GI tract, and to evaluate biomarkers of GI structure and function under PBI.
(iii) To establish a radiation dose dependent injury profile for GI-ARS in animals irradiated under PBI.

Experimental details are reported in Section V Materials and Methods.

IV. MILITARY RELEVANCE

Both early and late health effects of radiation exposure are major concerns for the military. Developing countermeasures to radiation exposure is a top priority for the US Department of Defense. Effective countermeasures would expand the options available to field commanders operating Ionizing Radiation (here defined as IR) threat environments, and improve the morale of personnel at risk of exposure to IR. DoD Defense Technology Objective MD.18 states: "Effective mitigation of health consequences [of IR] will (1) reduce casualty load at medical treatment facilities, (2) sustain a more effective operational force after a radiation exposure event, (3) allow commanders to conduct operations in radiation field environments without undue risk of decremented performance of personnel due to acute tissue injury, and (4) reduce the negative psychological impact on personnel tasked to operate in contaminated environments."
An important barrier to advanced development of radiation countermeasures is the paucity of large, long-lived, non-rodent animal models of radiation injury. The only choices at present are nonhuman primates (NHPs) and canines. The higher sentient status of NHPs, their expense and occupational health hazards, are also disadvantages of working with NHPs. Canines are notoriously sensitive to NSAID and vomiting, therefore providing oral medication may be an issue (Swindle et al. 2012). The addition of miniature swine as a large animal radiation injury model would facilitate development of promising countermeasures for both military and civilian use that could be used for prophylactic, mitigator and therapeutic use.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures

Male Göttingen minipigs will be obtained from Marshall BioResources, North Rose, NY, at age of 4-6 months, and at a weight 7-13 kg (see section V.3.). Minipigs may be group-housed during quarantine (see section V.5.1). Age and weight will be strictly controlled, which will avoid possible complications related to increasing weight and fatty mass (see section V.5.1.2).

Animals will be irradiated at an age of approximately 5 months. Minipigs will be single-housed but kept in visual contact with each other, and fed twice a day according to their weight, following recommendations from the vendor (Marshall Bioresources), and as described in section V.5. Veterinary care. After the required period of quarantine/acclimation (see Section V.5.1.), animals will be sedated as described in section V.4.1.2.1. Anesthesia / Analgesia / Tranquilization, transported to the radiation facility, placed on a sling or a table, irradiated and returned to their quarters, where they will be monitored until full recovery. A LINAC with field definition determined by a multi-leaf collimator, or a Cobalt-60 source with unilateral sequential exposure, will be used to irradiate the animals, depending on the experiment. A representative figure of a LINAC Elekta is reported in Figure 1.

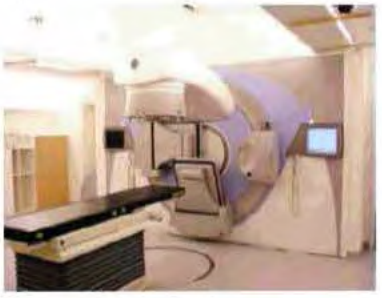


Figure 1: Example of LINAC Elekta with table.

All animals will receive preventative minimum supportive care, according to section V.4.1.2.2.

Survival will be monitored for 30-60 days, depending on the experiment; appropriate biomarkers to support the natural history of ARS will be collected at periodic time points. Blood for CBC/differential and plasma will be collected before irradiation and at several time points after irradiation, as described in the individual experiments and in section V.4.4.2 Biosamples. Time points may be shifted by 24 – 48 hour, or dropped in case of unforeseen circumstances. Additional blood draws may be taken, per veterinarian request, if needed to assess health of the animal. Blood will be collected from either a Vascular Access Port, or an externalized catheter, or from peripheral veins (V.4.3.2. Procedure(s) and V.4.4.2. Biosamples).

Moribund animals will be euthanized according to pre-established euthanasia criteria (section V.4.5. Study Endpoints); animals completing the survival study will be euthanized within 3 days from the completion of the observation period. Euthanasia will be carried out according to section V.4.6. Euthanasia. Tissues and blood samples may be collected for further analysis and tissue sharing, as outlined in section Tissue Sharing V.4.4.8.

Primary data-points of the study will depend on the Experimental Aims.

V.1.1. Experiment 1. To establish the dose dependent natural history of animals exposed at H-ARS doses to unilateral sequential (Cobalt-60), and to follow the development of radiation-induced coagulopathies.

Minipigs will be exposed to hematopoietic total body doses of radiation using unilateral sequential Cobalt-60 exposure. We will irradiate animals with four doses, between 1.6 Gy and 3.0 Gy, based on historical data from the (b)(4) corresponding to expected LD20/45, LD40/45, LD60/45 and LD80/45. Final doses will be provided to the IACUC via memo prior to commencement of the experiment. Survival will be monitored for 45 days after exposure. Simultaneously, we will follow the development of radiation-induced coagulopathies. Blood will be collected twice before irradiation, and, with day 0 the day of irradiation, at days +1, 3, 7, 10, 14, 17, 20, 23, 27, 30, 35, 40 and 45. Time points of blood collection may be subjected to change by up to +/- 24 hours. Blood volume will be within allowed guidelines: up to 1% of total blood will be drawn in a single day, and no more than 7% of total blood volume in a week (Diehl et al, 2001) (see V.4.4.2).

Coagulation assays are sensitive to dilution parameters. As part of Experiment 1, we will use control sham animals to optimize protocols and dilution factors for swine samples, and to obtain baseline values and measures of assay reliability.

Primary experimental endpoints

The primary endpoints will be 45-day survival, blood cell counts, general indicators of animal health, gross and histopathology (H&E) of major organs (heart, lung, small intestine, liver, spleen, kidney, lymph nodes).

Secondary experimental endpoints

We will assess clotting factors (i.e. AT, APTT, fibrinogen), thrombomodulin (TM)-protein C system, acute phase proteins, fibrin deposition/clots (i.e. PTAH), complement activation (i.e. C5a) and hemostasis molecular assays (i.e. soluble fibrin, D-dimer, TA complex, F1+2, anti-thrombin, angiotensin-converting enzyme, clot formation).

Number of animals for Experiment 1: 12 animals x 4 radiation doses (expected LD20 to LD80) + 3 animals (for pilot coagulation evaluation studies and GI function studies) = 51

V.1.2. Experiment 2. To establish the relationship between radiation-induced intestinal bleeding and function of the GI tract, and to evaluate biomarkers of GI structure and function

Minipigs will be exposed to irradiation geometries that will spare approximately 40-50% (head, forelimbs and thorax shielded) of bone marrow, using a LINAC at 0.5-0.6 Gy/min, and GI structure and function will

be followed by scheduled necropsy points out for 30 days. Necropsy will occur at day 6 (n=3 animals/dose), day 9 (n=3 animals/dose), day 15 (n=3 animals/dose), and day 30 (n=3 animals/dose). Animals will be exposed to either one of two doses, a lower one, between 8-12 Gy, or a higher one, between 13-16 Gy. Final doses will be established based on BARDA data from other minipig-funded studies; a memo will be submitted with the final radiation doses. In addition, we will use 3 sham-irradiated animals to optimize the procedures for the GI function assays (measuring metabolic mucosa function, protein digestibility and absorption of sugars) and to collect data from normal, un-irradiated animals, to compare to those obtained from irradiated animals

Metabolic mucosa function, protein digestibility, amino acid absorption test and xylose absorption test will be measured using tracers according to Engelen et al. (2013). Tracer is a substance (not naturally found in the system) added to the system to trace out a metabolic pathway (i.e. L-[ureido-13C-4,4,5,5-2 H5]-citrulline); the tracee is the native substrate being traced (citrulline).

Tracers for metabolic mucosa function, protein digestibility, amino acid absorption test and xylose absorption test will be administered as described in section V.4.4.7. Other Procedures – Administration of tracers and samples will be collected according to section V.4.4.2. Biosamples. Metabolic mucosa function and protein digestibility will be measured using a modified protocol with NON-radioactive stable isotopes of tracer amounts of the following macro-nutrients: L-[ureido-13C-4,4,5,5-2H5]-citrulline,15N-labeled spirulina protein and L-[1-13C]-Phenylalanine. Amino Acid absorption will be measured with tracer amounts of the NON-radioactive stable isotopes of the following inert amino acids: L-[13C6]-allo-isoleucine AND L-[D10-15N] - allo-isoleucine. Sugar absorption will be done using the standard D-xylose test.

Epithelial intestinal cell proliferation index will be measured using in vivo labeling with BrdU. BrdU will be administered according to section V.4.4.7 Other Procedures, and samples will be collected according to section V.4.4.2. Biosamples.

Timeline for the collection of CBC, plasma, and tissues for the GI function assays described above will be done according to Table 1.

Table 1

Exp2: time line and endpoints

Experimental group	Time points																												
	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Sham Control n=3	A		A		A																											
	B		B		B			C																							
Radiation Dose (cohort 1, n=3)	A		A		A																											
	B		B		B			C																							
Radiation Dose (cohort 2, n=3)	A		A		A		A		A																							
							B		B		C																					
Radiation Dose (cohort 3, n=3)	A		A		A		A		A			A		A																		
												B		B		C																
Radiation Dose (cohort 4, n=3)	A		A		A		A		A			A		A				A				A				A				A		
																		B				B				B				B		C

A = complete blood counts B= Spirulina, D-Xylose, Citrulline C = Brdu, Euthanasia, Tissue Collection

Blood samples volume At the time of blood collection, animals will be restrained, according to Section V.4.2. Prolonged Restraint. On the days when intestinal function is tested (labelled as B in Table 1), we will exceed the 1% daily (1% of 65 ml/kg) allowed total blood volume (Diehl et al, 2001). On those days, there will be 14 blood collections, for a total of approximately 25 ml of blood (14 time points x 1.7 ml + 0.5 ml for CBC). The 1.7 ml include initial 0.7 ml of fluid (blood mixed with saline or heparin used to lock the catheter) to be discarded. With the exception of experimental groups Sham and cohort 1, we will not exceed the 7% allowed in a one-week period. With regards to experimental groups Sham and cohort 1 we may exceed the 7% allowed in a one-week period (depending on the weight of the animal). For Sham and cohort 1 groups we will be collecting multiple blood samples on 3 different instances within 1-week time period. However, the animals will be euthanized within 1-4 days after the last blood collection.

Blood sample time points Blood samples for measuring citrulline will be taken before the single dose of citrulline, in a post absorptive state (16-18 hours overnight fasting), and again at 2, 5, 10, 20, 30, 60, 90, 120 min after administration of the single dose of citrulline (Figure 2). Time 0 (T=0min) starts at administration of citrulline.

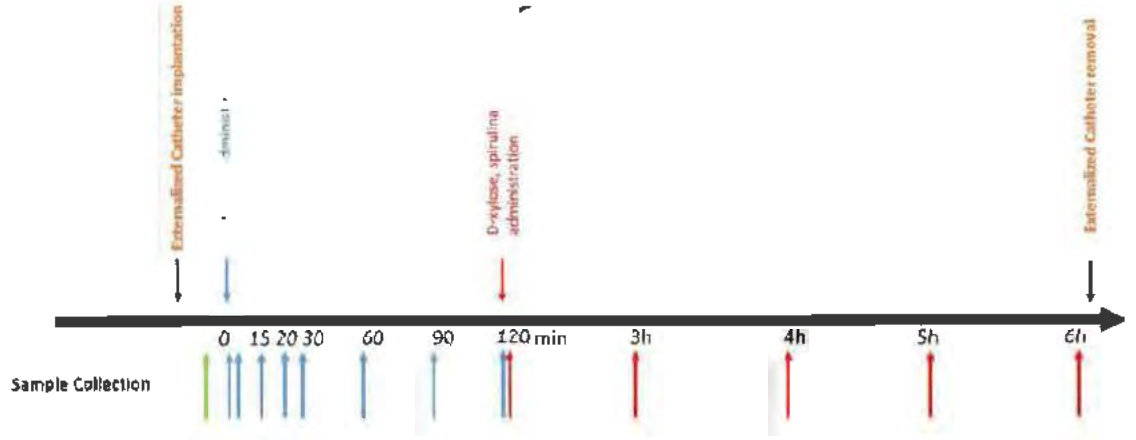


Figure 2: schematic of reagents administration and sample collection. Green: baseline sample before treatment. Blue: administration and plasma samples for citrulline kinetics. Red: D-xylose, spirulina, phenylalanine and allo-isoleucine administration and plasma samples for D-xylose, spirulina, phenylalanine and isoleucine kinetics. Black arrows bracket the entire duration of the experimental procedure, approximately 7 hours.

For the nutritional supplement with tracers, time zero is the time of oral administration of standard meal containing the tracers (15N-labeled spirulina, L-[1-13C]-phenylalanine, L-[13C6]-allo-isoleucine and D-xylose), and the simultaneous single dose administration of L-[D10-15N]- allo-isoleucine through the infusion set. This time corresponds to the 120th minute (T=120min) after administration of citrulline. Afterwards, blood sampling will continue on hourly basis, over the course of four hours. The first collection of these series will be 2 minutes after tracer's administration (five blood samples total per animal) (Figure 2). Blood samples (1 ml aliquots) will be put in Lithium-heparinized tubes, immediately placed on ice, processed to obtain plasma, divided in aliquots, and immediately frozen at -80 °C until further analysis.

At the time of scheduled euthanasia (day 6, 9, 15 and 30), animals will be infused with BrdU (see section V.4.4.7 Other Procedures),approximately one hour prior to humane euthanasia . Parts of GI tract (jejunum, duodenum, and ileum) will be fixed and analyzed for proliferative markers. Table 1 reports the timeline for sample collection and type of assay. Sham irradiation will be done before irradiation with Cobalt, in order to perfect techniques and assays prior to dealing with irradiated animals. For each of the two radiation doses, we will irradiate 12 animals during the same day. No more than 12 animals will be irradiated in one day. For each radiation dose, four cohorts, consisting of three animals each, will be followed over time; each cohort will be euthanized at either day 6 or 9 or 15 or 30.

Primary experimental endpoints. The primary experimental endpoints of the study will be structure and function of the GI, as measured by histological slides, percentage protein digestibility (Engelen et al. 2013), production rate of citrulline (Engelen et al. 2013), plasma concentration of D-xylose, and 30-day survival.

Secondary experimental endpoints. Repair of GI will be determined by BrdU labeling, as well as Ki67 and TUNEL assay.

Number of animals for Experiment 2: 3 animals x 4 time points x 2 doses TBD (8-12 Gy, and 13-16 Gy) + 3 control animals = 27

V.1.3. Experiment 3. To establish a radiation dose dependent injury profile for GI-ARS in animals irradiated under PBI using LINAC.

We will irradiate fifty-six animals under 40-50% bone marrow sparing, as described in Experiment 2 (head, forelimbs and thorax shielded). Animals irradiated at GI doses (5 12 Gy) show extensive hemorrhages and necrosis of the GI tract, in addition to villar blunting/fusion and loss of crypts, which may contribute to impaired absorption of nutrients. We will use histological measures as well as metabolomics technology to assess expression of biomarkers in the plasma of animals irradiated at GI doses, and to gain insights into the molecular pathways possibly involved in loss of GI function. Final doses are to be determined in consultation with (b)(4) a memo will be submitted with the final radiation doses. Survival will be monitored for 45 days after exposure. Blood will be collected twice before irradiation, and, with day 0 the day of irradiation, at days +1, 3, 7, 10, 14, 17, 20, 23, 27, 30, 35, 40 and 45. Time points of blood collection may be subjected to change by up to +/- 24 hours. Samples will not exceed the 7% total blood volume within one week time period (see V.4.4.2 Biosamples).

Primary experimental endpoints. The primary experimental endpoint of the study will be natural history of GI function over 45 days, metabolomics, and correlation with histological features.

Secondary experimental endpoints. The secondary experimental endpoint will be blood cell counts, and general indicators of animal health.

Number of animals for Experiment 3: 8 animals x 7 doses = 56 animals

V.2. Data Analysis

Experiment 1 and 2: the number of animal requested is the minimum necessary to allow for analysis of all proposed biomarkers and replication of results. This is a pilot study, therefore we cannot make any assumptions in terms of expected size effects or difference in means. Differences in means between groups will be assessed using Student's T-test or ANOVA (or corresponding non-parametric tests). Strength and direction of association between variables will be estimated using the Pearson Product-Moment correlation coefficient or the Spearman coefficient or the Kendall rank correlation (experiments 1-3).

Experiment 3: Kaplan Meyer curves and a preliminary probit will be calculated using 8 doses and 7 animals per dose, for the PBI-GI irradiation. Probit curves are usually constructed using a minimum 5 doses/6 animals per dose; 8 doses and 7 animals per dose will increase the power of analysis, and narrow the confidence intervals, increasing the overall predictive ability of the probit curve

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered The purpose of this project is to determine the effect of radiation on survival, GI function and coagulation. There is no computer model or tissue culture that can mimic the effect of radiation on the whole organism and predict lethality. Survival and organ function depend on an intricate network of organ cross-talks and cellular microenvironment that cannot be recreated *in vitro*. We do not know all of the cell types and tissues involved in radiation injury or how radiation injury causes cell and tissue damage. The concept of multi-organ failure has been suggested, but how the injured organs affect each other's vital functions is unknown. The present state of the art of tissue culture is still incapable of reproducing the *in vivo* relationships. Computer modeling is similarly limited by lack of full characterization of the biological elements and interactions under study.

These considerations are consistent with the FDA requirement ("Animal Rule') for preclinical research in both small and large mammals before granting approval for drug testing in humans.

V.3.2. Animal Model and Species Justification Large, long-lived, non-rodent animal models are required for drug studies submitted to the FDA for licensure applications. The close similarity to humans in anatomy and physiology of organs such as the liver, pancreas, kidney and heart has made the pig the primary species of interest as organ donors for xenographic procedures. Minipigs are routinely used for toxicity testing. As such, further and rapid characterization at physiological, molecular and genetic level of the minipig is likely to occur, thus increasing acceptance of this model and facilitating advanced drug development. We have been developing the Göttingen minipig as an additional large animal model for the study of the ARS and advanced development of radiation countermeasures. The current study will help to further characterize the model in terms of natural history of GI- and H-ARS, and to understand the reasons behind organ dysfunction. Studies on the Sinclair minipig will increase the choice of animal model available to mimic the condition in humans, overall increasing the chances of obtaining a FDA approved drug for the mitigation and treatment of the ARS and eventually decreasing the number of animals required for research. Our previous studies on H-ARS and GI-ARS have utilized male Göttingen minipigs to develop probit curves. Since we are using our own historical data as terms of comparison, we will continue to use male minipigs for this study.

V.3.3. Laboratory Animals Swine

V.3.3.1. Genus / Species *Sus scrofa domestica*

V.3.3.2. Strain / Stock Göttingen miniature swine

V.3.3.3. Source / Vendor Marshall BioResources/Marshall Farms Group Ltd, 5800 Lake Bluff Road, North Rose NY 14516.

V.3.3.4. Age 4-6 months

V.3.3.5. Weight 7-13 kg

V.3.3.6. Sex Male

V.3.3.7. Special Considerations NA

V.3.4. Number of Animals Required (by species)

Exp 1: 51

Exp 2: 27

Exp 3: 56

Total: 134

V.3.5. Refinement, Reduction, Replacement (3 R's)

V.3.5.1. Refinement

The study data-points currently mandated by the FDA for approval of radiation countermeasures under the Animal Efficacy Rule is mortality. Hence the primary study data-point in our development of Göttingen minipigs as a model for ARS is survival. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress, using an extensive set of criteria (see animal endpoints, paragraph V.4.5 – Lethargy, weight loss, temperature, respiratory distress, food intake, bleeding *etc.*). Animal care and use procedures will only be performed by trained personnel. We have considerable experience in the care and handling of minipigs. Extensive human interaction every weekday will reduce stress in the animals. Use of slings, supportive care, topical anesthetics, and injectable or inhalation anesthetics will minimize distress during blood collections and irradiation. For instance, topical lidocaine will be used for blood collection from VAPs and isoflurane anesthesia will be used for blood collection from peripheral veins. Animals are acclimated to the sling during the incoming physicals at which time we use positive reinforcement by offering them treats at the end of the physical exam. VAP design and the blood collection have been refined during multiple iterations of previous studies to minimize discomfort to the animals.

V.3.5.2. Reduction

Blood samples obtained from minipigs before irradiation will serve as controls, thus reducing the number of animals required for this study.

V.3.5.3. Replacement None

V.4. Technical Methods

V.4.1. Pain / Distress Assessment

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column C _____ (# of animals)

V.4.1.1.1.2. Column D ___6__ (# of animals)

V.4.1.1.1.3. Column E ___128__ (# of animals)

V.4.1.1.1.4. Pain Category Assignments

Exp. #	Experimental/Control Group	C	D	E	Totals
1	To establish the dose dependent natural history of animals exposed at H-ARS doses to unilateral sequential (Cobalt-60), and to follow the development of radiation-induced coagulopathies.		3	48	51
2	To establish the relationship between radiation-induced intestinal bleeding and function of the GI tract, and to evaluate biomarkers of GI structure and function		3	24	27
3	To establish a radiation dose dependent injury profile for GI-ARS in animals irradiated under PBI.			56	56
Totals:			6	128	134

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization

We are planning to use anesthesia/sedation at the time of blood sampling (if necessary), prior to irradiation, surgery (if applicable), and prior to euthanasia. Midazolam and Acepromazine may be used to sedate large and fractious animals prior to blood collection for the ease of handling and alleviating stress to the animals as well as handlers. Anesthesia/sedation regimen for the animal procedures is outlined in Table 2. Drug dose, frequency, route, site of injection, and needle/catheter size are outlined in Table 3 in

section V.4.4.1. Injections. Atropine will be added for any procedure that requires intubation. Cetacaine topical spray may be used to facilitate intubation

Table 2

*Anesthesia/sedation regimen**

Blood sampling, emergency medical care	i	Midazolam or Acepromazine, with or without isoflurane 1-5% (face mask or endotracheal tube)
		OR
	ii	Xylazine + Telazol or Telazol with or without isoflurane1-5% (face mask or endotracheal tube)
		OR
	iii	Isoflurane 1-5% (face mask or endotracheal tube)
		OR
	iv	Telazol + dexmedtomidine or ketamine + xylazine with or without isoflurane 1-5% (face mask or endotracheal tube)
Irradiation, Surgery, Euthanasia	v	Telazol with or without isoflurane 1-5% (face mask or endotracheal tube), Atropine (when intubated)
		OR
	viii	Telazol + dexmedtomidine or ketamine with or without isoflurane 1-5% (face mask or endotracheal tube), Atropine (when intubated)
		OR
	ix	Xylazine + Telazol with or without isoflurane 1-5% (face mask or endotracheal tube), Atropine (when intubated)

*Agents will be selected and doses may be refined based on veterinarian's discretion.

At the end of the irradiation procedure or surgery, once the animals are back in their cages, dexmedetomidine can be reversed by atipamezole *i.m.* (same volume as dexmedotmidine) to facilitate recovery.

V.4.1.2.2. Pre- and Post-procedural Provisions

Body weights and health check status

Body weights and standard animal health check status (hydration status, hair coat, skin quality, vitals) will be determined at least once prior to irradiation, and on days of blood collection. Data will be recorded on the Animals' Clinical Observation at Blood Collection Form (c) (see Section VIII Enclosures). Animals will be monitored daily for general indicators of animal health (activity, posture, stool, vomit, respiratory activity, respiratory rate, anorexia and temperature). Monitoring will occur at least once in the morning (am) and once the afternoon (pm) throughout the duration of the study. Monitoring in the critical period of sick animals will be performed with increased frequency, as described in V.5.2.1. Routine Veterinary Medical Care. Daily observations will be recorded on study specific form, Animals' Daily Observations Form (d) (see Section VIII Enclosures).

Supportive care

We will administer prophylactic empirical antibiotic regimen to reduce the risk of infection to all animals in the protocol between day 3-30 post irradiation. For the animals to be used for pilot coagulation evaluation studies (Experiment 1), we will administer the supportive care prior to initiation of the experimental procedures. Supportive care will be provided in the form of antibiotics (Gentamicin and Amoxicillin), antipyretic/anti-inflammatory (Carprofen), and gastrointestinal protectant (sucralfate) as per the dose and route outlined in the Table 3 in section V.4.4.1. Injections. Dietary supplementation and oral hydration will be offered in the form of moistened/liquid food, Metamucil, pumpkin mash, Pedialyte, and apple sauce. This supportive care was proven to improve survival in Göttingen minipigs irradiated with total body irradiation (Cobalt-60).

Preventative supportive care will follow a population based regimen, which will provide care to all animals on a fixed schedule, regardless of blood counts and body temperature values, starting 3 days after irradiation, and continuing through day-30. Emergency care on an individual basis may be added per veterinarian requirement

V.4.1.2.3. Paralytics NA

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched Altweb, AGRICOLA and Pubmed

V.4.1.3.2. Date of Search 12/2/2015

V.4.1.3.3. Period of Search 2000 - 2015

V.4.1.3.4. Key Words of Search swine, total body irradiation, partial body irradiation, ARS, sequelae, pain, distress, death as an endpoint, alternatives

V.4.1.3.5. Results of Search

Alternatives to toxicity testing in five animals have been proposed in the form of cell and tissue culture, which are not feasible for the purpose of this study where the cross-talk among organs and strain sensitivity is being investigated in relationship to survival after acute irradiation. Additionally, bioinformatics or *in silico* approaches applied to life science might be created ad hoc to validate studies; however these approaches cannot be adopted to model the complexity of multi-organ failure in a large animal, because the mechanisms of radiation injury are still for the most part unknown. Alternatives to irradiation to study the ARS (total body or partial body) do not exist. We will however provide anesthetics and analgesics for pain management at the time of VAP and catheter implantation (See VAP or externalized catheter placement under section **V.4.3.2. Procedure (s)**), as well as during the days that follow surgery, to alleviate the pain produced by the procedures.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification

Irradiation itself is not a painful procedure. Pain and distress are associated with the immune suppression, GI injury and bone marrow suppression post TBI. At equivalent TBI doses, pain and distress after PBI are expected to be less intense, because of the residual, spared, bone marrow which is still capable of producing blood cells. These animals may not have acute effects of irradiation (bleeding, infection *etc.*). Depending upon the irradiation dose, irradiated animals die due to compromised immune responses and opportunistic infections. The percentage of surviving animals is the indicator of the efficacy of a countermeasure. We cannot give systemic anesthetic agents to animals after the irradiation procedures, since they are known to interact with the immune system (see references in Jacobsen, K. O., V. Villa, V. L. Miner, and M. H. Whitnall. 2004. Effects of anesthesia and vehicle injection on circulating blood elements in C3H/HeN male mice. *Contemp Top Lab Anim Sci* 43:8-12.), and would confound the correlation of radiation dose with incidence of moribundity, resulting in a waste of animals. However, we are providing supportive care in terms of analgesics, antibiotics, and oral fluids/ nutritional support. Use of supportive care is expected to mitigate the pain associated to the sequelae of irradiation.

V.4.2. Prolonged Restraint

Only short periods of restraint in the sling (<30 minutes) will be necessary for irradiation (in Cobalt facility) procedure, physical examination and blood collections. To minimize discomfort, we have designed a new type of sling that uses softer, elastic material to support the animal, and utilizes a more efficient restraint design that maintains the animal in place while reducing manipulation by technicians and consequently stress to the animals. During the acclimation period, slings are used for physical examination. At the time of physical examination, animals are acclimated to the sling for 5-10 minutes. Acclimation to the sling is

very rapid and non-stressful; animals are petted and gently spoken to while restrained. It has been our experience that resting on the sling does not agitate the animal at all.
Animals are anesthetized/sedated and transported to the Cobalt Facility or LINAC holding area prior to irradiation (<20 minutes). Animals will be kept under continuous observation while anesthetized.
For Experiment 2, animals may be restrained for a maximum of 6 hours in cages with limited space (based upon the experimental design), that allow the minipig to sit or stand, but not turn around, or in the sling. The choice between cage or sling (or a combination of both) will depend upon animal's individual preference, based on vocalization and motion. Animals will be at first placed on the sling, for the samples to be taken in the first 1-2 hours. During restraint, animals will be under continuous monitoring.

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions

Animals will be fasted overnight (approximately 16 – 18 h) prior to VAP implantation surgery. For antibiotic, one dose of Cefazolin sodium or ampicillin sodium will be given just prior to the surgery, or intra-operatively, for prophylaxis. A single dose of Pre- or intra-operative buprenorphine or Carprofen will be provided for analgesia. Drug doses, frequency, route of administration, site of injection, and needle/catheter size are outlined in Table 3 in section V.4.4.1. Injections. Cetacaine topical spray will be used to facilitate intubation.

V.4.3.2. Procedure(s)

VAP implantation. VAP catheters (SoloPort subcutaneous access port, either PMID or MID model; Instech Solomon, PA) will be surgically implanted in the right external jugular veins under general anesthesia according to the principles of aseptic surgery. Animals will be lying either in dorsal or lateral recumbency; vital signs such as heart rate, respiration rate, and blood pressure will be taken at every 5 min, whereas, temperature and percent oxygen saturation will be measured at every 15 min by vet techs. The catheter tip will be introduced through a 2-3 mm incision in the right jugular vein and advanced to the junction of the external jugular vein and vena cava. Prior to insertion, the VAP catheter will be flushed with locking solution (heparin lock flush solution, 100 IU/ml). Prior to securing the VAP catheter within the vein, 1 preplaced 2-0 or 3-0 Vicryl (polyglactin 910) suture (Ethicon, Somerville, NJ) will be used to ligate the vessel just cranial to the insertion point of the catheter tip. A second and third vicryl ligature will be placed around the caudal portion of the vessel containing the VAP catheter and retention beads, with 1 ligature on either side of one of the beads. The port will be secured within the scapular or mid-dorsal incision site to the underlying musculature by use of 2-0 or 3-0 poldioxanone (PDS) (Ethicon, Somerville, NJ) or 2-0 or 3-0 Monocryl (poliglecaprone 25) at a minimum of 2 of the 5 anchor holes on the port. Catheter function will be confirmed intraoperatively through aspiration of heparinized saline and withdrawal of a blood sample via the septum. If the aspiration of a blood sample is not successful, or there are air bubbles indicating incomplete seal, another ligature around one of the beads will be added and catheter function will be re-checked. The port reservoir and catheter will be flushed again with saline

and locked with a heparin lock flush solution (100 IU/ml). All subsequent lock solutions will be heparin (100 IU/ml).

During closure of the surgical sites, special attention will be given to eliminating dead space around the port and around the catheter loop on the neck. The muscle and subcutaneous layers will be closed with 2-0 or 3-0 PDS II (polydioxanone suture; Ethicon) or 2-0 or 3-0 Monocryl (poliglecaprone 25) using a simple continuous pattern. Skin incisions will be closed with 2-0 PDS II (Ethicon) or 2-0 or 3-0 Monocryl (poliglecaprone 25) in a continuous intradermal pattern. Tissue glue (VetBond, 3M, St Paul, MN) may be used to help seal surgical sites as necessary to help prevent contamination.

Externalized catheter implantation. An externalized catheter (7 Fr, Polyurethane single or triple lumen catheter, approx. 60-70 cm) may be used in place of the VAP in the external jugular vein as described above in the VAP catheter placement. However, in this case, the catheter will be tunneled to an exit site on the dorsum of the animal without a VAP. A coil of the catheter will be left underneath the skin and a subcutaneous purse-string suture will be placed around the catheter to secure it at the exit site. Skin will be closed using 2-0 PDS or 2-0 or 3-0 Monocryl (poliglecaprone 25).

V.4.3.3. Post-surgical Provisions

VAP surgery or externalized catheter surgery. Animals will continue to be monitored until fully recovered from anesthesia; they will be returned to their home cage when ambulatory. Supplemental heat may be provided in the home cage post-operatively (e.g. Bair Hugger warm air blanket, heating pads).Additional post-operative analgesia will be given with oral Carprofen for three days starting the day after surgery, or using sustained release buprenorphine as recommended by an AFRRI veterinarian in consultation with the PI. Trimethoprim-sulfa will be given for 5 days or more as determined by veterinarian to lessen the risk of post-op infection. Alternatively, enrofloxacin can be given every 24 hrs for 3-5 days or more if needed as determined by the veterinarian. Excede (Ceftiofur) may be used as an antibiotic as determined by the veterinarian. The advantage of this antibiotic is that the single injection provides protection against the bacterial infection for seven days. Drug doses, frequency, route of administration, site of injection, and needle/catheter size are outlined in Table 3 in section V.4.4.1. Injections.

Postsurgical recovery and pain will be monitored twice daily by visual examination of the incisions and overall conditions of the animals. Parameters monitored will be food intake, activity, alertness, vocalization, guarding, and response to human contact. Topical antibiotic ointment may be applied at the surgical site. Animals will be allowed to fully recover from the surgery before irradiation is done. The recovery period may last for 2-4 weeks.

V.4.3.4. Location

VSD surgical suite

V.4.3.5. Surgeon

Surgeons will be AFRRI/HJF veterinarians.

V.4.3.6. Multiple Major Survival Operative Procedures NA

V.4.3.6.1. Procedures NA

V.4.3.6.2 Scientific Justification NA

V.4.4. Animal Manipulations

V.4.4.1. Injections

All injections will be administered as outlined in the Table 3 below -

Table 3

List of drugs to be administered, dose, frequency, route, site of injection and recommended needle/catheter size

Drug	Dose and Frequency	Route	Referenced	Needle Gauge and length	Catheter Gauge and length
Acepromazine	0.03-1.1 mg/kg PRN	*i.m.*[x]	Table 2	21-27 G, ½-1½"	N/A
Amoxicillin	10 mg/kg BID	*p.o. or i.v.*[a] or *i.m.*[y]	V.4.1.2.2. Pre- and Post-procedural Provisions	21-27 G, ½-1½"	18-27 G ¾-1¼"
Ampicillin sodium	10-20 mg/kg SID	*i.v.*[a]	V.4.3. Surgery	21-27 G, ½-1½"	18-27 G ¾-1¼"
Atipamezole (Antisedan, Pfizer)	same volume as dexmedetomidine	*i.m.*[y]	V.4.1.2.1. Anesthesia/Analgesia/Tranquilization	21-27 G, ½-1½"	N/A
Atropine sulfate	0.04 mg/kg SID	*s.c.*[g] or *i.m.*[y] or *i.v.*[a]	Table 2	21-27 G, ½-1½"	18-27 G ¾-1¼"
BrdU (Sigma Aldrich)	150 mg/kg SID	*i.v.*[a] *OR VAP using a gripper portal access needle/Huber needle OR externalized central venous catheter or intravenous catheter OR Central venous catheter*	V.4.4.7. Other Procedures	21-27 G, ½-1½" 20-22 G, ½-1½" (Huber) 21-23 G, 1-1½" (jugular cut down)	18-27G, ½-1½"
Buprenorphine	0.01 to 0.02 mg/kg PRN 0.12 – 0.27 mg/kg (sustained release) PRN	*s.c.*[g]	V.4.3. Surgery and V.4.4.7. Other Procedures	21-27 G, ½-1½"	N/A
Carprofen	2.2 mg/kg BID	*p.o. or s.c.*[g] or *i.m.*[y]	V.4.1.2.2. Pre- and Post-procedural Provisions; V.4.3. Surgery	21-27 G, ½-1½"	N/A
Cefazolin sodium	20 mg/kg SID	*i.v.*[a]	V.4.3. Surgery		18-27 G, ¾-1¼"
Cetacaine topical spray	N/A	*Topical*	V.4.3. Surgery	N/A	N/A
Dexmedetomidine (Dexdomitor, Pfizer)	0.05-0.1 mg/kg (w/ Telazol) PRN	*i.m.*[y]	Table 2	21-27 G, ½-1½"	N/A
Enrofloxacin	5-10 mg/kg SID	*p.o or i.v.*[a] or *i.m.*[y]	V.4.3. Surgery	21-27 G, ½-1½"	18-27 G ¾-1¼"
Euthasol (sodium pentobarbital)	minimum 1 ml/4.5 kg	*i.v.*[a] or *i.c.*	V.4.6. Euthanasia	19-27 G, ½-1½"	18-27 G ¾-1¼"
Excede (Ceftiofur)	5 mg/kg (sustained release), SID	*s.c.*[g]	V.4.3. Surgery	21-27 G, ½-1½"	N/A
Heparin lock, IV	1-3 ml volume (one sample collection), 0.5 ml volume (multiple sample collection), PRN (surgery)	*i.v.*[a]	V.1.2. Experiment 2, V.4.3. Surgery, V.4.4.2. Biosamples	21-27 G, ½-1½"; 20-22 G ½-1¼" (Huber)	18-27 G ¾-1¼"
Gentamicin	2 mg/kg SID	*p.o. or i.v.*[a] or *i.m.*[y]	V.4.1.2.2. Pre- and Post-procedural Provisions	21-27 G, ½-1½"	18-27 G ¾-1¼"
Isoflurane	1-5% PRN	*Inhalation*[w]	Table 2, V.4.6. Euthanasia, Appendix I	N/A	N/A
Ketamine	5-20 mg/kg (w/ Xylazine); PRN	*i.m.*[x]	Table 2	21-27 G, ½-1½"	N/A
L-[D10-15N] - allo-isoleucine (Cambridge Isotopic Laboratories, Andover, MA)	3.86 µmol/kg in a volume of 0.7 ml/kg SID	*i.v.*[a] *OR VAP using a gripper portal access needle/Huber needle OR externalized central venous catheter or intravenous catheter*	V.1.2. Experiment 2, V.4.4.7. Other Procedures	21-27 G ½-1½" OR 20-22 G ½-1¼" (Huber)	18-27 G ¾-1¼"
L-[ureido-13C-4,4,5,5-2H5]-citrulline (Cambridge Isotopic Laboratories, Andover, MA)	1.29 µmol/kg, in a volume of 0.7 ml/kg SID	*i.v.*[a] *OR VAP using a gripper portal access needle/Huber needle OR externalized central venous catheter or intravenous catheter*	V.1.2. Experiment 2, V.4.4.7. Other Procedures	21-27 G ½-1½" OR 20-22 G ½-1¼" (Huber)	18-27 G ¾-1¼"
Midazolam	0.1-0.5 mg/kg; PRN	*s.c.*[g] or *i.m.*[x]	Table 2	21-27 G, ½-1½"	N/A
Saline	1-5 ml volume (one sample collection), 1 ml volume (multiple sample collection), PRN (surgery)	*i.v.*[a]	V.4.3.2. Procedures, V.4.4.2. Biosamples, Appendix I	21-27 G, ½-1½", 20-22 G ½-1¼" (Huber)	18-27 G ¾-1¼"
Subcutaid	1 g/pig BID	*p.o.*	V.4.1.2.2. Pre- and Post-procedural Provisions	N/A	N/A
Telazol®	4.4 mg/kg (w/ Dexmedetomidine) or 2-6 mg/kg alone or w/ Xylazine; PRN	*s.c.*[g] or *i.m.*[x]	Table 2 V.4.6. Euthanasia	21-27 G, ½-1½"	N/A
Trimethoprim-sulfa	25-50 mg/kg (oral), 5 mg/kg (injectable); BID	*p.o. or i.m.*[y]	V.4.3. Surgery	21-27 G, ½-1½"	N/A
Xylazine	1 - 2 mg/kg (w/ Ketamine) or 2.2 mg/kg (w/ Telazol); PRN	*i.m.*[x] or *s.c.*[g]	Table 2	21-27 G, ½-1½"	N/A

[x] thigh/neck; [g] inguinal folds,axillary folds,behind ears, neck; [a] VAP, externalized catheter, peripheral catheter, peripheral veins (cephalic, saphenous, auricular, femoral, epigastric); [w] Face mask,ET tube

V.4.4.1.1. Pharmaceutical Grade Drugs

Lidocaine (5%), topical

Telazol®

Xylazine

Ketamine

Atropine sulfate

Cefazolin sodium

Ampicillin sodium

Heparin lock, IV

Saline

Buprenorphine

Carprofen

Metamucil

Enroflaxacin

Amoxicillin

Gentamicin

Midazolam

Acepromazine

Cetacaine topical spray

Atipamezole (Antisedan, Pfizer)

Dexmedetomidine (Dexdomitor, Pfizer)

Excede (Ceftiofur)

Isoflurane

Euthasol (sodium pentobarbital)

All the drugs listed above are ordered from veterinary product distributors by VSD or USUHS pharmacy, and they all are pharmaceutical grade drugs.

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

BrdU (Sigma Aldrich)

L-[ureido-13C-4,4,5,5-2H5]-citrulline (Cambridge Isotopic Laboratories, Andover, MA)

15N-labeled spirulina protein (Cambridge Isotopic Laboratories, Andover, MA)

L-[1-13C]-Phenylalanine (Cambridge Isotopic Laboratories, Andover, MA)

L-[13C6]-allo-isoleucine (Cambridge Isotopic Laboratories, Andover, MA)

L-[D10-15N] - allo-isoleucine (Cambridge Isotopic Laboratories, Andover, MA)

D-xylose (Sigma Aldrich)

V.4.4.2. Biosamples We will collect blood samples throughout the duration of the study, as well as tissue samples at necropsy, for use in our laboratory and for sharing with collaborators. Time points of

blood collection are subjected to change by up to +/- 24 hours. Blood will be used for CBC counts and isolation of plasma/serum for laboratory assays. Tissues will be used for histology as well as molecular laboratory techniques as applicable (*i.e.* Western blot, PCR, ELISA, *etc.*). Tissue samples, to include organs ((heart, lung, small intestine, liver, spleen, kidney, lymph nodes), blood and hair may be taken at the time of euthanasia for tissue blocks and for frozen tissue repository.

Mean blood volume for minipigs is 61-68 ml/kg. Up to 1% of total blood will be drawn in a single day, and no more than 7% of total blood volume in a week (Diehl, 2001), unless as otherwise specified in Experiment 2.

Collection of blood samples. We will obtain blood samples from externalized catheter (if applicable), or from VAPs (if applicable), or from peripheral veins namely cephalic, saphenous, auricular, epigastric, and/or femoral. Blood collection will be performed using same needle and catheter size as used for i.v. injections as indicated in Table 3 in section V.4.4.1. Injections.

Blood draw from VAP. Lidocaine (5%) cream may be applied topically to the site of venipuncture to minimize discomfort to the animals about 15 minutes before starting the procedure; site will be cleaned with 70% alcohol and povidone iodine or Chlorhexidine as described below.
The port will be accessed using a gripper portal access needle/Huber needle with or without infusion set. For Experiment 2, the needle (or infusion set) may be capped and left in place for up to 7 hours, to facilitate multiple blood draws separated by short time intervals and/or administration of multiple tracers. The needle will be maintained in place using clear skin patches (i.e. Tegaderm) or tape. Otherwise, a jacket/tether system may be used.

Before placement of a non-coring needle, the port injection site will be scrubbed with povidone iodine or Chlorhexidine and alternating with 70% isopropyl alcohol until all gross debris is removed (3 to 5 scrubs each). To collect a single sample, approximately 1 ml fluid will be withdrawn from the catheter and port until blood no longer appears to be mixed with saline. This fluid will be discarded. The blood sample will be collected into a new syringe and placed in appropriate vials for analysis. Next, 3 ml heparin lock flush solution (100 IU/ml) will be infused into the port and catheter as a locking solution.

When collecting multiple samples within the same day, the catheter will be flushed with 1 ml of saline solution and locked with 0.5 ml of heparin lock. At the time of blood collection, we will discard the first 0.7 ml of fluid (blood mixed with heparin) prior to collecting the blood sample.

A standard protocol will be followed for obtaining a sample from a problematic catheter. First, external pressure will be applied to the non-coring needle where it entered the port to ensure the tip of the needle is all the way through the septum and into the reservoir. Second, high-pressure turbulent flushing will be attempted with a change in the animal's position. This may result in the use of a higher volume of saline

flush (more than 5 ml). A second person will be needed to extend the neck of the animal by raising it or flexing it away from the side that contains the VAP (in case the VAP has been secured at the scapular incision). Third, the non-coring needle will be replaced; at times the blood may be clotted inside the lumen of the needle. Fourth, if the described series of interventions fail to return the VAP to functioning, the sample will be collected from accessible peripheral vessels (cephalic, saphenous, auricular, and/or femoral) using one of the anesthesia regimens as described in section V.4.1.2.1. (With the exception of Experiment 3 (time point 120 min) where anesthesia cannot be used.

Blood draw from externalized catheter. If an externalized catheter is implanted in the external jugular vein (section V.4.3.2) and used for blood collection, the catheter will be flushed with 1-5 ml saline and the first 0.5 ml of blood will be discarded. Blood samples will be collected using a new syringe. After blood collection, catheter will be flushed again and locked with 1-3 ml of heparin lock (100 IU/ml). When collecting multiple samples within the same day, the catheter will be flushed with 1 ml of saline solution and locked with 0.5 ml of heparin lock. At the time of blood collection, we will discard the first 0.7 ml of fluid (blood mixed with heparin) prior to collecting the blood sample.

Blood draw from peripheral veins. Alternatively, we will collect blood from peripheral veins such as auricular, femoral, cephalic, epigastric, and/or saphenous veins. An i.v. catheter may be placed in one of the peripheral veins. Animals may be sedated at the time of blood sampling according to section V.4.1.2.1 Anesthesia/Analgesia/Tranquilization, if necessary. All procedures will be entered into the experimental record. At blood collection, site will be cleaned with 70% alcohol.
Collection of samples will be performed with the minipig restrained in a sling or on a minipig in the cage; at the same time, we will take vitals. The entire procedure takes less than 30 min. After blood draw, animals will be returned to their cages and checked to ensure that the needle puncture wound is closed and the blood has clotted.

V.4.4.3. Adjuvants NA

V.4.4.4. Monoclonal Antibody (MAb) Production NA

V.4.4.5. Animal Identification Tattoos or ear tags and cage cards/tags

V.4.4.6. Behavioral Studies NA

V.4.4.7. Other Procedures

Temperature transponder. At the time of irradiation, VAP surgery, or catheter implantation, while the animal is anesthetized, a temperature microtransponder (Implantable Electronic ID Transponder IPPT-

300, Bio Medic Data Systems, Seaford, DE), will be implanted subcutaneously behind the neck, over the shoulder to facilitate body temperature measurements. Alternatively, body temperature will be measured using a minimally-invasive ear probe or a rectal probe.

Dosimetry. Animals will be sedated, and taken in covered transporting cages to the Cobalt or LINAC facility, where they will be placed on a sling or a table and body measurements will be taken. Additional sedation will be administered as appropriate.

Administration of tracers (L-[ureido-13C-4,4,5,5-2H2]-cit, L-[D10-15N]- allo-isoleucine, 15N-Spirulina, L-[1-13C]-Phenylalanine, L-[13C6]-allo-isoleucine, D-xylose)

All test substances and solutions will be freshly prepared. For i.v. administration the solutions will be sterile (by 0.22 µm filtration/or autoclaving) and isotonic. All solutions must be at room temperature prior to dosing. Animals will be fasted overnight (16 -18 hours). The catheter will be flushed with 5 times the dead volume immediately after the injection of a tracer. This may result in the use of a higher volume of saline flush (more than 5 ml). The dead volume of the VAP includes port plus internal catheter, and will be measured at the time of VAP implantation. The dead volume of the catheter or the infusion set will be measured prior tracer administration/blood collection, by ejecting the liquid into an Eppendorf tube and measuring with a pipette.

1) T=0min: citrulline NON-radioactive stable isotope (L-[ureido-13C-4,4,5,5-2H2]-cit, Cambridge Isotopic Laboratories, Andover, MA) will be delivered intravenously through the VAP (using a gripper portal access needle/Huber needle) or peripheral veins or central venous catheter or a peripheral intravenous catheter @1.28 µmol/kg, in a volume of 0.7 ml/kg. The needle and catheter sizes, and site of injection are outlined in Table 3 in section V.4.4.1. Injections.
2) T=120min (i.v. administration): L-[D10-15N]- allo-isoleucine NON-radioactive stable isotope (Cambridge Isotopic Laboratories, Andover, MA) will be delivered intravenously through the VAP (using a gripper portal access needle/Huber needle) or peripheral veins or central venous catheter or a peripheral intravenous catheter @ 3.86 µmol/kg in a volume of 0.7 ml/kg. The needle and catheter sizes and site of injection are outlined in Table 3 in section V.4.4.1. Injections.

   T=120min (oral administration): the following tracers will be administered orally in ENSURE: 15N-Spirulina, NON-radioactive stable isotope (Cambridge Isotopic Laboratories, Andover, MA) + L-[1-13C]-Phenylalanine, NON-radioactive stable isotope, (Cambridge Isotopic Laboratories, Andover, MA) + L-[13C6]-allo-isoleucine, NON-radioactive stable isotope, (Cambridge Isotopic Laboratories, Andover, MA) (all isotopes at 3.86 µmol/kg in a volume of 0.7 ml/kg) + D-xylose.

BrdU. BrdU is a synthetic analog of thymidine. BrdU is selectively incorporated into the newly synthesized DNA of replicating cells during the S phase of the cell cycle, and it is commonly used in the

detection of proliferating cells. Antibodies specific for BrdU can then be used in cells and in frozen or formalin-fixed paraffin embedded tissues to detect levels of BrdU and identifying actively replicating cells. BrdU has cytotoxic, teratogenic, and mutagenic properties; accidental exposure presents potential health and safety hazards to laboratory staff, animal handlers and other personnel. Primary routes of occupational exposure to BrdU include aerosol exposure, ingestion, accidental injection, and tissue absorption. Biosamples and anything that has been in contact with BrdU injected animals is considered hazardous to humans for 72 hours from the time of injection. A standard operating procedure for working with BrdU in minipigs at AFRRI has been developed and is attached as part of this amendment (see Appendix 1), together with the approved Safety Form 310.
VSD staff will be notified of the intention of using BrdU two weeks prior to the beginning of the experiment (see section V.5.1.Husbandry Consideration). Rooms will be posted and the SOP will be made available outside the animal room. At all time when handling animals injected with BrdU, personnel will have to wear proper PPE (see BrdU SOP in Appendix 1 and section V.5.1. Husbandry Considerations).

BrdU will be injected in sedated animals, according to the procedure attached in Appendix 1. This protocol has been optimized for the Göttingen minipig under (b)(6) Briefly, animals will be anesthetized (as described in V.4.1.2.1. Anesthesia / Analgesia / Tranquilization) and restrained on the sling. BrdU (150mg/kg) will be infused through the VAP (using a gripper portal access needle/Huber needle) or externalized central venous catheter or intravenous catheter. See Table 3 for needle and catheter size. Possible veins to be accessed will be peripheral veins (cephalic, auricular, saphenous, epigastric, and femoral), vena cava, or external jugular vein.
Central venous cut down may be performed in case percutaneous access is not possible. The site will be aseptically prepared and a 1-2 cm incision will be made under sterile conditions. Manual pressure will be applied on the vein for hemostasis after injection is made. Incision will be closed using either absorbable suture material (2-0 or 3-0 PDS) or staples. Buprenorphine (dose, route, site of injection, and needle size specified in Table 3 in section V.4.4.1. Injections) may be given as needed for pain management. BrdU will be injected at the rate of up to 5 ml/kg body weight through the cut-down vein. Needle size is specified in Table 3 in section V.4.4.1. Injections.

V.4.4.8. Tissue Sharing Bio-samples (as described in section **V.4.4.2. Biosamples**) will be collected, to be used for CBC counts, plasma/serum isolation, histo-chemistry/protein/molecular assays among others, and to be shared with collaborators upon request and as experiments permit.

V.4.4.9. Animal By-Products NA

V.4.5. Study Endpoints Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress. The determination of the moribundity must be made in consultation with a veterinarian.

Euthanasia will be carried out either after completion of the observation period (30 day for experiment 2, or 120 day for experiment 3), or when the animal(s) shows at least one of the absolute criteria for moribundity, or a combination of four criteria for non-absolute moribundity. Absolute criteria will be lack of responsiveness (lying down, no effort to get up when people enter the cage, no ability to stand without support), continuous prolonged dyspnea (>30 minutes), body weight loss (loss of greater than 20% of expected weight), hypothermia (<36°C) and anorexia (skip 3 consecutive meals, even if eating enrichment), Non-absolute criteria will be hyperthermia (>40°C), anemia/pallor, petechiae/ecchymosis, vomiting/diarrhea, lethargy, seizures or vestibular signs, and prolonged hemorrhage. Attainment of 4 non-absolute criteria will qualify for immediate euthanasia. Euthanasia of the animal with less than 4 non-absolute criteria will be made upon consultation and/or discretion of a veterinarian.

Absolute	
1	Non responsive, assuming the animal has recovered from anesthesia
2	Dyspnea (fast respiration rate, shallow breathing, labored breathing), continuous, prolonged >30 minutes)
3	Loss of 20% of expected weight**
4	Hypothermia (<36°C) (rectal temperature)
5	Complete anorexia (skip 3 consecutive meals – pig chow)
Non-absolute	
1	Hyperthermia (>40°C) (rectal temperature)
2	Anemia/pallor, CRT > 2 seconds.
3	Vomiting/diarrhea
4	Lethargy
5	Seizures or vestibular signs (falling, circling or head tilt, paddling)
6	Partial anorexia
7	Prolonged hemorrhage (hemorrhage = any sign of internal or external bleeding, including presence of petechiae/ecchymosis, bleeding from snout, eye, mouth, anus; presence of blood in the cage; presence of blood in the stool. Hemorrhaging can be also determined by drop in HCT %).
*Attainment of 4 non-absolute criteria will qualify for immediate euthanasia	
**Based on Marshall Bio-resources growth chart –weight versus age	

Animals will be euthanized according to current American Veterinary Medical Association (AVMA) guidelines.

V.4.6. Euthanasia Animals will be euthanized according to current American Veterinary Medical Association (AVMA) guidelines. Animals will be anesthetized with Telazol® or ketamine/xylazine or 1-5% isoflurane via mask (with 1-3 L/min O_2 flow rate) or any one of the combinations described in section V.4.1.2.1. Animals will then be euthanized with Euthasol® (sodium pentobarbital) or another commercial euthanasia solution. Animals will be deeply anesthetized with 1-5% isoflurane (via face mask) in addition to the injectable anesthesia during intra-cardiac injection of Euthasol. Drug doses, site of injection, route of administration, and needle/catheter size are specified in Table 3 in section V.4.4.1. Injections. In the rare event that a moribund animal is not responsive and necessitating of immediate euthanasia, Euthasol may be injected intravenous or intracardiac under 1-5% isoflurane without prior injectable anesthesia. Death will be confirmed by AFRRI veterinarian or veterinary technician via auscultation of the heart, with cessation of sounds for at least 2 minutes or longer as per current SOP. See item V.4.5 for euthanasia criteria before the end of the study.

V.5. Veterinary Care

V.5.1. Husbandry Considerations Minipigs will be housed in stainless steel cages/runs, in tactile, visual and olfactory contact with adjacent animals. During quarantine/acclimation period, up to day of surgery, minipigs may be group-housed. After surgery, they will be singly housed to prevent damage to the VAPs and to allow individual assessment of feed consumption and fecal/urine/blood production. Rooms will be maintained on a 12:12 h light (0600 to 1800) dark cycle with the temperature set to 27 to 29°C and 30% to 70% relative humidity. Commercial diet (Mini-Swine Diet 8753, Harlan Teklad Diets, Madison, WI) will be provided. Water will be provided ad libitum.

Handling and housing of animals administered with BrdU. Generally, special care has to be taken when handling animals and animal excreta for the 72 hours subsequent to BrdU administration. Cages must be lined with plastic sheets, and sheets must be covered with low-dust animal bedding. No change in bedding and no cage hosing is done for the 72 hours following BrdU administration. Drains are temporarily sealed during those 72 hours. After 72 hours, the bedding and the underlining plastic liners are collected in hazard waste bags, and picked up as hazardous waste by USUHS waste pick-up, as per SOP. Cages are rinsed and sent through cage wash. We plan to maintain the animals in the necropsy room (b)(6) after administration of BrdU, until euthanasia (approximately 1 hour after BrdU administration). If, in case of emergency, animals need to be returned to their home cages, housing will be performed according to instructions above.

V.5.1.1. Study Room

Minipigs will be located at AFRRI for the duration of the study. They will either be in a VSD surgical suite for VAP implantation, in transit to and from the cobalt /LINAC facility in a covered transport cage, in slings for irradiation in the cobalt/LINAC facility, or in their housing cage or a VSD prep room for blood collections or euthanasia. They will be housed in stainless-steel cages in an environmentally controlled and continuously monitored animal room as described in Section V.5.1. For BrdU injections, animals will be injected in VSD (b)(6) next to an eye wash station for protection of personnel.

V.5.1.2. Special Husbandry Provisions Tap water will be provided ad libitum. Diet will be controlled in accordance with vendor's recommendations so as to prevent excessive weight gain. Pumpkin mash will be provided starting at day 3 after irradiation to all animals. Liquid food (i.e. ProMod Liquid Protein) and/or moistened pellet will be offered if observed that the sick animal prefers it to dry food. No treats in the form of marshmallow, peanut butter, crackers will be offered to this animal, since they are on a calorie-restricted diet. Instead, fresh fruits, vegetables, and/or yogurt will be offered daily (excluding weekends and holidays).

V.5.1.3. Exceptions NA

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care Animals will be acclimated for at least 1 week prior to any procedure. Each animal will be observed at least twice daily for evidence of pain or distress, stool consistency, left-over food, demeanor and overall animal appearance. Body weights will be determined at least once prior to irradiation and on days of blood collection.
Animals will be qualitatively assessed at least twice daily (a.m. and p.m.) for general behavioral status and food consumption by VSD techs and/or research techs. Daily observations will be annotated on the Animals' Daily Observation Form (d). The heart rate will be measured at the time of each blood collection. If animals look dehydrated, supplemental oral fluids (Pedialyte, etc.) will be provided. Diarrhea has not been observed in minipigs irradiated at doses <8 Gy. Appropriate standard operating procedures will be followed by staff veterinarians in the event of pain or distress.

The critical period (period of time when animals show signs of ARS and experience increased morbidity and mortality) is defined as day 14-30 post-irradiation for animals irradiated at H-ARS, and day 5-10 post-irradiation for animals irradiated at GI-ARS doses (bilateral, total body irradiation). The window of the critical period may change for partially irradiated animals and for animals irradiated with unilateral sequential geometry.

Monitoring of sick animals will be performed with increased frequency during this time to include: an early morning check (before 9 a.m.), a mid-morning check (between 9 a.m. and noon), an afternoon check (between 1 p.m. and 3 p.m.), and a late afternoon check (between 4 p.m. and 6 p.m.).

If animal conditions are deteriorating rapidly during the day, an additional evening check (after 8 p.m.) will be done, followed by an early morning check at <10 hours apart. Rapidly deteriorating conditions are defined as follows: activity score of 2, >1°C change of body temperature per hour, respiratory activity of 1 with continuous dyspnea for more than 10 consecutive minutes, complete anorexia for more than 12 hours, single instance of vomiting/diarrhea, single instance of seizure or vestibular signs, mild hemorrhaging for longer than 6 hours.

Scoring Criteria

i.	Activity score: 0- Normal bright alert and responsive (BAR), 1-Periods of inactivity, quiet, alert and responsive (QAR), 2-Laying down, gets up with stimulus, 3- Non-responsive
ii.	Posture/Movement Score: 0- Normal gait and posture, 1- hunched appearance or slower movement, 2-sternal recumbency without dog sitting posture, 3-lateral recumbency, 4-dog-sitting posture, shoulders forward, 5-ataxia or uncoordinated movement
iii.	Stools: Y=1, N=0
iv.	Stool Consistency Score: 0 =Normal Formed Stool, 1- Formed Hard Stool, 2-Loose stool, 3- Diarrhea
v.	Vomit: Y=1, N=0
vi.	Respiratory Activity Score: 0- Normal Respiration, 1- Labored breathing, or intermittent cough 2- gasping, open mouth breathing, or persistent cough
vii.	Respiratory rate (RR): breaths per minute
viii.	Food (left in the cage): Y=1, N=0, partial =0.5
ix.	Temp: °C

V.5.2.2. Emergency Veterinary Medical Care Emergency veterinary medical care is available 24/7. The on-call veterinary officer will be available via telephone 24 hours a day. The VSD on-call roster will be located outside the VSD conference room (b)(6) on the bulletin board and at the security watch desk. Additionally, the on-call roster for the research staff will be located outside (b)(6)

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy

Physical and occupational enrichment will be done as per the VSD minipig enrichment SOP, except single housing after catheter/VAP implantation if applicable, and irradiation. Dietary enrichment will be done as per section V.5.1.2.

V.5.3.2. Enrichment Restrictions Minipigs will be single-housed after surgery (if applicable) and after irradiation until completion of the study, to avoid mechanical and infectious complications related to implanted catheters and irradiation, but they will be in visual, olfactory and auditory contact with other minipigs in the same room. Animals may be group-housed during quarantine.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)				1, 3, 4, 5, 7 (drug delivery)	(b)(6)
				1,2,3b,4, 5, 6 (VAP), 7 (in vivo BrdU labeling)	
				1, 3, 4, 5	
				1,3,4,5	

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)					(b)(6)
				1, 3, 4, 5	
				1,2,3,4,7	
				1,2,3,4	

NAME	DEGREE	TRAINING	DATE	PROCEDURES	YRS/MOS EXPERIENCE
(b)(6)					(b)(6)
				1,2,3,4,5	
				3b,4, minipig husbandry procedures	
				Minipig husbandry procedures	
VSD Back-up technicians					
(b)(6)				All veterinary technical procedures	(b)(6)
				All veterinary technical procedures	
				All veterinary technical procedures	

Procedure and manipulation codes:

Code 1= Animal handling and euthanasia

Code 2= Surgery (aseptic technique) pre- and post-operative care

Code 3= Injections (3a–ip, 3b-sc, 3c-gavage) - can say 3 if trained in all manipulations or add 3d-iv

Code 4= Blood collection under anesthesia (4a-cardiac, 4b-posterior vena cava)

Code 5= Tissue collection (after euthanasia) - doesn't need to be specific

Code 6 = Implantation (provide details)

Code 7 = Others - (provide protocol specific manipulations or procedures for e.g., retro-orbital bleeding, tail vein injection, or drug delivery)

VII. BIOHAZARDS/SAFETY NA

VIII. ENCLOSURES: Animals' Clinical Observation at Blood Collection Form (c), Animals' Daily Observations 630 Form (d) (see Section VIII Enclosures, and Protocol for in vivo labeling with BrdU.

References

Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, Vidal JM, van de Vorstenbosch C; European Federation of Pharmaceutical Industries Association and European Centre for the Validation of Alternative Methods. A good practice guide to the administration of substances and removal of blood, including routes and volumes. J Appl Toxicol. 2001 Jan-Feb;21(1):15-23.

(b)(4),(b)(6)

Engelen, M. P. K. J., Com, G., Anderson, P. J., & Deutz, N. E. P. (2014). New Stable Isotope Method to Measure Protein Digestibility and Response to Pancreatic Enzyme Intake in Cystic Fibrosis. Clinical Nutrition (Edinburgh, Scotland), 33(6), 1024–1032. http://doi.org/10.1016/j.clnu.2013.11.004

(b)(4),(b)(6)

X. ASSURANCES

Protocol Title: A STRAIN COMPARISON STUDY ON SURVIVAL, VASCULAR DAMAGE, AND LONG TERM HEALTH EFFECTS IN IRRADIATED MINIPIGS (*Sus scrofa domestica*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard / Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedure(s): I **AM / AM NOT** conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. If applicable, potential pain and/or distress **WILL / WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

_____	_____	_____
Principal Investigator (Printed Name)	Principal Investigator (Signature)	(Date

Animals' Clinical Observations at Blood Collection Form (c)

		Animal ID		Time Point	
	Rectal/Wand	Body Weight (kg):		Scale ID#	*Initials/Date:*
icore	(0) Normal: < 2 sec	(1) Mild: 2 sec	(2) Moderate: 2-3 sec	(3) Severe: >3 sec	*Initials/Date:*
ues Color	(0) Pink	(1) Pale	(3) Cyanotic		*Initials/Date:*
	Normal	Rough	Looses Hair		*Initials/Date:*
	(0) Normal/pink	(1) Dry	(2) Red		*Initials/Date:*
	(0) None	(1) Petechiae	(2) Bruising	(3) Hematoma	*Initials/Date:*
	(0) Normal	(1) Discharge (L) (R)	(2) Hemorrhage (L) (R)	(3) Porphyrin (L) (R)	*Initials/Date:*
	(0) Moist	(1) Dry	(2) Discharge	(3) Epistaxis	*Initials/Date:*
	Heart Rate:	beats in	seconds	= beats per min	
ystem	Rhythm:	(0) Regular	(1) Irregular		*Initials/Date:*
	Pulse quality:	(0) Strong	(1) Weak		
	(0) Normal	(1) Crackles	(2) Wheezes	(3) Stridor	*Initials/Date:*
itation	(0) Normal	(1) Distended			*Initials/Date:*
ion	% Isoflurane	Acepromazine	Medazolam		*Initials/Date:*

Observations recorded by (initials/date):

nd date each entry)

Observation Form (d)

Date		**Animal ID**		**Time Point**	
Temp °C ;	**Rectal/Wand**	**Body Weight (kg):**		**Scale ID#**	***Initials/Date:***
Skin Tent Time Score	(0) Normal: < 2 sec	(1) Mild: 2 sec	(2) Moderate: 2-3 sec	(3) Severe: >3 sec	***Initials/Date:***
Mucous Membranes Color	(0) Pink	(1) Pale	(3) Cyanotic		***Initials/Date:***
Hair coat	Normal	Rough	Looses Hair		***Initials/Date:***
Skin quality	(0) Normal/pink	(1) Dry	(2) Red		***Initials/Date:***
Hemorrhages	(0) None	(1) Petechiae	(2) Bruising	(3) Hematoma	***Initials/Date:***
Eyes	(0) Normal	(1) Discharge (L) (R)	(2) Hemorrhage (L) (R)	(3) Porphyrin (L) (R)	***Initials/Date:***
Nose	(0) Moist	(1) Dry	(2) Discharge	(3) Epistaxis	***Initials/Date:***
Cardiovascular System	Heart Rate:	beats in	seconds	= beats per min	***Initials/Date:***
	Rhythm:	(0) Regular	(1) Irregular		
	Pulse quality:	(0) Strong	(1) Weak		
Lung Sounds	(0) Normal	(1) Crackles	(2) Wheezes	(3) Stridor	***Initials/Date:***
Abdominal Palpitation	(0) Normal	(1) Distended			***Initials/Date:***
Anesthesia/Sedation	% Isoflurane	Acepromazine	Medazolam		***Initials/Date:***

Observations recorded by (initials/date):

NOTES (initial and date each entry)

Animal's Daily Observation Form (d)

SEE SCORING CRITERIA AT THE FRONT COVER OF THE RECORD

Animal ID#:	**Radiation Dose (Gy):**
Study time point	**Supportive care:** Y N

Obs. time	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)			(viii)	(ix)	*Initial*	*Date*
	Activity	Posture Movement	Stool	Stool Consist	Vomit	Respiratory Activity	Breaths	Sec	Rpm	Food left	Temp		
Early am													
am													
pm													
Late pm (after 4pm)													
Night (after 8pm) *													

The critical period is defined as day 14-30 post-irradiation. Monitoring of sick animals will be performed with increased frequency during this time to include: an early morning check (before 9 am), a mid-morning check (between 9am and noon), an afternoon check (between 1pm and 3pm), and a late afternoon check (between 4pm and 6pm).

* If animal conditions are deteriorating rapidly during the day, an additional evening check (after 8pm) will be done, followed by an early morning check at < 10 hours apart. Rapidly deteriorating conditions are defined as: activity score of 2, >1°C change of body temperature per hour, respiratory activity of 1 with continuous dyspnea for more than 10 consecutive minutes, complete anorexia for more than 12 hours, single instance of vomiting/diarrhea, single instance of seizure or vestibular signs, mild hemorrhaging for longer than 6 hours).

Appendix 1

PROTOCOL: in vivo labeling of 5-Bromo-2'-deoxyuridine (BrdU)

GENERAL PROCEDURE

1- Preparation and mixing of BrdU (final concentration 31 mg/ml in PBS)

Preparation and mixing of BrdU will be performed ONLY in a fume hood. Any work with BrdU needs to be done over disposable absorbent bench top spill pads. Any laboratory equipment or surfaces that have come in contact with BrdU will be disposed of or decontaminated. This can be done by wiping them down with soap and water and paper towels. Keep a copy of BrdU safety form and MSDS close by.

- Order 5-Bromo-2'-deoxyuridine from SIGMA (cat. No. B5002, 5 grams, ≥99% pure by HPLC); BrdU can be stored at -20°C as powder or in solution.
- PPE: Disposable lab coat; two pairs of powder-free, disposable nitrile gloves, with the outer one covering the gown cuff whenever there is risk of exposure to hazardous chemicals; eyewear providing chemical splash protection; appropriate lab attire (full-length pants, closed toe shoes, etc.)
- Notify the Open Lab (b)(6) in advance (by email) for the days we will be using the hood for mixing BrdU and that BrdU is a potential carcinogen. Reserve the hood on the day of use.
- Prepare the fume hood by covering the surface with disposable absorbent bench top spill pads. Place inside the hood a stirrer/hot plate, one pipetman, 10 mL pipettes, paper towels, sterile syringes with Luer-Lock™ fittings, sterile syringe

caps, one Nalgene filter unit with 0.2 microns filter (Nalgene® vacuum filtration system (250ml), from Sigma cat no. Z370606), 2 ziplock bags (gallon size), aluminum foil cut in 8x8" pieces for wrapping syringes. The hood will be posted with a sign "CAUTION - CANCER SUSPECT AGENT".

- BrdU stock (powder) is stored at -20ºC. Place one bottle (5 grams) in a ziplock bag, contained inside a polystyrene box covered with absorbent material and sufficiently large to contain a 500 mL beaker. Transport the box to the hood. Place box inside hood.
- Prepare a beaker (250 ml) by adding 160 mL of sterile PBS and a disposable stirring bar. Place a thermometer inside the beaker.
- Place the beaker on the hot plate. The temperature should reach between 40º-50ºC.
- Open the container of BrdU; aspirate about 5 ml of warm PBS from the beaker and add it to the bottle of BrdU (careful not to spill!). Try to mix – the Brdu powder will clog the tip of the pipette. Return the pipette to the beaker, and immerge the tip into the warm PBS. Expel any content and mix by pipetting up and down a couple of time. Again, aspirate about 5 ml of warm PBS from the beaker and add it to the bottle of BrdU and repeat the procedure described above until all the BrdU is dissolved.
- Once the BrdU is completely dissolved, connect the filter unit to the vacuum. Close the empty bottle of BrdU and place in an empty ziplock. Place the filter unit inside the polystyrene box. Carefully, pour the BrdU solution into the filter unit and open the vacuum. Once all the solution has been filtered, close the vacuum,

remove the top portion of the filter unit and discard in the ziplock bag together with the empty BrdU bottle.

- When loading the syringes, first un-wrap one syringe and one cap for each reiteration. Load the syringe with the required volume (i.e. 5 syringes with 10 ml + 1 syringe with 5 mL), recap the syringe, wrap in aluminum foil, and place in a clean ziplock bag.
- Discard empty receiving unit of the Nalgene filter unit in the ziplock with the empty BrdU bottle. Place ziplock with syringes in the polystyrene box. Place box on a cart and wheel to VSD.
- To clean the hood: immediately dispose of all contaminated supplies in a biohazard box (triple-bag). Wipe down surface with 10% bleach. Fill the beaker with 10% bleach, let it sit a few minutes, and rinse with soap and water. Immediately close biohazard box, label with "caution carcinogen suspect agent and "incineration only". Send it for incineration.

2- In vivo labeling – 150 mg/kg final concentration

VSD staff will be notified of the intention of using BrdU two weeks prior to the beginning of the experiment. Rooms will be posted with: CAUTION - CANCER SUSPECT AGENT, AUTHORIZED PERSONNEL ONLY. The SOP will be made available outside the animal room. At all time when handling animals injected with BrdU, personnel will have to wear proper PPE.

3- Required PPE

To prevent skin exposure, appropriate personal protective equipment (PPE) will be worn when handling any amount of BrdU. Ensure that all exposed skin is covered.

- Tyvek coveralls
- Double-nitrile gloves. Use two pairs of powder-free, disposable nitrile gloves, with the outer one covering the gown cuff whenever there is risk of exposure to hazardous chemicals
- Face shield for protective eyewear that provides chemical splash protection
- Appropriate lab attire (scrubs, closed toe shoes, etc.)
- Face mask
- Two pairs of shoe covers. One can be discarded inside the animal room

4- Animal room set up and handling

- Animals will be NPO for BrdU injections. Post a NPO sign outside the room door day before the injections.
- The animal room will be set up with twice as many cages as animals. One half of the cages will be set on one side of the room, and will be used during the days preceding the experiment. The remaining half will be set up on the other side of the room as described below to house animal after BrdU injection.
- Underneath each animal cage set up for BrdU experiment, plastic lined sheets (Custom-cut Diamond TEK liners, dimensions: 24" by 32", Harlan Laboratories Inc., Indianapolis, IN) will be put on the floor first, and then the sheets will be covered with low-dust animal bedding (Product #7092, 1/8" corn cob, Harlan Laboratories Inc., Indianapolis, IN). Also, some bedding will be put on the floor all around the cage to absorb any potential fluid draining from the cage. Additional corn husk bedding may be added during the 72 h period as needed.
- Special care will have to be taken when handling animals and animal excreta for the 72 h subsequent to BrdU administration. Animal room will have an MPW (medical pathological waste) box lined with two biohazard plastic bags to discard used PPE.

- There will be no change in bedding and no cage hosing for the 72 h following BrdU administration. Drains will be temporarily sealed during those 72 h. The weekend caretaker will need to arrive early during the weekend to feed the animals when they are in the first 72 hours period from injection, since VSD personnel is not trained for that. After 72h, the bedding and the underlining plastic liners will be collected in triple bagged biohazard boxes. Each box will be labelled appropriately by identifying the hazard (BrdU animal waste – biohazard/chemical hazard), date, and investigator's name. Put two "Incinerate Only" stickers on the box (one at the top and one at the side), and store the box into VSD (b)(6) cold room for pick up.
- The boxes will be picked up as hazardous waste by USUHS waste pick-up. The regular pick-up schedule is every Wednesday. Pick up services can be contacted at (b)(6) if needed.

5- Necropsy Room set up

Necropsy room will be set up for BrdU injection and to perform necropsy after euthanasia.

Materials:

- Materials required for physical, and injection of telazol, BrdU, and euthasol will be as follow:

  Animal records books, thermometer, timer, clock, syringes and needles, heavy duty packaging tape for biohazard boxes and sharps container, surgical tape for securing the catheter, saline flush, alcohol swab, externalized catheters, disposable 18 Micron filters (Homo-Nate® filter, P/N 50783, Utah Medical Products, Inc., Midvale, UT), telazol, euthasol, isoflurane anesthesia machine with appropriate sized mask, stethoscope, sling, plastic backed absorbent sheets, sharps container, and waste boxes lined with three biohazard plastic bags (01waste box and 01 sharp container for each injection day, and 02 waste boxes and 01 sharp container for each necropsy day).
- For necropsy, materials needed will be: 1 bucket filled with 3 liters of formalin labeled (Animal ID, Species, BrdU, (b)(6) Date, Protocol (b)(4)

(b)(4) scissors, scalpels, forceps, blades. Place "Formalin Caution" stickers on the bucket

6- Procedure for BrdU injection

- Animal will be transported from the animal room to the necropsy room using animal transport cage.
- Once in the necropsy room, animal will be put and secured on the sling.
- The physical will be performed by doing the overall assessment, and taking vital signs.
- If collecting blood prior BrdU injection, animal will be sedated with isoflurane. Blood will be collected from peripheral vein, and then telazol will be injected using dose, route, site of injection and needle size as outlined in Table 3 in section V.4.4.1. Injections.
- If blood is not going to be collected, telazol will be injected after the physical examination. Once animal is completely sedated, catheter and filter will be placed on the animal ear's vein, the flush syringe will be attached to the catheter to check for patency of the vessel.
- If the vein is patent, catheter will be secured by using pieces of surgical tape and BrdU (150 mg/kg) will be injected through the filter. The starting and ending time for BrdU injection will be recorded in the animal record book.
- In case of non-terminal BrdU injection, the catheter will be removed from the ear and animal will be returned back in the room using transport cage. Once in the animal room, animal will be transferred into its cage set up for BrdU experiment (with liners and bedding on the floor) and will be monitored until complete recovery.
- In case of terminal BrdU injection (one hour prior to euthanasia), catheter on the ear will be left in place, animal will be moved from the sling to a transport cage during the one-hour period. Animal will be closely observed during this period. Towels will be used to provide thermoregulation in case animal is hypothermic. Either telazol bump (approximately 0.1 ml) or isoflurane will be given if animal begins to gain consciousness. After one hour, animal will be humanely euthanized as outlined in section V.4.6. Euthanasia.

- Five minutes after injection, auscultate for cessation of heart sounds, and check mucous membrane for cyanosis.

7- Procedure for Necropsy

- Before starting necropsy, wear a PAPR (Powered Air Purifying Respirator) in addition to PPE listed above.
- Put the bucket with formalin for tissue sample collection into the fume hood and turn the air flow on.
- Move the animal from the transport cage to the necropsy table. The table will be covered entirely with the spill absorbent pads.
- Animal is placed in dorsal recumbency. A ventral midline incision is made extending from the manubrium to the pelvic canal. Skin and underlying musculature is reflected to the side. A small incision is made into the peritoneum and abdominal cavity is opened. About 20 cm long section of jejunum is snipped from the small intestine coils on the right side of the abdomen using straight Mayo scissors. The section is laid on the cutting board and cut into two halves. On one half, small horizontal incisions at a distance of 10-20 mm are made along the length using curved metzenbaum scissors and the tissue is dropped into formalin by holding it from the mesentery with thumb forceps (bucket labelled as "short"). The remaining section is opened along the entire length and dropped into formalin. Duodenum and ileum are collected in similar fashion. However, the total length of duodenum collected is about 10-12 cm and therefore one section that is cut open along entire length is approximately 6-8 cm and shorter one with horizontal incisions along the length is about 4 cm. Intestinal contents from the sections that are opened along the entire length are removed gently without touching the mucosae before putting them in formalin.
- Following small intestine tissue collection, sternum is collected using Mayo scissors.
- The tissues collected will stay in fixative for 48 hours at room temperature.
- The sternum is taken out of formalin after 48 hours, as with the other tissues. Then, the sternum is placed in Immunocal (Formic Acid bone decalcifier) solution for 24-48 hours. Finally, it is placed on the tissue processor

overnight, embedded, cut and stained. So, it takes a slightly longer time (3-4 days) to produce the sternum slides compared to lungs and small intestines.

- After necropsy is completed, discard carcass in triple bagged biohazard box, and store into VSD cold room. The box will be appropriately labelled by identifying the hazard (BrdU carcass – biohazard/chemical hazard), date, species, and investigator's name. 02 "Incinerate Only" stickers will be put on the box (one at the top and one at the side).
- Tissues will be ready for immunostaining a week following the necropsy. Submit the tissues (FFPE blocks) for immunostaining.
- Immunostaining - Blocks will be sent out to HISTOSERV, INC., 19526 Amaranth Drive, Germantown, MD 20874, PHONE (301) 972-2600, FAX (301)972-2627, Email: histoserv@histoservinc.com

8- Sanitizing Animal Room and Necropsy Room after BrdU Procedure

Materials needed: scrub brush, 10% bleach, sterilant solution (i.e. Vindicator), miltex instrument cleaner, miltex instrument lubricant, hose and sprayer

For the animal room, cages will be pre-washed with sterilant solution and rinsed using hose and sprayer before sending to cage wash.

For the necropsy room, all materials used for necropsy will first be soaked into 10% bleach, then rinsed. They will be soaked again into water with miltex instrument cleaner added (Alimed Inc., Dedham, MA), and will be allowed to sit for at least 15 min. Following the pre-soak, instruments will be scrubbed and rinsed with water. The clean instruments will be spread out on an absorbent sheet and miltex instrument lubricant (Alimed Inc., Dedham, MA) will be evenly sprayed.

All walls and floor will be pre-sprayed with 10% bleach than scrubbed through using a scrub brush, and rinsed. Then, walls and floor will be sprayed for the second time with sterilant solution that will be allowed to sit on the surfaces for at least 10 minutes. Using the hose and sprayer, all walls and floor will be rinsed and excess water will be squeegeed down the drain.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

Institutional Animal Care and Use Committee
Phone: (b)(6)
Fax: (b)(6)

April 15, 2015

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF ANESTHESIOLOGY

SUBJECT: IACUC Approval of Protocol - Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on April 15, 2015:

Animal Protocol Title: "Serotonin Targeted Biomarker and Therapeutic Approach in the Treatment of Traumatic Brain Injury (blast) and Posttraumatic Stress Disorder"

USUHS Protocol Number: (b)(6)

Expiration Date: April 14, 2018

Supporting Grant(s) Number: TBD

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc: Office of Research

Revised

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Serotonin Targeted Biomarker and Therapeutic Approach in the Treatment of Traumatic Brain Injury (blast) and Posttraumatic Stress Disorder

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6) Ph.D.

FUNDING AGENCY: DARPA

EARLIEST ANTICIPATED FUNDING START DATE: December 22, 2014

PRINCIPAL INVESTIGATOR:

(b)(6)	ANE	(b)(6)	12/15/2014
Principal Investigator Signature	Department	Office Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	Chair	(b)(6)	12/15/2014
Dept. Head Signature	Title	Telephone	Date

Typed Name: (b)(6) MD, MPH
CAPT, MC, USN

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

	MPS	(b)(6)	12/15/2014
Statistician Signature	Department	Telephone	Date

Typed Name: (b)(6) Ph.D.

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)	LAM	(b)(6)	2/18/15
Attending/Consulting Veterinarian Signature	Department	Telephone	Date

Typed Name: (b)(6) VMD

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

ANIMAL PROTOCOL TITLE: Serotonin Targeted Biomarker and Therapeutic Approach in the Treatment of Traumatic Brain Injury (blast) and Posttraumatic Stress Disorder in the Rat (*Rattus norvegicus*)

GRANT TITLE (if different from above): Serotonin Targeted Biomarker and Therapeutic Approach in the Treatment of Traumatic Brain Injury (blast) and Posttraumatic Stress Disorder

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6) Ph.D., (b)(6) Ph.D..

TECHNICIANS(S): (b)(6)
(b)(6)

I. NON-TECHNICAL SYNOPSIS:

Taken together, a critical factor in blast-induced traumatic brain injury (bTBI) and posttraumatic stress disorder (PTSD) lies with the association of cognitive deficits and mitochondrial metabolic integrity in the brain. Serotonin is one of the key neurotransmitter, which is affected by depression and anxiety (Gardner & Boles, 2011). How serotonin levels modulate the stress following bTBI or PTSD is entirely unknown. Our **long-term goal** is to identify the blood-based brain specific protein biomarkers of brain injury progression and to determine the potential targets for safe and effective therapeutic strategies. **The objectives** of this current proposal are to **a)** understand the relationship of serum serotonin levels, psychological, and biological effects of pre-existing stress on the severity of blast injury, and **b)** examine the potential intervention of sodium pyruvate that help to prevent, attenuate, and treat the effects of bTBI and PTSD. The work outlined below will test the **hypothesis** that low serum serotonin levels are associated with the severity of stress-related symptoms following recurrent bTBI and PTSD, which can be improved by increasing the serotonin level with pyruvate treatment.

II. BACKGROUND

II.1. Background:

Disturbances in the neurotransmitter system regulated by the hypothalamic–pituitary–adrenal (HPA) axis constitute a leading cause of neurophysiologic long-term sequelae after traumatic brain injury (TBI; J. Llompart-Pou et al., 2008; McIntosh, 1993). These are the same regions implicated in the etiology of PTSD, suggesting that although the initiating events of brain injury may differ, there may be a common etiological pathway resulting in the overlapping their clinical symptoms (Sumpter & McMillan, 2006).

Part of the problem in the effective treatment of bTBI is the lack of biomarkers to distinguish between PTSD and bTBI due to their overlapping impaired cognitive symptoms. We will compare the serum serotonin, cognitive behavior, and neuropathological markers of bTBI in rats with stress alone, or combined pre-existing stress with bTBI followed by pyruvate treatment to address the questions: **1)** If low serum serotonin can be used as a biomarker of cognitive deficit related to PTSD; **2)** If combined pre-existing stress and TBI also affect serum serotonin levels; and **3)** What is the efficacy of pyruvate treatment in PTSD-like symptoms following stress or stress+bTBI?

In our preliminary study, we found that low serum serotonin is associated with increased anxiety and depression in stressed rats. It is not known if stress associated with blast may also be affected by decreased serotonin levels. Experiments proposed will address the questions: (1) can serum serotonin be used as a biomarker of bTBI, and (2) what is the efficacy of pyruvate treatment in bTBI?

Our published results also suggest a protective role of pyruvate in ischemic astrocytes through the mitochondrial mechanism (Sharma, Karian, Sharma, Liu, & Mongan, 2003; Sharma, Walsh, Kerr-Knott, Karaian, & Mongan, 2005). These neuroprotective properties of pyruvate may be attributable to pyruvate's role in improving neuronal energy via intracellular calcium buffering (Villalba et al., 1994).

Serotonin (5-hydroxytryptamine; 5HT) is a monoaminergic neurotransmitter, produced by its precursor tryptophan. However, the exact origin of serotonin levels around the injured brain area is not known because about 90% of the body's serotonin resides in intestine, blood platelets and pre synaptic endings (Da Prada & Picotti, 1979). Serotonin exerts its multiple physiological actions by its recycling mechanisms through a variety of receptors in brain (5-HT2A, 5-HT3, 5-HT5A and 5-HT7; Barnes & Sharp, 1999).

It is widely believed that brain serotonin deficiency plays a role in depression. Brain serotonin levels in humans can be measured by conventional method of positron emission tomography (PET; Visser et al., 2011). The disadvantage of this method is that tracer used in PET imaging can enter into the kynurenine pathway under inflammatory conditions and thus provide a false signal. In rats, in-vivo serotonin levels can also be measured by voltametery with carbon fiber microelectrodes implanted into the brain. This is used frequently to monitor sub-second dopamine release in freely moving and anesthetized rats (Hashemi et al., 2009). This technique has its own technical difficulties such as electrode deterioration and increased sensitivity to 5-HT measurement. Using commercial ELISA kit, we have recently demonstrated that compared with control animals, low serum serotonin levels in rats are associated with increased anxiety and damaged brain mitochondria in stressed animals with or without mTBI (using fluid percussion). We don't know if serotonin blood levels reflect the brain's level of serotonin. Also, researchers don't know whether the dip in serotonin causes the depression or the depression causes serotonin levels to drop. Therefore, *in this current proposal, we will measure serotonin levels in plasma, CSF, and brain from rats subjected to stress alone, bTBI alone, or stress+bTBI (compared to controls).*

The most common drugs to treat depression are SSRIs (selective serotonin reuptake inhibitors) such as Celexa, Lexapro, Prozac, Paxil and Zoloft. However, serious side effects (dizziness, nausea, lethargy, headache, anxiety, and agitation) have

been reported after the withdrawal of prolonged SSRI treatment. This discontinuation syndrome results from neurophysiologic readjustment in the central nervous system to compensate for the pharmacologic activity of the SSRI (Visser et al., 2011; Bond, 2005). *Now the question is, are there any alternatives that are effective but safer than SSRI?* Based on our preliminary data about the protective effects of pyruvate in increasing the serum serotonin and in reducing the depressive symptoms in PTSD rats, we plan to extend our study on pyruvate effects in bTBI and PTSD on serotonin, cognitive deficits and neuropathology.

Pyruvate: Glucose is an obligatory metabolic fuel for brain. The metabolism of glucose by brain cells requires several coordinated steps involving different cell types and different cellular compartments, either of which may be markedly disrupted by the time a head injured patient is able to recognize the consequences of his/her TBI. Furthermore, experience with the precarious use of glucose to treat head injured patients necessitates the development of a more targeted metabolic and anti-inflammatory treatment strategy (Desagher, Glowinski, & Premont, 1997; Matsumoto, Yamada, Kohmura, Kinoshita, & Hayakawa, 1994).

A decrease brain serotonin following TBI and PTSD is also known to cause reduced cellular metabolism through impaired mitochondrial oxidative phosphorylation, increased inflammation and oxidative stress (Gardner & Boles, 2011). Therefore, in this proposed study; use of sodium pyruvate in the treatment of bTBI and PTSD to elevate the brain serotonin levels through its mitochondrial mechanisms, anti-oxidative and anti-inflammatory property is a novel avenue (Lee, Kim, & Koh, 2001; Matsumoto et al., 1994; Mongan, Capacchione, Fontana, West, & Bunger, 2001; Mongan, Karaian, Van Der Schuur, Via, & Sharma, 2003; Moro, Ghavim, Hovda, & Sutton, 2011; Neigh, Bowers, Pyter, Gatien, & Nelson, 2004; O'Connell et al., 2005; O'Donnell-Tormey, Nathan, Lanks, DeBoer, & de la Harpe, 1987; Sharma, Benford, Li, & Ling, 2009; Sharma et al., 2003; Sharma & Mongan, 2009; Vilau, Pascu, & Kitulescu, 1967). Ours is the first study to examine the effects of pyruvate on serum and brain serotonin levels, cognitive deficits and neuropathology in response to bTBI with/out pre-existing stress.

II.2. Literature Search for Duplication

II.2.1. Literature Source(s) Searched: Medline (PubMed), PsycInfo, F1000Biology (and electronic journal review service), BRD, and RePORT

II.2.2. Date of Search: December, 2014

II.2.3. Period of Search: No Period Restriction

II.2.4. Key Words and Search Strategy: Traumatic brain injury, BOP (blast overpressure), PTSD (post-traumatic stress disorder), rats, age differences, locomotion, activity, anxiety, depression, serotonin, pyruvate (words are entered separately and in combination; rat is entered with each term) have been searched in data base searches. We also read electronic and paper journals regularly and search for phrases, titles, and abstracts relevant to our work.

II.2.5. Results of Search: There has been extensive work on traumatic brain injury, and the various behavioral measures included in our work. However, there has been little work combining these variables with our rat stress model of "Warrior Stress Paradigm" and blast TBI. Overall, literature searches on the topics to be covered in our experiments indicate the value of our approaches, and have not revealed existing

literature or data that would supersede our experiments on blood based biomarkers of brain injury severity in response to repeat blast TBI with/out pre-existing stress and pyruvate treatment.

III. OBJECTIVE\HYPOTHESIS:

The objectives of this current research proposal are: a) Understand the spectrum of changes in serum serotonin levels, psychological and neuro-pathological effects of pre-existing stress on the severity of blast TBI, and b) Examine the potential effects of sodium pyruvate that may prevent, attenuate and treat the effects of repeat blast on TBI and PTSD. The work outlined in this proposal will test the **hypothesis** *that low serum serotonin levels are associated with the severity of stress related symptoms following recurrent bTBI and PTSD, which can be improved by increasing the serotonin level with pyruvate treatment.*

IV. MILITARY RELEVANCE:

The use of improvised explosive devices (IEDs) during the recent Iraq and Afghanistan wars has led to an injury, which is different from that in previous US wars (Traumatic Brain Injury in the United States: A Report to Congress. Emergency Department Visits, Hospitalization, and Deaths, 2002-2006, Atlanta, 2010, CDC report 2010). The Department of Defense and the Defense and Veteran's Brain Injury Center estimate that 22% of all combat casualties from the recent Afghanistan and Iraq conflicts are brain injuries (Hoge et al., 2008).

It is likely that the mild TBI and cognitive impairments observed among many of the troops returning from OIF and OEF result from repeated exposures to blast overpressure (Belanger, Kretzmer, Yoash-Gantz, Pickett, & Tupler, 2009; Sayer et al., 2008; Traumatic Brain Injury in the United States: A Report to Congress. Emergency Department Visits, Hospitalization, and Deaths, 2002-2006, Atlanta, 2010, CDC report 2010). While there are some reports to suggest that PTSD like symptoms in our troops might be due to the war related exposure to psychological trauma (Belanger, Kretzmer, Vanderploeg, & French, 2010; Vanderploeg, Belanger, & Curtiss, 2009). The intriguing questions is "why not all soldiers with mTBI have PTSD?" The diagnostic dilemma and ineffective treatment of mTBI and PTSD offer a great challenge to the clinicians due to the overlapping neurobehavioral symptoms such as insomnia, irritability, social withdrawal, impaired concentration and short-term memory deficit and complex metabolic and biochemical manifestations. If not diagnosed and treated promptly, PTSD quickly manifests itself into a socio-economic burden on society. Hence, it is very important to identify the blood -based biomarkers of TBI and PTSD.

V. MATERIALS AND METHODS

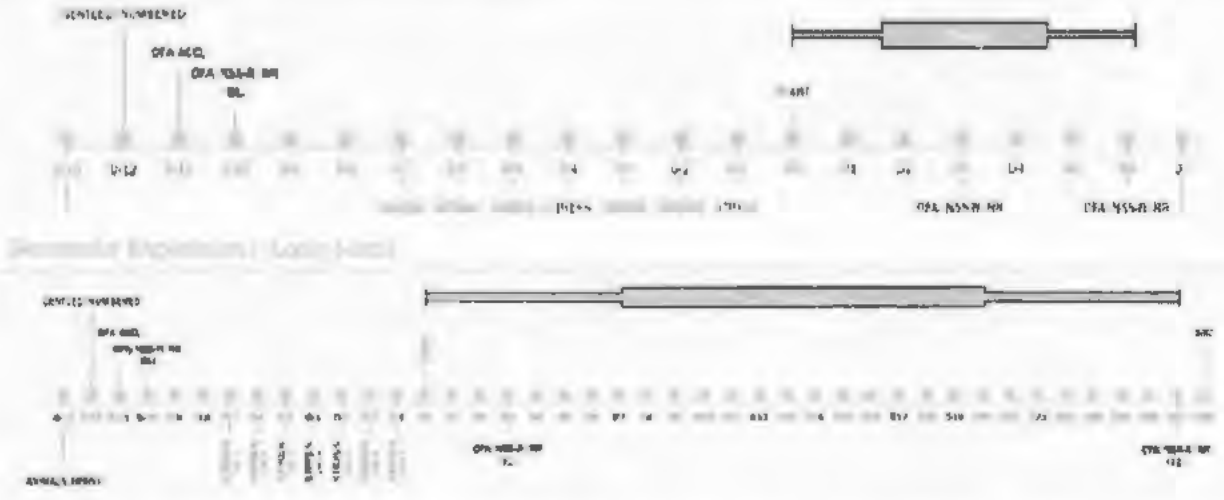
V.1. Experimental Design and General Procedures:

The experimental design for the 2 experiments is: 2 (no blast, blast) x 2 (no stress, stress) x 2 (no pyruvate, pyruvate) x 2 (short-term [7 days], long-term [28 days]) yielding 16 "cells" each of which will include 12 rats (10-12 rats are required for appropriate statistical power, so 12 rats are planned for each cell in case of loss of data due to equipment failures or unforeseen problems). In addition, animals are necessary for

training and piloting procedures (additional 20 rats). Therefore, this protocol requests authorization of 212 rats (see section V.3.4).

Group	Explanation
1	No blast, No Stress, No Drug
2	No blast, No Stress, Drug
3	No blast, Stress, No Drug
4	No blast, Stress, Drug
5	Blast, No Stress, No Drug
6	Blast, No Stress, Drug
7	Blast, Stress, No Drug
8	Blast, Stress, Drug

Both aims will be run simultaneously in counter-balanced cohorts to minimize experimental error. Please see below Tables for timelines of the biological and behavioral data collection. The behavioral test will include 1. Open field activity (OFA)- This test is a measure of motor performance, anxiety and general health of the animal, 2. Neurological severity Score- Revised (NSS-R)- These measures include observation of a righting response, leg flexion, balance, and sensory responses to mild stimuli (e.g., touching ear with cotton-tipped stick, response to light). These measures are similar to those described in CNRM SOP #2, and 3. Rotarod (RR)- The rotarod test measures an animal's fatigue and ability to maintain balance by coordinating the movement of all four feet and making the necessary postural adjustments. It also measures the animal's ability to improve on these skills with practice.



V.2. Data Analysis: Samples sizes for each experiment were based on two strategies: (1) sample sizes of previous experiments in our laboratory and in the published literature that have yielded statistically significant findings; and (2) power analyses of the dependent measures based on data from ongoing experiments and

data reported in the relevant literature following the procedures of (Cohen, 1988; Keppel, 1991; Keppel, Saufley, & Tokunaga, 1992). Estimates of effect size in the population were determined by calculating an estimated omega squared. Phi statistics then were calculated to determine the ratio of treatment variance to error variance for a given sample size. Using phi and power tables, the necessary sample size to achieve 0.80 power was determined. Sample sizes of 12 are appropriate for the behavioral and biological measures. The data will be analyzed by ANOVA or MANOVA (with and without covariates). Tukey HSD post hoc tests or Dunnett's t will be used to compare differences between conditions. All analyses will be two-tailed with alpha = 0.05.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered: This research project relies on biological and behavioral responses in living organisms. Rats are subjects in this study because their responses are similar and predictive of human responses. Non-animal alternatives such as computer modeling and cell cultures were considered but are not suitable to assess biology and behaviors in organisms.

V.3.2. Animal Model and Species Justification:

Rats were chosen as subjects because they provide a valid and reliable model for the questions under study. There is extensive similarity between rodents and humans at the genetic level and there exists an extensive research literature using rats in animal models of a variety of human conditions including stress and mental and physical health. In addition, rodents are commonly used in investigations of TBI.

Dr. (b)(6) laboratory has used rats, mice, other rodents, and primates in their research over the past 30 years. Their findings with rats have been reliable (in their laboratory and in other laboratories) and have proven to be valid predicting effects in human subjects and human populations with regard to the topics that we study. Dr. (b)(6) has also been using rats in her laboratory for over 18 years and has found reliable results.

V.3.3. Laboratory Animals: All animals that will visit the Army Institute of Research (WRAIR) vivarium as part of collaborative studies must be shown to meet WRAIR Veterinary Services Program (VSP) health criteria IAW *UWN-501, Quality Control of Rodents and Rabbits* and/or *UWN-015, Receipt and Quarantine* prior to arrival. Diagnostic results, sentinel data, health certificates and prior notification and coordination with VSP will be required as applicable. The attending veterinarian will deny receipt of animals that do not meet required entry criteria.

		Species #1	Species #2
V.3.3.1.	Genus & Species:	*Rattus norvegicus* (Rat)	
V.3.3.2.	Strain/Stock:	Sprague-Dawley	
V.3.3.3.	Source/Vendor:	Charles River	
V.3.3.4.	Age:	Young adult/ adolescents (about 55 days)	
V.3.3.5.	Weight:	250– 300 g	
V.3.3.6.	Sex:	Males	
V.3.3.7.	Special	N/A	

Considerations:

V.3.4. Number of Animals Required (by Species) 212

V.3.5. Refinement, Reduction, Replacement (3 Rs)

V.3.5.1. Refinement: Animals are carefully monitored to limit or avoid pain and distress. Animals are observed daily for any behavioral signs of distress, including vocalizations, weight loss/gain will be evaluated by periodic weighing every alternate day, changes in food consumption, changes in reactivity, and any signs of injury. If there are any indications of pain or distress, then the LAM staff is consulted. With regard to logistical and technical refinements, many of the behavioral assessments use automated equipment interfaced with computers to allow for the collection of many parameters simultaneously and non-invasively. The use of sophisticated software and computerized technologies allows us to collect fine-grained and complete data sets for every animal included in the experiment, eliminating the need for additional studies that might otherwise have been necessary. Dr. (b)(6) many years of behavioral investigations regarding stress and various drugs also have allowed us to minimize the sample sizes as we have refined testing to be sensitive and reliable.

V.3.5.2. Reduction: The sample sizes for the proposed work were derived from power analyses of pilot data and past work in our laboratory and in other laboratories. These samples sizes therefore constitute the minimum number of animals necessary to obtain meaningful results and represent a useful application of knowledge of statistical effect size and the concept of power. In addition, in each experiment multiple dependent variables are measured. Measurement of many variables in the same animals markedly reduces the total number of experiments conducted. This strategy is used to reduce the number of subjects as much as possible without compromising the science.

V.3.5.3. Replacement: There are no appropriate replacement methods for the proposed work. See CNRM SOP #1 for additional information indicating that replacements are not available for this type of project.

V.4. Technical Methods

V.4.1. Pain / Distress Assessment:

Animals will be anesthetized during brain injury. Animals will be observed daily by investigators and by LAM staff to check their health and well-being and to insure that there is no undue pain or distress based on behaviors (e.g., excessive grooming, shivering, or other unusual motor behaviors) and vocalizations (e.g., squealing or excessive squeaking). Any indications of pain or distress will be dealt with by alleviating the cause of the problem (with the exception of experimentally-induced behavioral distress) or by contacting LAM personnel for assistance and guidance.

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1.	**Number of Animals:**	**Species #1**	**Species #2**
V.4.1.1.1.1.	**Column C:**	48	
V.4.1.1.1.2.	**Column D:**	68	
V.4.1.1.1.3.	**Column E:**	96	

The number of animals in each category has been broken down as follows (please refer to table in Section V.1. for group assignments):

Category C:

- Animals that no not receive blast, stress (Groups 1, 2)

Category D:

- Animals that receive blast but no stress (Groups 5, 6)
- Pilot animals are included in this group to teach techniques

Category E:

- Animals that receive stress (Groups 3, 4, 7, 8)

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

Rats will be anesthetized by inhalation with 5% Isoflurane mixed with compressed medical air (21% O2) at 1 liter/min for 2 minutes prior to TBI using an induction chamber. The inhalant anesthesia does provide sufficient anesthesia while in the blast tube. Animals are placed directly from the induction chamber, into the blast tube, blast occurs, and animal is placed back in home cage. The time it takes once the animal is anesthetized to the time to blast is less than 2 minutes and all animals remain anesthetized. If they wake up too early, they are placed back in the anesthesia.

After 2 min, the rat is tested for consciousness by pinching its tail. If the rat responds to the tail pinch, anesthesia is continued for an additional minute. If the rat does not respond, the anesthetized rat is transferred and secured in the rat holder and secured in the blast tube. Further details regarding this procedure appear in (Yarnell et al., 2013).

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

The non-surgical procedures that may induce momentary discomfort (e.g., tail pinch) are included for scientific purposes and are short in duration (see time course listed for each dependent variable; Section V.4.4.6). Animals are observed daily by investigators and by LAM staff to check on their health and well-being and to assure that there is no undue pain or distress. Animals also are carefully monitored during behavioral testing procedures. In addition, animals are closely monitored during all procedures that involve any pain or potential distress (e.g., during and after minor surgery; during and after injections). Pain/distress is assessed based on behaviors and vocalizations. Any indications of pain or distress are dealt with by alleviating the cause of the problem (with the exception of experimentally-induced behavioral distress) or by contacting LAM personnel for assistance and guidance.

Repeat Blast exposure: Blast exposures will be conducted in the morning; animals will be exposed to 3 blasts (at 75 kPa each) with 30 minutes in between each blast. Animals will be monitored continuously, by the PI and/or authorized staff member for an additional three hours post-blast or until they are fully recovered from anesthesia at WRAIR. After recovery, animals will be brought back to USUHS. After blast exposure day they will be monitored at least twice daily, by the research staff and LAM staff, until the study endpoint for that animal is reached.

Pain Assessment Score Sheet (please see attachment): This instrument will be used for all post blast evaluations to ensure that pain symptoms are assessed in a

relatively objective manner. If the pain assessment score sheet indicates, the attending veterinarian at WRAIR will be contacted promptly to provide their clinical opinion and consult with PI and/or authorized research staff to determine disposition. If an animal is euthanized as a result of this process, the appropriate pain category numbers will be reported to the IACUC. We typically do not observe signs of pain or distress post-blast – especially at the blast intensity specified.

Rat stress model: We developed a non-painful "Warrior Stress Paradigm" to model impending threat of attack in novel environments based on previous studies, many of which were conducted in our laboratory (Yarnell, 2013). Briefly, in this stress paradigm, predator stress is manipulated by introducing a cotton ball with commercially purchased synthetic fox urine into a test cage with the rat. Unpredictable, non-painful stressors include noise, flashing light, and cage shaking. The animal is transferred from its home cage to the stress cage with lid. The fox urine (10mL) is absorbed by a large cotton ball and placed in varying spots in the stress cage. The procedure is conducted in a room separate from the housing room and the behavioral rooms. A bright fluorescent overhead light remains on during the procedure. This procedure lasts 20 minutes. The stimuli are presented for 7 days in a manner designed to minimize habituation of the response. Each manipulation occurs no more than once per day. A combination of these stimuli avoids habituation of the response.

Pyruvate administration: Sodium pyruvate (1g/kg) will be started within 30 min after the completion of injury/stress protocol (on day 0), and then it will be administered every 24h until the end of the experiment, by oral gavage in 2 ml sterile distilled water. At the same time, control animals will receive the similar volume of distilled water.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Sources Searched: Medline (PubMed), PsycInfo, and F1000 Biology, BRD, RePORT.

V.4.1.3.2. Date of Search: December, 2014

V.4.1.3.3. Period of Search: All years available on each database are searched (from recent work up to 20 years in the past)

V.4.1.3.4. Key Words of Search: brain injury, brain injury model, blast injury, pain, rat, anesthesia (these words are searched in carious combinations)

V.4.1.3.5. Results of Search: No alternatives are available to avoid using live animals for the assessment of biological and behavioral effects of brain injury and in intact rats and the procedures that are followed. The blast overpressure procedure (See section V.4.4.7) is described in detail in a recent paper (Yarnell, et al., 2013). This paradigm is considered to be the best animal model for exposure to improvised explosive devices. Animals are monitored closely for > 1 hour after brain injury and are monitored daily during each behavioral measure as well as during daily rounds by investigators.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

This study is designed to examine and treat the effects of pre-existing stress on blast TBI in terms of neuropatholical and neurobehavioral parameters. Yes, our stress

procedure will cause temporary distress during animal exposure to light, sound, and predator scent. But this non-painful stress procedure will cause less disstress than other existing stress protocols such as "repeat tail shock or animal exposure to the close vicinity of live predators". Our proposed stress model of "**Warrior Stress Paradigm**" is to simulate the impending threat of attack in novel military environments.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery N/A
V.4.3.1. Pre-surgical Provisions: N/A
V.4.3.2. Procedure: N/A
V.4.3.3. Post-surgical Provisions: N/A
V.4.3.4. Location: N/A
V.4.3.5. Surgeon: N/A
V.4.3.6. Multiple Major Survival Operative Procedures: N/A
V.4.3.6.1. Procedures: N/A
V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations
V.4.4.1. Injections: N/A
V.4.4.2. Biosamples:

Blood for biochemical analysis: Just prior to the beginning (day -10) and at the termination of the experiments, using a 22 Gauge BD Vacutainer® Eclipse™ blood collection needle, 100-150 µl blood will be drawn from the tail vein into an anticoagulant-free (red top) Vacutainer®. 50 uL of blood will be immediately used for ISAT machine for determination of blood gases, acid-base parameters, glucose, lactate and pyruvate. We will allow the remaining blood to clot for 15-20 minutes at room temperature. We will centrifuge the Vacutainers® for 10 minutes at 2,500-2,800 revolutions per minute (rpm) (1,500-2,000 x g) to separate the plasma.

Collection of CSF for brain injury evaluation: Just before the termination of experiment, rats anesthetized with isoflurane will be secured in a stereotactic frame in such a way as to allow the head to move up and down but not to move side-to-side. The back of the head and neck will be shaved clean of fur, and wiped with alcohol swabs. The head will be flexed in order to clearly display the occipital protuberance in the neck. After making a skin incision on the midline above the cervical vertebrae and the occipital protuberance, the atlantooccipital membrane will be evidenced by blunt dissection. A 25G needle will be used to puncture the membrane and collect the cerebrospinal fluid. The fluid will be cleared by centrifugation and the supernatant saved at -80°C.

Collection of brain parts for molecular biology, enzyme assays and serotonin measurements: Deeply anesthetized animals (8 rats/group) will be decapitated and brains will be quickly removed and placed on ice; the frontal cortex, amygdala, and the dorsal and ventral hippocampus will be dissected, weighed, flash frozen, and stored at -80C until use. Brain regions will be processed individually for protein extraction. In addition to the brain, we will also collect intestine, lungs, and spleen for further studies to examine the impact of blast exposure and stress on serotonin levels.

Collection of whole brain tissue for histology: Deeply anesthetized animals (4 rats/group) will be transcardially perfused with normal saline, followed by 4% paraformaldehyde in phosphate buffered saline (PBS) into the left ventricle. Following fixation, immediately thereafter, the brain, spinal cord, lungs, spleen, and intestine will be removed, and post-fixed for further evaluation. Organs will be post-fixed in 4% paraformaldehyde and 10% sucrose in PBS overnight at 4°C. The next day, brains will be transferred to 30% sucrose solution to complete the cryoprotection; after 24 hours or when organs no longer floats, they will be stored at -80°C.

V.4.4.3. **Adjuvants**: N/A

V.4.4.4. **Monoclonal Antibody (MAbs) Production**: N/A

V.4.4.5. **Animal Identification**: Cages are numbered corresponding to the animals in that cage and rat tails are coded with a marker using a stripe system that corresponds to units of hundreds, tens, or ones, depending on the location of the mark on the tail.

V.4.4.6. **Behavioral Studies**: Behavioral testing of rats will be performed in Laboratory for Animal Research (LAM) at USUHS. All rats will undergo the following neurobehavioral tests to determine 1) what kinds of behaviors are affected by the brain injury and/or stress; and 2) the outcomes of various treatments to see if there is an improvement after the treatment. Data from tests that utilize categorical (non-continuous) scoring (e.g., 5-point scales) should be analyzed by a non-parametric statistical method that does not make any assumptions concerning the normality of data distribution. All behavioral measures will occur on the same day and will occur directly after the previous one is complete (wait times of approximately 15-45 min will occur while equipment is being cleaned and while other animals are being tested).

1) **Open field activity (OFA)**. OFA is a collection of sensitive unconditioned behaviors that occur when an animal moves in its environment. These measures have been used in rat studies in Dr (b)(6) laboratory for many years (including recent studies of single blast-induced and other brain injuries) and have provided reliable and valuable data (Bowen, Eury, & Grunberg, 1986; Elliott, Faraday, Phillips, & Grunberg, 2004; Elliott & Grunberg, 2005; Faraday, Elliott, Phillips, & Grunberg, 2003; Faraday, O'Donoghue, & Grunberg, 2003; Faraday, Scheufele, Rahman, & Grunberg, 1999; Grunberg & Bowen, 1985; Morse et al., 1997). Horizontal activity provides information about gross motor performance and general health; center time provides an index of anxiety-related behavior (with an inverse relationship between center time and anxiety); vertical activity provides an index of depression-related behavior (with escape behaviors inversely related to depression). Locomotor activity is measured using an Accuscan Electronics infrared photocell system (Accuscan Electronics, Columbus, OH), located in a dedicated room within the animal facility. This room is constructed of cinderblock walls, acoustic tile ceiling and steel doors so that sound is kept to a minimum. One-hour activity measurements will be obtained during animals' active or dark cycle. Animals will be placed singly in a 40 x 40 x 30 cm clear Plexiglas arena and a Plexiglas lid with multiple holes to allow air flow. The lid ensures that subjects have adequate ventilation but cannot escape during data collection. A photocell array measures horizontal

locomotor activity and vertical activity. Data will be automatically gathered and transmitted to a computer equipped with "Fusion" software (from Accuscan Electronics). Once subjects are placed in the test areas, the experimenter turns off the lights and leaves the room. Chambers are cleaned between subjects with a clidox-s solution. Duration, frequency of repetition, and number of test days: The duration of locomotor activity testing is a maximum of 60 min per day. Animals will be acclimated to the equipment and then tested prior to blast exposure (baseline), 3 days, and either 6 or 27 days post exposure. OFA will be performed around the same time at each time point.

2) **Neurobehavioral observation.** Observations will be made and recorded using the Neurobehavioral Severity Scale-Revised (NSS-R) that was developed in the PI's laboratory (Grunberg, Yarnell, Chwa, Hutchison, & Barry, 2011) and based on neurological severity scales in the literature (Hamm, 2001; Shohami, Novikov, & Bass, 1995) using a Likert-format scoring system. These measures include observation of a righting response, leg flexion, balance, and sensory responses to mild stimuli (e.g., touching ear with cotton-tipped stick, response to light). The measures are similar to those described in CNRM SOP #2.
The NSS-R is a specific, continuous sequence of behavioral tests and observations based on the Neurological Severity Score (NSS). The 10 tasks assess reflex suppression, general movement, and postural adjustments in response to a challenge. The tests are ordered to ensure that subsequent measures are not affected by the preceding measure. Each task is scored using a three-point Likert scale, in which a normal, healthy response is assigned a "0," a partial or compromised response is assigned a "1," and the absence of a response is assigned a "2." Scores are totaled (max score of 20; where higher scores reflect greater extent of injury) and analyzed. Duration, frequency of repetition, and number of test days: The duration of neurobehavioral testing is a maximum of 5 min per day. Animals will be tested prior to blast exposure (baseline), 3 days, and either 6 or 27 days post exposure. Neurobehavioral observations will be performed around the same time at each time point.

3) **Rotarod.** The rotarod test measures an animal's fatigue and ability to maintain balance by coordinating the movement of all four feet and making the necessary postural adjustments. It also measures the animal's ability to improve on these skills with practice. Each Rota-Rod treadmill consists of a motor-driven drum with constant speed or accelerating speed modes of operation. The drum (7.0 cm diameter) allows each animal to maintain a suitable grip. It is divided into four test zones (8.9 cm wide) so that up to four animals may be tested at the same time. The device consists of a smooth hard plastic cylinder with concentric circular plastic sides (39.7 cm diameter) attached to prevent the rat from climbing off the cylinder laterally. The cylinder is connected to a variable speed reversible motor, allowing the speed and direction of rotation of the cylinder to be changed.
An accelerated rotarod task will be used with a Med Associates rat rotarod (Med Associates, Inc, St. Albans, VT). The equipment consists of a metal frame with polyvinyl chloride (PVC) dividers. Rats will be placed on the device facing the wall with the rod rotating in the direction opposite from the animal (so that the rat has to use its

paws to pace forwards to avoid falling off the rod). When a rat falls of the rotating drum (height of 26.7 cm), it breaks a photobeam, stopping the timer associated with that chamber. Once all four photobeams have been broken, the drum will cease rotating. Rats will be tested 3 times/time point on this device. In each trial, the speed of rotation will be slowly increased from 0 revolutions per minute (rpm) to 35 rpm for a maximum of 5 min. The mean duration on the device, expressed in seconds will be recorded for scoring each rat. Each animal's performance will be evaluated, including time spent on the rotarod at each rotation speed. The procedures will be consistent with other reports in the literature (e.g. McPhee, et al., 2005; Rustay et al., 2003).
Duration, frequency of repetition, and number of test days: The duration of neurobehavioral testing is a maximum of 15 min per day. Animals will be tested prior to blast exposure (baseline), 3 days, and either 6 or 27 days post exposure. Neurobehavioral observations will be performed around the same time at each time point.

V.4.4.7. Other Procedures:

For blast over pressure (BOP), rats will be transported to WRAIR and back to USUHS in a closed van provided by WRAIR and driven by an insured WRAIR driver. Rats will be housed in transportation cages. Cages will be secured from sliding and room temperature will be maintained. The vehicle will be cleaned before and after transport. WRAIR requires that we use their van and driver (this procedure of animal transport to WRAIR is similar with our current ongoing studies).

For BOP, there will be no surgery. Exposure to BOP will be conducted at WRAIR under the direction of Dr. (b)(6) following procedures described by our research group (Yarnell et al., 2013). The blast tube consists of a 2.5 ft long compression chamber separated from a 15 ft long expansion chamber (both of 1 ft diameter) by a polyester Mylar sheet (Du Pont, Wilmington DE). Anesthetized rats will be placed singly in a secured holder within the mouth of the blast tube. Using an air compressor, the pressure is then increased in the compression chamber until it reaches a critical value determined by the thickness of the Mylar sheet at which point the Mylar ruptures, causing a blast pressure wave to pass down the expansion tube. Available membranes will be calibrated to rupture at a compression pressure generating an exposure pressure of ~ 75 kPa. This level of blast exposure produces a mild traumatic brain injury. Rats will be exposed to three consecutive blasts with about 30 minutes in between each blast (three blast exposures in one day). After injury the rats will be returned to their home cages and monitored until consciousness is regained. They will be observed at WRAIR for at least 30 min after exposure to BOP. Rats will be kept at WRAIR for less than 12 hours.

The temperature of the transport vehicle will be maintained between 68-72°F. Once rats have received their blast exposure, they will be monitored for 30 minutes after they have recovered righting reflexes and will be examined to determine that they are not showing any signs of severe stress in excess of the symptoms expected for recovery from anesthesia before they are transported back to USU. An approved member on the protocol will accompany the rats at all times at WRAIR and monitor the rats for any signs of severe stress. If any signs of severe stress occur while the animals are away from USU, the accompanying investigator will euthanize the animal within the

WRAIR facilities.

BOP injuries will be conducted at WRAIR, Forest Glen. Behavioral measures will be conducted in the laboratories of Dr. (b)(6) in LAM at USUHS.

V.4.4.8. **Tissue Sharing**: N/A

V.4.5. Study Endpoint:

The study endpoint is euthanasia in each experiment. Rats will be euthanized on either 7 or 28 days after injury (injury day = day 0 [i.e., all animals including sham will be euthanized on days 7 or 28]; depending on if they are in the short- or long-term injury group). If any animals show evidence of pain or distress at any time, then LAM staff will be consulted regarding the most appropriate treatment or procedures. If the condition is not ameliorated in the judgment of a LAM veterinarian, then the rat will be euthanized after consultation with the PI.

V.4.6. Euthanasia:

Subjects will be euthanized by increasing dose of isoflurane followed by necropsy for organ harvesting. For transcardial perfusion, deeply anesthetized animals will be transcardially perfused with normal saline first followed by 4% paraformaldehyde in phosphate buffered saline (PBS) into the left ventricle.

If any signs of severe stress occur while the animals are away from USU, the accompanying investigator will euthanize the animal within the WRAIR facilities. WRAIR has a similar setup for euthanizing animals, as USU and the same procedures will be followed.

V.5. Veterinary Care

The WRAIR IACUC Collaboration Protocol Signature Sheet (UWZ-1001 Processing, Reviewing and Maintenance of Extramural and Collaborative Proposals) must be signed by the Institute PI, the Business Office, the Joint Safety and Environment Office (JSEO) and the Attending Veterinarian or designee prior to protocol approval. This form indicates the responsible parties have reviewed and can support the collaborative proposal.

V.5.1. Husbandry Considerations:

Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. **Study Room:**

Building(s) LAM facility (b)(6) Room Number(s) (b)(6)

(b)(6) (b)(6)

V.5.1.2.

Special Husbandry Provisions:

Food Restriction:	Yes	_____	No	X
Fluid Restriction:	Yes	_____	No	X

V.5.1.3. **Exceptions**: None

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care:

Animals will be observed and cared for by the Center for LAM veterinary technicians and veterinarians according to USUHS Center for LAM SOPs.

V.5.2.2. Emergency Veterinary Medical Care:

All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy:

Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Name of person performing activity	Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
(b)(6) **Ph.D.**	Animal Handling	18+ years' experience with rat surgery	USUHS Investigator Training and Animal Handling courses (Several sessions, including session in 2000). CITI tarininh 2014
	Oral gavage	18+ years	
	Anesthesia	18+ years	
	Blood collection	18+ years	
(b)(6) **Ph.D.**	Animal Handling	30+ years experience with rats and other rodents	USUHS Investigator Training and Animal Handling courses (several sessions, including sessions in fall, 1980 and again in fall, 1990); NIH/NIMH training courses (several in fall, 1981-4)
	Oral gavage	30+ years experience with rats and other rodents	USUHS Investigator Training and Animal Handling courses, 1980,1990; NIH/NIMH training

			courses 1980s
	Behavioral Measures	30+ years experience with rats and other rodents	NIH/NIMH training courses1980s
	Biological sample collection	30+ years experience with rats and other rodents	NIH/NIMH training courses 1980s
	Anesthetized Decapitation	30+ years experience with rats; 1000+ decapitations performed	NIH/NIMH training courses1980s
	Blast tube procedures	5 years experience	WRAIR collaboration 2009 - present
(b)(6) **Ph.D.**	Will not be handling animals	N/A	N/A
	Animal Handling	4 years experience with rats	USUHS Investigator Training and Animal Handling courses (Fall, 2010); also training by Dr. (b)(6) and lab personnel (Fall, 2010)
	Oral gavage	Limited experience with rats Will be trained by PI	USUHS Investigator Training and Animal Handling courses; training by Dr. (b)(6) and lab personnel (Fall, 2010)
	Behavioral Measures	4 years experience with rats	Training by Dr. (b)(6) and lab personnel (Fall, 2010). Additional training by Dr. (b)(6) as needed.
	Biological sample collection	4 years experience with rats	Training by Dr. (b)(6) and lab personnel (Fall, 2010)
	Anesthetized Decapitation	4 years experience with rats	Training by Dr. (b)(6) and lab personnel (Fall, 2010)
	Blast tube procedures	4 years experience	WRAIR collaboration 2010-present
(b)(6)	Animal Handling	1 year experience with rats	USUHS Investigator Training and Animal Handling courses (Fall, 2013); training by Dr. (b)(6) and lab personnel (Fall & Winter, 2013)
	Oral gavage	limited experience with rats Will be trained by PI	USUHS Investigator Training and Animal Handling courses; training by Dr. (b)(6) and lab personnel (Fall, 2013)
	Behavioral Measures	1 year experience with rats	Training by Dr. (b)(6) and lab personnel (Fall, 2013)

	Biological sample collection	1 year experience with rats	Training by Dr. (b)(6) and lab personnel (Spring, 2014). Additional training by Dr. (b)(6) as needed.
	Anesthetized Decapitation	1 year experience with rats	Training by Dr (b)(6) and lab personnel (Spring, 2014)
(b)(6)	Animal Handling	4 years experience with rats	USUHS Investigator Training and Animal Handling courses (Spring, 2011); training by Dr. (b)(6) and lab personnel (Spring, 2010)
	Oral gavage	limited experience with rats Will be trained by PI	USUHS Investigator Training and Animal Handling courses; training by Dr. (b)(6) and lab personnel (Fall, 2010)
	Behavioral Measures	4 years experience with rats	Training by Dr. (b)(6) and lab personnel (Spring, 2010)
	Biological sample collection	4 years experience with rats	Training by Dr. (b)(6) and lab personnel (Fall, 2010). Additional training by Dr. (b)(6) as needed.
	Anesthetized Decapitation	4 years experience with rats	Training by Dr (b)(6) and lab personnel (Fall, 2010)
	Blast tube procedures	4 years experience	WRAIR collaboration 2010-present
(b)(6)	Animal Handling	.5 years experience with rats	USUHS Investigator Training and Animal Handling courses (Spring, 2014); training by Dr. (b)(6) and lab personnel (Spring, 2014)
	Oral gavage	limited experience with rats Will be trained by PI	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel (Spring, 2014)
	Behavioral Measures	.5 years experience with rats	Training by Dr (b)(6) and lab personnel (Spring, 2014)
	Biological sample collection	.5 years experience with rats	Training by Dr (b)(6) and lab personnel (Spring, 2014). Additional training by Dr. (b)(6) as needed.
	Anesthetized Decapitation	.5 years experience with rats	Training by Dr. (b)(6) and lab personnel (Spring, 2014)
(b)(6)	Animal Handling	3 years experience with rats	USUHS Investigator Training and Animal Handling courses (Spring, 2011); training by Dr. (b)(6) and lab personnel

			(Spring, 2011)
	Oral gavage	limited experience with rats Will be trained by PI	USUHS Investigator Training and Animal Handling courses; training by Dr. (b)(6) and lab personnel (Fall, 2011)
	Behavioral Measures	3 years experience with rats	Training by Dr. (b)(6) and lab personnel (Spring, 2011)
	Biological sample collection	3 years experience with rats	Training by Dr (b)(6) and lab personnel (Fall, 2011). Additional training by Dr. (b)(6) as needed.
	Anesthetized Decapitation	3 years experience with rats	Training by Dr. (b)(6) and lab personnel (Fall, 2011)
	Blast tube procedures	1 year experience	WRAIR collaboration (Fall, 2013)
(b)(6)	Animal handling, blood, tissue collection. Oral gavage	Research Assistant	Rodent handling class and Investigator Training, 2009, 2015. CITI training 2015
(b)(6)	Blood collection, terminal procedure and formalin fixation, histochemistry	Co-investigator, 4 year experience	Rodent handling class and Investigator Training, 2004 CITI training 2014
(b)(6)	Animal Handling	1 year experience with rats	USUHS Investigator Training and Animal Handling courses (Fall, 2013); training by Dr. (b)(6) and lab personnel (Fall & Winter, 2013)
	Oral gavage	limited experience with rats Will be trained by PI	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel (Fall, 2013)
	Behavioral Measures	1 year experience with rats	Training by Dr (b)(6) and lab personnel (Fall, 2013)
	Biological sample collection	1 year experience with rats	Training by Dr. (b)(6) and lab personnel (Faill, 2013). Additional training by Dr. (b)(6) as needed.
	Anesthetized Decapitation	1 year experience with rats	Training by Dr. (b)(6) and lab personnel (Fall, 2013)
(b)(6)	Blast Tube operation	3 years experience with blast tube operations	WRAIR Aseptic Training (Feb., 2011); Rat Handling (Feb., 2011); IACUC Guidelines for PI's and Staff (Feb., 2011)

(b)(6)	Animal Handling	3 year experience with rats	USUHS Investigator Training and Animal Handling courses (Fall, 2011); training by Dr. (b)(6) and lab personnel
	Oral gavage	limited experience with rats Will be trained by PI	USUHS Investigator Training and Animal Handling courses; training by Dr. (b)(6) and lab personnel (Fall, 2011)
	Behavioral Measures	3 year experience with rats	Training by Dr. (b)(6) and lab personnel (Fall, 2011)
	Biological sample collection	3 year experience with rats	Training by Dr (b)(6) and lab personnel (Fall, 2011). Additional training by Dr. (b)(6) as needed.
	Anesthetized Decapitation	3 year experience with rats	Training by Dr (b)(6) and lab personnel (Fall, 2011)
(b)(6)	Blood collection, terminal procedure and formalin fixation	Research Technician, >15 years experience	Rodent handling class and Investigator Training, 2009, CITI training 2014
(b)(6)	Blast Tube operation	5 years experience with blast tube operations	WRAIR Aseptic Training (Feb., 2013); Rat Handling (Feb., 2013); IACUC Guidelines for PI's and Staff (Feb., 2013)
(b)(6)	Animal Handling	1 year experience with rats	USUHS Investigator Training and Animal Handling courses (Fall, 2013); training by Dr. (b)(6) and lab personnel (Fall & Winter, 2013)
	Oral gavage	limited experience with rats Will be trained by PI	USUHS Investigator Training and Animal Handling courses; training by Dr. (b)(6) and lab personnel (Fall, 2013)
	Behavioral Measures	1 year experience with rats	Training by Dr. (b)(6) and lab personnel (Fall, 2013)
	Biological sample collection	.5 year experience with rats	Training by Dr. (b)(6) and lab personnel (Winter, 2013). Additional training by Dr. (b)(6) as needed.
	Anesthetized Decapitation	.5 year experience with rats	Training by Dr (b)(6) and lab personnel (Spring, 2014)
(b)(6)	Animal Handling	2 year experience with rats	USUHS Investigator Training and Animal Handling courses (Fall, 2012); training by Dr. (b)(6) and lab personnel

			(Fall & Winter, 2012)
	Oral gavage	limited experience with rats Will be trained by PI	USUHS Investigator Training and Animal Handling courses; training by Dr. (b)(6) and lab personnel (Fall, 2012)
	Behavioral Measures	2 year experience with rats	Training by Dr. (b)(6) and lab personnel (Fall, 2012)
	Biological sample collection	2 year experience with rats	Training by Dr (b)(6) and lab personnel (Winter, 2013). Additional training by Dr. (b)(6) as needed.
	Anesthetized Decapitation	2 year experience with rats	Training by Dr (b)(6) and lab personnel (Winter, 2013)

VII. BIOHAZARDS/SAFETY: During animal handling, all personnel will wear personal protective equipment including lab coats, gloves and face- mask to minimize the exposure to rat allergens. All personnel included in this protocol will also annually visit the USUHS health nurse to discuss their needs and suggestions regarding their safety.

All animal perfusions with formaldehyde will be performed in fume hood or a ventilated table in LAM necropsy room to avoid inhalation of toxic fumes from formaldehyde. In addition, all protocol members will wear appropriate protective equipment.

VIII. ENCLOSURES:

1. See attached letter of support for access to the BOP tube at WRAIR from Dr. (b)(6)
2. See attachment for WRAIR's pain assessment score sheet that is used after BOP procedure to determine the level of animal discomfort.

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) 02 | 18 | 2015

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

TBD

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing

animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) [redacted]

02 | 18 | 2015

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress WILL or WILL NOT be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) [redacted]

02 | 18 | 2015

Principal Investigator Signature Date

X. **PROTOCOL ABSTRACT:**

A. **Animal Protocol Number:**

B. **Animal Protocol Title:** Serotonin Targeted Biomarker and Therapeutic Approach in the Treatment of Traumatic Brain Injury (blast) and Posttraumatic Stress Disorder

C. **Principal Investigator:** (b)(6) PhD

D. **Performing Organization:** Uniformed Services University of the Health Sciences

E. **Funding:** DARPA

F. **Objective and Approach:** Taken together, a critical factor in blast-induced mTBI and PTSD lies with the association of cognitive deficits and mitochondrial metabolic integrity in the brain. Serotonin is one of the key neurotransmitter, which is affected by depression and anxiety (Gardner & Boles, 2011). How serotonin levels modulate the stress following bTBI or PTSD is entirely unknown. Our **long-term goal** is to identify the blood-based brain specific protein biomarkers of brain injury progression and to determine the potential targets for safe and effective therapeutic strategies. **The objectives** of this current proposal are to **a)** understand the relationship of serum serotonin levels, psychological, and biological effects of pre-existing stress on the severity of blast injury, and **b)** examine the potential intervention of sodium pyruvate that help to prevent, attenuate, and treat the effects of bTBI and PTSD. The work outlined below will test the **hypothesis** that low serum serotonin levels are associated with the severity of stress-related symptoms following recurrent bTBI and PTSD, which can be improved by increasing the serotonin level with pyruvate treatment.

G. **Indexing Terms (Descriptors):** traumatic brain injury (TBI), post-traumatic stress disorder (PTSD), stress, blast overpressure (BOP), serotonin, pyruvate, behavioral/biological responses of rats to TBI/PTSD/stress

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April 3, 2012

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS

SUBJECT: IACUC Approval of Protocol - Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via designated member review on April 3, 2012:

Animal Protocol Title: (b)(4)
(b)(4)

USUHS Protocol Number: (b)(6)

Expiration Date: April 2, 2015

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Vice Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: (b)(4)
(b)(4)

GRANT TITLE (if different from above): (b)(4)
(b)(4)

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: (b)(4)

EARLIEST ANTICIPATED FUNDING START DATE: 10/16/2011

P (b)(6) **R:** (b)(6)

P

APG
Department

Office/Lab Telephone

02/23/12
Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)
Research Unit
Typed Name:

Signature
MD, PhD

Professor/Chair
Title

(b)(6)
Telephone

02.23.12
Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)
Statistician Signature
Typed Name (b)(6) Ph.D.

PMB
Department

Telephone (b)(6)

Date 2/23/12

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)
ignature
DVM

(b)(6)

LAM
Department

(b)(6)
Telephone

3/16/12
Date

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: (b)(4) (b)(4)

GRANT TITLE (if different from above): (b)(4) (b)(4)

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: (b)(4)

EARLIEST ANTICIPATED FUNDING START DATE: 10/16/2011

PRINCIPAL INVESTIGATOR: (b)(6)

Principal Investigator Signature | Department | Office/Lab Telephone | Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

Research Unit Chief / Dept. Head Signature | Title | Telephone | Date
Typed Name: (b)(6) MD, PhD

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

Statistician Signature | Department | Telephone | Date
Typed Name: (b)(6) Ph.D.

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

Attending/Consulting Veterinarian Signature | LAM Department | Telephone | Date
Typed Name: (b)(6) DVM

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6)

ANIMAL PROTOCOL TITLE: (b)(4)
(b)(4)

GRANT TITLE (if different from above): (b)(4)
(b)(4)

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6)

TECHNICIANS(S): (b)(6)
(b)(6)

I. NON-TECHNICAL SYNOPSIS: (b)(4)
(b)(4)

II. BACKGROUND:

II.1. Background:

The effects (b)(4)

The possibility of a terrorist attack against civilians, or military troops deployed overseas is at present in the minds of both citizens and government officials. Mass casualties can be inflicted with chemical or biological toxins/weapons. Nerve agents are lethal chemical weapons that have been used in war and in terrorist attacks, with devastating consequences. Organophosphate nerve agents display a very high affinity for brain acetylcholinesterase (AChE) (9, 10, 11, 63). Inhibition of AChE by OPs and the resulting accumulation of acetylcholine can initiate seizures by activating nicotinic and muscarinic receptors, which are widely distributed throughout the brain. These receptors are present not only at postsynaptic sites in cholinergic synapses, but also on presynaptic terminals of both excitatory and inhibitory synapses, regulating the release of glutamate and gamma-aminobutyric acid (GABA) (37, 108). Therefore, it is conceivable that OP-induced inhibition of AChE can tilt the balance between the two major excitatory and inhibitory neurotransmitter systems—glutamatergic and GABAergic, respectively (38, 55). One of the clinical manifestations of exposure to nerve agents is seizure activity and status epilepticus (SE), which can lead to death or brain damage, with long-term cognitive and other behavioral consequences (8, 35, 56, 91, 99) (b)(4)
(b)(4)
(b)(4) Current medical countermeasures against nerve agent

poisoning are not adequately effective in preventing seizure-induced brain damage (57, 89, 96). (b)(4)

(b)(4)

(b)(4) This is very important because in an emergency situation there is no opportunity for pretreatment. Our ultimate goal is to develop a medical countermeasure against nerve agents that will effectively stop seizures and protect from brain damage and the resulting behavioral deficits, and do so without significant acute and/or long-term adverse effects. (b)(4)

(b)(4)

The necessity to study immature, aged, and female rats

Having demonstrated the effectiveness of the (b)(4)

(b)(4) we now propose to test if these compounds are equally effective in young and aged rats, as well as in female rats. Are there reasons to expect age- and/or gender-related differences in the (b)(4)

(b)(4) First, let us examine if the existing literature suggests differential propensity for seizure generation, and, thereby, differential susceptibility to seizurogenic agents between genders and in different ages. It is well documented that young animals and humans are more prone to generating seizures than adults, but the neuropathology induced by seizure activity is less severe and more transient in the developing brains (41, 42, 67). It is particularly relevant to mention that pilocarpine-induced SE causes inflammation and epileptogenesis in rats, but not in infant rats (64). Aged humans (45, 58) and animals (68,110) are more susceptible to both seizures and the associated neuropathology compared to adults. Gender-related differences in seizure susceptibility exist in animals and humans (47), but more research is needed to clarify and categorize these differences. For example, in humans, males have a greater susceptibility to SE, but females have a higher incidence of idiopathic epilepsies (23, 74). In animals, male rats also are more susceptible to limbic seizures and SE than female rats (94, 83), but aged female mice are more sensitive to kainic acid-induced seizures (113). There are only few studies on the age- and gender-dependency of the susceptibility to nerve agent toxicity. In guinea pigs, the lethal potencies of VX and sarin are significantly higher in the adult males than in any other age or in females (30). In rats, the lethal potency of soman is greater in 5 day-old rats than in 30 day-old rats (106), and in the young (up to 30 days) and the aged males, compared to the adults (101,106), suggesting that the toxicity of soman during an animal's life span approximates a U-shaped curve (101). Our proposed studies will yield data on the age- and gender-related susceptibility to soman toxicity and lethality. Based on the limited information available, as reviewed above, our working hypothesis is that the LD50 will be lower in the immature and aged rats (greater susceptibility/lethality to

soman), greater in female rats or similar to the LD50 in males, but the soman-induced neuropathology –which will be examined at multiple points, 24 hours, 1 week, 1 month, and 3 months after soman exposure– will be lower in the immature rats, and the recovery of the damage will be faster (the reverse will probably be true in the aged rats). It is also possible, however, that the soman-induced SE in the early postnatal rats will produce disruption in the normal brain development, which will become evident in the behavioral studies proposed here.

(b)(4)

(b)(4) On an acute basis, GABA agonists in developing rats can facilitate instead of suppressing seizures (82). In response to metabotropic glutamate receptor-anticonvulsants, different responses have been observed even in a narrow range of young ages (7 to 25 day-old rats) (66), and, in general, young rats are more responsive to NMDA and non-NMDA receptor antagonists used as anticonvulsants compared to adult rats (67). Aged animals and humans also differ from other age groups in their responses to anticonvulsants, one reason being the different pharmacokinetics in the aged organism (45, 58, 68, 110). (b)(4)

(b)(4)

Significance of studying the protection of the amygdala, the hippocampus, and the prefrontal cortex

(b)(4)

(b)(4) There are two reasons that we choose to study these structures. First, the amygdala and the hippocampus are seizure-prone structures that play a central role in temporal lobe epilepsy, which is the most common form of epilepsy, the (5, 6, 7, 76, 85). It is not surprising therefore that the amygdala and the hippocampus also appear to play a central role in the generation of seizures induced by nerve agents (4), as suggested by the rapid increases in extracellular glutamate in these brain regions after nerve agent exposure (52, 53, 54), and the profound damage these structures suffer by exposure to nerve agents (2, 4, 12, 35, 40, 46, 80, 102, 103, 104, 111). (b)(4)

(b)(4)

(b)(4)

(b)(4) The second reason is that the amygdala, the hippocampus and the prefrontal cortex are the most important brain regions in affective and cognitive functions. The amygdala is best known for its central role in emotional behavior, being a key component of the brain's neuronal network that determines the emotional significance of external events (26, 29, 61). Many emotional/psychiatric disorders are associated with pathophysiological changes in the amygdala (21, 28, 88, 109). In addition, both the amygdala and the hippocampus play a pivotal role in mediating or modulating cognitive functions (26, 61). The amygdala modulates memory acquisition and consolidation, decision-making, and interpretation of the emotional significance of external and internal events via extensive, reciprocal connections with the prefrontal cortex (36, 84) (the brain area considered to be the center for cognitive functions) and the hippocampus (76). Long-term memory storage is thought to occur in neocortical areas, but the amygdala appears to be the site for storage of fear-memory (29, 65), whereas the hippocampus is the storage site of short-term memory and the gateway for consolidation of long-term memory in neocortex (20, 33, 60). Therefore, damage to these regions is likely to be significantly responsible for the long-term cognitive and behavioral deficits that follow nerve agent exposure, in both animals (9, 50, 51, 69, 70, 71, 72, 73) and humans (18, 27, 77, 81, 97). For these reasons it is important to determine the neuropathological and pathophysiological changes induced in the amygdala, hippocampus and prefrontal cortex by soman. (b)(4)

(b)(4)

Progress from the previous protocol

(b)(4)

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: Databases searched: BRD (Biomedical Research Database), DOAC (DTIC Online Access Controlled)* Technical Reports, DOAC Research in Progress, FEDRIP, PubMed, Web of Science

II.2.2. Date of Search: 30 January 2012; Search #: P20120119

II.2.3. Period of Search: 1985-2012

II.2.4. Key Words and Search Strategy: (b)(4)

(b)(4)

II.2.5. Results of Search:

Database	Number of Results
PubMed*	37
Web of Science*	27
DOAC	199 (includes 0 in BRD)
FEDRIP	1
Total all databases	261

No citations were found, other than our own work, to use (b)(4)

(b)(4)

III. OBJECTIVE\HYPOTHESIS: (b)(4)

(b)(4)

female rats. The 16-20 month category is designated as "aged" by the supplier, and even 8 month-old male rats have been shown to be more sensitive to soman than immature or adult rats (101). After exposure to soman, the animals will be euthanized at 24 hours, 1 week, 1 month, or 3 months to evaluate nerve agent-induced pathology. Neuronal loss, using design-based stereology, and neurodegeneration, using Fluoro-Jade-C staining will be studied in the amygdala, hippocampus, and prefrontal cortex, at 24 hours, 1 week, 1 month, and 3 months after soman exposure.

In specific aim 3 we will determine whether treatment of immature (7 to 14 days-old) and adult (50 to 70 days-old) male and female rats with (b)(4) protects the animals against the behavioral alterations that are observed after (b)(4) (b)(4) At 1 month and 3 months after soman exposure, we will perform a comprehensive neurobehavioral evaluation in these animals. These tests will evaluate motor, cognitive, mnemonic and emotional abnormalities known to be associated with (b)(4) Motor performance will be evaluated using the roto-rod test; performance on this test has been shown to be reduced in rats following soman exposure (32). Cognitive impairment will be determined using tests for novelty preference in the open field, and mnemonic cognitive processes will be evaluated using the t-maze test, performance in which has also been shown to be affected by soman exposure (32, 78). Finally, emotional behavior will be evaluated by measuring the acoustic startle response/pre-pulse inhibition, and with tests of unconditioned fear (light/dark boxes, elevated plus-maze); increases in these responses have been seen following exposure to soman in rats (25).

In specific aim 3 we will also determine whether treatment of immature (7 to 14 days-old) and adult (50 to 70 days-old) male and female rats with (b)(4) protects the animals against alterations in synaptic plasticity and neuronal excitability that are observed after (b)(4)
(b)(4)

Behavioral and pathophysiological studies will be performed in both the immature and the adult male and female rats, but not in the aged animals, where variability among rats in cognitive/behavioral and physiological functions may be very high even in the control group, making it difficult to determine the deficits resulting from the (b)(4)
(b)(4)

IV. MILITARY RELEVANCE: (b)(4)
(b)(4)

(b)(4)

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

(b)(4)

(b)(4)

V.1.1. Experiment 1:

(b)(4)

(b)(4) (7 to 14 days old), adult (50 to 70 days old), and aged (16 to 20 months old) male rats.

(b)(4)

(b)(4)

A week prior to the experiments, 50% of the adult and aged, male and female rats in experiment 1 will be implanted with cortical electrodes for electroencephalogram (EEG) recordings. These EEG recordings will be used to confirm the onset and intensity of behavioral convulsions observed in the rest of the animals. For the EEG recordings, along with behavioral observations, the timing (time to onset and duration) and the intensity of seizures will be measured via continuous EEG monitoring. Animals will be placed in individual recording chambers, and a baseline EEG will be obtained before administration of any drug.

The immature rats will not be implanted with cortical electrodes and the timing (time to onset and duration) and the intensity of seizures will be measured via behavioral observations.

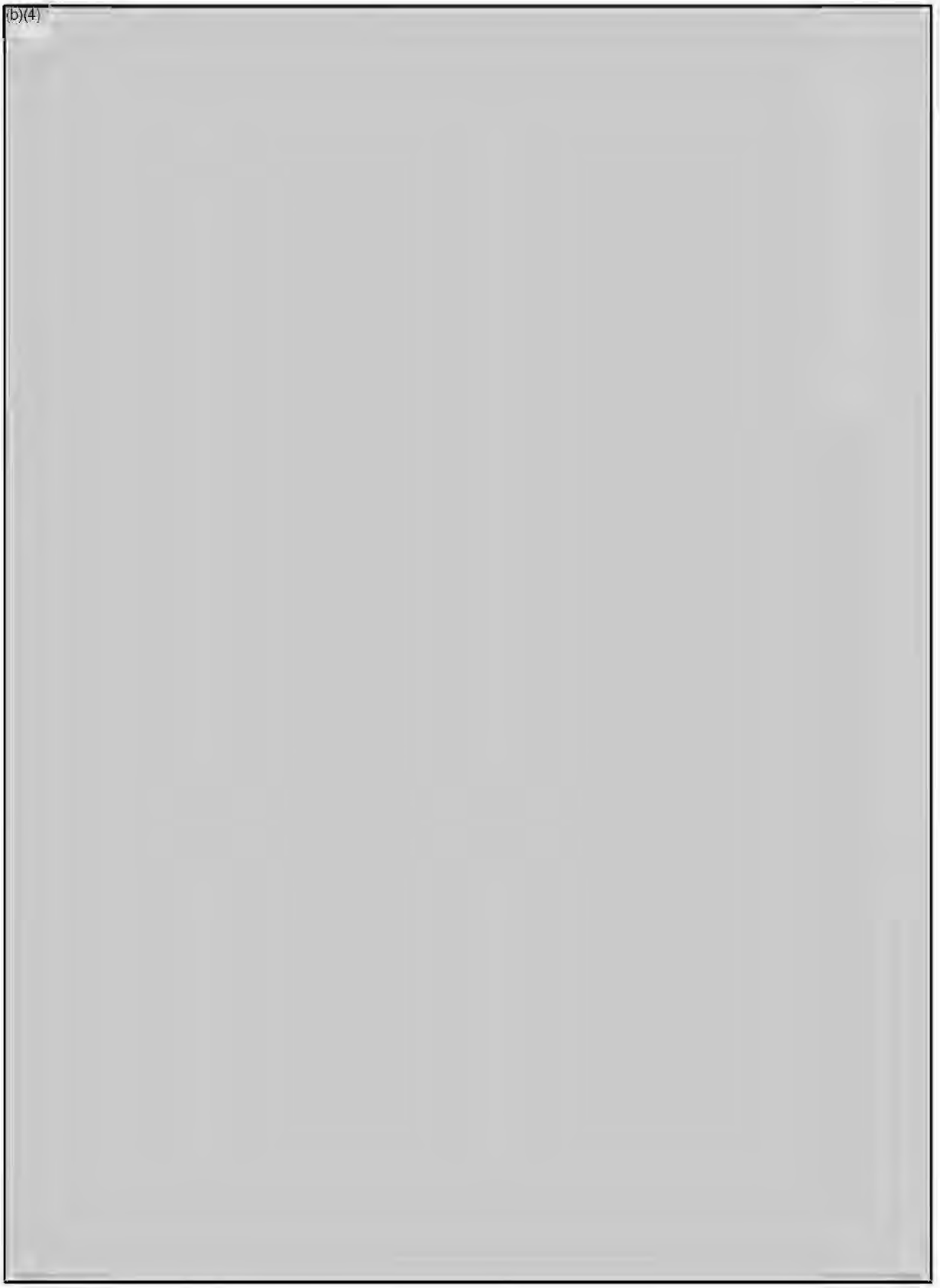
(b)(4)

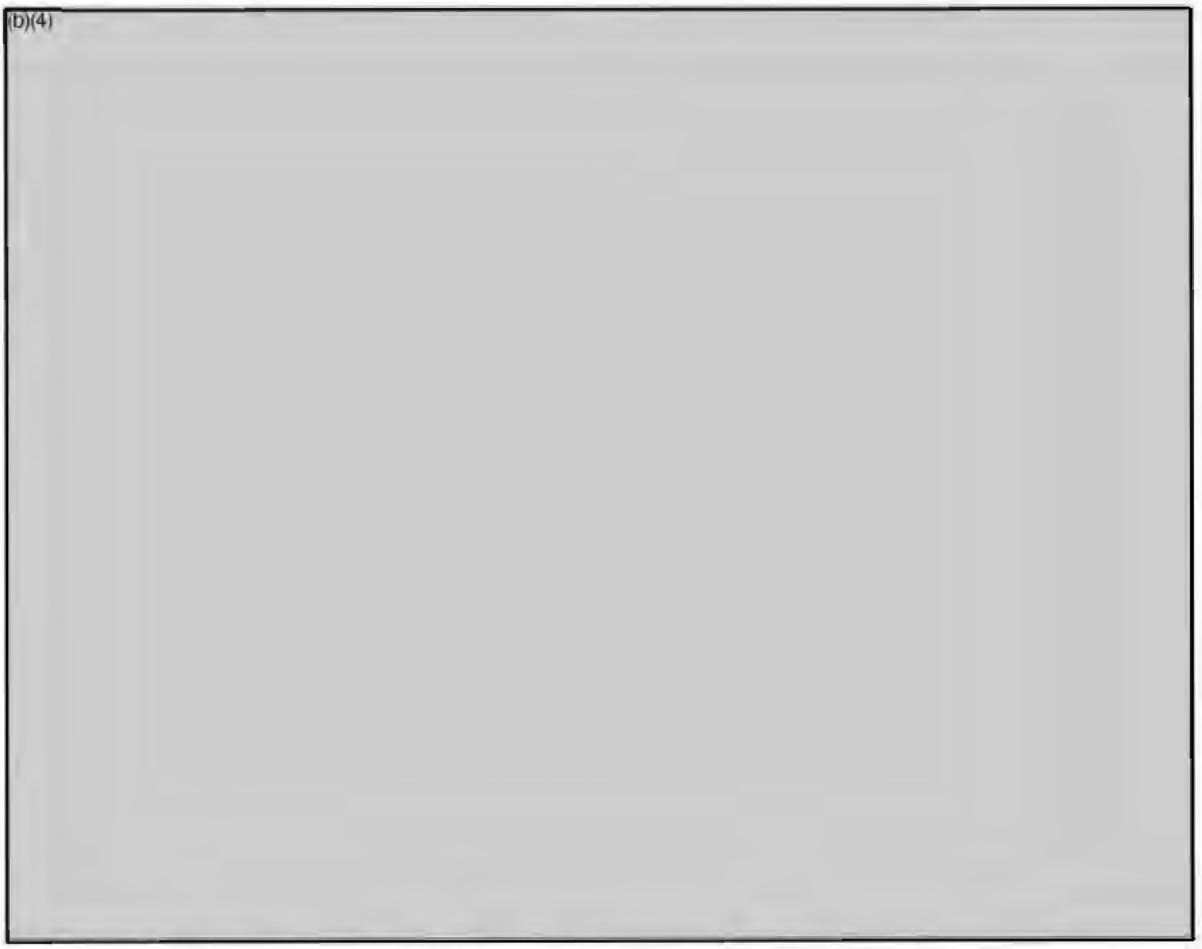
(b)(4) The determination to classify a seizure as being terminated will be the progressive diminution and eventual elimination of repetitive high amplitude spikes or sharp waves in the EEG and the absence of obvious signs of clonic convulsive behaviors. For a seizure to be considered terminated the EEG will have to be free of repetitive high amplitude spikes or sharp waves within the 3-hour EEG recording period on the day of exposure and during the 30-min EEG sample obtained 24 hours after exposure.

The following dependent measures will be used to establish the effectiveness of the drug treatments: the speed (latency) at which the EEG "normalizes" following drug treatment, the overnight change in body weight and the extent of the neuropathological damage at 24 hours, 1 week, 1 month, and 3 months after soman exposure. With the drug treatment protocol described above, we expect a very high survival rate. However,

(b)(4)

(b)(4)





Considerations regarding Experiment 1:

1) In a previous protocol we have obtained neuropathology data for adult male rats at 24 hours and 1 week after soman (b)(4) exposure, but not at 1 month and 3 months after exposure. In this study we will perform additional experiments in adult male rats at 24 hours and 1 week after soman, which are necessary for comparisons with other ages and the females.

2) The age we chose for the immature rats (7 to 14 days old at which time they open their eyes) coincides with the rapid developmental changes in the brain. The gender of the immature rats may not be possible to determine at the time of soman exposure, but it will be determined at 1 week, 1 month and 3 months after soman exposure, when neuropathology and behavioral studies will be carried out. The same issue applies to the (nominally female) immature rats in Experiment 2, and it is expected that the numbers of male and female animals will even out between the 2 experiments. If not, additional animals will be ordered.

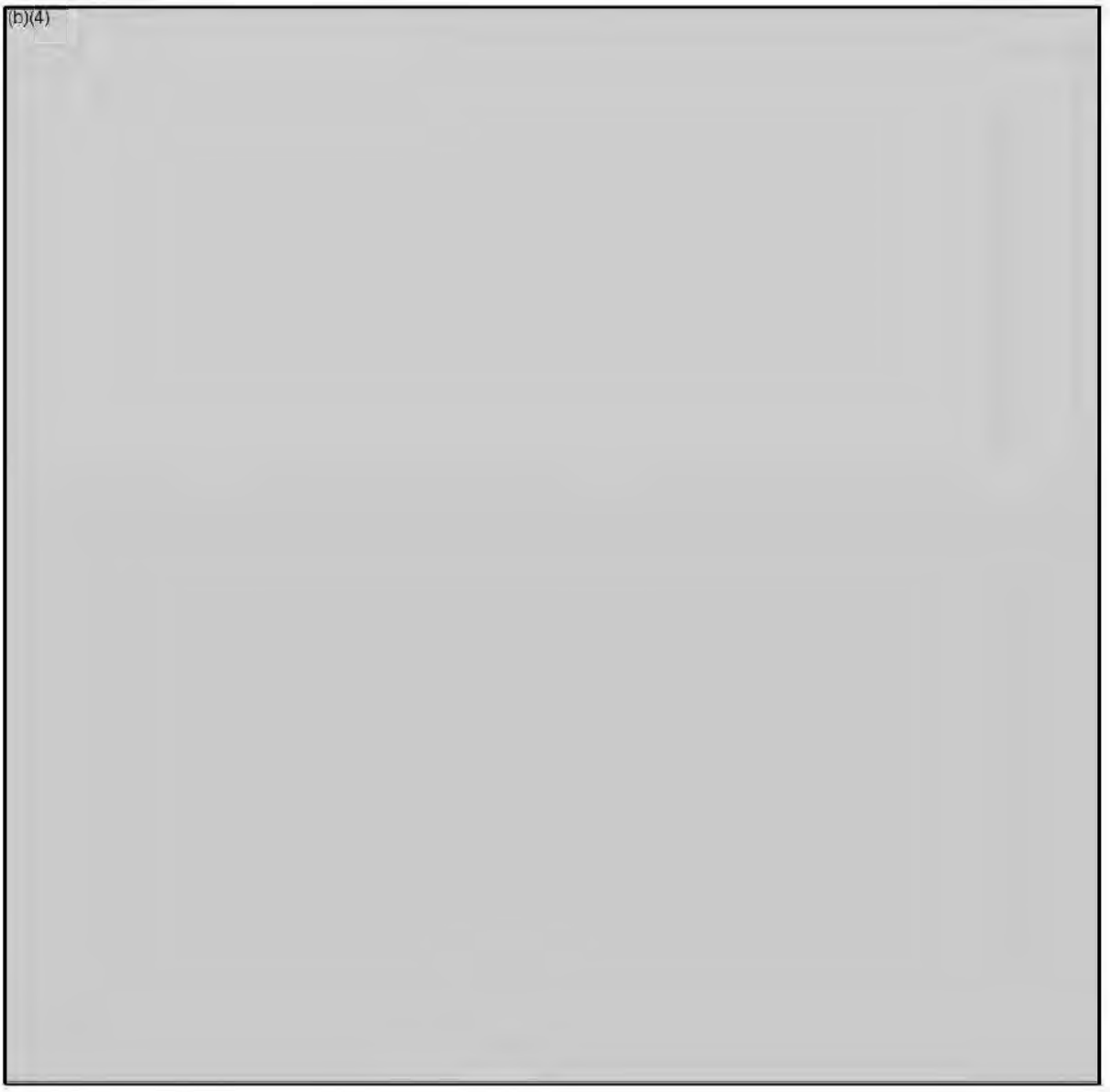
V.1.2. Experiment 2:

The experimental design of Experiment 2 is exactly the same as described in Experiment 1 with the exception of the use of **female** rats.

(b)(4)

Immature, adult, and aged **female** rats will be randomly assigned to groups per Tables 4, 5, and 6 below:

(b)(4)



Considerations regarding Experiment 2:

1) The age we chose for the immature rats (7 to 14 days old at which time they open their eyes) coincides with the rapid developmental changes in the brain. The gender of the immature rats may not be possible to determine at the time of soman exposure, but it will be determined at 1 week, 1 month and 3 months after soman exposure, when neuropathology studies will be carried out.

2) In the female rats, the estrous cycle will be monitored, and if the results show that it influences the variables under study (susceptibility to the soman-induced SE and behavior in particular), then the estrous cycle will be taken into account in the analysis and presentation of the results.

V.1.3. Experiment 3:

In specific aim 3 we will determine whether treatment of immature (7 to 14 days old) and adult (50 to 70 days old) male and female rats with (b)(4) protects the animals against the behavioral alterations that are observed after nerve agent exposure (**Experiment 3A**). At 1 month and 3 months after soman exposure, we will perform a comprehensive neurobehavioral evaluation in these animals. These tests will evaluate motor, cognitive, mnemonic and emotional abnormalities known to be associated with exposure to soman and other organophosphates. Motor performance will be evaluated using the roto-rod test; performance on this test has been shown to be reduced in rats following soman exposure (32). Cognitive impairment will be determined using tests for novelty preference in the open field, and mnemonic cognitive processes will be evaluated using the t-maze test, performance in which has also been shown to be affected by soman exposure (32, 78). Finally, emotional behavior will be evaluated by measuring the acoustic startle response/pre-pulse inhibition, and with tests of unconditioned fear (light/dark boxes, elevated plus-maze); increases in these responses have been seen following exposure to soman in rats (25).

In specific aim 3 we will also perform electrophysiological experiments to determine whether treatment of immature (7 to 14 days old) and adult (50 to 70 days old) male and female rats with (b)(4) protects the animals against alterations in synaptic plasticity and neuronal excitability that are observed after nerve agent exposure (**Experiment 3B**). At 1 month and 3 months after soman exposure, rats will be euthanized, and brain slices containing the amygdala, hippocampus and prefrontal cortex will be prepared. Alterations in neuronal excitability and synaptic plasticity (long-term potentiation) in these three brain regions will be performed using both whole-cell recordings and extracellular, field potential recording techniques.

Behavioral and electrophysiological experiments will be conducted in both immature and adult male and female rats, but not in aged animals. Variability among aged rats in cognitive/behavioral and physiological properties may be very high even in the control group, making it difficult to determine the functional deficits resulting from the exposure to soman and the protection conferred by the (b)(4)

(b)(4)

Experiment 3A: To determine whether treatment of immature (7 to 14 days old) and adult (50 to 70 days old) **male and female** rats with (b)(4) protects the animals against the behavioral alterations that are observed after nerve agent exposure.

(b)(4)

(b)(4)

A week prior to the experiments, 50% of the adult and aged, male and female rats in experiment 1 will be implanted with cortical electrodes for electroencephalogram (EEG) recordings. These EEG recordings will be used to confirm the onset and intensity of behavioral convulsions observed in the rest of the animals. For the EEG recordings, along with behavioral observations, the timing (time to onset and duration) and the intensity of seizures will be measured via continuous EEG monitoring. Animals will be placed in individual recording chambers, and a baseline EEG will be obtained before administration of any drug.

The immature rats will not be implanted with cortical electrodes and the timing (time to onset and duration) and the intensity of seizures will be measured via behavioral observations.

(b)(4)

(b)(4) The determination to classify a seizure as being terminated will be the progressive diminution and eventual elimination of repetitive high amplitude spikes or sharp waves in the EEG and the absence of obvious signs of clonic convulsive behaviors. For a seizure to be considered terminated the EEG will have to be free of repetitive high amplitude spikes or sharp waves within the 3-hour EEG recording period on the day of exposure and during the 30-min EEG sample obtained 24 hours after exposure.

(b)(4)

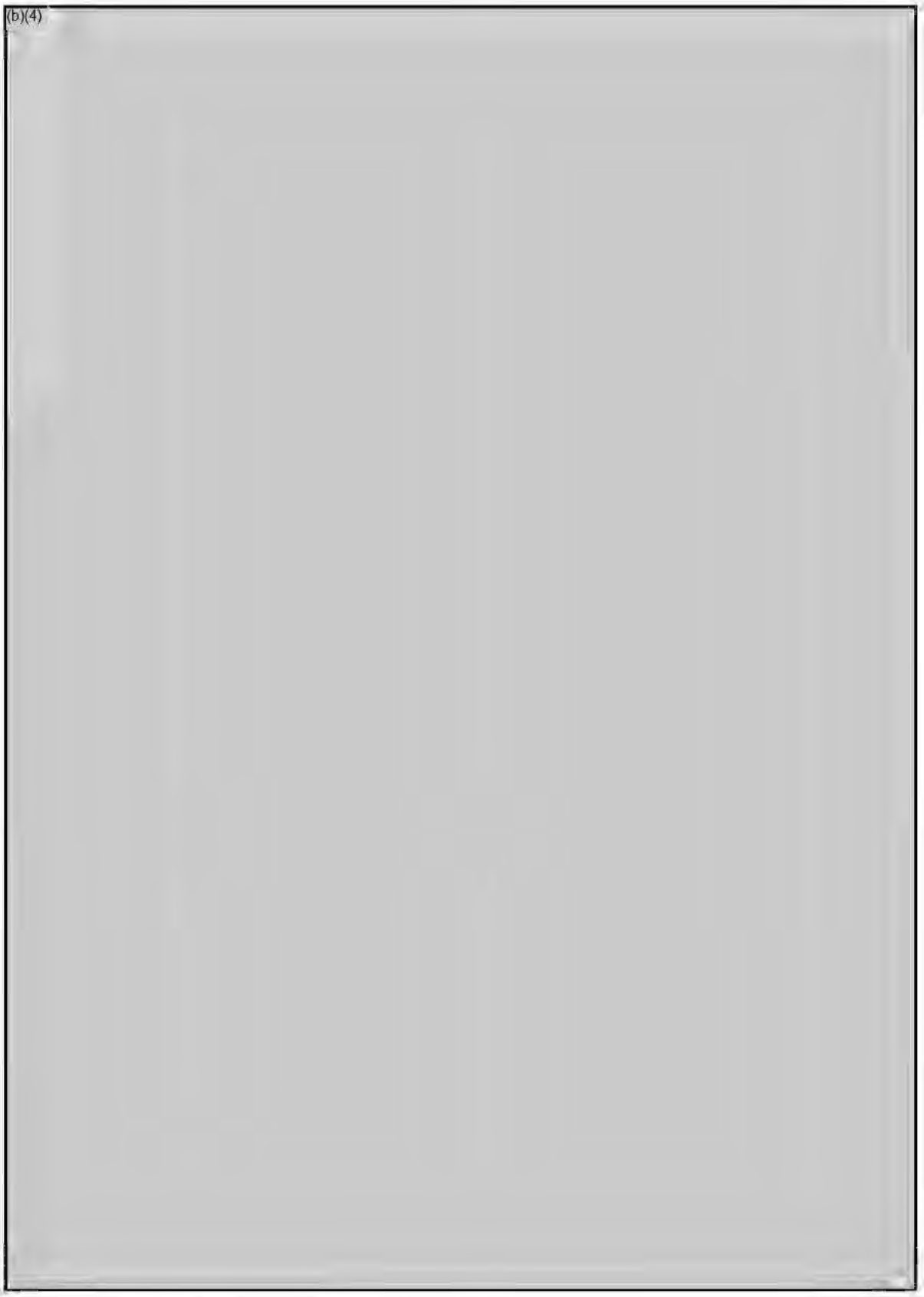
We will perform a comprehensive battery of behavioral and neurological tests (see Section V.4.4.6. Behavioral Studies) on the (b)(4) rats, the (b)(4) (b)(4) 1 month and 3 months after soman exposure. These tests will evaluate motor, cognitive, mnemonic and emotional abnormalities known to be associated with (b)(4) (b)(4) Motor performance will be evaluated using the roto-rod test; performance on this test has been shown to be reduced in rats following (b)(4) (b)(4) Cognitive impairment will be determined using tests for novelty preference in the open field, and mnemonic cognitive processes will be evaluated using the t-maze test, performance in which has also been shown to be affected by (b)(4) (b)(4) Finally, emotional behavior will be evaluated by measuring the acoustic startle response/pre-pulse inhibition, and with tests of unconditioned fear (light/dark boxes, elevated plus-maze); increases in these responses have been seen following (b)(4) in rats (25).

(b)(4)

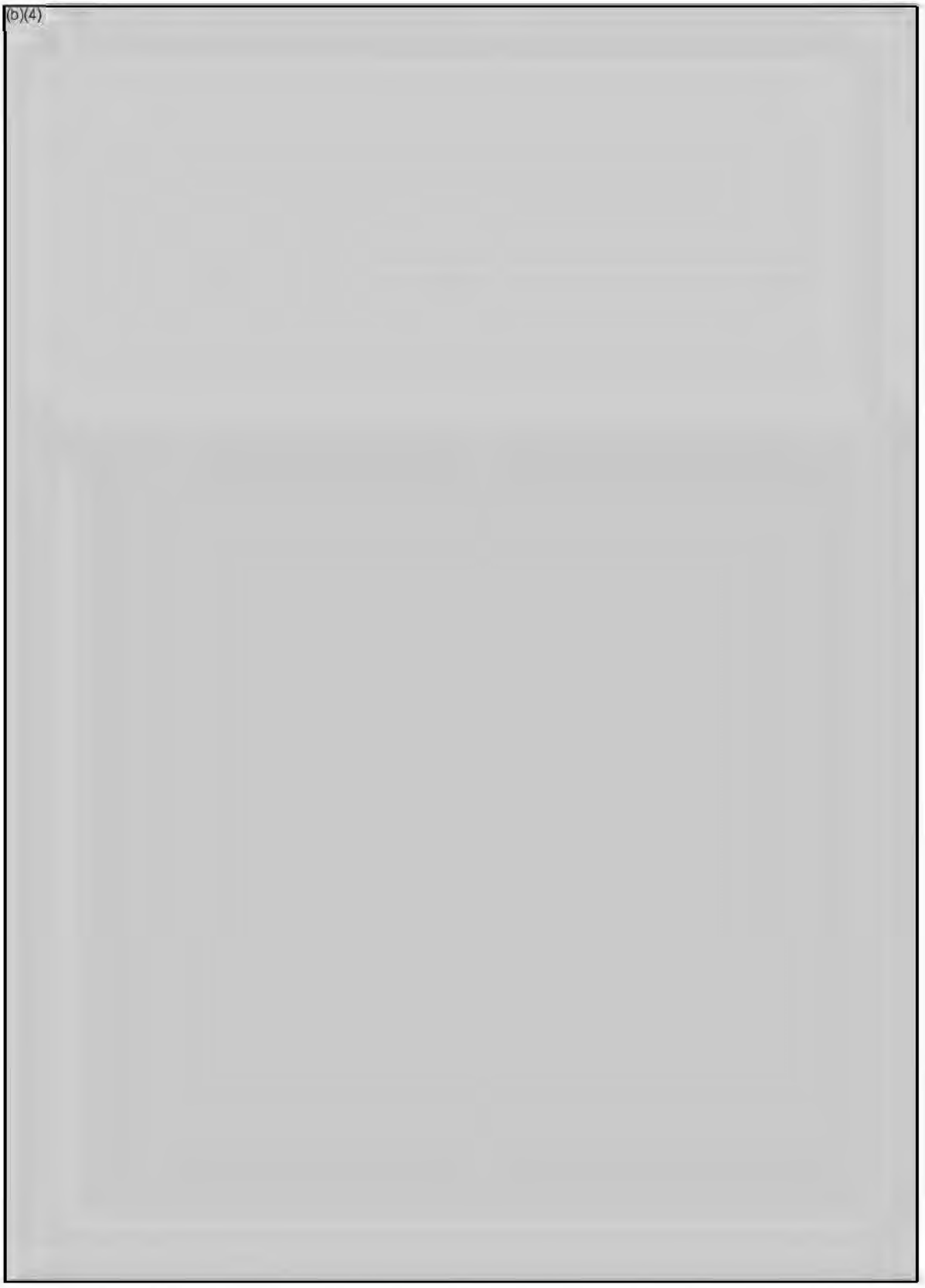
Immature and adult male and female rats will be randomly assigned to groups per Tables 7, 8, 9, 10, 11, and 12 below:

(b)(4)

(b)(4)



(b)(4)



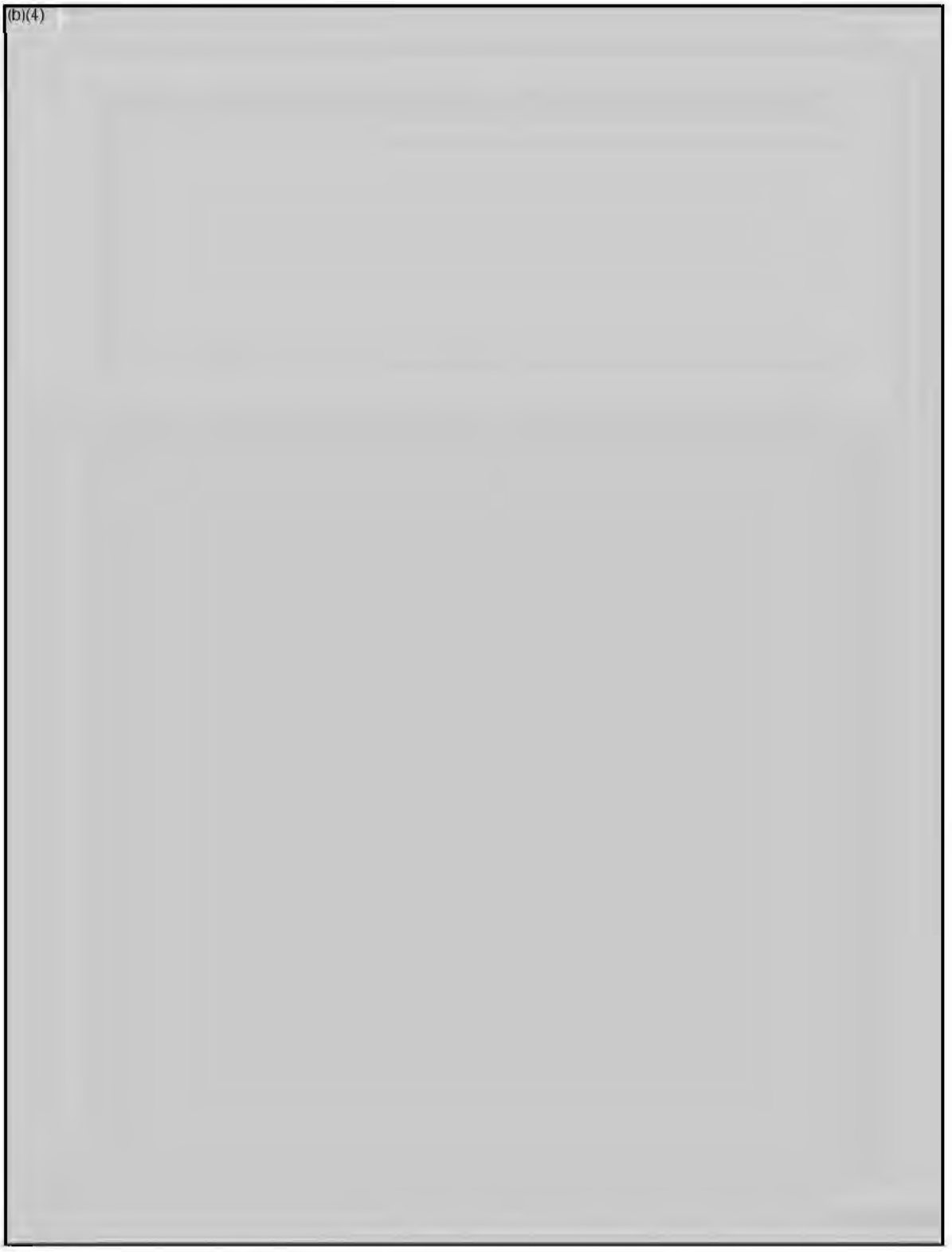
(b)(4)

Immature (7 to 14 days old), and adult (50 to 70 days old) **male and female rats** will be (b)(4) One month or three months after (b)(4) electrophysiological experiments will be conducted in the (b)(4)

Pathophysiological alterations will be studied in *in vitro* brain slices containing the amygdala and hippocampus and in slices from the prefrontal cortex. These studies will be performed using both extracellular, field potential recording techniques and whole-cell recordings.

(b)(4)

(b)(4)



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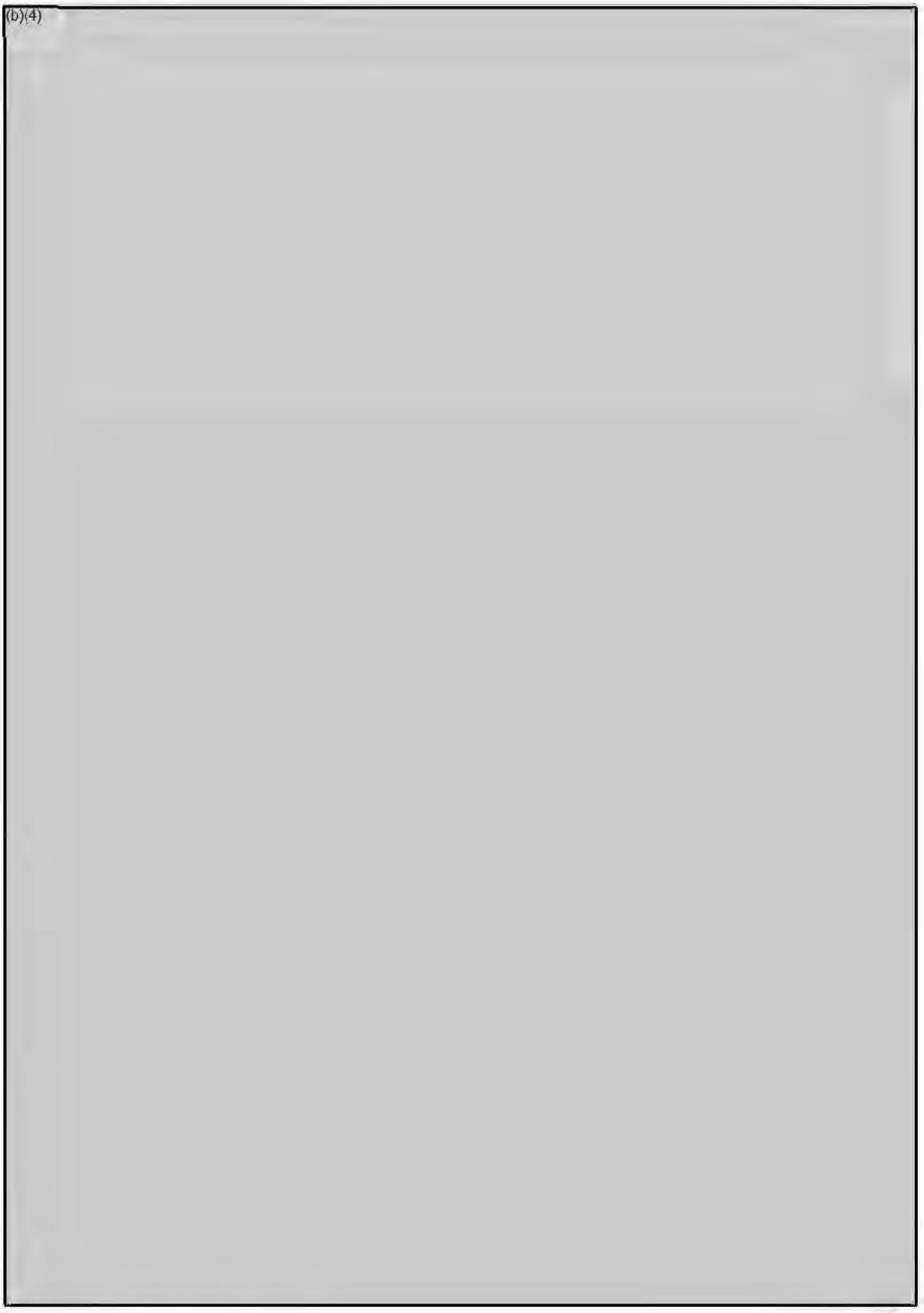


(b)(4)

vivo, information obtained using non-animal models may not accurately reflect a drug's action in a whole animal.

V.3.2. **Animal Model and Species Justification**: Immature (7 to 14 days old), adult (50 to 70 days old), and aged (16 to 20 months old) Male and Female Sprague-Dawley (SD) rats (*Rattus norvegicus*) will be used for these experiments. SD rats have been chosen as a suitable species and animal model for this research because they have been extensively used in nerve agent research to evaluate new medical countermeasures. Also, the SD rat is a primary model used in studying the pathophysiology of the amygdala, hippocampus, and prefrontal cortex. Consequently, it is helpful to use the same animal to make comparisons with the literature. Rats can provide up to eight 400-µm thick brain slices per animal. By using the SD rats, we can produce high quality slices and thus facilitate and expedite the acquisition of data. The rat brain slice preparation allows stable whole-cell patch clamping recording and various pharmacological manipulations under controlled conditions. Thinner slices can easily be prepared for morphological staining. Lactating surrogate mothers will be required to accompany the rat pups that have not yet been weaned.

V.3.3. **Laboratory Animals:** Note that all animals used in Experiments 1, 2 and 3A and B will be purchased and received by the principal investigator (b)(6) at USUHS.

		Species #1	Species #2
V.3.3.1.	**Genus & Species:**	*Rattus norvegicus*	
V.3.3.2.	**Strain/Stock:**	Sprague-Dawley (b)(4)	
V.3.3.3.	**Source/Vendor:**	(b)(4)	
V.3.3.4.	**Age:**	7-14 days (males and females) 50-70 days (males and females) 16-20 months (males and females) Adult lactating retired breeders (age to be determined by vendor)	
V.3.3.5.	**Weight:**	Appropriate for age at time of delivery	
V.3.3.6.	**Sex:**	Male and Females for experimental animals Female for lactating surrogate mothers	
V.3.3.7.	**Special Considerations:**	Lactating surrogate mothers (retired breeders	

from the supplier) will be required to travel with the immature rats that have not yet been weaned and to be housed with pups at USUHS. Single housing is required after EEG surgery on adult and aged rats to prevent animals from interfering with implant healing. Single housing is also required after agent exposure when animals may still be sick or debilitated. Animals may be pair-housed until the day of surgery, after which they must be singly housed.

V.3.4. Number of Animals Required (by Species):

(b)(4)

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:
Rats will be anesthetized with isoflurane and reach a surgical plane of anesthesia for restraint purposes before decapitation for preparation of brain slices. All EEG surgery will be performed under appropriate anesthesia and pre-and post-surgical analgesics will be used to relieve pain. Any rat exhibiting signs of pain or impairment beyond that from this model will be euthanized at an early endpoint per paragraph V.4.5.

V.3.5.2. Reduction:
Use of an *in vitro* brain slice preparation in Experiments 3B allows the harvest of multiple brain slices per rat, thus significantly reducing the number of animals needed to obtain the proposed data. Power calculations have been performed to determine the minimum number of rats needed to reach statistical significance for each experiment. If the first 6 animals in *in vivo* treatment groups (Experiments 1, 2 and 3A and B) survive, the remaining 4 animals will not be ordered.

V.3.5.3. Replacement:
Biological response to drug treatment is highly complex; no alternative to animal experimentation is available or practical. We are using a rodent model (rat) versus a

the first 6 animals in *in vivo* treatment groups (Experiments 1, 2 and 3A and B) survive, the remaining 4 animals will not be ordered.

V.3.5.3. Replacement:
Biological response to drug treatment is highly complex; no alternative to animal experimentation is available or practical. We are using a rodent model (rat) versus a nonhuman primate model. The rat model is the least sentient and smallest animal model we could use for this study and still obtain acceptable results. In addition, we will perform a series of morphological measurements that require a sufficiently large animal to assure that there is sufficient brain mass for multiple samplings. Mice would not be sufficient.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

In this protocol 288 lactating mothers from Experiments 1, 2, and 3A and B will be available for transfer for training or experimental purposes, or euthanized if they cannot be used (Column B). Six hundred eight (608) male SD rats from Experiment 1 and 3A and B and 608 female SD rats from Experiment 2 and 3A and B will receive an injection of saline instead of nerve agent in combination with (b)(4) and therefore will experience no pain (Column C). Eight hundred (800) male SD rats from Experiment 1 and 3A and B and 800 female SD rats from Experiment 2 and 3A and B will be injected with nerve agent and experience intense seizures. While the drugs/treatments under investigation may reverse some of these signs during certain phases of these experiments, these animals will experience some level of seizure or physical stress and discomfort for some period of time during the experiments and will be included in Column E (Non-alleviated pain).

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	(b)(4)	
V.4.1.1.1.2. Column D:	0	
V.4.1.1.1.3. Column E:	(b)(4)	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:
For surgical implantation of cortical electrodes for EEG recordings in Experiment 3, animals will receive one dose of buprenorphine (0.05 mg/kg s.c.) before any incisions are made. The animals will be anesthetized with isoflurane (3% for induction, 2.0-2.5% for maintenance; with oxygen). Anesthesia will be induced by isoflurane vapor in 100% oxygen in a commercially available induction chamber. Once anesthesia is induced,

animals will be transferred to the designated surgery area and anesthesia will be maintained via nose cone. To monitor the depth of anesthesia (including during surgery time) we will test whether the animal has lost its toe pinch (pedal withdrawal) reflex. Prior to incision, animals will receive bupivicaine at electrode placement sites (infusion of surrounding tissue), at a maximum of 0.2 ml within any one animal, and buprenorphine HCl (0.05 mg/kg, s.c.) to assist in post-operative pain management.

Following surgery for implantation of cortical electrodes, animals will receive buprenorphine HCl (0.05 mg/kg, s.c.), which will be given at a minimum one time at the end of the day of surgery, just before close of business. This is in addition to the preemptive dose given prior to surgery. All surgeries should be performed in the AM. Additional doses may be given every 8-12 hours, up to 3 days after the surgery as needed, in consultation with the attending veterinarian.

The buprenorphine dose may be increased, up to a dose of 0.25 mg/kg i.m. or s.c. at each buprenorphine dosing time point, depending on the pain status of the animal. Dose will not be adjusted without approval of the attending veterinarian.

For brain pathology studies, the animals will be anesthetized with 75-100 mg/kg, i.p., sodium pentobarbital prior to euthanasia.

For amygdalo-hippocampal and prefrontal cortex slice preparations, rats will be anesthetized by isoflurane inhalation under a hood until they are non-responsive to toe-pinch, (b)(4)

(b)(4)

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

(b)(4)

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: : BRD (Biomedical Research Database), DOAC (DTIC Online Access Controlled)* Technical Reports, DOAC Research in Progress, FEDRIP, PubMed, Web of Science

V.4.1.3.2. Date of Search: 30 January 2012; Search #: P20120119

V.4.1.3.3. Period of Search: 1985-2012

V.4.1.3.4. Key Words of Search: (b)(4)

(b)(4)

V.4.1.3.5. Results of Search:

Database	Number of Results
PubMed*	4
Web of Science*	23
FEDRIP	5
Total all databases	32

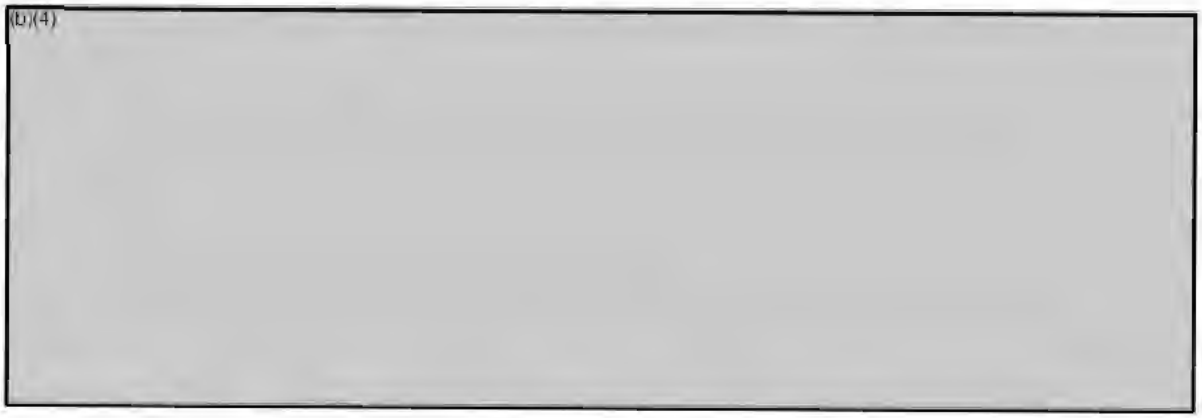
*Duplicate citations removed in PubMed/Web of Science bibliography

The references found in this search cited the use of several *in vitro* models, including primary neuronal cultures and acute and organotypic hippocampal slices. The cited studies investigated protection from neurotoxicity, release or uptake of neurotransmitters, gene expression, and electrophysiological effects of ACh stimulation. We will use hippocampal, amygdala, and prefrontal cortex slices in this protocol. None of the other models offer any advantage over the slice preparations we propose to use. None are able to duplicate the behavioral convulsions, electrographic seizures, or brain damage produced in an *in vivo* animal model, which are necessary to evaluate anticonvulsant and neuroprotective drugs.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
There is no alternative for inducing brain damage in rats that mimics the damage caused by nerve agents to determine whether a compound is neuroprotective. The only way to determine the neuroprotective efficacy of a putative drug is to cause actual status epilepticus in an animal model to induce damage prior to administration of the neuroprotective compound and to determine the number of surviving neurons.

(b)(4)

(b)(4)



V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions:
A week prior to the experiments, 50% of the adult and aged, male and female rats in experiment 1 will be implanted with cortical electrodes for electroencephalogram (EEG) recordings. These EEG recordings will be used to confirm the onset and intensity of behavioral convulsions observed in the rest of the animals. For the EEG recordings, along with behavioral observations, the timing (time to onset and duration) and the intensity of seizures will be measured via continuous EEG monitoring. Animals will be placed in individual recording chambers, and a baseline EEG will be obtained before administration of any drug.

The immature rats will not be implanted with cortical electrodes and the timing (time to onset and duration) and the intensity of seizures will be measured via behavioral observations.

The surgical procedures will be performed aseptically following the guidelines of the SOP from USUHS (VSD SOP 22-300). Animals will receive one dose of buprenorphine (0.05 mg/kg s.c.) before any incisions are made. Anesthesia will be induced by isoflurane vapor in 100% oxygen in a commercially available induction chamber. Once anesthesia is induced, animals will be transferred to the designated surgery area and anesthesia will be maintained via nose cone. See Section V.4.1.2.1 Anesthesia/Analgesia/Tranquilization for details.

When the animal is anesthetized, its head will be shaved with a hair clipper. The head will be cleaned with betadine and alcohol, each repeated 3 times alternately. Then the animal will be placed into a stereotaxic frame (appropriate frame size will be used for rats) after treating its pressure points such as ears and nose with lidocaine jelly. A heated pad with thermister-controlled feedback will be put between the animal's body and the base of the stereotaxic frame. Ophthalmic ointment will be applied to the eyes to prevent dryness. The body will be covered with a sterile drape with an opening to expose the head. Prior to incision, animals will receive bupivicaine at electrode placement sites (infusion of surrounding tissue) to assist in pain management. The

injection volume will be limited to the minimum required for pain control, to minimize health risks. Care will be taken to avoid systemic effects.

V.4.3.2. Procedure:

The skin will be incised longitudinally along the midline from eye level to the neck level and retracted to the sides with hemostats. The periosteum will be scraped off from the skull. Five stainless steel machine screws (the smallest possible size that provide adequate anchoring) will be used as electrode contact and simultaneous anchoring points for the headpiece that will be built from acrylic cement to secure the female electrode connector in place. The screws will be manually positioned in the frontal or parietal bone plates and one over the cerebellum. The screws will be partially screwed in manually without penetrating the dura mater.

V.4.3.3. Post-surgical Provisions:

Excess cement will be removed from the headpiece to make its edges smooth. If necessary, skin will be sutured at both ends of the incision line. The sutures (suture thread - non-absorbable nylon) will be performed with the help of atraumatic needles with suture size #0. The animal will be released from the stereotaxic frame and placed in a recovery cage on a chemical pocket warmer wrapped in cloth or paper until the animal fully recovers from anesthesia. Alternatively, the animals may be placed in a forced-air recovery chamber warmed to 34 °C. Once fully awake and performing purposeful movements, the animal will be moved back to the holding area. At least 7 days will be allowed for recovery after surgery before any experimentation. Removal of stitches will occur 7 days after surgery.

Only the rats that maintain good health following the surgery will be used for experiments. This decision will be jointly made by the animal-care personnel and the investigators. All efforts will be made to provide all animals with the best care possible. Each animal will be individually monitored throughout the study by experienced personnel from our laboratory. We will also closely work with the veterinarian and animal-care staff of the animal facility to ensure that adequate treatment is provided in a timely manner should it be deemed necessary.
After surgery, the animals will be placed in a clean cage provided with a recirculating warm-water veterinary heating pad or in a forced-air recovery chamber warmed to 34 °C to maintain proper body temperature. After recovery from anesthesia (evidence of righting reflex and purposeful movements) the animal will be given at least one dose of an analgesic, buprenorphine HCl (0.05 mg/kg s.c.), for post-operative analgesia, which will be given every 8-12 hours up to 3 days after the surgery. The medication will be administered by the by PI or by qualified PI staff. If necessary, based on consultation with the attending veterinarian, additional buprenorphine may be given as needed.

Additional doses of buprenorphine will be given if required, based on assessment of PI and attending veterinarian of animal's condition, which will include assessment of the presence of the following signs of pain/distress: hunching, abnormal gait, abnormal attitude (increased or decreased aggression), inability to move to food/water, excessive porphyrin staining of eyes/nose, shuddering or twitching, unkempt appearance, and vocalization when handled.

Following at least 1 hour on the warming pad or in a forced-air recovery chamber, upon full recovery in which the animal can maintain an upright posture and is ambulatory, the animal will be returned to its holding room cage. Laboratory personnel will provide the care of the animal until full recovery if it has not already occurred. All animals will be checked at least once 30 minutes after return to home cage, to ensure recovery is continuing, and at least once again just prior to the end of the work day to ensure animals are recovering normally. Animals will be examined at least once daily by the PI staff for one week post-surgery to ensure that no complications occur. Observations will be recorded for each animal in a place that is accessible to the animal care staff (post-surgery cage cards may be used for this purpose). PI staff observations will be separate from routine observations conducted by the animal care staff.

V.4.3.4. Location:
Surgeries (implantation of electrodes) will be performed at USUHS in LAM, Room (b)(6) Euthanasia and perfusions will be performed in room (b)(6) at USUHS. Euthanization will occur in chemical hoods in (b)(6)

It is anticipated that all of the EEG surgery will be performed at USUHS (LAM).

V.4.3.5. Surgeon:
(b)(6)

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:
All drug injections (including drugs injected to induce anesthesia/euthanasia) will be performed using 1-ml syringes fitted with 25-26 gauge, 1/2"or 5/8" needles. Injections on
(b)(4)
(b)(4) utilized in this protocol will be prepared at concentrations to deliver volumes of 0.5 ml/kg. Anesthesia and analgesia will be administered as described in Section V.4.1.2.1 Anesthesia/Analgesia/Tranquilization.

V.4.4.2. Biosamples:
(b)(4)

(b)(4)

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification:
Cage cards, headpieces numbered with nontoxic ink. This will be the responsibility of the USUHS research team.

V.4.4.6. Behavioral Studies:
The following procedures will be performed at the USUHS:

Rotarod test: It measures an animal's ability to maintain balance by coordinating movement and making postural adjustments. It also measures the ability to improve these skills with practice. Each rat rotarod treadmill consists of a motor-driven drum with constant speed or accelerating speed modes of operation. The drum (7.0 cm diameter) allows each animal to maintain a suitable grip. It is divided into four test zones (8.9 cm wide) so that up to four animals may be tested at the same time. The device consists of a smooth hard plastic cylinder with concentric circular plastic sides (39.7 cm diameter) to prevent the rat from laterally climbing off the cylinder. Rats are placed on the device facing the wall with the rod rotating in the direction opposite from the animal (so that the rat uses its paws to pace forward and avoid falling off the rod). When a rat falls off the rotating drum (height of 26.7 cm), it breaks a photobeam, stopping the timer associated with that zone. In each trial, the speed of rotation is increased from 0 revolutions per minute (rpm) to 35 rpm for a maximum of 3 or 5 min. The mean duration on the device (based on 3 consecutive trials) is recorded.

Preference for Novelty (Open Field): Testing will be performed in a Plexiglas cage (56cm x 34cm x 20cm) with the floor divided in 18 equal squares. Three identical aluminum cubes will be evenly distributed in the cage in fixed positions. Three other cubes will make up the novel objects. One novel object will be similar to the neutral cubes in that its top will be uneven with tracks making up a square pattern (tactile stimuli). One novel object will be identical with the neutral objects except that a slight smear of cheese will be placed on the side facing the experimenter (olfactory stimuli). The third object will be similar to the neutral blocks except that it will be slightly smaller with uneven sides (visual stimuli). Locomotor activity will be measured using an Omnitech Electronics Digiscan infrared photocell system. One-hour activity measurements are obtained during animals' active or dark cycle.

T-maze: The task is based on the premise that animals have evolved an optimal strategy to explore their environment and obtain food with a minimum amount of effort. The T-maze apparatus is made of white Perspex. The runway of the maze is 80 cm long and its side arms are 30 cm long. The width of the maze is 10 cm and the walls are

10 cm high. A guillotine door is used to open the start box and two additional doors are used to block access to the arms, as necessary. Distinctive extra-maze cues are placed in the surrounding walls. Habituation sessions will be performed during 2 consecutive days, allowing free exploration of the maze for 15 min each day. Each rat will receive 10 precision pellets of food, scattered evenly throughout the maze, on each of the habituation sessions. During training each trial consists of a sample run and a test run. In the sample run, rats will be placed at the start position and required to make a forced choice by blocking the access to one of the arms. Two pellets will be positioned at the end of the open arm. The rats will be allowed to eat the pellets and will be immediately placed in a holding cage for 10 s before being returned to the maze for the test run. In the matching-to-place (MTP) and the non-matching-to-place (NMTP) versions of the task the reinforced choice in the test run is either the previously visited arm (MTP) or the arm opposite (NMTP). Each rat will perform 1 block of 10 trials each day. The sequence of arm visits (sample runs) will be randomized for each experimental subject.

Acoustic Startle Response: ASR testing will be conducted with a Startle Response Acoustic Test System (Coulbourn Instruments, PA). This system consists of a weight-sensitive platform in a sound-attenuated chamber. The animals' movements in response to stimuli will be measured as a voltage change by a strain gauge inside each platform and will be converted to grams of body-weight change following analog to digital conversion. These changes will be recorded by an interfaced computer as the maximum response occurring within 200 ms of the onset of the startle-eliciting stimulus. All acoustic stimuli will be administered by an amplified speaker mounted 24 cm above the test cage. During testing, animals will be individually placed in holding cages (14.5 × 7 × 6.5 cm) that are small enough to restrict extensive locomotion, but large enough to allow the subjects to turn around and make other small movements. These will then be placed on the weight-sensitive platform. Following placement of the animal into the chamber, the chamber lid will be closed, leaving the subject in darkness. A three-minute adaptation period occurs in which no startle stimuli is presented. Startle stimuli consist of 120 dB Sound Pressure Level (unweighted scale; re: 0.0002 dynes/cm2) noise bursts of 20 msec duration. In addition to the 120 dB stimuli, the 110 dB level will also be used for comparison to insure that the 120 dB stimulus level not only will meet a response threshold but also will not reach a leveling out of the maximum response. Thus confirmed, results will be given for the 120 dB level only. Decibel levels will be verified by a Radio Shack Sound Pressure Machine Model 2800 (Provo, UT). Each stimulus will have a 2-ms rise and decay time such that onset and offset will be abrupt, which is a primary criterion for startle. Each trial type (120 dB, 110 dB, or no stimulus) will be presented eight times. Trial types will be presented at random to avoid order effects and habituation. Inter-trial intervals will range randomly from 15 to 25 s.

Light-Dark Task/Open Field Behavior: Assessment of Anxiety and Locomotor Response to Novelty. The light-dark task will be performed in Hamilton-Kinder activity monitors (40 x 40 x 40 cm) fitted with "dark" inserts which make half the volume of the box dark (0-2 lux). The rest of the chamber is open to ambient lighting (85-125 lux). A small arch-shaped opening allows the rats to move freely between the two sides. The floor of both halves of the box is covered with corncob bedding. Infrared beams surrounding each box detect the animals' positions. Infrared beam breakages are then converted into distance and position by Hamilton-Kinder software running on a personal

computer. The test session begins when the rat is placed gently in the light side of the chamber. Rats are allowed to move freely about the chamber for 15 min. The following measures are scored 1. Total distance traveled (cm). Distances in the light and dark halves are summed to obtain "total distance," which is a measure of locomotor activity. 2. Time spent in the light half of the chamber(s). This is one of two indicators of anxiety in this task. Rats that spend less time in the light are deemed to be more anxious. 3. Emergence from the dark (emergence interval; s). This is a second measure of anxiety. Rats that take longer to emerge from the dark after their first entry are deemed to be more anxious.

V.4.4.7. Other Procedures:
On the day of exposure to the nerve agent, the animals will be connected to recording leads via the connection plug on their heads. Brain EEG activity will be recorded while they are in cylindrical plastic recording chambers, 16" in diameter and 26" high with Iso-pad bedding on the floor, where they are free to move about. The period of confinement in the recording chambers will not exceed 3 hours on the experimental day or 1 hour on the day after.

The following 5 procedures will be performed at the USUHS:

Histology: Twenty-four hours, 1 week, 1 month, or 3 months after soman exposure, the animals will be deeply anesthetized with 75-100 mg/kg, i.p., sodium pentobarbital, until a surgical plane of anesthesia is reached (non-response to strong toe-pinch) and then exsanguinated via perfusion with normal saline followed by 10% neutral buffered formalin. The brains will be removed and post-fixed overnight at 4° C, then transferred to a solution of 30% sucrose in PBS for 72 hours, and frozen with dry ice before storage at –80° C until sectioning. Sections containing the rostro-caudal extent of the BLA, the hippocampus, and the prefrontal cortex will be cut at 40 µm on a sliding microtome. One series of sections will be mounted on slides (Superfrost Plus; Daigger, Vernon Hills, IL) in PBS for Nissl staining with cresyl violet. An adjacent series of sections will also be mounted on slides for Fluoro-Jade C staining.

Fluoro-Jade C staining (Histo-Chem, Jefferson, AR) will be used to identify irreversibly degenerating neurons in the amygdala, the hippocampus, and the prefrontal cortex. Mounted sections will be air-dried overnight and then immersed in a solution of 1% sodium hydroxide in 80% ethanol for 5 min. The slides will then be rinsed for 2 min in 70% ethanol and 2 min in deionized water (dH20), and incubated in 0.06% potassium permanganate solution for 10 min. After a 2-min rinse in dH20, the slides will be transferred to a 0.0001% solution of Fluoro-Jade C dissolved in 0.1% acetic acid for 10 min. Following three 1-min rinses in dH20, the slides will be dried on a slide warmer, cleared in xylene for at least 1 min and coverslipped with DPX (Sigma). Fluoro-Jade C stained sections undergo tissue shrinkage, so they cannot be used for stereological quantification.

Stereological quantification: Design-based stereology will be used to quantify the total number of neurons on Nissl-stained and GAD67-stained sections. Sections will be viewed with a Zeiss Axioplan 2ie (Oberkochen, Germany) fluorescent microscope with a motorized stage, interfaced with a computer running StereoInvestigator 7.5

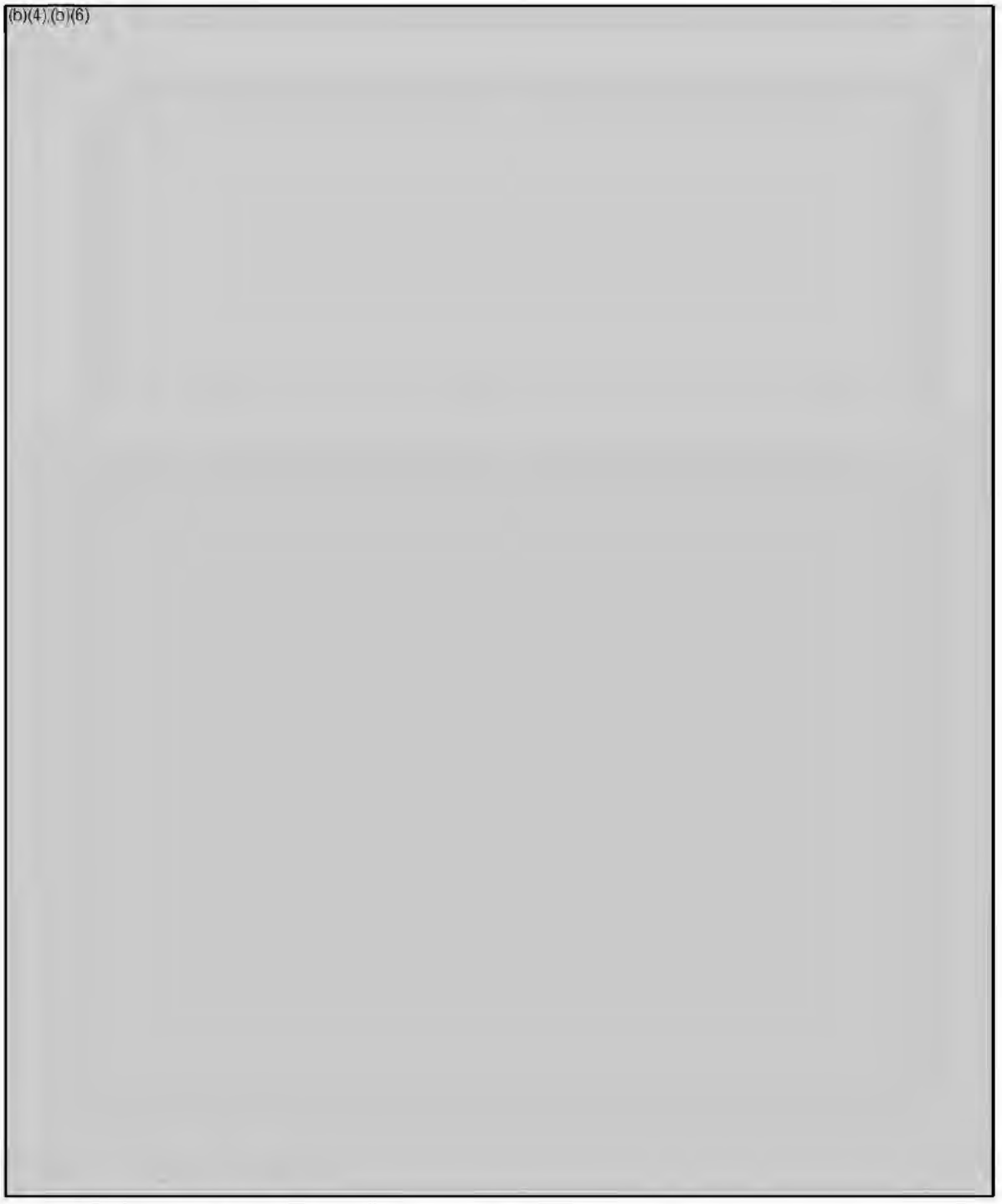
(MicroBrightField, Williston, VT). Estimated totals will be determined using the fractionator probe, and all sampling will be done under a 63× oil immersion objective.

Glutamic acid decarboxylase-67 (GAD67) immunohistochemistry. Our previous studies have demonstrated delayed loss of GABAergic interneurons in the BLA (31, 86). To determine what proportion of the lost neurons are GABAergic interneurons, we will combine stereological quantification with GAD67 immunohistochemistry. To label GAD67-immunoreactive neurons, a I-in-5 series of free-floating sections will be collected from the cryoprotectant solution, washed three times for 5 min each in 0.1 M phosphate buffered saline (PBS), then incubated in a blocking solution containing 10% normal goat serum (NGS; Chemicon, Billerica, MA) and 0.5% Triton X-100 in PBS for one hour at room temperature. The sections will then be incubated with mouse anti-GAD67 serum (1:1000, MAB5406; Chemicon), 5% NGS, 0.3% Triton X-100, and 1% bovine serum albumin, overnight at 4°C. After rinsing three times in 0.1% Triton X-100 in PBS, the sections will be incubated with Cy3-conjugated goat anti-mouse antibody (1:1000; Jackson ImmunoResearch, West Grove, PA) and 0.0001% DAPI (Sigma-Aldrich, St. Louis, MO) in PBS for one hour at room temperature. After a final rinse in PBS, sections will be mounted on slides, air dried for 30 min, then coverslipped with ProLong Gold antifade reagent (Invitrogen, Grand Island, NY).

Brain slice electrophysiology: Coronal slices containing the amygdala and the hippocampus, and slices containing the prefrontal cortex will be prepared from experimental and control rats, 1 month and 3 months after soman exposure. The rats will be anesthetized with isoflurane and then decapitated. The brain will be rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM) 125 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, and 22 glucose, bubbled with 95% O_2 and 5% CO_2 to maintain a pH of 7.4. Coronal slices containing both the hippocampus and the amygdala, and coronal slices containing the prefrontal cortex will be cut at 400 µm with a Vibratome (Series 1000; Leica Microsystems, Bannockburn, IL). Slices will be kept in a holding chamber containing oxygenated ACSF at room temperature, and recordings will be initiated ≥1 hr after slice preparation. For whole-cell recordings, slices will be transferred to a submersion-type recording chamber, where they will be continuously perfused with oxygenated ACSF, at a rate of 3–4 ml/min. Neurons will be visualized with an upright microscope (Nikon Eclipse E600fn; Nikon, Tokyo, Japan) using Nomarski-type differential interference optics through a 60× water immersion objective. Tight-seal (>1 GΩ) whole-cell recordings will be obtained from the cell body of pyramidal-shaped neurons in the BLA region, the pyramidal layer in the CA1 hippocampal area, and the layer III and V pyramidal cells of the prefrontal cortex. Patch electrodes will be fabricated from borosilicate glass to have a resistance of 1.5–5.0 MΩ when filled with a solution containing (in mM) 135 Cs-gluconate, 10 $MgCl_2$, 0.1 $CaCl_2$, 1 EGTA, 10 HEPES, 2 Na-ATP, 0.2 Na3GTP, pH 7.3 (285–290 mOsm). Neurons will be voltage-clamped using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA); some recordings will be obtained in the current-clamp mode. Access resistance (5–24 MΩ) will be regularly monitored during recordings, and cells will be rejected if resistance changes by >15% during the experiment. The signals will be filtered at 2 kHz, digitized (Digidata 1322A; Molecular Devices), and stored on a computer using pClamp9 software (Molecular Devices). Analysis of recorded currents will be performed using pClamp9 software and

the Mini Analysis Program (Synaptosoft, Inc., Leonia, NJ). Extracellular field potentials will be obtained from the BLA, while stimulus pulses are applied to the external capsule, the CA1 hippocampal area, while stimulation is applied to the Schaffer collaterals, and layer III and V of the prefrontal cortex slices, while stimulation is applied to the subcortical white matter. Recording electrodes will be filled with ACSF (resistance: 4 to 7 MΩ). To determine alterations in neuronal and network excitability, whole-cell recordings will be performed in the current-clamp mode to determine changes in the intrinsic excitability parameters of pyramidal cells in the BLA region of the amygdala, the CA1 hippocampal area, and layers III and V of the prefrontal cortex, in slices obtained from the experimental and control groups, at 1 and 3 months following exposure to soman (or saline, for the controls). The following parameters will be measured: resting membrane potential, neuronal input resistance, action potential threshold, action potential characteristics, spontaneous firing, and firing characteristics in response to depolarizing current pulses passed via the recording electrode. Whole-cell recordings in the voltage-clamp mode will also be performed to determine differences, among experimental and control groups, in spontaneous GABAergic and glutamatergic activity in the BLA region of the amygdala, the CA1 hippocampal area, and layers III and V of the prefrontal cortex. These experiments will provide an insight into soman-induced alterations in the intrinsic excitability of pyramidal neurons in the BLA, the CA1 region, and the prefrontal cortex, as well as alterations in the basal, spontaneous activity of the two major neurotransmitter systems that control neuronal network excitability (the GABAergic and the glutamatergic system), and the extent to which the (b)(4) (b)(4) administered 30 or 60 min after soman exposure prevent these alterations. For alterations in synaptic plasticity, we will test the hypothesis that soman-induced seizures and the resulting neuronal damage alter synaptic plasticity in the amygdala, the hippocampus, and the prefrontal cortex, and that termination of the seizures by administration of the (b)(4) will minimize these alterations. Synaptic plasticity will be studied by investigating the characteristics of long-term potentiation (LTP) of synaptic transmission, as we have done previously in both amygdala slices (3) and cerebral cortex slices (2a). In brain slices obtained from rats from experimental and control groups, sacrificed 1 or 3 months after exposure to soman (or saline, for the control groups), field potentials and compound EPSCs will be recorded in the BLA, the CA1 area, and in the prefrontal cortex in response to electric stimulation of the external capsule, the Schaffer collateral/commisural pathway, and the subcortical white matter, respectively. Because LTP is considered to be the cellular mechanism underlying learning and memory, these experiments may provide an explanation for the memory impairment and other cognitive deficits that result from exposure to nerve agents, and will determine if the (b)(4) can prevent alterations in synaptic plasticity by soman.

(b)(4)



V.4.4.8. Tissue Sharing:
Tissues not used in this study may be made available to other investigators as required. Lactating mothers will be available for transfer for training or experimental purposes, or euthanized if they cannot be used. Every effort will be made to transfer these animals to another protocol.

V.4.5. Study Endpoint:

For **Experiments 1, 2, and 3A and B** the endpoint is euthanasia of the male and female rats for neuropathological studies, preparation of brain slices, or at the end of the behavioral experiments, within 4 months of receipt at USUHS. The study endpoint for the surrogate mothers is the time when the pups are weaned or removed for experiments.

(b)(4)

Early Endpoint: Any of the following will represent a reason for early euthanasia: inability to ambulate or reach food or water, persistent seizures (development of continuous (>10 sec) rhythmic spike and/or sharp wave activity at least 2X greater than the baseline amplitude and that lasts more than 30 min), persistent tremors, self mutilation, continuous circling, or persistent labored respiration or gasping, loss of 20% of weight below baseline, prostration/inactivity for up to 24 hours, or a significant decline in vital signs. These signs, if observed by animal facility personnel and no study personnel can be reached, may be taken by the veterinarian as cause for euthanasia. Some behavioral changes may be caused by nerve agent-induced seizures and will not represent early endpoints: lethargy, weakness, inability to walk straight and uncertain ambulation (as long as it does not interfere with the animal's ability to eat and drink), intermittent abnormal breathing pattern. The enclosed Pain/Distress Criteria scoring sheet may be used as a guide.

V.4.6. Euthanasia:

If any of the experimental animals develop serious complications due to the agent exposure and/or associated treatments while at USAMRICD, the animal may be euthanized early with an overdose of pentobarbital (>75 mg/kg, IP) or carbon dioxide administered from a cylinder upon consultation between the PI and the facility veterinarian.

For brain pathology studies, at USUHS the animals will be anesthetized with 75-100 mg/kg, i.p., sodium pentobarbital, until a surgical plane of anesthesia is reached (non-response to strong toe-pinch) and then exsanguinated via perfusion with normal saline followed by 10% neutral buffered formalin.

For brain slice preparations, rats will be anesthetized with isoflurane inhalation under a chemical fume hood and euthanized by decapitation. Isoflurane will be administered via a precision vaporizer. When the animal has lost its toe pinch (pedal withdrawal) reflex and eyelid blink (palpebral) reflex, and is immobilized, it will be removed from the vaporizer and decapitated with a guillotine.

Following the collection of tissues the remaining carcass will be disposed of IAW the most recent version of VMSB SOP VM-08-301 (114), "Animal Euthanasia."

Comments on the use of decapitation: The guillotine will be maintained in a sharpened condition to ensure rapid and clean decapitation. In the past, one guillotine has been effective for approximately 200 rat decapitations without sharpening. Commercial sharpening will be performed after every 200 procedures or sooner if needed to ensure continued sharpness. The PI has utilized guillotines in two other protocols as co-investigator and three as PI and is proficient in their use. Any other personnel on this protocol will not assist until they have been trained in the use and safety considerations by the PI and are proficient. Decapitation is preferred over other euthanasia procedures because it minimizes residual medications that might affect neuronal responses to stimulation.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

The rats will be maintained under an AAALAC accredited animal care and use program. They will be acclimated upon arrival for 5 days. Animals will be housed in an approved animal holding room after release from acclimation. They may be pair-housed but must be individually housed post-surgery to ensure no interference with the surgical sites. Food and water will be freely available except during the exposure period. The veterinary staff will provide daily checks of general health and provisions of food and water.

(b)(4)

V.5.1.1. Study Room:

Building(s): (b)(6) Room Number(s): (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No X

Fluid Restriction: Yes _____ No X

V.5.1.3. Exceptions: (b)(4)

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
The PI or research staff will observe the rats at least twice a day following all procedural manipulations. The veterinary staff will observe the rats at least once a day for general health and husbandry condition. Any rat displaying any other signs of pain, distress or illness will be evaluated by the PI and/or attending veterinarian and, if necessary, euthanized as previously described.

Animal monitoring: Animals will be examined after return to their home cage, approximately 30 minutes after return, and once again that day near end of the work day by the USUHS research team. Animals will be checked at least twice daily for one week post-exposure by the USUHS PI and staff. Observations will be recorded somewhere accessible to animal care staff. USUHS PI and staff observations for specific signs related to surgery will be in addition to routine observations conducted by the animal care staff.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

If any rat experiences non-study related distress or pain prior to the study, the PI or co-PI will consult the attending veterinarian. If necessary, the rat will be treated or euthanized as directed by the attending veterinarian. Euthanized rats will not be entered into the study and additional rats may be requested as replacements.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets for rodents; balls, toys and food enrichment treats for rodent species.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Animal handling, agent handling, injections, anesthesia, EEG surgery, guillotine usage, euthanasia, electrophysiology	(b)(6)	PhD in neuroscience. 20+ yr rodent handling, surgery, tissue dissection, electrophysiological experimentation. Over 200 rats euthanized via guillotine. 6 years performing EEG electrode surgery in rodents.	Investigator training Dr. (b)(6) has performed hundreds of surgeries, euthanasia, and anesthesia.
Animal handling, agent handling, injections, anesthesia, EEG surgery, guillotine usage, euthanasia, electrophysiology		PhD in physiology 30+ yr animal handling, injections, surgery, tissue dissection, electrophysiological experimentation. Over 200 rats euthanized via guillotine.	Investigator training Small animal handling workshop 30+ years experience and on-the-job training
Animal handling, anesthesia, EEG surgery, guillotine usage, euthanasia, perfusions, electrophysiology		PhD in neuroscience. 20+ yr rodent handling, surgery, tissue dissection, and electrophysiological experimentation. Over 200 rats euthanized via guillotine	Dr (b)(6) was retrained as an animal investigator at USUHS in 2007. 20+ years experience and on-the-job training, anesthesia, euthanasia, tissue samples, small animal surgery, perfusions, electrophysiological experimentation.
Animal handling, injections, EEG		PhD in neuroscience More	Investigator training in small animal

surgery, perfusion, histological preparation, neuropathology	(b)(6)	than 15 years of experience in small animal handling, injections, perfusion, histological techniques, and assessment of neuropathological damage	handling and experimentation, including behavioral tests.
Animal handling, injections, EEG surgery and recordings		Ph.D. student at USUHS. Two+ years experience in handling small laboratory animals, tissue samples and small animal surgery including EEG and osmotic minipumps.	Investigator training and small animal handling workshop to be completed. Extensive experience and on-the-job training
Animal handling, injections, EEG surgery and recording		Lab Technician, Ms. (b)(6) has one year of on-the-job training in animal experimental procedures, Rats perfusions and surgery.	Investigator training Small Animal Handling Workshop.
Animal handling, agent handling, injections, anesthesia, EEG surgery, guillotine usage, euthanasia, electrophysiology		PhD in physiology 20+ yr animal handling, injections, surgery, tissue dissection, electrophysiological experimentation. Over 200 rats euthanized via guillotine.	Investigator training Small animal handling workshop 20+ years experience and on-the-job training

VII. BIOHAZARDS/SAFETY:

XCSM agent, decontaminated XCSM agent waste, chemicals, and hazardous waste used in this protocol will be handled in accordance with all applicable state and federal guidelines, regulations, and standing operating procedures.

Use of XCSM: The laboratories follow the standard operating procedures for the use and disposal of XCSM agents, as described IAW the latest version of SOP# 87-201-RS-

01, SOP# 87-335-VA-12, and APGR 200-60. These include initial and quarterly monitoring, as well as daily checks, of hood safety.

Use of formalin: All operators using formalin must wear proper personal protection equipment, i.e., gloves, safety glasses, lab coat, and they will also use a portable chemical hood. Exposure to 10% neutral buffered formalin solution for perfusion is limited to no more than 400 mL/day. The 10% neutral buffered formalin waste (mixed with blood) will be disposed of down the sink in rm 6, bldg E-3244, and documented in a sink waste log.

Minimal PPE for animal handling includes nitrile gloves, mask worn in the slung position or readily available, safety glasses with sideshields or goggles, and fully buttoned lab coat. Double gloves will be worn during agent exposure. All agent work will be done in a certified chemical fume hood in a laboratory that has been appropriately permitted IAW USAMRICD Memorandum 385-6.

Waste anesthetic gas (isoflurane) will be scavenged either by evacuation into a chemical fume hood or by extraction with a filter system in a recirculating air cleaning device (Extract-All system, VetEquip Inc., Pleasanton, CA).

VIII. ENCLOSURES:

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Tra

(b)(6) [redacted]

Pri

03/20/12
Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and

(b)(6) [redacted]

Princip

03/20/12
Date

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL** or **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods a[redacted]ol, I have determined that alternative procedures [redacted]he objectives of this proposed experiment.

Prin[redacted] 02/23/12
Date

X. PROTOCOL ABSTRACT:

A. **Animal Protocol Number**:

B. **Animal Protocol Title**: (b)(4)

(b)(4)

C. **Principal Investigator**: (b)(6)

D. **Performing Organization**: USUHS and USARRICD

E. **Funding**: (b)(4)

F. **Objective and Approach**: The purpose of this work is to develop improved therapies to treat seizures caused by exposure to nerve agents and to prevent the associated neuropathology.

We propose to accomplish our goals by using in vivo exposure of rats to soman. The (b)(4)

G. **Indexing Terms (Descriptors)**: (b)(4)

(b)(4)

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(b)(4) (b)(6)

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(b)(4),(b)(6)

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September 7, [illegible]

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on September 7, 2012:

Animal Protocol Title: "Epigenetic biomarkers of post-traumatic stress disorder in mouse model in constant pressure environment."

USUHS Protocol Number: (b)(6)

Expiration Date: September 6, 2015

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Epigenetic biomarkers of post-traumatic stress disorder in mouse model in constant pressure environment

GRANT TITLE (if different from above): Epigenetic biomarkers of stress at (b)(4) (b)(4) conditions

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: AFMSA, DOD

EARLIEST ANTICIPATED FUNDING START DATE: July 9, 2012

PRINCIPAL INVESTIGATOR:

(b)(6) _____ APG (Department) (b)(6) (Office/Lab Telephone) 07.09.2012 (Date)

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) _____ Professional Chair (Title) (b)(6) (Telephone) 07.09.2012 (Date)
Research Unit C[illegible]gnature
Typed Name: (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the

(b)(6) _____ APG (Department) (b)(6) (Telephone) 07.09.2012 (Date)
Typed Name:

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) _____ LAM (Department) (b)(6) (Telephone) 24 JUL 12 (Date)
Attending/Consulting Veterinarian Signature
Typed Name: (b)(6) (b)(6)

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) PhD.

ANIMAL PROTOCOL TITLE: Epigenetic biomarkers of post-traumatic stress disorder in mouse model in constant pressure environment

GRANT TITLE (if different from above): Epigenetic biomarkers of stress at (b)(4) (b)(4) **conditions**

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6)

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

At present, there is no definitive treatment and no cure for post-traumatic stress disorder (PTSD). Brain function of PTSD victims is strongly impacted especially with changes in how neurons in the brain communicate between each other. In this proposal we will continue to use mice as an animal model to address an important challenge facing clinical research in its quest to help PTSD victims. This proposal is building on the previous proposal (Animal Study Proposal/ USUHS IACUC (b)(6) where we have investigated one of the epigenetic mechanisms: microRNAs (miRNAs) expression in mice subjected to various levels of stress conditions. Epigenetic mechanisms are not related to mutations in DNA but they affect DNA encoded read-outs, which include messenger- and micro- RNAs and therefore translation into proteins. As miRNAs are complementary to one or more mRNA molecules they can interfere with expression of a number of target genes and influence gene regulatory networks and therefore affect signaling pathways underlying pathophysiology and behavior changes caused by PTSD. Because complex epigenetic changes may underlie diverse phenotypes of PTSD and contribute to PTSD pathophysiology, a novel more complete approach than has been used previously is needed. This novel approach has to take into account individual variations between subjects because different life experiences and epigenetic modifiers affect mice during their development and impact their behavioral characteristics. In our PTSD model, we will expose mice to one of several stress levels: no stress, 3 hours of restraint stress, or 3 hours of restraint stress plus intermittent tail shock. US Air Force personnel may face intermittent changes in atmospheric pressure when performing their mission therefore our model will include (b)(4) chamber, where mice will be under controlled pressure environment for determined time period.

We hypothesis that epigenetic characteristics play a major role in the communication between neurons after a stressful environmental or psychological insult by regulating mechanisms underlying activity dependent molecular processes. Epigenetic signatures in the central nervous system appear critical for controlling cell identity and play an important role in the development of brain function. Some of the end targets of epigenetic regulation include signaling proteins that play important roles in communication between neurons, brain function and respective behavior. Abnormalities in the epigenetic modifications will result in the disruption of the translation of mRNAs affecting proteins that mediate brain function and alert neuronal communication. Therefore understanding the way the epigenetic profiles impact the brain and influence PTSD will allow for therapeutic intervention. We believe that in the future we will be able to develop

therapeutic applications for the reversal of brain malfunction caused by stress. This will enable a reduction of anxiety episodes in military and/or civilians associated with PTSD and exposure to (b)(4) pressures associated with (b)(4)

II. BACKGROUND:

II.1. Background:

Interactions between an individual's genetic make-up and environmental exposure results in differential disease outcomes in response to the same environmental insults. One such example is post-traumatic stress disorder (PTSD) where individuals exposed to trauma display heterogeneous responses, including the development of diverse psychological symptoms and differential rates of recovery. This heterogeneous breakdown of neurocognitive systems is one of the most important indicators of the involvement of gene-environmental interaction in the etiology of PTSD.

Environment can interact with the genome in non-mutagenic manner to produce gene expression changes caused by epigenetic mechanisms during development and adulthood. Epigenetic factors including histone modifications, DNA methylation and microRNA (miRNA) expression can dynamically change genome function under acute and chronic environmental influence, thereby impacting neuroplasticity, learning and memory [4, 5]. Chromatin modifications and miRNA interference emerges as fundamental molecular mechanisms for the altered transcriptional regulation involved in brain injury, neurodegenerative and stress related disorders [6-8]. Stress, infectious agents, environmental toxins and "harsh" environmental conditions are well recognized chromatin modifiers that may alter the PTSD outcome and the efficacy of treatment [9]. Recent years brought growing evidence supporting the specific impact of environmental toxins on chromatin [10, 11].

During deployments/operations, US Air Force (USAF) personnel are exposed to extreme environmental conditions including low-oxygen, low-ambient air pressure, environmental toxins, etc. Most, or perhaps all individuals develop a spectrum of (b)(4) symptoms known as acute or chronic mountain sickness, including the more severe conditions of cerebral and lung edema [12]. In addition, deployment and other rescue operations can lead to PTSD among USAF personnel as depicted from the recently reported increase in the incidence of PTSD among USAF personnel after deployment to Iraq/Afghanistan [1]. Furthermore, the environmentally induced PTSD symptoms have also been observed in active-duty USAF noncombatants [13, 14]. Thus the deployment to any war zone in combination with extreme environmental conditions can cause increased mental health problems. Differential PTSD outcomes and treatment response among the USAF personnel occurs due to the underlying complex gene-environmental interactions. The understanding of how genes and environmental factors interact to cause disease states will thus lead to novel strategies for the diagnosis, treatment and prevention of complex human disorders.

A number of association studies that have been performed to date clearly support our hypotheses on the polygenic nature with strong environmental component as the underlying causes for the etiology of (b)(4) sickness and adaptation [15]. Recent genome-wide association studies designed to identify genetic contributions to human adaptation a(b)(4) have revealed a subset of genes related to the HIF (b)(4) nducible factor) pathway [16]. HIF, the key regulator of oxygen homeostasis, constitutes a set of heterodimeric transcription factors (HIF1, HIF2, HIF3) comprised of different alpha and beta subunits [17]. HIFα subunits are

stabilized by (b)(4) thereby regulating the expression of variety of genes that control a switch from oxidative to glycolytic metabolism, cause inhibition of cellular proliferation, elicit an increase in the production of red blood cells & hemoglobin in blood, and stimulate oxygen delivery through erythropoiesis and angiogenesis [18, 19]. It is interesting to note that hematological response (higher hemoglobin concentration, increased hematocrit values, erthyocytosis etc.) caused by acute exposure or adaptation to (b)(4) vary significantly among endemic/ethnic populations suggesting genetic component (b)(4) and possible environmental factors (b)(4) [20, 21]. Under (b)(4) conditions, HIF1 activates the transcription of Jumonji domain containing histone demethylase enzymes (JMJD1A- demethylates H3K9, JMJD2B- demethylates H3K36, JMJD2C, PLU-1), thereby affecting the downstream expression of their target genes [22, 23]. SIRT1 (deacetylates H3K9) and SIRT6 (deacetylates lysine residues) are also known to inactivate HIF1α under (b)(4) [24, 25]. A recent study has shown (b)(4) mediated upregulation of histone methyl transferase - G9a and histone deacetylases [26]. HIF transcription factors also affect the expression of miRNAs (considered a part of epigenome), which are short ~22 nucleotide RNA sequences that bind to complementary sequences in the 3' UTR of multiple target mRNAs mostly resulting in their silencing [27, 28]. In cancer cell lines (b)(4) triggers a specific pattern of miRNAs expression, suggesting a (b)(4) impact on epigenome in the tumor cells [29]. Also in non-proliferating cells, HIF1α/ HIF1β induction alter miR-101 expression, which in turn downregulates the expression of EZH2, a polycomb protein that catalyzes H3K27 trimethylation [30]. All these studies support the combinatorial role of epigenetic mechanisms and HIF factors in regulating (b)(4) stress response. In addition to HIF-mediated pathways, expression of genes linked to (b)(4) sickness is also epigenetically regulated in a tissue specific manner [15, 16, 31].

We hypothesize that physiological conditions caused by (b)(4) such as (b) air pressure and low oxygen, trigger epigenetic modifications in various organs that are comorbid to stressful deployment activity. We will implement an integrated approach using molecular epigenetics and behavioral techniques to unravel the underlying role of epigenetic modifications, which affect brain function, neuronal circuitries, hematological and pulmonary functions and behaviors associated with PTSD after exposure to (b)(4) insults. Behavioral phenotyping of mice is critical in order to identify biomarkers of stress under (b)(4) conditions in relation to subject specific response to stress and traumatic events. (b)(4) (b)(4) Building upon our previous experience, we will categorize the mouse behavioral phenotypes using the results of their performance in rotarod, fear conditioning and acoustic startle response tests. Comprehensive genome-wide epigenetic profiling (ChIP-Seq for histone modifications, RRBS-Seq for DNA methylation, miRNA-Seq) and expression profiling (RNA-Seq) will be implemented via next-generation sequencing approach, that yields unprecedented quality sequencing data with the highest signal to noise ratio. We will build our approach based on our experience gained during yet to be completed project focused on the epigenetic signature of (b)(4) (b)(4) we generated PTSD mouse model by exposing mice to two stress levels: 3 hours of restraint stress plus intermittent tail shock (severe stress level) or 3 hours of restraint stress (low stress level). Based on the acoustic startle response, we have categorized these mice into three behavioral groups (consistent, frozen and hyperactive). Interestingly, among these miRNAs, which are deregulated in response to stress, miR-106a is predicted to inhibit HIF-1α expression. The miR-199a-5p, with observed down-regulation in restraint + shock mice, has been experimentally proven to inhibit HIF-1α expression [33]. In the study proposed here, we will build on these findings by employing the same PTSD mouse model, and

exposing animals to (b)(4) conditions in a chamber mimicking the (b)(4) environment.

In the proposed project, we will establish a genome wide list of targets affected by exposure to (b)(4) conditions alone and in combination with stress. We will perform bioinformatics functional pathway analysis to unravel signaling pathways involved and propose future treatment targets for PTSD. In the follow-up study, we plan to determine epigenetic markers altered in blood samples from PTSD USAF personnel post- and pre-deployment and compare them with the best hits from animal studies; and validate therapeutic options based on the outcome of these studies.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:

Biomedical Research Database (DoD)
Pubmed
NIH REPORTER

II.2.2. Date of Search:

July 3, 2012

II.2.3. Period of Search:

PubMed – all, NIH RePORT – all, DoD Biomedical Research Database Search 1999-2009 (all available).

II.2.4. Key Words and Search Strategy:

Source	Keywords		Hits
PubMed	(b)(4)	epigenetics	1
		PTSD	1
		ChIP-Seq	0
		microRNA	2
	(b)(4)	PTSD	2
		epigenetics	0
		microRNA	0
		ChIP-Seq	0
		PTSD, epigenetics	0
		stress, epigenetics	0
		stress, microRNA	0
		"whole genome"	0
		"whole-genome"	0
NIH RePORTER	(b)(4)	epigenetics	0
		PTSD	0
		microRNA	1
		"Chip-Seq"	0
	(b)(4)	epigenetics	0
		PTSD	0
		microRNA	
		"Chip-Seq"	0
	(b)(4)	stress, epigenetics	
DoD Biomedical Research		stress, epigenetics	0

Database			
	(b)(4)	PTSD	0
		epigenetics	0
		chromatin	0

II.2.5. Results of Search:

Investigations of epigenetic modifications resulting in physiological adaptation to (b)(4) environments and their corresponding interactions with PTSD susceptibility have yet to be investigated. A search for (b)(4) and "epigenetics" in PubMed returned one article that is a perspectives paper suggesting that epigenetic studies would be valuable in assessing adaptation mechanisms. A search for (b)(4) and "PTSD" in PubMed also returned a single paper. This study investigated the use of (b)(4) exposure in desensitization therapies for flying phobias. Searching (b)(4) and "microRNA" returned two manuscripts that examined the impact of (b)(4) on cells grown in culture and variations in (b)(4) related transcription factors in the yak. Changing the search term (b)(4) returned a few additional papers with respect to PTSD. Both examined the potential anxyolytic effects of (b)(4) exposure in rats. Thus, no currently published research indicates a duplication of studies proposed in our experiments.

With respect to ongoing funded projects, examination of NIH RePORTER and the Department of Defense Biomedical Research Database revealed only a single study combining (b)(4) or (b)(4) conditions with epigenetic modifications. This study is investigating mechanisms contributing to vasculature blockage in patients with sickle cell disease. No funded research was found that examines the epigenetic changes induced by (b)(4) environments or its contributions to PTSD onset.

III. OBJECTIVE\HYPOTHESIS:

In the proposed project, we will establish a genome wide list of targets affected by exposure to (b)(4) conditions alone and in combination with stress. We will perform bioinformatics functional pathway analysis to unravel the signaling pathways involved and propose future treatment targets for PTSD. We propose the following Specific Aims:

Specific Aim 1 - Test the impact of differential time course of (b)(4) exposure on mouse behavioral phenotypes as well as hematological, pulmonary and central nervous system pathologies.

Specific Aim 2 - Determine genome-wide epigenetic profile in mouse brain, blood and lung tissues exposed to (b)(4) pressure/oxygen conditions.

Specific Aim 3 - Identify genome-wide epigenetic profile in brain, blood and lung tissues of PTSD mouse exposed to (b)(4) pressure/oxygen conditions.

IV. MILITARY RELEVANCE:

Understanding the interaction between a demanding environment and the dynamic human genome is one of the most challenging and pressing areas of military and clinical research that in the long run should help to alleviate the debilitating consequences of military activity resulting from interactions between harsh environment, demand for high achievement and enduring stress. Recent reports found increasing rates of PTSD among those serving in the harsh and stressful

environments of Iraq and Afghanistan [1]. The proposed project aims to determine epigenetic marks or novel regulatory pathways affected by (b)(4) environment and stress, which may further help to identify individuals at risk for PTSD, assess mental health readiness among those who remain active in service and also enable targeted treatment for a wide spectrum of PTSD cases.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:
FLOW CHART OF EXPERIMENTAL PROCEDURES:

V.1.1. Specific Aim 1

Experiment 1.1: Pilot experiment to assess proper chamber ventilation during (b)(4) exposure.

In all experiments covered by this protocol we will utilize the compact (b)(4) chamber developed by (b)(4) (b)(4) They have extensive experience in creating research level chambers designed for small animals such as mice that enable proper monitoring of animal health during experimental conditions. Our chamber will be used in combination with a pressure controlled purge system to mimic (b)(4) conditions. We are working closely with the company to ensure our chamber is capable of achieving our experimental goals while maintaining the most naturalistic living environment the mice (beyond those variables related to (b)(4) environments). The final design will be discussed with (b)(6) to ensure the safety and well-being of the mice and if required further modification of this protocol will be submitted.

The (b)(4) Research Chamber- Modified Vickers Special to be used will be supplied by (b)(4) and is equipped with the vacuum source. The chamber was originally built as a medical (b)(4) chamber for (b)(4) oxygen therapy by Vickers and has 23" inch inside diameter with an approximately 6'8" interior length. The overall length is about 7 feet. A removable full-length tray can be used to support 2-3 normal mouse cages within the chamber. The tray is designed to slide out onto a removable dolly. (The dolly is needed only if the tray is to be pulled out more than 1/3 of its length.) A control panel enables continuous ventilation at known controlled rates with system panel to monitor pressure and temperature inside the chamber. Within the chamber we will maintain the (b)(4) using constant volume fraction air removal by the vacuum pump. We will maintain the constant air flow to maintain the adequate ventilation. The system is able to provide ventilation upto 300 l/min if necessary (usually 240 l/min for human exposure). We will monitor the build-up of condensation-moisture and during these preliminary experiments we will also monitor CO2 levels. Based on the experience of the renowned expert in pulmonary rodent physiology our consultant/collaborator Dr. (b)(6) (b)(6) we estimate that 15 mice will produce 1700 ml per hour of CO2, which should be easily compensated by appropriate ventilation flow.

Upper limit of CO2 production rate: 0.075 ml/g per minute or 4.5 ml/g per hour [42]. Thus, for 15 mice weighing 25g each, a high estimation for the rate of CO2 production will be: (15 mice) x (30g/mouse) x (4.5ml/g per hour) = 1700 ml per hour. During the active dark cycle oxygen consumption in C57Bl6 mice is 160 (ml/kg per min) (PMID: 22653990) [43]. This equals 9.6 ml/g per hour or approximately twice the rate of CO2 production. Thus, based on high

activity consumption rates, adequate ventilation can be achieved with an exchange rate of 4 liters per hour.

The pilot experiment will have 2 phases (short 15 min and 3 hour duration). During the first 15 min two mice will be exposed to (b)(4) psia pressure respectively and MouseOx Pulse Oximeter/Physiological Hub (Starr Life Sciences, Oakmont, PA) will be used to record non-invasively vital signs (arterial oxygen saturation, heart and breath frequencies (f_h & f_b), and motion status) periodically throughout the duration of the experiment. Mice vitals will be monitored continuously for 15 min at the normal pressure (baseline), during ascent and at the designated pressure and then during descent (the rate of ascent and descent to reach specified simulated (b)(4) condition will be performed at a rate of 200m/min, and for 1500 m this will take ~8 min and for 5000m this will take ~25 min respectively). After reaching normal pressure we will continue monitoring vitals for another 15 min. Based on previous experience of experts in pressurized chamber physiology CAPT (b)(6) (b)(6) Walter Reed National Military Medical Center Pulmonary/Critical Care Medicine, Silver Spring) and Dr. (b)(6) (Duke University) we do not expect any problems with mouse physiological processes during these experiments. We also do not expect bigger than 1% changes in CO2 level during these experiments. In order to minimize potential gasses build up over time conventional cages with the filter covers removed will be used. NH4 levels will be monitored inside the chamber using portable NH4 monitor and then indicator paper positioned inside the cage will be used immediately before cage changes during the longer than 24 hours exposure time. This cage level monitoring will continue throughout the duration of the project if the buildup is significant, otherwise it will be terminated after pilot experiments, and after approval from the veterinarian.

In the second phase of pilot experiment we will verified the accuracy of calculated ventilation flow with 3 cages (5 mice per cage) in the chamber, when the mice will be visually monitored constantly by (b)(6) for 3 hours under (b)(4) psia pressures respectively.

Once in the chamber mice will be monitored for sign of distress (such as hyper or hypoactivity, inner ear discomfort indicated by excessive grooming or scratching, vocalizations, self-trauma, unkempt appearance, respiratory distress, reduced food and water intake, head tilting or shaking) and we will consult with Veterinarian staff if any sign of abnormal behavior is noted. If abnormal breathing is noted mice will be descended to normoxic pressure. The chamber is transparent and therefore monitoring will be easy to achieve. As in the 15 min exposure experiments we also do not expect any problems with mice behavior, accumulation of moisture and CO2 level during these 3-hour exposure experiments.

The final design of the Reimers Systems modified chamber will be discussed with Dr. (b)(6) and the Veterinarian staff and if required modification of this protocol will be submitted for IACUC approval.

This experiment uses a total of 15 mice. Two mice will be exposed for 15 minutes to each pressure (normoxic, (b)(4) psi) every 24 hours therefore we can monitor well-being of mice the day of exposure and the day afterwards. Then same mice plus the thirteen will be exposed for three hours at three pressures every 24 hours so we can monitor well-being of mice the day of exposure and the day afterwards.

In summary, we will wait at least 24 hours between exposures and will not re-expose mice who display the symptoms of distress outlined in section V.4.5. Study Endpoint.

Total mice number will be 15 and mice will be used in these pilot experiments. There will be at least 2 one day interval between different exposure pressures.

Experiment 1.2: Impact of (b)(4) exposure on mouse behavioral motor phenotypes as well as hematological, pulmonary and central nervous system pathologies.

In these experiments we will implement the (b)(4) chamber with parameters determined in the pilot experiments (Exp.1.1). C57BL/6J (B6) mice will be exposed to simulated atmospheric pressure of (b)(4) psia corresponding to (b)(4) sea level. Atmospheric pressure of (b)(4) psia (equivalent to sea level) will be applied as normobaric normoxic environment. The rate of ascent and descent to reach specified simulated altitude and sea level condition will be performed at a rate of 200m/min. Mice will be exposed to (b)(4) for 3 hrs, 6 hrs, 24 hrs, 1 week and three weeks. 15 mice per group each for three diverse pressure levels and 5 different exposure periods will be used. Total mice number will be 15x 3 pressure levels (b)(4) (b)(4) x 5 exposure periods (3 hrs, 6 hrs, 24 hrs, 1 week and 3 weeks) = 225 total mice.

Behavioral impact of (b)(4) conditions will be determined using the rotarod performance test, where the mouse will be placed on a rotating cylindrical rod and subjected to forced motor activity. The time period for which the given mouse stays on this rotating rod is a measure of its balance, coordination, physical condition, and motor-planning. The limited supply of oxygen at (b)(4) could result in a potential loss of coordination and balance.

For rotarod performance test, baseline line values will he recorded 24 hrs prior to the (b)(4) (b)(4) exposure and final test values will be measured immediately at the end of exposure at normoxic pressure.

Brain, blood and lung tissues collected from the mice after (b)(4) exposure for different time points, will be processed using standard histopathological, hematological and immunohistological techniques to determine the pathological effects of (b)(4) environment as compared to sea level in coordination with the AFRI veterinarian pathologist, Dr. (b)(6) MAJ, U.S. Army Veterinary Corps. Particular attention will be paid to histological signs of pulmonary edema (see our recent study [28]), pulmonary fibrosis, hemopoietic abnormalities, morphological changes in carotid body and brain vasculature in order to identify the underlying pathological effects of low-pressure. If necessary our long-term collaborator Dr (b)(6) will be consulted for the outcome of behavioral studies.

Time period of exposure that showed the most significant alterations (in terms of behavioral, pathological and hematological) in response to (b)(6) conditions during the minimal time will he then used in Specific Aim 2. We expect that 1 week of exposure will be a sufficient time period.

Organ and blood isolation: Mice male (8-10 week old) will be anesthetized. Under anesthesia mice will be cervically dislocated, decapitated with scissors and then the brain and lung will be removed and then fixed with 4% of paraformaldebyde (10 mice for each group). For hematological evaluation the blood will be removed from tail or if required for more extensive hematological analysis using cardiac puncture.

Justification and rational for mice number see above.

V.1.2. Specific Aim 2

Experiment 2.1: Genome-wide epigenetic profile in mouse brain, blood and lung tissues exposed to (b)(4) conditions.

C57BL/6J (B6) and Balb/cJ (Balb) male mice will be exposed to (b)(4) psia corresponding to (b)(4) or normobaric normoxia for a time period that showed the most significant alterations (in terms of behavioral, pathological and hematological) in response to (b)(4) conditions, as evaluated in Specific Aim 1. We will use 80 mice per strain each for 2 different pressure levels (b)(4). Total # mice will be 2 strains x 2 conditions x 80 = 320 mice

We selected these two inbred strains as previous comparative studies show their large differential susceptibility to (b)(4) and environmental challenges [35, 36]. Specifically, ventilatory response to acute (b)(4) challenge is significantly greater in Balb compared with B6 mice due to a greater increase in (b)(4) breath frequency (fb) [35] and changes in lung structures between these strains [36]. In addition, B6 mice show a greater exercise tolerance in (b)(4) compared to Balb mice, suggesting that mechanisms of altitude adaptation favored acclimation in B6 mice [37]. In collaboration with our consultant/collaborator Dr. (b)(6) total body plethysmography will be performed to measure breathing tidal volume (VT) and inspiratory time (T_I) in mice [35]. Minute ventilation (VE)= f_b*VT, and mean inspiratory flow (VT/TI) as a surrogate of neural activity in breathing regulation will be calculated. Metabolic data will also be measured by assessing O_2 consumption (VO_2) and CO_2 production (VCO_2) using indirect open circuit calorimetric system (provided by Dr (b)(6) in-line with the barometric chambers. Reference gas measurements will be obtained intermittently to correct for sensor drift, and VO_2 and VCO_2 data will be normalized to standard temperature and pressure conditions, and to the body weight of each animal. The baseline VE will be referenced to VO_2 and VCO_2 to assess the coupling between ventilation and metabolic rate. Increased VE/VO_2 indicates greater ventilation for a given level on O_2 delivery, suggesting that gas exchange in the lung is impaired. Total body plethysmography will be performed before mice enter the (b)(4) chamber, and then immediately after exposure period. Animals will be placed in the plethysmography chamber (17cm x 6cm x 6cm; ~600 cm^3) situated close to (b)(4) chamber and will be allowed to acclimate to the chamber (exploration, and self-grooming is expected) for at least 30 min and only after the animal became quiescent the breathing frequency, tidal volume, and inspiratory time will be recorded. The total recording time in chamber is not expected to exceed 30 min and then animals will be weighed afterwards.

Behavioral phenotyping will be done using the rotarod test as specified in Specific Aim 1 at 24 hours before the beginning of exposure and then immediately after total body plethysmography.

Tissue collection:
Tissue samples will be collected from each group of mice. Briefly, mice will be anesthetized with isoflurane placed in a transparent desiccator jar under a perforated floor in order to separate the mouse from the isoflurane. This will be done in a fume hood present in our lab (b)(6)

(b)(6) Once the mouse is adequately anesthetized, demonstrated by lack of the pedal withdrawal reflex, the live mouse will be removed from the desiccator jar. Alternatively, IP injection of anesthetic will be administrated with 25 - 30 gauge needles in the caudal abdomen with sodium pentobarbital 50 mg/kg (1.5 mg per 30 g mouse) (or, if sodium pentobarbital creates interference with signaling pathways, a mixture of ketamine 100mg/kg and xylazine 20mg/kg will be used) before perfusion. The level of anesthesia will be assessed by toe pinching. Following anesthesia, the mouse chest will be cut open with scissors to expose the beating heart and then perfused through the left ventricle of the heart with heparinized phospbate-buffered saline (10% v/v) for 5 min. Once perfusion is complete, tissues will be dissected out. Tissue samples will be immediately fixed in 10% formalin and would be handed over to AFFRI, veterinary pathologist (10 mice for each group).

The tissue samples from the remaining 60 animals from each group will be collected to perform epigenetic studies. We will quantify histone H3/H4 acetylation and histone H3-Serine10 phosphorylation or other relevant epigenetic targets by quantitative Western blot, chromatin immunoprecipitation (ChIP) assays or genome-wide ChIP-sequencing. Quantitative real time PCR will be used to verify significant findings of high-throughput approach. We will also measure methylation differences by RRBS and changes in miRNA profiles in response to (b)(4) exposure. The brain (cortex, hippocampus), blood and lung tissues will be collected from these mice will be utilized to perform whole genome epigenetic profiling. Histone modifications will be determined through chromatin immunoprecipitation assays (ChIP-Seq) [38] using anti-acetyl histone H3, anti trimethyl histone H3K4, anti trimethyl histone H3K27, anti acetyl histone H3K18 and normal rabbit IgG antibodies. Genome-wide DNA methylation will be determined using reduced representation bisulphite sequencing (RRBS-Seq) approach, a bisulfite-based protocol that enriches CG-rich parts of the genome particularly the gene promoters [39]. For hematological evaluation 10 mice in each group the blood will be removed using cardiac puncture. Whole-genome profiling for miRNA (miRNA-Seq) and mRNA (RNA-Seq) will be performed with RNA isolated from brain, blood and lung tissues. Bioinformatics pipelines to statistically analyze these results have already been developed in (b)(6) Top hits from chromatin/expression and methylation profiling will be validated using quantitative real-time PCR/RTPCR and pyrosequencing respectively.

We plan to establish genome wide list of targets affected by exposure to (b)(4) conditions that are epigenetic biomarkers for (b)(4) condition,

Mice total per group per strain= 80 = 10 for pathology evaluation + 60 for CNS and lung epigenetic evaluation and 10 for blood epigenetic evaluation.

We have 2 pressure groups (sea level + 5000m) and 2 strains = total 320 mice

V.1.3. Specific Aim 3

Experiment 3.1 - Identify genome-wide epigenetic profile in brain, blood and lung tissues of PTSD mouse exposed to (b)(4) pressure/oxygen conditions.

To generate the PTSD mouse model, we will expose C57BL mice to 2 stress levels: 3 hours of restraint stress plus intermittent tail shock (severe stress), 3 hours of restraint stress (lower level stress), and naïve-control (putatively unstressed) (for details of stress protocol see below). This will be carried out before exposure of mice to the (b)(4) conditions (b)(4) corresponding to (b)(4) or normobaric normoxia. The period of (b)(4) exposure post stress will be same as specified in Specific Aim 1. We will use 60 mice for each three different levels of stress and two diverse pressure values (b)(4) (total number is = 60x 3x 2= 360 mice). Acoustic startle (ASR) and fear conditioning behavioral tests will be performed with

PTSD mice exposed to stress before and immediately after exposure to (b)(4) or normobaric condition. ASR system consists of weight-sensitive platform inside sound-attenuated chamber, where mouse movements in response to stimuli (sound) are measured as a voltage change by a strain gauge. ASR baseline line values will be recorded 24 hrs prior to the (b)(4) exposure and final test values will be measured immediately at the end of exposure. Brain (cortex, hippocampus), blood and lung tissues dissected from the PTSD and naïve control mice exposed to (b)(4) or normobaric conditions will be utilized to perform whole genome epigenetic profiling (ChIP-Seq, RRBS-Seq, miRNA-Seq) and RNA-Seq similar to Specific Aim 2. Comprehensive blood analysis and pathological assessment of each PTSD mouse model subjected to (b)(4) conditions will also be performed. Top hits from chromatin/expression and methylation profiling will be validated using quantitative real-time PCR/RTPCR and pyrosequencing respectively. In addition, top hits will be also verified in amygdala, a brain region implicated in response to (b)(4) and stress. Whole genome approach cannot be applied to RNA/DNA extracted from amygdala because of its relative small size and therefore a small yield of RNA/DNA. In general we plan to establish comprehensive genome wide list of targets affected by exposure to (b)(4) conditions alone and in combination with stress that are epigenetic biomarkers for stress under (b)(4) environment
This should help us to unravel signaling pathways involved and propose future treatment targets for PTSD.

Stress protocol in the mice and perform acoustic startle response (ASR) testing in order to determine the impact of the stressing procedures on behavioral phenotype.

Stress model:

Male mice 5-6 week old will be exposed to immobilization and tail shocked for 2 h per day for 3 consecutive days. This procedure will be done during the dark phase of the light–dark cycle (adapted for mice from [54]. Mice will be restrained but wrapped in a cloth jacket and immobilized in a ventilated plexiglass tube to avoid increase in the intrinsic temperature of mouse. In addition the room where the stress will be applied will be well ventilated. (b)(4) electric shocks (b)(4) GrassS88 stimulator, [59]) will be delivered to their tails at semi-random intervals of 150 to 210 s. This stress protocol is based on the helplessness paradigm in which animals undertake an aversive experience without possible adaptive response ([56,57], see [54]). The duration of stress is based on previous demonstrations that repeated stress sessions for 3 days are more effective than a single stress session in producing lasting physiological and behavioral abnormalities, such as elevations in basal plasma corticosterone levels, exaggerated acoustic startle responses and reduced body weight [55,59]. Stress sessions lasting beyond 3 days do not appear to produce greater physiological and/or behavioral changes [55,59]. The weight of mice will be monitored for stressed and control groups.

ASR testing will be conducted with a Startle Response Acoustic Test System (Coulbourn Instruments, PA). The system is computer-based and all stimulus parameters are specified by entries in Windows based software with protocols saved to disk files. The audio source module placed above the test cage is used to provide acoustic stimuli. The software allows the use of either stimulus type (tone or noise). For tone stimuli, amplitude, frequency and duration, and for white noise stimulus duration of noise bursts is computer controlled.

Two days before the stress protocol mice will be placed in ACS chamber with no sound applied for 15 min in order to acclimate them to the equipment. Then one day before exposure to

stress protocol, mice will be placed on the weight-sensitive platform in a sound-attenuated chamber and after chamber lid is closed, mouse will be left in darkness for 3-5 min adaptation period (without startle stimuli). Then startle stimuli will be applied (100 &110 dB) as a noise bursts of 20 ms duration, preceded or not by 100-150 ms pre-pulse with 68 dB intensity. Sound intensities will be verified by a sound meter. Six types of stimulus will be applied: 100 &110 dB alone, with pre-pulse, pre-pulse alone and no stimulus. Each trial type will be presented eight times. Trial types will be presented in random order to avoid order effects and habituation. Inter-trial intervals range randomly from 15 to 25 s. The mice movements in response to stimuli will be measured as a voltage change inside platform and then will be converted to grams of body weight change after analog-digital conversion. These changes will be recorded as the maximum response occurring within 100-200 ms of the onset of the startle-eliciting stimulus. All acoustic stimuli will be administered by an amplified speaker placed above the test cage. Mice will spend not more than 15 minutes in the chamber. The chamber used to ASR experiments will be sanitized with soap, water and then alcohol after each animal.

All animals will be tested 1 day following the final day of the stress procedure and then the same day after the results will be compared to reaction that startle stimuli evoked before exposure to stress. During an experimental session, the software displays both numeric and response waveform data. A post-hoc viewer is included for critical examination trial-by-trial response data.

Rational for number of mice

This proposal constitutes our initial award on exposure to (b)(4) conditions therefore limited or no preliminary data are available. Based on our experience in areas hippocampal signaling pathways and electrophysiology, and our previous and then published results on stress by Dr. (b)(6) and on brain microRNA [46,47] we used following criteria to estimate group size.

The tissue samples from the remaining x animals from each group will be collected to perform epigenetic studies as mentioned in our previously approved protocols. We will quantify histone H3/H4 acetylation and histone H3-Serine10 phosphorylation or other relevant epigenetic targets by quantitative Western blot, chromatin immunoprecipitation (ChIP) assays or genome-wide CHIP-sequencing. Quantitative real time PCR will be used to verify significant findings of high-throughput approach. We will also measure methylation differences in response to exposure to (b)(4) conditions. The animal number is determined by the requirement to provide sufficient amount of tissue required for *in-vitro* analysis (ChIP experiment required many animals due to small sample amount obtained per animals). The group size is based on results of previous experiments, published literature and an effect size and power analysis calculation previously performed [41].

The data generated from the above experiments will be analyzed by ANOVA and differences with $P< 0.05$ will be considered statistically significant. All data will be subjected to statistical analysis to determine significant differences between control and various metal treated tissues. For direct comparison of 2 samples, students T-test will be used. For quantitative analysis of Western blots, optical intensity of bands will be evaluated with MultiGuage. Gel loading variation will be normalized with total H2A. Unpaired T-test will be used to detect changes in histone modifications in metal exposed tissues as compared to controls. If there are any queries about the statistics and data used, then Dr (b)(6) (biostatistician) will be consulted.

V.1.4. Table

Expt	Treatment	Organs and blood	Analysis	Number
1	(b)(4) chamber	Lung,CNS, blood	Pathological evaluation, motor test (rotarod)	15
2	chamber	Lung,CNS, blood	Pathological evaluation, motor test (rotarod)	225
3a	chamber C57BL	Lung, CNS, blood	ChIPSeq, RNA-Seq,mi-RNA profiling, RRBS, motor test (rotarod), total body plethysmography	160
3b	(b)(4) chamber Balb/cJ	Lung, CNS, blood	ChIPSeq, RNA-Seq,mi-RNA profiling, RRBS, motor test (rotarod), total body plethysmography,	160
4	Sham/Stressed C57BL	Hippocampus & Cortex	acoustic startle response ChIPSeq, RNA-Seq, mi-RNA profiling, RRBS, motor test (rotarod),	360
			Total	920

V.2. Data Analysis:

For quantitative analysis of Western blot, image analysis will be performed [60]. Data will be compared across sham and/or stressed mice. All data will be subjected to statistical analysis to determine significant differences between various treatments for stressed and sham mice. For direct comparison of 2 samples, Student's T-test will be used. If several parameters are being compared then ANOVA will be run. The significance will be determined at a probability level of $P < 0.05$. If there are any queries about the statistics and data used, then Dr. (b)(6) (biostatistician) will be consulted.

For behavioral experiments, 15 animals per group are sufficient to detect a difference of 1.1 standard deviations between groups with 80% power, based on a 2-group t test with a 5%, two-sided significance level. Previous behavioral studies with similar outcomes have observed differences in this range [30].

We will assess differences between different groups of mice in several different parameters.

The software program a statistical package Sigma Stat from Jandel Scientific and GraphPadInStat from GraphPad will be used for statistical analysis. Significance will be defined as $P < 0.05$.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

The physiological effects of stress (PTSD) are best understood in the context of the whole organ. Cell lines do not provide a good model for PTSD because neuronal/glia and neuron/neuron connectivity has undergone extensive rearrangement during the stress. Computer models are not a viable alternative since the complexity of CNS can be model only at the levels of small neuronal networks with simplified electrophysiological characteristics and without taken into account metabolic and signaling pathways. Tissue culture cannot model stress responses that are related to complex interactions of sensory and motor systems. Cell and tissue cultures with permanently transfected receptors were considered, but these systems lack the complex cellular interactions and physiologic responses found in whole organisms and needed to answer our experimental questions.

We have not come across any other non-animal alternatives that would mirror complexity of the whole animals and could model expression of mRNAs, micro-RNAs, epigenetic modification of respective promoter networks and its subtle interactions with neuronal networks, neuronal channel and receptors and signaling pathways.

V.3.2. Animal Model and Species Justification:

Mice have been successfully used to model complex human neurological pathology and genetic conditions (for comprehensive review see book by Crawley, What's wrong with my mouse?; 2nd edition, publisher John Wiley and Sons 2007). We also considered rats as an alternative model, which Drs (b)(6) used in their study of PTSD, however the potential of obtaining knockout or transgenic mice in the future targeting molecules involved in synaptic plasticity was a decisive factor in selection of mouse vs. rat. Our laboratory has extensive experience with murine models. (b)(4) (b)(4) In addition extensive comparative data are available for studies of C57Bl mouse (see e.g. Crawley 2007 pages 19-29 and 188-191, www.jax.org). In order to avoid a potential confounding effect of estrous cycle male mice will be used. In the future studies females will be used for more complete evaluation.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	Mus musculus	Mus musculus
V.3.3.2. Strain/Stock:	C57Bl	Balb/cJ (Balb)
V.3.3.3. Source/Vendor:	(b)(4)	

V.3.3.4.	**Age:**	4-6 weeks	4-6 weeks
V.3.3.5.	**Weight:**	20-40 g	20-40 g
V.3.3.6.	**Sex:**	Males	Males
V.3.3.7.	**Special Considerations:**	N/A	

V.3.4. Number of Animals Required (by Species):
Total 160 Balb/cJ and 760 C57Bl mice will be used under this protocol see Table in V.1.4 for details.

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: Novel tailshock delivery device incorporating coiled wire electrodes embedded in soft agar and affixed with flexible parafilm wrap will be employed (following suggestions of Dr. (b)(6) [54]). This would allow us to avoid the traditional "alligator clip" and reduce injury to animal with increasing efficacy of shocks delivered.

Mice may display different behaviors to indicate pain or distress. These include hyper- or hypoactivity, inner ear discomfort indicated by excessive grooming or scratching, vocalizations, self-trauma, unkempt appearance, respiratory distress, reduced food and water intake, head tilting or shaking. No distress is expected beyond the temporary discomfort during the tail shock and restraint periods (PTSD model). Mice that display distress behaviors that induce overt injuries (self-mutilation or aggression injury) will be euthanized. Mice that signs of respiratory distress will be monitored until symptoms improve or euthanized after 8 hours if distress persists. Mice with unkempt appearance or abnormal activity levels (including head tilting or shaking) will be referred to veterinary staff for assessment if the condition persists for more than 8 hours. When possible, treatment options will be pursued before euthanization.

V.3.5.2. Reduction: If possible, tissue from each mouse will be used in more than one Western Blot/mi-RNA profiling, qPCR test thereby reducing the overall number of animals required. In order to optimize RNA/ChIP(DNA) yield different brain regions (like cortex) will be considered in testing. Statistical tests will be constantly applied to the data generated, to ensure that the minimal number of animals necessary to obtain significant data is used. No other reduction alternatives were considered.

V.3.5.3. Replacement:

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. **Column C**:	520	160
V.4.1.1.1.2. **Column D**:	0	
V.4.1.1.1.3. **Column E**:	240	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: Mice will be anesthetized with isoflurane before euthanasia using the drop method in a transparent bell chamber. Once the mouse is adequately anesthetized, demonstrated by lack of the pedal withdrawal reflex, the live mouse will be removed from the desiccator and cervically dislocated. Although isoflurane is our preferred method of anesthesia, it has been noted that isoflurane may influence the quality of neuronal survival in acute slices. Therefore if micro-RNA yield is affected we will anesthetize mice by IP injection of anesthetic administrated with 22-25 gauge needles in the caudal abdomen with sodium pentobarbital 50 mg/kg (1.5 mg per 30 g mouse) (or if sodium pentobarbital creates interference with signaling pathways mixture of ketamine 100mg/kg/ and xylazine 20mg/kg or other anesthetics recommended by Veterinarian will be used). This procedure will be performed by Drs (b)(4) (or personnel trained and supervised by them) and the level of anesthesia will be assessed by toe pinching.

Mice will be anesthetized prior to transcardial perfusion or brain removal by one of the following methods: overdose of isoflurane anesthesia in a "open drop" bell jar with a perforated floor to separate the mouse from the liquid anesthetic or 4% isoflurane/pure oxygen in an induction chamber; IP injection of sodium pentobarbital 50 mg/kg (1.5 mg per 30 g mouse); or a mixture of ketamine 100mg/kg and xylazine 20mg/kg. IP injections will be administered with 25 to 30 gauge needles and in a volume less than 0.5 cc. This procedure will be performed by Drs. (b)(4) (or personnel trained and supervised by him) and the level of anesthesia will be assessed by toe pinching (lack of a pedal withdrawal reflex). For *in vivo* experiments, mice will remain anesthetized with either isoflurane, or ketamine/xylazine throughout the recording session. Anesthesia will be supplemented as needed to maintain unresponsiveness to noxious stimuli (toe pinch to check pedal withdrawal reflex). Mice will be euthanized by anesthetic overdose at the end of the experiment (in the case of isoflurane, 4% in oxygen will be administered until breathing ceases) followed by cervical dislocation.

Although we do not anticipate any more than momentary pain or distress from any of the procedures, topical anesthetics will be applied to the tips of tails after tissue sampling if the animal appears to be in pain. A topical anesthetic spray (such as ethyl chloride spray) will be applied to the tail tip, followed by a small amount of EMLA anesthetic cream applied to the distal end of the tail. This will be repeated if the mice continue to exhibit signs of discomfort.

For general inhalation anesthesia during recovery procedures (blood spotting), oxygen will be mixed with isoflurane delivered via precision vaporizer using a rodent anesthesia machine. Mice will be placed in an induction chamber and exposed to a mixture of O2 and isoflurane (2-4%) to induce general anesthesia. Following induction, mice will be removed from the chamber and maintained on 0.25-2% isoflurane administered by nose cone. We will monitor the depth of anesthesia by noting response to toe pinch (pedal withdrawal reflex), mucus membrane color, and rate and quality of respirations. Tail snip procedures will begin once there is no pedal reflex.

Waste anesthetic gases will be actively scavenged through the central vacuum system or passively scavenged using a charcoal canister.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Pre-stress Provision: We will identify the mice that will be used in our stress protocol and we will request LAM staff for additional information with regard to their behavior and appearance (e.g. skin lesions and external pathological changes). Mice that exhibit any sign of excessive aggressive behavior or any visible pathological changes will be excluded from studies. Mice will be handled and exposed to behavioral equipment for few minutes one day prior to experiments to minimize any stress effects that might result from routine handling during the behavioral testing and novel environment stress effects.

Post-stress Provision: After stress procedure,, mice will be clearly identified and LAM personnel will be notified and arrangements made so that stressed mice will be checked at least twice a day by LAM personnel and lab personnel to observe signs of excessive pain and severe distress. These signs include self-mutilation, gnawing at limbs, abnormal breathing, unkempt appearance, self-imposed isolation, abnormal posture, muscle rigidity or lack of muscle tone, and twitching/trembling/tremor. Mice that loose weight 20% and show signs of excessive distress will be removed from the study and euthanized. While inside the (b)(4) chamber where weight measurements are not possible, we will use the body condition scoring technique described Ullman-Culleré and Foltz (Body Condition Scoring: A Rapid and Accurate Method for Assessing Health Status in Mice, (1999) *LAS 49(3)319-23*). We will consult veterinary staff for mice with a body condition score of 2+ for treatment.

Based on our literature search results, isoflurane, and ketamine/xylazine are commonly used anesthesia agents in *in vivo* recordings and are capable of maintaining adequate levels of anesthesia. Transcardial perfusions following anesthetic overdose are also commonly performed to efficiently remove blood from tissue and quickly fix the tissue when perfused with formaldehyde. The anesthetic overdose ensures that the animal experiences no discomfort. Microwave fixation is not appropriate for our needs as blood left in the tissue can skew analysis of biochemical pathways.

V.4.1.2.3. Paralytics: None

V.4.1.2.3. Paralytics: None

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

Source	Keywords	PubMed
PubMed	Non-painful PTSD model	0
	"in vitro" model PTSD	2
	"in vitro" animal model PTSD	0
	Mouse odorant stress	9

	Mouse odor stressor	20
	Non-painful acoustic startle	1
	Non-painful electric shock	5
	Mouse anesthesia	3954
	Mouse ketamine xylazine	164
	Mouse isoflurane	656
	Mouse euthanasia	301
	Mouse humane euthanasia	19
	Mouse transcardial perfusion	14
	Mouse fixation	5217
	Mouse microwave fixation	69
	Mouse paraformaldehyde	815

A search for *in vitro* models of PTSD returned only two results, one of which examined the effect of cancer diagnosis on PTSD-like symptoms in humans and the second was a review that cited the first article. Several studies have successfully used odorants, often based on odors associated with predators, to induce stress responses in mice. Currently, no *in vitro* models of PTSD are available. Computer modeling of PTSD is also currently not feasible since the biological mechanisms leading to this condition are not sufficiently understood. Searches for alternatives to animal models on the USDA's Animal Welfare Information Center (awic.nal.usda.gov) and the Johns Hopkins Bloomberg School of Public Health (altweb.jhsph.edu) did not identify any viable alternatives to using mice in these studies.
Anesthesia in mice is frequently induced with a mix of ketamine and xylazine or isoflurane. Both approaches provide sufficient levels of anesthesia for prolonged surgical procedures beyond the timeframes needed for our own experiments. Microwave fixation following anesthetization is not appropriate for our studies as blood removal prior to fixation is required. Anesthetic overdose followed by transcardial perfusion of physiological saline and paraformaldehyde is a widely used method of fixation.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PubMed, USDA Animal Welfare Information Center, and Johns Hopkins Bloomberg School of Public Health site for alternatives to animal models.

V.4.1.3.2. Date of Search: July 6

V.4.1.3.3. Period of Search: All

V.4.1.3.4. Key Words of Search:

PubMed Search **Non-painful PTSD model**	0
PubMed Search **in vitro model PTSD**	2
PubMed Search **in vitro animal model PTSD**	0
PubMed Search **Non-painful acoustic startle**	1
PubMed Search **"Non painful" acoustic startle**	1
	19

PubMed Search **Non-painful "electric shock"** 1
PubMed Search **"Non painful" "electric shock"** 4

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

The proposed model of PTSD is based on the helplessness paradigm in which animals undertake an aversive experience without possible adaptive response [54,56,57]. The duration of stress is based on previous demonstrations that repeated stress sessions for 3 days are more effective than a single stress session in producing lasting physiological and behavioral abnormalities, such as elevations in basal plasma corticosterone levels, exaggerated acoustic startle responses and reduced body weight [55,59] and stress sessions, beyond the 3 days, do not appear to produce greater physiological and/or behavioral changes [55,59]. A similar stress model was also applied to mice [64]. These protocols do not produce any damage to the tail tissue. If such unlikely event occurs, this mouse would be removed from the studies, anesthetic or analgesic would be applied to alleviate momentary pain and then mice would be euthanized. The model of PTSD based on a learned helplessness paradigm requires exposure of the animal to unalleviated distress through the administration of restraint and tail-shock.

Study endpoint is survival for 4 weeks post-stress and subsequent behavioral testing. Mice will be euthanized by lab personnel or LAM personnel. Any mice that lose 20% weight (mice will be weigh only outside the pressure chamber and it will be done once a week or more often if recommended by Veterinarian staff) or show three or more following signs of excessive distress that include abnormal breathing, unkempt appearance, self-imposed isolation, abnormal posture, muscle rigidity, lack of muscle tone, and twitching/trembling/tremor, will be removed from the study and euthanized by lab or LAM personnel within 8 h of signs of excessive distress appearing. While inside the (b)(4) chamber where weight measurements are not possible, we will use the body condition scoring technique described Ullman-Culleré and Foltz (Body Condition Scoring: A Rapid and Accurate Method for Assessing Health Status in Mice, (1999) *LAS 49(3)319-23*). We will consult veterinary staff for mice with a body condition score of 2+ for treatment.

No analgesia will be applied since this would compromise the goal of this study because of the potential interference with mice behavior status and plasticity of the brain [50,51,52].

V.4.2. Prolonged Restraint:

No restraint in excess of three hours will be applied during this study. The total time mice will be restraint consists of no more than five minutes prior and post stressed whilst restrained for a 2-h (total restraint time= 5 min prior + 2 hour of restraint and tailshock + 5 min post = 130 min). The immobilization and tail-shocks will be applied during the dark phase of the light-dark cycle. Animals will be restrained in a cloth jacket with their head and torso immobilized in a ventilated plexiglass tube. The duration of stress is effective at producing lasting physiological and behavioral abnormalities. One month after stress, mice will be euthanized and the amygdala and hippocampus excised and frozen.

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: IP injection of anesthetic will be administrated with 25 – 30 gauge needles in the caudal abdomen before perfusion with sodium pentobarbital 50 mg/kg IP (1.5 mg per 30 g mouse). If sodium pentobarbital creates interference with signaling pathways, a mixture of ketamine 100mg/kg and xylazine 20mg/kg IP will be used.

V.4.4.2. Biosamples:

Except blood No tissue will be collected when animals are still alive. Tissue (brain) will be collected after death. Brain will be dissected from mice after euthanasia.

Blood collection and platelet preparation

We will follow an established procedure described e.g. [65,66]. Mice will be anesthetized with a mixture of ketamine and xylazine (100 mg/kg ketamine and 20 mg/kg xylazine), or sodium pentobarbital (50 mg/kg) administered intraperitoneally. Blood will be collected into citrated syringes from the vena cava or by cardiac puncture. These are terminal procedures performed with a large gauge needle and we will collect as much blood as we can recover from each animal.

Red blood cells will be removed by centrifugation and platelet-rich plasma will be recovered and subsequently platelets will be pelleted by second centrifugation. Half of the platelets will be resuspended in modified Tyrode's buffer and then platelet aggregation characteristics will be determined turbidimetrically as described [66].

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Ear tag, tattoo and cage cards.

V.4.4.6. Behavioral Studies: Male mice 5-6 week old will be exposed to immobilization and tail shocked for 2 h per day for 3 consecutive days. This procedure will be done during the dark or active phase of the light–dark cycle (adapted for mice from (b)(6) (b)(6) Mice will be restrained but wrapped in a cloth jacket and immobilized in a ventilated plexiglass tube. (b)(4) electric shocks (b)(4) Animal Test Cage Grid Floor Shocker, Coulbourn Instruments, USA, [59]) will be delivered to their tails at semi-random intervals of 150 to 210 s (Graphic State Notation software, Habitest Universal Link, Coulbourn Instruments, USA). This stress protocol is based on the helplessness paradigm in which animals undertake an aversive experience without possible adaptive response [54, 56, 57]. The duration of stress will be based on previous demonstrations that repeated stress sessions for 3 days are more effective than a single stress session in producing lasting physiological and behavioral abnormalities, such as elevations in basal plasma corticosterone levels, exaggerated acoustic startle responses and reduced body weight [55,59] and stress sessions, beyond the 3 days, do not appear to produce greater physiological and/or behavioral changes [55,59]. The efficiency of the stress will be tested using behavioral testing known as acoustic startle response (ASR). After stress animals will be assessed by an heightened acoustic startle and weight loss.

Inescapable tail shock model of traumatic stress causes chronic hyperarousal [58,59] and exaggerated ASR [53,54]. ASR is the unconditioned response to short acoustic stimuli that is used to characterize the CNS response to various treatments. Augmented startle response is a frequent symptom of PTSD and is likely related to changes in the limbic system associated with the stress [61].

ASR testing will be conducted with a Startle Response Acoustic Test System (Coulbourn Instruments, PA). The system is computer-based and all stimulus parameters are specified by entries in Windows based software with protocols saved to disk files. The audio source module placed above the test cage is used to provide acoustic stimuli. The software allows the use of either stimulus type, (tone or noise). For tone stimuli, amplitude, frequency and duration, and for nosie simulus duration of noise bursts is computer controlled.

Before the stress protocol, mice will be placed in ACS chamber with no sound applied for 15 min in order to acclimate them to the equipment. Then one day before exposure to stress protocol, mice will be placed on the weight-sensitive platform in a sound-attenuated chamber and after chamber lid is closed, mouse will be left in darkness for 3-5 min adaptation period (without startle stimuli). Then startle stimuli will be applied (100 &110 dB) as a noise bursts of 20 ms duration, preceded or not by 100-150 ms pre-pulse with 68 dB intensity. Sound intensities will be verified by a sound meter. Six types of stimulus will be applied: 100 &110 dB alone, with pre-pulse, pre-pulse alone and no stimulus. Each trial type will be presented eight times. Trial types will be presented in random order to avoid order effects and habituation. Inter-trial intervals range randomly from 15 to 25 s. The mice movements in response to stimuli will be measured as a voltage change inside platform and then will be converted to grams of body weight change after analog-digital conversion. These changes will be recorded as the maximum response occurring within 100-200 ms of the onset of the startle-eliciting stimulus. All acoustic stimuli will be administered by an amplified speaker placed above the test cage. Mice will spend in the chamber not more than 15 min. The chamber used to ASR experiments will be sanitized with soap, water and then alcohol after each animal.

All animals will be tested 1 day following the final day of the stress procedure and the results will be compared to reaction that startle stimuli evoked before exposure to stress. During an experimental session, the software displays both numeric and response waveform data. A post-hoc viewer is included for critical examination trial-by-trial response data.

Rotarod test. The rotarod is used to evaluate motor coordination. The mouse is placed upon the apparatus, which has a rotating rod. The rod is slowly accelerated from 4 rpm to 40 rpm over 5 minutes, The number of seconds during which each mouse remains on the rod is recorded.

V.4.4.7. Other Procedures:

Cardiac Perfusions: The mice will be anesthetized as previously described before the cardiac perfusions. They will be checked by toe pinching to ensure that the anesthetic has taken effect. Then the mouse chest will be cut open with scissors to expose the beating heart and then perfused through the left ventricle of the heart with heparinized phosphate-buffered saline (10% v/v) for 5 min. followed by 4% (v/v) paraformaldehyde in 0.1 M sodium phosphate at pH 7.4. The animals will be euthanized with overdose of sodium pentobarbital. Tissue will be then cryoprotected by immersion overnight at 4°C in a solution of 25% (w/v) sucrose in PBS. Frozen sections 20-30 microns thick will be cut on a sliding microtome with a freezing stage and will be stored in PBS containing 0.1% sodium azide. Following a previously approved protocol we will investigate the bleeding time at the time of mouse genotyping.

V.4.4.8. Tissue Sharing: We do not anticipate that will have any extra tissue from our experiments.
Any tissue not needed for our experiments will be made available to other investigators upon request.

V.4.5. Study Endpoint: Study endpoint is survival for 4 weeks post-stress and subsequent behavioral testing. Mice will be euthanized by lab personnel or LAM personnel. Any mice that show signs that include self-mutilation, gnawing at limbs, abnormal breathing, unkempt appearance, self-imposed isolation, abnormal posture, muscle rigidity or lack of muscle tone, and twitching/trembling/tremor, mice that lose 20% of initial body weight (measured during the weekly cage change as outline in Special Husbandry Provisions V.5.1.2) and show signs of excessive distress will be removed from the study and euthanized and euthanized by lab or LAM personnel within 8 h of their signs of excessive pain appearing. No analgesia will be applied since this would compromise the goal of this study. While inside the (b)(4) chamber where weight measurements are not possible, we will use the body condition scoring technique described Ullman-Culleré and Foltz (Body Condition Scoring: A Rapid and Accurate Method for Assessing Health Status in Mice, (1999) *LAS 49(3)319-23*). We will consult veterinary staff for mice with a body condition score of 2+ for treatment. The study will also be terminated early if we are unable to maintain an adequate environment inside the chamber as outlined in the Special Husbandry Provisions V.5.1.2.

V.4.6. Euthanasia:
Decapitation after anesthesia with anesthetization as previously described (section V.4.1.2.1.).
The scissors used for decapitation will be maintained in good working order and the blades checked routinely for sharpness. Mice used for immunohistochemistry will be will be deeply

anesthetized with injectable anesthetics then perfused transcardially as previously described. If additional euthanasia methods are necessary, mice will be given an overdose (100-150 mg/kg) of sodium pentobarbital IP as previously described. Mice used for RNA harvest will be decapitated with sharp scissors while deeply anesthetized (*see V.4.1.2.1*). Mice used for blood collection will be euthanized by exsanguination after anesthetic overdose as outlined in V.4.4.2. Mice euthanized by LAM personnel will be euthanized by an overdose of cylinderized CO_2. Euthanasia will be performed in the LAM CAF. Animals will be placed in a chamber that is not overcrowded. With animals in the chamber, the flow rate will displace at least 20% of the chamber volume per minute. Gas flow will be maintained for at least one minute after apparent clinical death. Animals will be verified as dead (lack of heart beat, lack of respirations) before removal of the chamber. A supplemental method to ensure death (cervical dislocation or bilateral thoracotomy) may be used. These methods are in compliance with the most current report of the AVMA Panel on Euthanasia.

V.5. Veterinary Care: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each mouse in this protocol. Mice will be housed in standard cages up to 5 animals/cage before protocol manipulations.

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) _____ Room Number(s) _____

V.5.1.2. Special Husbandry Provisions: Since the shock protocol should be administered during the dark phase of the light cycle therefore we request that our animals will be kept under reverse light cycle conditions. Mice will be maintained in the (b)(4) environment (b)(4) chamber) for up to one week at which point the cages, food, and water will be changed. We request standard filter top cages to minimize odorant stressors between cages. For the two and three week exposure periods, mice will be replaced in the (b)(4) chamber after the weekly cage, food and water change. We request an exception to the bi-weekly cage change requirement to extend the time period to one week. If a cage runs out of water or food at any point or bedding becomes soaked with water, the experiment will be temporarily suspended until the chamber can be opened and the problem corrected. The experiment will then be continued.

A small digital meter with a highly visible readout will be placed inside the chamber to monitor the internal temperature and humidity. As ambient air is used to continuously refresh the internal environment, we expect minimal differences between the chamber and room temperature and humidity. We will consult veterinary staff if the chamber humidity rises above 70% or temperature exceeds 80°F. A similar device will be included to monitor CO_2 levels.

Food Restriction: Yes _____ No _____

Fluid Restriction: Yes No

V.5.1.3. Exceptions:

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be observed at least once daily by Center for LAM personnel, and attending veterinary care will be provided by DLAM vets as needed. PI or research staff will monitor the animals at least once a day during and following stress to monitor for signs of excessive pain. All mice that reveal 20% weight loss, (mice will be weigh only outside the (b)(4) chamber and it will be done once a week or more often if recommended by Veterinarian staff) ruffled fur, lethargy, loss of righting reflex will be removed from the study and euthanasia request will be sent to LAM staff. In the event of any adverse reaction during the protocol procedures PI and/or his designee will be notified and they will consult with the Veterinarian on duty to take the most appropriate action.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

Protocol activity or procedure	Name of person performing activity	Qualifications of person performing activity	Specific training in this activity or procedure
Intraperitoneal injections	(b)(6)	18 yrs experience	1, 2, 4
Intraperitoneal injections		8 yrs experience	1, 3, 5
Intraperitoneal injections		0 yrs experience (will be supervised by PI)	1, 3
Intraperitoneal injections		0 yrs experience (will be supervised by PI)	1, 3
Intraperitoneal injections		0 yrs experience (will be supervised by PI)	1, 3
Intraperitoneal injections		0 yrs experience (will be	1, 3

	technician	supervised by PI)	
gas anesthesia administration	(b)(6)	18 yrs experience	1, 2, 4
gas anesthesia administration		7 yrs experience	1, 3, 5
gas anesthesia administration		3 yrs experience (will be supervised by PI)	1, 3
gas anesthesia administration		3 yrs experience (will be supervised by PI)	1, 3
gas anesthesia administration		2 yrs experience (will be supervised by PI)	1, 3
gas anesthesia administration		3 yrs experience (will be supervised by PI)	1, 3
Cervical dislocation		18 yrs experience	1, 2, 4
Cervical dislocation		8 yrs experience	1, 3, 5
Cervical dislocation		0 yrs experience (will be supervised by PI)	1, 3
Cervical dislocation		0 yrs experience (will be supervised by PI)	1, 3
Cervical dislocation		0 yrs experience (will be supervised by PI)	1, 3
Decapitation		18 yrs experience	1, 2, 4
Decapitation		8 yrs experience	1, 3, 5
Decapitation		0 yrs experience (will be supervised by PI)	1, 3
Decapitation		0 yrs experience (will be supervised by PI)	1, 3
Decapitation		0 yrs experience (will be supervised by PI)	1, 3
Decapitation		0 yrs experience (will be supervised by PI)	1, 3
Acoustic startle		3 yrs experience	1, 2, 4
Acoustic startle		3 yrs experience	1, 3
Acoustic startle		0 yrs experience (will be supervised by PI)	1, 3, 5
Acoustic startle		3 yrs experience	1, 3
Acoustic startle		0 yrs experience (will be supervised by PI)	1, 3
Acoustic startle		0 yrs experience (will be supervised by PI)	1, 3
Stress procedure		3 years experience	1, 2, 4
Stress procedure		0 yrs experience (will be supervised by PI)	1, 3, 5

Stress procedure	(b)(6)	3 yrs experience	1, 3
Stress procedure		0 yrs experience (will be supervised by PI)	1, 3
Stress procedure		0 yrs experience (will be supervised by PI)	1, 3

1) Training from USU investigator training course

2) Training from NIH staff during previous employment at NIH and use of rodents on approved protocols

3) Training from PI/Lab staff

4) Training from postdoctoral experience in Italy (Dr. (b)(6)

5) Training from veterinary staff at Cornell University and University of Maryland School of Medicine

(b)(6)

VII. BIOHAZARDS/SAFETY: Isoflurane/oxygen used for anesthesia will be used under fume/chemical hood to prevent exposure of personnel to the vapors. When handling animals all personnel will wear personal protective equipment including lab coats, face masks and gloves to minimize exposure to rodent allergens. Paraformaldehyde, used in perfusions, will be weighed out and used in a fume/chemical hood, with personnel wearing gloves, lab coats and face masks to protect from noxious fumes. All perfusions will also with similar precautions to minimize exposure to vapors.

VIII. ENCLOSURES:

References:

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IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User

(b)(6) _____ 07.09.2012
Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____
_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 07.09.2012

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedu(b)(6) lish the objectives of this proposed experiment.

[redacted]

07.09.2012
Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: Epigenetic biomarkers of post-traumatic stress disorder in mouse model in constant pressure environment

C. Principal Investigator: (b)(6) PhD

D. Performing Organization: Department of Anatomy, Physiology and Genetics

E. Funding: AFMSA, DoD

F. Objective and Approach: In the proposed project, we will determine a genome wide list of targets affected by exposure to (b)(4) conditions alone and in combination with stress. We will perform bioinformatics functional pathway analysis to unravel the signaling pathways involved and propose future treatment targets for PTSD. We will test the impact of differential time course of (b)(4) exposure on mouse behavioral phenotypes as well as hematological, pulmonary and central nervous system pathologies. We plan to determine genome-wide epigenetic profile in mouse brain, blood and lung tissues exposed to (b)(4) (b)(4) pressure/oxygen conditions. We will identify genome-wide epigenetic profile in brain, blood and lung tissues of stressed mouse exposed to (b)(4) pressure/oxygen conditions.

G. Indexing Terms (Descriptors): Mouse, Stress, Hippocampus, Amygdala, ChIPSeq, RNASeq, mi-RNA-Seq, Methylation, PTSD, Signaling Pathways, (b)(4) (b)(4)

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

June 19, 2013

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY AND GENETICS

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Full Committee Review on June 19, 2013:

Animal Protocol Title: "Targeting the 2-AG Hydrolyzing Enzymes MAGL and ABHD6 for the Treatment of EAE (rat and mouse)"

USUHS Protocol Number: (b)(6)

Expiration Date: June 18, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6). This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Vice-Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

Revised

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE:
Targeting the 2-AG Hydrolyzing Enzymes MAGL and ABHD6 for the Treatment of EAE (rat and mouse)

GRANT TITLE (if different from above):
Role of $iPLA_2$ in Oligodendrocyte Toxicity Induced by Reactive Microglia

USUHS PROJECT NUMBER: USUHS Grant (b)(6) DMRDP Research Grant (b)(6)

FUNDING AGENCY:
The Defense Medical Research and Development Program (DMRDP), USUHS

EARLIEST ANTICIPATED FUNDING START DATE: Current

PRINCIPAL INVESTIGATOR:

(b)(6)
P

APG | (b)(6) | 05/24/13
Department | Office/Lab Telephone | Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review (b)(6) od scientific research practice.

Professor | (b)(6) | 05.28.2013
Research Unit Chief / Dept. Head Signature | Title | Telephone | Date
Typed Name: (b)(6) MD, Ph.D

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)
Stat

APG | (b)(6) | 05/24/13
Department | Telephone | Date
Typed Name:

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if

(b)(6)

LAM | (b)(6) | 05/28/13
Department | Telephone | Date
Attending/Consulting Veterinarian Signature
Typed Name (b)(6) VMD

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR:
(b)(6) Anatomy, Physiology, and Genetics, (b)(6)

ANIMAL PROTOCOL TITLE:
Targeting the 2-AG Hydrolyzing Enzymes MAGL and ABHD6 for the Treatment of EAE (rat and mouse)

GRANT TITLE (if different from above):
Role of iPLA2 in Oligodendrocyte Toxicity Induced by Reactive Microglia

USUHS PROJECT NUMBER: USUHS Research Grant (b)(6) DMRDP Research Grant, (b)(6)

CO-INVESTIGATOR(S):
(b)(6)

TECHNICIANS(S):

I. NON-TECHNICAL SYNOPSIS:

Enhancement of endocannabinoid signaling has recently emerged as an alternative and attractive strategy for the treatment of multiple sclerosis (MS) and other neurological diseases. It is generally believed that boosting the endogenous levels of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) can activate cannabinoid receptors in a site-and event- specific manner without causing psychotropic side effects. Although 2-AG is the dominant endocannabinoid in the brain and spinal cord, and has been shown to regulate cell viability, differentiation, synaptic plasticity and immune function, it is still unclear how modulating its biosynthetic and hydrolytic enzymes can affect the pathogenesis of MS. We hypothesize that there is an altered expression of 2-AG signaling components in experimental autoimmune encephalomyelitis (EAE) and that boosting the endogenous levels of 2-AG by targeting its hydrolyzing enzymes, monoacylglycerol lipase (MAGL) and alpha/beta hydrolase domain 6 (ABHD6), can ameliorate EAE by both shared and independent mechanisms. We aim to use primary cultures of olidendrocytes derived from mouse or rat brain to elucidate the role of 2-AG in the development and treatment of EAE with pharmacological and genetic tools.

II. BACKGROUND:

II.1. Background:

MS is one of the most common neurological diseases in young adults. It affects 250,000-350,000 people in the United States and 2-3 million people worldwide (1, 2). The pathological hallmarks of MS are multifocal inflammation, demyelination, gliosis, oligodendrocyte death and axonal injury. Accumulating evidence indicates that neurodegeneration occurs not only as a late consequence of axon demyelination in MS, but also as a very early event (3, 4). Therefore, agents targeting both inflammation and neurodegeneration at the early stage might be desirable for the treatment of MS.

A growing body of evidence suggests that cannabinoids, either exogenous (plant-derived and synthetic) or endogenous, are efficacious for the treatment of several animal models of MS (5). The regulatory action of cannabinoids in EAE-induced neurodegeneration is largely due to cannabinoid type 2 (CB2) receptor-mediated modulation of the inflammatory response and CB1 receptor-mediated neuroprotection (6, 7). However, the clinical application of synthetic and plant-derived cannabinoid agents in MS is still limited by the potential psychoactive effects caused by CB1 receptor activation. Although CB2 receptor activation is supposed to avoid the psychotropic effects, it is difficult to separate the action of CB1 and CB2 receptors exerted by exogenous cannabinoids because of the limited selectivity of the compounds and the interaction between CB1 and CB2 receptors (8, 9). Therefore, boosting the endogenous levels of cannabinoids is now becoming a novel and promising strategy to halt the progression of neurological diseases.

In this animal protocol, we will use the primary cultures of microglia and oligodendrocytes from mouse or rat brain and the mouse EAE model of human MS to elucidate the significance of different pools of 2-AG in the development and treatment of MS through selectively targeting the 2-AG hydrolytic enzymes, MAGL and ABHD6.

Refererences:

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2. Kurtzke JF (1993) Epidemiologic evidence for multiple sclerosis as an infection. Clin Microbiol Rev 6(4):382-427.
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5. Kubajewska I & Constantinescu CS (2010) Cannabinoids and experimental models of multiple sclerosis. Immunobiology 215(8):647-657.
6. Maresz K, et al. (2007) Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB1 on neurons and CB2 on autoreactive T cells. Nat Med 13(4):492-497.
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9. Callen L, et al. (2012) Cannabinoid receptors CB1 and CB2 form functional heteromers in brain. J Biol Chem 287(25):20851-20865.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:
BRD; RePORT; PubMed.

II.2.2. Date of Search:
04/19/2013

II.2.3. Period of Search:
1983 – 04/19/2013

II.2.4. Key Words and Search Strategy:

Experimental autoimmune encephalomyelitis (EAE), anandamide (AEA), 2-arachidonoylglycerol (2-AG), monoacylglycerol lipase (MAGL), α/β hydrolase domain 6 (ABHD6), cannabinoids, mice, rats.

BRD: experimental autoimmune encephalomyelitis (EAE): (0).
RePORT: EAE (243)
PubMed:
EAE (9384)
monoacylglycerol lipase (626)
ABHD6 (20)
2-arachidonoylglycerol + EAE (5)
monoacylglycerol lipase + EAE (0)
α/β hydrolase domain 6 + EAE (0)
cannabinoids + EAE (51)

II.2.5. Results of Search:

There is a growing body of evidence that cannabinoid compounds are effective in the animal models of MS, and sativex, a cannabis-based medicine composed of delta-9-tetrahydrocannabinoid and cannabidiol, has been used to treat pain and plasticity in Canada and some European countries for MS patients. However, the use of sativex in MS patients has not yet been approved in US a major concern is that this drug may still cause psychotropic effect via activation of CB1 receptor, although it has been clearly demonstrated to reduce pain and spasticity in MS patients. Although the use of CB2 receptor selective agonists can avoid the psychotropic effect and is also shown to be effective in the animal models of MS, there is a general consensus that activation of CB2 receptor mainly suppresses the inflammatory and autoimmune response, whereas activation of CB1 receptor provides neuroprotective effects. Therefore, targeting both CB1 and CB2 receptors might be necessary for the maximum therapeutic effects of cannabinoids. It is known that endocannabinoids are synthesized on demand and enhancement of their endogenous levels at the site of injury can exert protective effects and prevent activation of CB1 receptor non-discriminately. However, this theory has not been tested in MS studies because of the lack of selective pharmacological and genetic tools. Several selective and highly potent inhibitors of 2-AG hydrolysis have been developed in recent years, but so far no studies have been reported regarding their efficacy in the animal models of MS. In this protocol, we will test the role of the selective MAGL inhibitor JZL184 and the ABHD6 inhibitor WWL70 in the EAE mouse model.

III. OBJECTIVE\HYPOTHESIS:

Objective: To examine the expression pattern of 2-AG signaling components and determine the therapeutic efficacy of inhibition of 2-AG hydrolyzing enzymes MAGL and ABHD6 in EAE.

Hypothesis: Boosting the endogenous levels of 2-AG by targeting its hydrolyzing enzymes, MAGL and ABHD6, can ameliorate EAE.

IV. MILITARY RELEVANCE:

Multiple sclerosis is a chronic demyelinating disease of the central nervous system. The cause remains unknown but probably involves a combination of genetic and environmental

factors. The average age of onset is between 20 and 40 years of age. Currently there are about 350,000 people in the United States suffering from MS. MS is also relative frequent in the military population. It has been reported that the rate of multiple sclerosis is rising among Gulf War Veterans. Currently more than 25,000 veterans from all wars have been diagnosed with MS, which is now recognized as one of the service connected chronic diseases in the United States.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

The long-term objective of the proposed research is to elucidate the role of 2-AG in the development and treatment of EAE. Our central hypothesis is that boosting the endogenous levels of 2-AG by targeting its hydrolyzing enzymes, MAGL and ABHD6, can ameliorate EAE.

To test our hypothesis, we will test 1) the therapeutic efficacy of inhibition of MAGL and ABHD6 in EAE; and 2) whether mice with targeted gene deletion of DAGLα are vulnerable, whereas mice with either MAGL and ABHD6 knockout or knockdown are resistant to EAE.

Several of the mouse strains used in this protocol will be bred under a separate breeding protocol because the models are not commercially available or the animals are not available at the age, size, quantity, etc., required for this research project. Founder animals will be obtained from the sources listed in the breeding protocol.

Our detailed experiments are listed as follows:

(b)(4)


(b)(4)

V.1.3. Experiment 3: Test the therapeutic efficacy of inhibition of MAGL and ABHD6 in EAE mice.

JZL184 has been shown to selectively inhibit the activity of MAGL and increase the endogenous levels of 2-AG in a dose-dependent manner. Acute inhibition of MAGL by JZL184 at 40 mg/kg elicits a 10-fold increase of brain levels of 2-AG and produces cannabimimatic effect. Chronic treatment with JZL184 at 40 mg/kg for 6 consecutive days sustains the brain levels of 2-AG but causes CB1 receptor desensitization and behavioral tolerance. However, treatment with JZL184 at 8 mg/kg for 6 consecutive days remains its anesthetic and anxiolytic properties, suggesting at optimal concentration, the inhibitor of MAGL might be more therapeutically beneficial. Consistently, our studies have suggested that chronic inhibition of ABHD6, which is known to cause a moderate increase of 2-AG in the CNS, is effective in a mouse model of traumatic brain injury. To test and compare the therapeutic effectiveness of the inhibition of MAGL and ABHD6, female 7- to 9-week-old C57BL/6 mice will be used for EAE induction and then treated with JZL184 and WWL70. JZL184 at 2, 4 and 8 mg/kg and WWL70 at 2, 4 and 8 mg/kg will be given intraperitoneally starting at the immunization (day 3) or at the disease onset (usually occurs around day 10) after EAE induction, and then once a day for 28

days. All the drugs will be dissolved in 200 µl 1% DMSO and given intraperitoneally. Our preliminary studies showed that treatment with WWL70 at 10 mg/kg or JZL184 at 10 mg/kg daily (i.p.) for 3 weeks reduced neurological signs of EAE and did not show any toxic effect by the drugs *per se*. Therefore, we anticipate at the dose range chosen for this study, no drug toxicity should be observed in the treated animals.

At 14 and 28 days after EAE induction, animals will be euthanized and the spinal cord tissues and coronal sections will be used for western blot and immunohistochemistry (IHC), respectively. To determine the role of JZL184 and WWL70 on inflammatory response, T cell infiltration and microglia/macrophage accumulation in the spinal cord white matter will be examined using IHC. The expression of iNOS, components of NADPH oxidase (gp91phox and p67phox), COX-1/2, PGES-1 and the EP1-4 receptors in spinal cord will be assessed by western blot and IHC. The expression of GLT-1, a glutamate transporter which maintains the glutamate in the synaptic cleft below the toxic levels, will be also examined by western blot. The role of JZL184 and WWL70 on EAE-induced demyelination and axonal injury will be examined by analyzing the immunoreactivity of MBP, NF-200 and SMI32. Oligodendrocyte progenitor cells (OPC) and mature OLs will be examined using antibodies against NG2 and CC1, respectively.

This study will require 7 animals per group to have 80% power to detect a difference of 2 points on a 0-5 point scale of clinical signs, using a t-test with a 5%, 2-sided significance level. We will use 10 mice/group because we expect 7 out of 10 to develop EAE. Therefore, the total number of animals used in this experiment (10 animals/group x 2 time points for treatment/group x 8 groups =160) is listed below:

Treatment	Number of animals
Control	20
EAE/vehicle	20
EAE/JZL184 (2 mg/kg)	20
EAE/JZL184 (4 mg/kg)	20
EAE/JZL184 (8 mg/kg)	20
EAE/WWL70 (2 mg/kg)	20
EAE/WWL70 (4 mg/kg)	20
EAE/WWL70 (8 mg/kg)	20

V.1.4. Experiment 4: Test whether inhibition of the 2-AG hydrolytic enzymes affect the 2-AG signaling components with/without EAE induction.

To determine whether treatment with the MAGL and ABHD6 inhibitors can affect the expression and function of the 2-AG signaling components, EAE mice will be given JZL184 (8 mg/kg, i.p.) and WWL70 (8 mg/kg, i.p.) starting at day 3 post-immunization and then once daily until day 28. The same concentration of drugs will be also given to control animals without EAE induction. Animals will be euthanized at 14 and 28 days and the brain and spinal cords are immediately dissected, immerged in liquid nitrogen and kept in -80°C freezer. The expression of 2-AG synthetic and hydrolytic enzymes DAGLα, DAGLβ, MAGL and ABHD6 and the expression of CB1 and CB2 receptors in the spinal cord will be assessed by real time PCR and western blot. Cannabinoid receptor density and function will be also examined by measuring the radioactive activity of [^{3}H]-CP55940 binding and CP55940 stimulated ^{35}S-GTPγS binding,

respectively. The levels of 2-AG, arachidonic acid (AA) and prostaglandins (PGs) in lipid extracts from spinal cord will be analyzed by reverse phase HPLC coupled with tandem mass spectrometry (LC/MS/MS). The total number of animals (10 animals/group x 2 end time points x 6 groups = 120) is as follows:

Treatment	Number of animals
Vehicle	20
JZL184 (8 mg/kg)	20
WWL70 (8 mg/kg)	20
EAE/vehicle	20
EAE/JZL184 (8 mg/kg)	20
EAE/WWL70 (8 mg/kg)	20

V.1.5. Experiment 5: Determine the cannabinoid receptor dependency of the MAGL and ABHD6 inhibitors in the treatment of EAE.

To determine whether the therapeutic effects of the MAGL and ABHD6 inhibitors are mediated by activation of CB1 and CB2 receptors, we will perform another set of study to treat animals with JZL or WWL70 together with CB1 receptor antagonist AM281 and CB2 receptor antagonist AM630 alone or their combination. For this experiment, the optimum dose of JZL184 and WWL70 obtained from the Experiment 3 will be used. All drugs will be dissolved in 1% DMSO and will be given intraperitoneally at the immunization (day 3) or at the disease onset (usually occurs around day 10) after EAE induction. All the drugs will be dissolved in 200 µl 1% DMSO and given intraperitoneally. The treatment groups and the total number of animals (10 animals/group x 2 time points for treatment x 10 groups =200) are listed as follows:

Treatment	Number of animals
Control	20
EAE/vehicle	20
EAE/JZL184	20
EAE/JZL184 + AM281 (3 mg/kg)	20
EAE/JZL184 + AM630 (3 mg/kg)	20
EAE/JZL184 + AM281 + AM630	20
EAE/WWL70	20
EAE/WWL70 + AM281 (3 mg/kg)	20
EAE/WWL70 + AM630 (3 mg/kg)	20
EAE/WWL70 + AM281 + AM630	20

V.1.6. Experiment 6: Use of ABHD6 siRNA injection in EAE mice.

At 14 and 28 days after EAE induction, the expression of ABHD6, MAGL, CB1 and CB2 receptors in the lumbar spinal cord of both ABHD6 siRNA and RISC-free siRNA administered

(i.v.) mice will be examined using real time PCR, western blot, and IHC. 2 mg/kg siRNA in phosphate-buffered saline (PBS) will be injected into the tail vein as described (Yang et al. Ann Neurol. 2010; 67:498-507). We will use radiolabeled cannabinoid receptor agonist and antagonists to determine whether the expression and function of cannabinoid receptors are altered in siRNA injected mice. At 14 and 28 days after EAE induction, the spinal cord levels of 2-AG, AA and PGs and ABHD6 activity will be also evaluated as described above. The total number of animals (10 animals/group x 2 time points/group x 4 groups = 80) is listed below:

siRNA	Number of animals
ABHD6 siRNA	20
RISC-free siRNA	20
ABHD6 siRNA/EAE	20
RISC-free siRNA/EAE	20

V.1.7. Experiment 7: Use of iPLA2 inhibitor in EAE mice.

Our preliminary results found that bromoenol lactone (BEL), a selective calcium independent PLA2 (iPLA2) inhibitor, significantly attenuated peroxynitrite formation in reactive microglia and microglia induced toxicity to OLs. This result suggests that BEL may ameliorate the disease progression of EAE. Female C57BL/6 wild type mice (8- to 10 week old) will be used for the induction of EAE and then treated either with BEL or vehicle alone (equal volume of DMSO, used to dissolve BEL). Mice will be treated with 0.5 mM or 2 mM BEL (200 µl, i.p.) starting on day 3 after the first 2 days of EAE induction or on day 12 after the disease onset, followed by daily injection of the inhibitors (200 µl at 0.5 mM or 2 mM) until day 28. Behavioral monitoring will continue up to 60 days. The clinical scores will be compared in each group.

The study will require 7 animals per group to have 80% power to detect a difference of 2 points on a 0-5 point scale of clinical signs, using a t-test with a 5%, 2-sided significance level. We will use 10 mice/group because we expect 7 out of 10 to develop EAE. Therefore, the total number of animals used in this experiment (10 animals/group x 2 time points for treatment/group x 6 groups =120) is listed below:

Treatment	Number of animals
Vehicle	20
BEL (0.5 mM)	20
BEL (2 mM)	20
EAE	20
BEL (0.5 mM)/EAE	20
BEL (2 mM)/EAE	20

V.1.8. Experiment 8: Cultures of oligodendrocytes from neonatal rats and mice

Oligodendrocyte cultures from control and transgenic mice (C57BL6/NTac, DAGLα

-/-, 129SvEv/C57BL6J, DAGLβ -/- and MAGL -/-) will be prepared according to methods that have been used routinely in our lab to produce highly enriched stage specific rat oligodendrocyte cultures. Postnatal day 0-3 rats and mice will be anesthetized with hypothermia in an ice water bath and then decapitated. The brains will be removed, cleaned of meninges, dissociated to single cells suspensions, and plated under conditions for growth of oligodendrocyte cultures. For cultures of premyelinating OLs (preOLs), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) are added in the culture medium. For the cultures of mature OLs, the growth factors are switched to T3 and CNTF after 7 days in the culture medium for preOLs.

For cell cultures using transgenic mice, we estimate that we may require approximately 8 pups per primary culture. We expect to use approximately one primary culture every other month, or 6 per year, for a total of approximately 20 (which include 10% additional for unexpected problems or contamination within the culture, i.e.18 + 2 = 20) over the 3 years of our study. 20 primary cultures per strain x 5 strains x 8 pups per primary culture = 800 pups over the 3 years will be needed for culture preparation. For cell cultures using rats, we estimate that we may require 10 rat pups (and one mother rat) per primary culture. We expect to use one primary culture every other week (26 primary cultures per year x 3 years x 10 pups per culture AND 1 dam per culture = 780 pups and 78 dams = 858 rats) for testing the response of different stages of oligodendrocytes to oxidative stress. The total number of animals required for culture experiments is listed as follows:

Animals	**Numbers over 3 years**	**Age**
Rat	858	Pups (780); mother (78)
Mouse	800	Pups
Mouse	980	Adults

V.2. Data Analysis:

Statistical methods and other calculations used to determine the group's sizes and total numbers of animals are listed under the individual experiments. Prism statistical software and graphing, from GraphPad, will be used for data analysis. Student t-tests will be used to compare the cell density measures. The chi square test will be used to compare the expression differences of proteins interested. ANOVA with post hoc analysis will be used to monitor the neurological deficits across the multiple disease time points.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

We will use the primary oligodendrocyte cultures derived from rat or mouse brain to study the signaling mechanisms of oxidative stress induced toxicity. However, because of the complex cell-cell interactions and multiple signaling pathways involved in the real setting of the disease models, an intact animal model system is required. Our in vitro systems lack the complexity required to comprehensively evaluate the disease process and the interrelated effects of various manipulations on the disease course. We will use several transgenic mouse models to study whether targeted signaling molecules in the pathways of oligodendrocyte toxicity found in vitro play critical roles in the pathogenesis of multiple sclerosis.

V.3.2. Animal Model and Species Justification:

Complete Freund's adjuvant (CFA) is used for emulsifying the myelin proteins and induction of EAE. Although several alternative adjuvants (monophoshoryl lipid A/trehalose decorynomycolate, aluminum hydroxide, titermax, ribi, syntax adjuvant formulation) have been used to introduce peptides, antibodies or vaccines in modulating the immunological responses in several model systems, none of these adjuvants has been used in inducing EAE. The injection of myelin peptides emulsified in incomplete Freund's adjuvants can, in fact, render the mice resistant to the induction of EAE (Marusic and Tonegawa, 1997). To study the pathogenic mechanisms and the pharmacological intervention of the disease activity, we will use myelin proteins emulsified in complete Freund's adjuvant to induce EAE, a commonly used and well established model system.

Myelin oligodendrocyte glycoprotein (MOG) is one of the major myelin proteins used to induce EAE in mice, which resembles the human disease multiple sclerosis. The greater incidence in female population and the better immune response observed in female mice suggest that female mice will be ideal in use for the study of mechanisms of the disease. We have chosen the specific transgenic mouse lines to help us gain more direct evidence for testing the involvement of specific molecules in the pathogenesis of the disease. JZL184 has been shown to selectively inhibit the activity of MAGL and increase the endogenous levels of 2-AG in a dose-dependent manner. Acute inhibition of MAGL by JZL184 at 40 mg/kg elicits a 10-fold increase of brain levels of 2-AG and produces cannabimimatic effect. Recent genetic evidence demonstrates that DAGLα and MAGL are the principal enzymes for 2-AG synthesis and degradation, respectively. 2-AG is the dominant endocannabinoid and a key regulator of synaptic communication in the CNS. ABHD6 is a newly identified 2-AG hydrolyzing enzyme and responsible for only 4% 2-AG inactivation in brain homogenate. However, it contributes to 40% of 2-AG hydrolysis in neurons and has an additive effect with MAGL. Although ABHD6 is known to be expressed and attributable to the 2-AG hydrolysis in BV2 cells, its expression and function in primary cultures of microglia have not been confirmed. With specific gene knockout mice and siRNA injection into mice, we will get a better understanding of the inhibition of 2-AG hydrolyzing enzymes in the development and treatment of EAE.

Rats will be used to prepare cell cultures since neonatal rats brains result in a much higher cell yield than mice brains. Therefore, we will first use rat cultures to study the signaling molecules involved in the toxicity of oxidative stress to oligodendrocytes and then use mice cultures derived from transgenic mice to see whether knockout of the specific molecules affects the toxicity. We have chosen Sprague-Dawley rats because they are widely used for oligodendrocyte and other nerve tissue cultures. We have used SD rats previously in similar work and need to be able to relate results across studies.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	Rattus norvegicus	Mus musculus

V.3.3.2. Strain/Stock:	Sprague-Dawley	C57BL/6J DAGLα$^{-/-}$ DAGLβ$^{-/-}$ MAGL$^{-/-}$
V.3.3.3. Source/Vendor:	(b)(4) or other approved vendor	C57BL/6J (b)(4) (b)(4) DAGLα$^{-/-}$, DAGLβ-/- MAGL$^{-/-}$ In-house breeding
V.3.3.4. Age:	0-3 d pups + mother	Cultures: 0-3 d pups EAE mice: 8-10 wks
V.3.3.5. Weight:	As for age	As for age
V.3.3.6. Sex:	Mixed in litters	Cultures: mixed litters EAE mice: female
V.3.3.7. Special Considerations:	Dam should arrive with 10 pups, 0-3 days old, may or may not be litter mates. Alternately, gestational day 17-18 timed-pregnant rats may be ordered.	Cultures: Dam should arrive with 8 pups, 0-3 days old, may or may not be litter mates. Alternately, gestational day 17-18 timed-pregnant mice may be ordered. EAE: General facility and specific pathogen-free barrier
V.3.4. Number of animals Required (by species)	Pups (780) + Mother (78)	Pups (800) +adult mice for EAE study (980)

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:

EAE will be induced with MOG peptides emulsified with CFA. Any animals that develop open lesions or infection secondary to any of the injection methods will be removed from the research groups and be euthanized at an early endpoint. Additionally, animals that are observed to reach the stage 4 behavioral criteria will be euthanized at an early endpoint. No other refinement alternatives are considered.

V.3.5.2. Reduction:

The dose dependent studies of several inhibitors in the lipid metabolic pathways in culture will suggest to us the doses suitable for the animal study. Therefore, we can reduce the groups of animals used for testing the efficacy of the drugs. However, considering the reports that chronic inhibition of MAGL can cause receptor desensitization and downregulation and the differences in vitro and in vivo, a set of dose-dependent study is also proposed for better understanding the mechanisms of drugs' action. We will prepare tissues in a way that allows us to use a given tissue for multiple assays rather than euthanizing a separate animal for each method. No other reduction alternatives are considered.

V.3.5.3. Replacement:

Biological response to multiple sclerosis is highly complex. The in vitro culture system lacks the complexity required to comprehensively evaluate the disease process and the interrelated effects of various manipulations on the disease course. Computer models can also not be utilized as basic information and knowledge is lacking to construct computer simulations. Therefore, the mouse model is a necessary smaller animal model we could use for this study and still obtain acceptable results. No alternative to in vivo experimentation is available.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	780	800
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:		980

Column C includes the rat and mouse pups for cell culture. **Column E** includes the 980 adult mice undergoing CFA injection and potential paralysis during the EAE induction. The USDA Animal Care Policy #11 describes paralysis as a condition that may cause distress in the animals. This policy also describes the use of CFA as a procedure that may cause pain in the animals, although that pain may range from only slight or momentary to more significant pain. *Although pain was thought to be rare and minor symptoms in MS patients in the past, recent investigations indicate pain is common in MS patients, which is possibly caused or triggered by inflammation, demyelination and axonal injury (Pollmann W and Feneberg W. CNS Drugs. 2008; 22:291-324; O'Connor AB et al. Pain. 2008; 137:96-111).* We will observe the animals and report any differences in pain category during our annual reports.

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquillization:

For culture preparation derived from neonatal mouse and rat brains, 0-3 day old rats or mice will be anesthetized with hypothermia by cooling in an ice water bath prior to decapitation. The

animals will not be directly in contact with the ice water bath since they will be placed in the finger of a latex glove. Sufficient anesthesia will be determined as lack of movement and lack of response to pinching the toes of the forepaw. Once the pups are sufficiently anesthetized, they will be euthanized by decapitation.

For EAE induction, a 200 µl portion of emulsion containing a 1:1 ratio of MOG/CFA will be administered to each mouse, the use of anesthesia for s.c. immunizations is not required. Although recent studies suggest that EAE mice may also experience pain during the onset or the disease progression (Olechowski et al. Pain. 2009: 141:156-164; Jung et al. J. Neurosci. 2009: 29:8051-8062), the post-procedural administration of an opiate analgesic or a NSAID or related drugs cannot be used to alleviate any possible pain, as most of these agents affect the immune and the inflammatory response and thus the disease severity of EAE. The use of analgesic or NSAID drugs will make it difficult to elucidate the pathogenic mechanisms of EAE and the therapeutic efficacy of several compounds (such as cannabinoid compound) tested.

For transventricular perfusion, mice will be anesthetized once with ketamine (60-90 mg/kg)/xylazine (10-15 mg/kg) by intraperitoneal injection into the caudal ventral abdomen using a 23-28 gauge needle. If 10 mg/ml ketamine and 20 mg/ml xylazine are used for the peritoneal injection and the average mouse weight is 30 grams, the injection volume is (60 mg/kg x 0.03kg /10 mg/ml) + (10 mg/kg x 0.03 mg / 20 mg/ml) = 0.18 ml + 0.015 ml ~ 0.2 ml. Even if the mouse requires a supplemental injection the total IP injection volume will generally not exceed 0.5 ml. Once the animals are deeply anesthetized (as indicated by recumbency, slow, regular breathing, and lack of response to toe pinch) the transventricular perfusion procedures will begin.

Dre (b)(6) **a graduate student, will perform the anesthetic procedures in this protocol.**

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

Although we do not expect any complications as a result of the procedural manipulations, we will monitor the mice in their home cages at least once a day for 2 or 3 days following IP injections. If the mice exhibit any signs of peritonitis, to include lethargy, anorexia, hunched posture, unusual vocalizations, appearance of significant weight loss, unkempt appearance, etc., the animals will be removed from the experimental group and euthanized. Mice will be monitored at least once a day for the development of abscesses, open sores, or other lesions at the site of SQ adjuvant injection. *We have found less than 5% of animals undergoing SQ adjuvant inject may develop open lesions near the inject sites.* If there are open lesions or peritonitis due to the adjuvant injections, the animals will be removed from the research groups and euthanized at an early endpoint. At 10 days following induction of EAE, we will monitor the neurological scores of the mice twice a day, including weekends.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: Pubmed for EAE mice model and transventricular perfusion; Altweb for review of transventricular perfusion method.

V.4.1.3.2. Date of Search: PubMed 04/19/2013; Altweb 04/19/2013

V.4.1.3.3. Period of Search: 1966 - Present

V.4.1.3.4. Key Words of Search:

Complete Freund's adjuvant, paralysis and perfusions, mice.

Complete freund's adjuvant (10214)
Complete freund's adjuvant + EAE (573)
Complete freund's adjuvant + alternative adjuvant (122)
Complete freund's adjuvant + alternative adjuvant + EAE (3)
Complete freund's adjuvant alternatives + EAE (0)

Paralysis + EAE (278)
Paralysis + pain (4421)
Paralysis + pain + EAE (3)
Paralysis + distress (489)
Paralysis + distress + EAE (0)

Complete Freund's adjuvant and those listed below:

	Most Recent Queries	Time	Results
#55	Search **intracardiac perfusion and *formaldehyde**	11:33:51	8
#41	Search **cardiac perfusion and *formaldehyde**	11:32:11	49
#35	Search **cardiac perfusion and euthanasia**	11:16:54	16
#34	Search **intracardiac perfusion and euthanasia**	11:16:36	0
#33	Search **transcardiac perfusion and euthanasia**	11:16:09	0
#22	Search **transcardial perfusion* and mice**	10:48:35	16
#11	Search **cardiac perfusion* and mice**	10:34:40	23
#1	Search **intracardiac perfusion and mice**	10:21:44	23

V.4.1.3.5. Results of Search:

Complete Freund's adjuvant is used for emulsifying the myelin proteins and induction of EAE. Although several alternative adjuvants (monophoshoryl lipid A/trehalose decorynomycolate, aluminum hydroxide, titermax, ribi, syntax adjuvant formulation) have been used to introduce peptides, antibodies or vaccines in modulating the immunological responses in several model systems, none of these adjuvants has been used in inducing EAE. The injection of myelin peptides emulsified in incomplete Freund's adjuvants can, in fact, render the mice resistant to the induction of EAE (Marusic and Tonegawa, 1997). To study the pathogenic mechanisms and the pharmacological intervention of the disease activity, we will use myelin proteins emulsified in complete Freund's adjuvant to induce EAE, a commonly used and well established model system.

Demyelination is one of the hallmarks of EAE, which can lead to paralysis. Although pain or distress can be associated with paralysis, MS patients usually do not experience pain or

distress because of the paralysis. We can speculate that EAE mice will not experience pain, but it's possible that they may become distressed.

Transventricular perfusion, after deep anesthesia, is a method commonly used to prepare the tissues appropriately for later histological and immunological analyses.

Based on the literature search results, cardiac perfusion and formaldehyde fixation are commonly performed procedures in a wide variety of research applications. The relevant results obtained from the above search described use of this procedure for fixation and analysis of a number of tissues (to include spleen, kidney, liver, lung, stomach, small intestine, lymph nodes, heart, brain, bone marrow, eyes, nasal cavities, cochlea, and uterine tissue) with the majority describing brain fixation. Unrelated articles described transcardial routes of compound delivery and use of cardiac perfusion to clear vessels of blood with or without residual compounds. One article described a minimally invasive technique of transthoracic cardiac infusion without thoracotomy. The authors claimed this procedure yielded higher quality tissue preservation and faster tissue harvesting capabilities without the artifacts of brain swelling and ventricular dilation seen with direct cardiac perfusion. While this less invasive method may offer these advantages, the procedure would be potentially painful or distressful, so animals would still be anesthetized. Until the research staff became proficient with the technique, these benefits may not be readily apparent. Additionally, these benefits may not be recognized with tissues outside the central nervous system. With the possible exception of microwave fixation in select applications, there currently is no other less painful alternative to perfusion to ensure adequate distribution of fixative to the tissues of interest. Microwave euthanasia/fixation is not a viable alternative for our study.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

In the past, pain was thought to be a rare and minor symptom in patients with Multiple sclerosis. However, recent investigations suggest pain is in fact quite common in MS patients (Pollmann W and Feneberg W. CNS Drugs. 2008; 22:291-324; O'Connor AB et al. Pain. 2008; 137:96-111). The causes and the pathogenic mechanisms of the pain are still unclear, although inflammation, demyelination and axonal injury may lead to pain. A very recent study using EAE mouse model also suggests that T lymphocytes infiltration and microglia/macrophage activation in spinal cord may be the cause of neuropathic pain (Olechowski et al. Pain. 2009; 141:156-164). In addition, USDA Animal Care Policy #11 considers paralysis in animals to be a distressful condition. In order to thoroughly assess the disease course in these mouse models and determine the role of the molecules of interest in this disease, we must follow the disease progression through various phases, including paralysis. However, it is not possible to administer anti-inflammatory drugs or opiate analgesics to lesson any pain that may be associated with the CFA injections; these compounds are known to affect the immune response (Schneemilch et al. Best Pract Res Clin Anaesthesiol. 2004; 18:493-507; Scholz and Woolf. Nat Neurosci. 2007; 10:1361-1368; Kurosawa and Kato. J Anesth. 2008; 22:263-277) that is an essential component of the model. Morphine and other opioids have immunomodulating effects on nearly all measurable parts of the immune system (macrophages, granulocytes, nk-cells; mediators like Interleukin 1, 2 and 6, TNFα). These will directly interfere with the mechanism involved in the induction of EAE. Although it is possible that Tylenol may be used for reducing pain without affecting the inflammatory response, considering the cannabinoid compounds have been shown to reduce both inflammatory and neuropathic pain, we opt to not using Tylenol in our experiments, in order to better understand the therapeutic potential of targeting the endocannabinoid system for EAE.
The mice will be euthanized once they reach stage 4 of the behavioral criteria.

V.4.4.2. Biosamples:

Following euthanasia by transventricular perfusion, the brain and lumbar spinal cord will be removed from EAE mice. These tissues will be fixed and cut using a cryostat into sections for further analysis as described in Experiment #2.

A postmortem tail biopsy immediately after the brain is removed from postnatal day 0-3 mice will be used to isolate genomic DNA and genotype each mouse by PCR analysis. The terminal 0.5 -1 cm of the tail will be quickly cut with a pair of sterile, sharp surgical scissors. DNA will be extracted from the tail samples for PCR analysis.

V.4.4.3. Adjuvants:

200 μg of MOG in 0.1 ml PBS will be emulsified with 0.1 ml complete Freund's adjuvant (Incomplete Freund's adjuvant containing 0.5-1 mg/ml of heat-inactivated Mycobacterium tuberculosis) will be administered at day 0 (total 0.2 ml injection volume) SQ divided into two sites between the shoulder blades and/or along the back. At day 7, the incomplete Freund's adjuvant will be similarly administered to enhance the immunization of MOG. The use of CFA will be performed accordance with the IACUC policy #2. Research personnel will observe the animals at least once a day following adjuvant injection to monitor for abscesses, open lesions, etc. If open lesions are noted, they will be treated with topical disinfectants and/or antibiotics. Any mice with lesions that are non-responsive to treatment will be removed from the research groups and euthanized at an early endpoint.

As part of EAE induction, an IP injection of 200 ng of pertussis toxin is administered on days 0 and 1 following MOG/adjuvant injection to further enhance the immunogenicity of the MOG. Monitoring after IP injections is as described earlier.

Complete Freund's adjuvant is used for emulsifying the myelin proteins and induction of EAE. Although several alternative adjuvants (monophoshoryl lipid A/trehalose decorynomycolate, aluminum hydroxide, titermax, ribi, syntax adjuvant formulation) have been used to introduce peptides, antibodies or vaccines in modulating the immunological responses in several model systems, none of these adjuvants has been used in inducing EAE. The injection of myelin peptides emulsified in incomplete Freund's adjuvants can, in fact, render the mice resistant to the induction of EAE (Marusic and Tonegawa, 1997). To study the pathogenic mechanisms and the pharmacological intervention of the disease activity, I will use myelin proteins emulsified in complete Freund's adjuvant to induce EAE, a commonly used and well established model system.

V.4.4.4. Monoclonal Antibody (MAbs) Production: None

V.4.4.5. Animal Identification:

Individual mouse will each be given an ear tag with an identification number. Mice will be assigned to several experimental groups depending on the design and recorded based on their identification numbers on ear tags. Each cage will be given a cage card that is numbered by LAM staff. Cage card numbers will be recorded.

V.4.4.6. Behavioral Studies:

Animals will be monitored at least twice a day blind by two independent observers from day 10 following MOG injection and neurological signs will be assessed as follows: 0, normal mouse; 1, piloerection, tale weakness; 2, tail paralysis; 3, tail paralysis plus hindlimb weakness/paralysis; 4, tail, hind and fore limb paralysis; 5, moribund/dead. We expect the disease course to progress over one to two months, so animal behavioral monitoring will continue for up to 60 days after induction of EAE.

V.4.4.7. Other Procedures:

Transventricular perfusions: Weights of mice in EAE experimental groups will be taken to determine appropriate dose of anesthesia prior to transventricular perfusion. For perfusion, the adult mice will be deeply sedated and lack the response to toe pinch. The skin of the chest will be incised with sharp scissors to allow the rib cage to be raised. The heart will be visualized. Perfusate will be passed through the vasculature by inserting a blunted 20g canula through a nick made in the left ventricle and positioning the cannula opening in alignment with the ascending aorta. The right atrium will be nicked to allow exit of blood and perfusate.

V.4.4.8. Tissue Sharing:

We will offer any animal not needed on this study and excess tissues to other investigators.

V.4.5. Study Endpoint:

The endpoint for adult mice on this study is survival until various time points after induction of EAE and treatment with inhibitors/placebo, after which the mice will be euthanized for tissue collection and evaluation. The endpoint for mouse and rat pups on this study is survival for 0-3 days after birth, at which time they will be euthanized and tissues collected for cell culture. The study endpoint for mother rats is survival until pups are removed, after which they will be offered to other investigators or euthanized. If there is inflammation or peritonitis due to the IP injections or non-healing open lesions from the adjuvant injections, the animals will be euthanized at an early endpoint. Any animal observed to be at stage 4 for behavioral monitoring criteria (tail, forelimb and hindlimb paralysis) will be euthanized at an early endpoint.

V.4.6. Euthanasia:

Following deep anesthesia, EAE animals will be euthanized by exsanguinations as a result of the perfusion procedures as described above. These procedures will be performed by Drs. (b)(6) a graduate student.

Carbon dioxide will be used to euthanize the mother rats after the pups are taken away for the cell cultures. Any animal that must be removed from study because of illness or complication from the study will also be euthanized with carbon dioxide. Animals will be euthanized by Center for LAM personnel or research personnel using cylinderized CO2 in compliance with the most current report of the AVMA guidelines on Euthanasia. Euthanasia will be performed in the LAM CAF. If possible, animals will be euthanized in their home cage. If not, a clean rodent cage will be used. Animals will be placed in the cage so that it is not overcrowded (each animal can place all four feet on the bottom of the cage.) With animals in the chamber, the flow rate of CO2 will displace approximately 20% of the chamber volume per minute. Gas flow will be maintained for at least one minute after respiration has stopped before

removing animals from the cage. A supplemental method to ensure death (cervical dislocation or bilateral thoracotomy) may be used.

For culture preparation, 0-3 day old rats or mice will be anesthetized by cooling in an ice water bath and then euthanized by decapitation with sharp scissors. These scissors will be tested for sharpness by cutting a piece of gauze as recommended by Roboz Surgical Instrument Company, Inc. The scissors will be sharpened or replaced as necessary, and a log book will be maintained to describe maintenance or replacement. These procedures will be performed by Drs. (b)(6)

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6) and/or a procedure room as assigned by LAM.

V.5.1.2. Special Husbandry Provisions: When the mice have paralysis and cannot reach the cage top sources after induction of EAE, wet rodent chow and transgel will be provided on cage floor as a source of food and fluids.

Food Restriction:	Yes	_____	No	X
Fluid Restriction:	Yes	_____	No	X

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:

Research personnel will observe the animals at least once a day for the first 2-3 days following IP and IV injections, and daily following SQ injections of adjuvant. Research personnel will also observe animals twice daily, beginning 10 days post-EAE induction, to assess behavioral criteria as described earlier. Animals will be observed at least once a day for general health and husbandry conditions by LAM personnel. Any animal found to be exhibiting signs of illness not related to the EAE (i.e., phlebitis, peritonitis, etc.) will be examined and removed from the research groups. Moribund animals may be euthanized for welfare reasons at the discretion of the veterinarian.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary

technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Anesthesia	(b)(6)	21 years experience	Trained in Hebrew University,1992, Harvard Medical School (HMS), 1999, and USUHS, 2006
Anesthesia		4 year experience	Trained in NIH, USUHS
Neonatal anesthesia and CO_2 euthanasia		21 years experience	Trained from HMS, 1999, and USUHS LAM staff, 2006.
Neonatal anesthesia and CO_2 euthanasia		8 years experience	Trained in USUHS, 2006, and UMDNJ LAM staff, 2005.
Perfusions		21 years experience	Trained in HMS, 1999, and Hebrew University, 1992
Perfusions		4 year experience	Trained in USUHS, 2009
Perfusion		4 year experience	Trained in NIH, USUHS
EAE model		8 year experience	Trained in HMS, 2005
EAE model		3 years experience	Trained in USUHS, 2010
EAE model		5 year experience	Trained in USUHS, 2007
EAE model		1 year experience	Trained in USUHS

Cell culture (rat/mice pups)	(b)(6)	8 years experience	Trained in USUHS, 2006, and UMDNJ, 2005
Cell culture (rat/mice pups)		14 years experience	Trained in HMS, 1999
Cell culture (rat/mice pups)		3 years experience	Trained in USUHS, 2010
Cell culture (rat/mice pups)		4 years experience	Trained in USUHS,2006, and UMDNJ, 2005
Intraperitoneal injections		13 years experience	Trained in HMS, 1999 and Hebrew University, 1996
Intraperitoneal injections		4 year experience	Trained in NIH, USUHS
Intraperitoneal injections		2 years experience	Trained in USUHS, 2011
Intraperitoneal injections		5 years experience	Trained in USUHS, 2007
Subcutaneous injections		9 years experience	Trained in HMS, 2000
Subcutaneous injections		1 year experience	Trained in USUHS
Subcutaneous injections		3 years experience	Trained in USUHS, 2010
Subcutaneous injections		2 years experience	Trained in USUHS, 2007
Intravenous injections		13 years experience	Trained in HMS, 1999 and Hebrew University, 1996
Intravenous injections		4 year experience	Trained in NIH, USUHS
Intravenous injections		2 years experience	Trained in USUHS, 2011

VII. BIOHAZARDS/SAFETY:

200 μg killed mycobacteria (SQ) and 200ng of pertussis toxin (IP) will be used in the induction of EAE. Care will be taken to reduce the potential for needle stick exposure to mycobacterial organism to prevent sensitization of research personnel.

Exposure to animal allergens is minimized according to the precautions and PPE designations for the room animals in which the mice and rats are housed within the LAM facilities. Lab coat, gloves, and masks will be worn when handling animals both in the CAF and in labs.

For preparation of tissues, animals are perfused with 4% paraformaldehyde as a fixative agent. Perfusions will be conducted in a fume hood and gloves, labcoat, and eye protection will

C. **Statistical Assurance:** I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. **Biohazard\Safety:** I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. **Training:** I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. **Training:** I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 6/19/2013
Date

G. **Training:** The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

H. **Responsibility:** I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 6/19/2013
Date

I. **Painful Procedure(s):**

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

Pr (b)(6) _____ 6/19/2013
Date

PROTOCOL ABSTRACT:

A. Animal Protocol Number: (b)(6)

B. Animal Protocol Title Targeting the 2-AG Hydrolyzing Enzymes MAGL and ABHD6 for the Treatment of EAE (rat and mouse)

C. Principal Investigator: (b)(6) M.D., Ph.D.

D. Performing Organization: Uniformed Services University of the Health Sciences.

E. Funding: The Defense Medical Research and Development Program (DMRDP); USUHS

F. Objective and Approach:

Cannabis-based medicine has a great potential for the treatment of multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system associated with demyelination and neurodegeneration. Through activating both CB1 and CB2 cannabinoid receptors, cannabinoids contribute to anti-inflammation, anti-oxidation and anti-excitotoxity. It is generally believed that boosting the endogenous levels of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) can activate cannabinoid receptors in a site-and event- specific manner without causing psychotropic side effects. However, due to the limited availability of selective pharmacological inhibitors and knockout mice, this theory has not been rigorously tested

2-AG is the dominant endocannabinoid in the brain and spinal cord, and has been shown to regulate cell viability, differentiation, synaptic plasticity and immune function. Monoacylglycerol lipase (MAGL) and α/β hydrolase domain 6 (ABHD6) are 2-AG hydrolyzing enzymes. Our preliminary data have shown that inhibition of those two enzymes significantly reduced the neurological signs of the disease. However, it is still unclear how modulating 2-AG's biosynthetic and hydrolytic enzymes can affect the pathogenesis of MS. In this proposal, we plan to use pharmacological and genetic tools to elucidate the role of 2-AG in the development and treatment of EAE.

Hypothesis: Expression of 2-AG signaling components in EAE change and boosting the endogenous levels of 2-AG by targeting its hydrolyzing enzymes, MAGL and ABHD6, can ameliorate EAE by both shared and independent mechanisms.

Objective: Endocannabinoids play an important role in the pathogenesis of EAE.

Approach: We will use EAE mouse model to study the participation of 2-AG in EAE and the effect of the modulation of 2-AG production on the progression of EAE in the mice. Specifically we will study the role of 2-AG hydrolyzing enzyme inhibitor WWL 70, JZL in the anti-inflammatory protection of EAE mouse model. We will use pharmaceutical and genetic methods to downregulate MAGL and ABHD6 expression to study 2-AG protective role in the EAE.

G. Indexing Terms (Descriptors):

Demyelination, toxicity, MAGL, ABHD6, cannabinoids, 2-AG, AEA, oligodendrocyte, EAE, multiple sclerosis, mice, rat.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
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BETHESDA, MARYLAND 20814-4712
http://www.usuhs.mil

June 18, 2013

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on June 18, 2013:

Animal Protocol Title: "Development of Neuroprotection Strategies for Brain Injury Using Rat Models"

USUHS Protocol Number: (b)(6)

Expiration Date: June 17, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET
IACUC Date Stamp

Revised

PROTOCOL NUMBER: (b)(6) (for triennual review)

PROTOCOL TITLE: Development of Neuroprotection Strategies for Brain Injury using Rat models.

GRANT TITLE (if different from above): 1) N-Acetylaspartate as a Biomarker for TBI in Body Fluids (BAA-MRMC, pending), 2) Combined Metabolic Therapy for the Treatment of Traumatic Brain Injury (BAA-MRMC, pending).

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: BAA-MRMC (pending).

EARLIEST ANTICIPATED FUNDING START DATE: May-June, 2013

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6)
Principal Investigator Signature

APG
Department

(b)(6)
Office/Lab Telephone

4/29/2013
Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)
Research Unit Chief / Dept. Head Signature
Typed Name:

Chief
Title

(b)(6)
Telephone

04/27/2013
Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

Statistician Signature
Typed Name:

Department

~~Telephone~~

~~Date~~

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

LAM (b)(6)

(b)(6) LAM (b)(6) 29 Apr 2013

Attending/Consulting Veterinarian Signature | Department | Telephone | Date

Typed Name: (b)(6) DVM

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

ANIMAL PROTOCOL TITLE: Development of Neuroprotection Strategies for Brain Injury using Rat models.

GRANT TITLE (if different from above): 1)N-Acetylaspartate as a Biomarker for TBI in Body Fluids (BAA-MRMC, pending) and 2) Combined Metabolic Therapy for the Treatment of Traumatic Brain Injury (BAA-MRMC, pending).

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): Drs (b)(6) Ph.D. and (b)(6)
TECHNICIANS(S): Ms. (b)(6) B.S., (b)(6) M.S.

I. **NON-TECHNICAL SYNOPSIS:** Traumatic brain injury (TBI) has been called the "signature injury" of the Iraq and Afghanistan wars. Since Afghanistan operations began in 2001, and Operation Iraqi Freedom in 2003, the Department of Defense reports that the most debilitating wounds suffered by military personnel are traumatic brain injuries. According to the Department of Defense Report, "Report to Congress in Accordance with Section 1634 (b) of the National Defense Authorization Act for Fiscal Year 2008," the Military Health System (MHS) has recorded 43,779 patients who have been diagnosed with a TBI in calendar years 2003 through 2007. Thanks to new helmet materials, up-armored vehicles and other new technologies, many combat personnel are surviving head injuries that in previous wars would have been fatal. However, the aftermath of some of these head injuries lead to chronic and debilitating conditions, including when the injuries are not diagnosed due to a lack of clear protocols and biomarkers for assessing milder forms of head injury. Clearly, development of simple and inexpensive adjunct treatment strategies that improve long term outcomes would be highly advantageous, and could even be given to personnel in a prophylactic manner when they are subjected to milder blast or concussive forces, and before any symptoms appear. In the ongoing studies, we showed that intragastric administration of glyceryltriacetate (GTA) improves levels of N-acetylaspartate (NAA) and ATP, markers of brain energy status, and Rotarod balance performance in the controlled cortical impact model (CCI) of TBI in the rat. We propose to extend these studies by including 1) melatonin, a lipid soluble pineal hormone that has anti-inflammatory and antioxidant properties and 2) ketone body molecules that have been found to be neuroprotective in a number of CNS injury models. Furthermore, we propose to investigate the role of NAA as a biomarker for brain injury in body fluids.

II. BACKGROUND:

II.1. Background: There are two separate components In this protocol, one aimed at developing neuroprotection strategies and methods and the second aimed at

developing NAA as a biomarker for TBI. They are discussed under two separate headings.

A. Neuroprotection Strategies for TBI.

Increased production of free radicals via a number of pathologic cascades is one of the many secondary effects of traumatic brain injury (TBI). After TBI, excitotoxicity caused by increased glutamate release leads to increased intracellular calcium in neurons. Furthermore, the release of inflammatory cytokines and intracellular calcium induce activation of enzymes including phospholipase A2 and cycloxygenase -2, which stimulate the eicosanoid cascade. The calcium influx alters mitochondrial membranes disrupting the electron transport chain, leading to accumulation of electron-reduced oxygen intermediates. Production of free radicals resulting from these complex interactions generates a state of oxidative stress. It appears that this oxidative stress plays a significant role in TBI, raising the possibility that antioxidant therapy using nontoxic antioxidants such as melatonin could be very effective in a clinical setting (1-3).

Melatonin as a Neuroprotectant: Melatonin, a product of tryptophan metabolism, was initially identified as a pineal hormone with roles in the control of circadian rhythms and reproductive physiology mainly in seasonally breeding animals. However, recent studies indicate that melatonin is also synthesized in many organs and tissues of the body in which it might act as a paracrine hormone or a signaling molecule (2,3). The best known actions of melatonin, currently supported by experimental and clinical data, include antioxidant and anti-inflammatory abilities, some of them involving transcriptional regulation of a number of related enzymes. Most of the beneficial effects of melatonin administration seem to depend on its effects on mitochondrial physiology. Melatonin effects on mitochondria include increased membrane fluidity, increased activity of electron transport chain complexes and ATP production, increased mitochondrial membrane potential, reduced oxidative stress and closing of the mitochondrial permeability pore (4)

Melatonin is known to protect against oxidative stress in cells. Antioxidant actions of melatonin are observed at different levels including attenuation of radical formation, which is also referred to as radical avoidance. Although melatonin efficiently interacts with various reactive oxygen and nitrogen species as well as with organic radicals, it also upregulates antioxidant enzymes and down regulates pro-oxidant enzymes (1). Efficacy of melatonin as an antioxidant is significantly higher than that of other antioxidants including vitamins E and C. Besides, the metabolites formed when melatonin functions as a free radical scavenger, namely N1- acetyl, N2- formyl, 5-methoxykynuramine (AFMK) and N1- acetyl, 5-methoxy kynuramine (AMK) also possess significant antioxidant and anti-inflammatory activity (5,6). Melatonin is highly lipid and somewhat water soluble and thus melatonin enters all cells and subcellular compartments with highest concentrations possibly being present in the nuclei and mitochondria.

Melatonin has been administered in both physiological and pharmacological amounts to humans and there is widespread agreement that it is a non-toxic molecule(7). In human volunteers, oral administration of melatonin in doses 1-300 mg or 1g of melatonin daily for 30 days resulted in no observable negative side effects (8). In

another study, a randomized double blind clinical trial in healthy adult male subjects by oral administration of melatonin (10 mg/day for 28 days or a placebo) found no evidence of toxicity (9). Also, giving female rats 200mg melatonin/kg body weight (equivalent to an average weight human consuming 14 g melatonin daily) throughout pregnancy caused no maternal or fetal toxicity (10). Exogenous melatonin administration has been shown to provide neuroprotective effects in preclinical models of traumatic brain injury, reducing histological damage and cerebral edema as well as improving neurobehavioral outcomes in mice and rats (11-13) . Also, endogenous melatonin increases in cerebrospinal fluid of patients after severe traumatic brain injury and this increase correlates with oxidative stress and metabolic impairment. Taken together, melatonin appears to be a highly promising candidate to be tested as a neuroprotectant in the proposed studies.

Ketogenic Diet for Neuroprotection: Recent studies have shown that ketogenic diet (KD) and ketone bodies are neuroprotective in a variety of CNS injury models (14). The list includes 1) glutamate toxicity *in vitro* and *in vivo*, 2) kainic acid toxicity in mice, 3) hypoxia/anoxia in mice, 4) global ischemia in rat, 5) focal cerebral ischemia in rat, 6) traumatic brain injury in rat, 7) Alzheimer's mouse model, 8) Parkinson's mouse model, 9) Amyotrophic lateral sclerosis mouse model and 10) brain tumor-astrocytoma in mice. The neuroprotective effect of KD appears to be due to the unique properties of ketone metabolism which make it a more suitable cerebral fuel under various neuropathological conditions. Properties of ketone metabolism that might contribute to neuroprotection are outlined below. First, only three enzymes are involved in the sequential metabolism of the ketone body beta-hydroxybutyrate (β-OHB) to acetyl CoA compared to the 12 enzymatic steps involved in the case of glucose. Second, ketone bodies are more efficient in the mitochondrial energy production via multiple mechanisms. Third, ketone bodies decrease production of free radicals by decreasing the reduced form of coenzyme Q, which decreases its reaction with O_2 to form superoxide $O_2^{\cdot}$. Other mechanisms such as glutathione reduction, which aids in the destruction of H_2O_2, might also play important roles under these conditions. The mechanisms given above relate closely to the increased energy hypothesis (metabolic hypothesis) proposed to explain the antiepileptic actions of KD.

Side Effects of Ketogenic Diets and Ketone Body Esters as Potential Solutions: Adverse side effects have been noted with chronic use of KD in animal studies as well as in human populations. A majority of these findings are derived from pediatric studies due to the greater use of KD to control childhood epilepsy (14). Early problems include dehydration, nausea/vomiting, diarrhea, constipation and hypoglycemia. Later problems include growth retardation due to insufficient protein levels, hepatic failure, vitamin/mineral deficiencies, immune dysfunction, renal stones, hypercholesterolemia and cardiomyopathy. In animal studies, it was found that rats that were fed KD for one month had significantly impaired visual-spatial learning compared with rats that were fed normal diet (15). While KD had minimal effect on activity level and emotionality, brain growth was impaired significantly under strict KD conditions.

Based on studies done in animals with defects in fatty acid oxidation it seems likely that long chain fatty acids and their derivatives play critical roles in multiple aspects of the side effects mentioned above including cardiomyopathy. One way to overcome these long chain fatty acid mediated side effects is to use the ketone bodies

β-OHB and acetoacetate directly, in place of KD. However, infusion of sodium salts of ketone bodies would result in a dangerous sodium overload. This can be overcome by administering ketone bodies in their esterified form. These esters are pH-neutral, sodium-free and easily diffusible precursors of ketone bodies. This approach has the added advantage that their uptake into the brain will be limited only by their concentration in the blood, and not by specific uptake mechanisms (blood brain barrier) due to the hydrophobic nature of the corresponding esters. These esters are hydrolyzed to their corresponding ketone bodies by the action of nonspecific intracellular esterases. As mentioned above, we have tried this approach using the triacetate ester of glycerol (GTA) and have discovered that it is protective against TBI in the CCI model in the rat (16). Also, in a related study, no toxicity was detectable on its chronic usage for as long as 4 months (17).

In earlier studies, different types of ketone body esters have been synthesized and tested for parenteral nutrition as replacement for emulsions of log-chain triacylglycerols. Of these, two ketone body esters were found to be effective with little or no detectable toxicity. They are 1) 1,3-butanediol diacetoacetate and 2) glyceryl bisacetoacetate (18,19). As indicated earlier, metabolism of these esters involves hydrolysis of the esters by nonspecific esterases present in all tissues. In the case of 1,3-butanediol diacetoacetate, the 1,3-butanediol formed as result of the ester hydrolysis is subsequently oxidized in the liver in a sequential fashion, first by alcohol dehydrogenase and then by aldehyde dehydrogenase to form a racemic (R,S) β-OHB which will subsequently form the physiological ketone body acetoacetate (20,21). Intravenous infusion studies have shown that the ketone bodies can be increased in the blood effectively without any notable side effects (21). Based on these studies, the major advantages of administering the ketone bodies in the ester form were determined to be 1) stabilization of acetoacetate which decomposes spontaneously to acetone and CO_2, 2) pH neutrality of the infusate, which minimizes damage to peripheral vein and 3) absence of sodium in the infusate. In some studies done in the pig, the plasma concentrations of the nonhydrolyzed esters were found to be very low and dropped to zero as soon as the infusion was stopped. This reflects the high esterase activity of plasma and tissues of pig. One way to overcome this potential problem is to mix the ketone body esters with anti-esterase flavenoids, such as those found in grapefruit juice, and this will reduce premature hydrolysis in the plasma and liver, increasing delivery of the esters to the brain (22).

B. N-Acetylaspartate: A novel biomarker for traumatic brain injury in body fluids.

The rapid neuronal and glial damage after TBI is resistant to any known neuroprotective regimes so far available for clinical use. Even though it can be reduced to some extent with dedicated intensive care management, the delayed secondary effects of brain trauma can be very severe due to inflammation, edema, diffuse axonal injury and impaired neuronal energy metabolism. Because of these reasons, it is of great importance to start early management tailored according to the extent of injury. For the early determination of severity of injury and assessing the prognosis during and after intensive care treatment there is a critical need for a simple and accurate diagnostic marker for the brain injury. N-acetylaspartate (NAA) has attracted much attention as a noninvasive marker of functional integrity of neurons in traumatic brain injury (TBI) using magnetic resonance spectroscopy (23-27). Not only has NAA been

identified as a marker in traumatic brain injury, its functional roles in neuronal energy metabolism have given it a central role in several forms of progressive and non-progressive neuronal afflictions (28,29). In TBI, the levels of both NAA and ATP in specific areas of brain decrease soon after cerebral injury and their levels return to normal in parallel to the recovery phase (26,27). The reduction in NAA is a reliable measure of injury severity and brain specific mitochondrial dysfunction (27, 30). Interestingly, cyclosporine treatment which prevents mitochondrial injury also prevents the decrease in brain NAA levels (31). Using mass spectrometric method for analysis, it has been shown that the NAA can be used as a serum marker in ischemic stroke as early as 24 hours (32) and that it increases during reperfusion after an ischemic episode (33). NAA can be measured in all body fluids (blood, cerebrospinal fluid and urine) accurately using mass spectrometric analysis (34) and an increase in NAA in the urine is used as a reliable diagnostic marker for Canavan disease, a genetic disease in which NAA is increased in the brain (34, 35). However, NAA has not been tested as a biomarker of TBI in any body fluid. The central hypothesis is that changes of NAA in the brain is reflected in its levels in the body fluids- blood, cerebrospinal fluid and urine-, and that changes in NAA levels in one or more of these body fluids can be used to monitor brain injury and recovery in TBI, from a diagnostic as well as prognostic angle.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s)

Literature Sources Searched: PubMed, BRD, FEDRIP

II.2.2. Date of Search: 2-19-2013

II.2.3. Period of Search: 1970-present

II.2.4. Key Words and Search Strategy: Glyceryltriacetate, traumatic brain injury, blast overpressure injury, rat, animal model, ketone body ester, melatonin, N-acetylaspartate, TBI biomarker.

II.2.5. Results of Search: ***A. Neuroprotection Strategies for TBI.*** *PUBMED:* Eighty three articles were found when keywords traumatic brain injury and melatonin were combined. None of them involves the proposed work. One article was found when key words traumatic brain injury and ketone body esters were combined. However, 33 articles were found when ketone body instead of ketone body ester was used. None of them involves the proposed work. No additional articles were found when traumatic brain injury was replaced with blast over pressure injury. One article was found when key words traumatic brain injury and glyceryltriacetate (GTA) were combined. This article was a recently published article from the PI's laboratory on the ongoing work. *BRD:* None. *FEDRIP:* One article was found when traumatic brain injury and melatonin were combined. This work was unrelated to the proposed work.

B. N-Acetylaspartate: A novel biomarker for traumatic brain injury in body fluids. PUBMED: Twenty five articles were found when keywords traumatic brain injury, N-acetylaspartate (NAA) and biomarker were combined. All those articles describe the

use of NAA as a biomarker for TBI in the brain by MRS techniques, but not in the body fluids. Thus, none of them involves the proposed work. BRD: None. FEDRIP: None.

Therefore, the proposed research and the goals do not duplicate earlier research in these areas of research.

OBJECTIVE\HYPOTHESIS:

A: Neuroprotection Strategies for TBI. The **central hypothesis** is that melatonin and esters of the ketone bodies are very useful as neuroprotectants for clinical use because of their effectiveness, lack of toxicity and lipophilic properties that help to overcome the blood brain barrier. We propose the following specific aims. First, we will synthesize esters of β-OHB and acetoacetate using glycerol as well as 1,3-butane-diol separately, and test their relative effectiveness as well as that of melatonin alone as well in combinations employing our ongoing CCI model of TBI in the rat. Second, we will determine whether or not these esters and melatonin exhibit acute or chronic toxicity by analyzing blood chemistry and tissue pathology. Subsequently, we will determine whether the effectiveness can be increased by combining with 1) GTA, the acetate ester we have used previously and 2) grapefruit juice, which contains anti-esterase flavenoids that will reduce premature hydrolysis of the esters in the gut and liver, thus increasing delivery of intact esters to the brain. Based on the outcome from the CCI model of TBI, we plan to test selected strategies in the blast over pressure model of brain injury as well.

B. N-Acetylaspartate: A novel biomarker for traumatic brain injury in body fluids:.NAA is present in the human brain at a concentration of approximately 10 mM, and an approximately 1000-fold gradient exists between the intracellular and extracellular compartments. NAA has been found to be a reliable biomarker in ALS patients with an approximately 5 fold higher serum concentration than in healthy age matched controls .Our central hypothesis is that brain injury causes cellular damage and NAA efflux from neurons to the extracellular space and subsequently to the bloodstream. The NAA efflux can be directly measured in the cerebrospinal fluid and will be reflected in body fluids such as blood and urine. Changes in NAA levels in one or both of these body fluids can be used to monitor brain injury and recovery after TBI to aid in diagnosis as well as prognosis. Our immediate goal is to develop a simple and reliable method for analyzing NAA in serum and urine after TBI for diagnostic purposes,validating its specificity to TBI and demonstrating that the NAA measurement can be converted to a point-of-care(POC) test. The long term goal of this proposal is to adapt the NAA biomarker method to a simplified hand held biosensor device for use on injured soldiers in combat support hospitals as well as for use in civilian hospital emergency rooms

III. MILITARY RELEVANCE:

A. Neuroprotection Strategies for TBI

Successful outcomes in the current studies will provide an adjunct therapy to the current TBI regimen of sedatives and anti-inflammatory agents. The most effective ketoesters and GTA can be used intravenously when TBI patients arrive at a medical facility, and can be given orally when appropriate. Bypassing both glucose and N-

acetylaspartate (NAA) metabolism in TBI patients by directly providing acetate and ketone bodies for brain energy production would help compensate for the substantial reductions in NAA and ATP levels observed after head injury. Providing additional acetate for nuclear histone acetylation reactions would help maintain transcriptional control of gene expression during cellular repair and recovery. Prolonged treatment with GTA after brain injury could also contribute to remyelination of injured white matter, and improve long term outcomes. Melatonin can act as an antioxidant and as an anti-inflammatory agent with little or no toxicity on long term usage.

Currently there is no effective pharmacological treatment for reducing the delayed, secondary neurological damage resulting from various forms of traumatic head injury. The proposed ketone body and acetate therapy is a non-pharmacological, nutrient-based method for improving brain energy status and facilitating CNS repair and recovery. Because GTA and the ketoesters are hydrophobic molecules, they will pass through the blood brain barrier, and even reach brain areas that have reduced blood flow resulting from injury. These compounds are inexpensive to manufacture and easy to administer in a field hospital setting. Based on our previous results with GTA we anticipate both improved long term neurological outcomes, and increased recovery rates of soldiers suffering from diffuse (blast overpressure) or traumatic brain injuries. An optimized ketoester and GTA treatment cocktail, possibly including anti-esterase flavenoids, could be ready for phase I trials quickly, and for field trials shortly thereafter. Melatonin also can be very helpful for this purpose.

B. N-Acetylaspartate: A novel biomarker for traumatic brain injury in body fluids: TBI has been called the "signature injury" of the Iraq war. Since Operation Iraqi Freedom began five years ago, the Department of Defense reports that more than 1,500 U.S. soldiers have died and more than eleven thousand American troops have been wounded. Among the most debilitating wounds are traumatic brain injuries. Thanks to such new helmet materials and technology, many war fighters in the global war on terror are surviving head injuries that in previous wars would have been fatal; however, they subsequently are plagued by TBI, which can be a chronic and debilitating condition without proper diagnosis and treatment. Also veterans with less severe brain damage are not getting the medical attention they need due to lack of diagnostic techniques. In view of the above situation, development of a sensitive and cost effective biomarker which could be used for screening hence identifying mild injuries as well as for predicting the progression and prognosis of moderate to severe injuries will be of tremendous importance to the soldiers, their family and the nation as a whole.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

A. Neuroprotection Strategies for TBI

Experimental Plan: A summary chart of the experimental plan is shown in Figure 1 below. As mentioned above, we plan to test four ketoesters for efficacy in our TBI model system. They are 1) 1,3-butane-diol-diaceoacetate (BDAA), 2) 1,3-butane-diol-di-β-hydroxybutyrate (BDHB), 3) glyceryl triacetoacetate (GTAA) and 4) glyceryl tri β-hydroxybutyrate (GTHB). Initially we plan to do a dose-response study in control rats

to test their effectiveness in increasing ketone body concentrations in the brain. We will use the molar dose range we have used earlier for similar studies we have done using GTA (16,17). An HPLC method we have described earlier will be used to simultaneously measure acetoacetate, β-OHB and acetate levels in the brain to determine the optimal dose for each ketoester. Subsequently, we also will do a time course study with the optimal dose obtained from the dose-response study. Groups of five male rats will be used for each time point or dose. This study will be done for all the four ketoesters before they are used in the TBI study. The experimental plan will be adjusted as needed depending on the dose-response and time-course studies. For example, if the time course studies show rapid metabolism of certain esters, then the number of doses per day will be adjusted accordingly. This study also will reveal whether or not any of these ketoesters have any yet unknown acute toxic effects. Brain Injuries will be performed as described in the previous protocol and as detailed below.

Similar dose response and time course studies are not planned for melatonin since we already know the effective dose ranges based on earlier studies in other model systems. We plan to select three doses in the range for the proposed experiments. Also, we plan to use intraperitoneal route in the case of melatonin based on previous studies. In contract, the ketoesters will be given intragastrically using a blunt end syringe as we have done earlier for GTA administration.

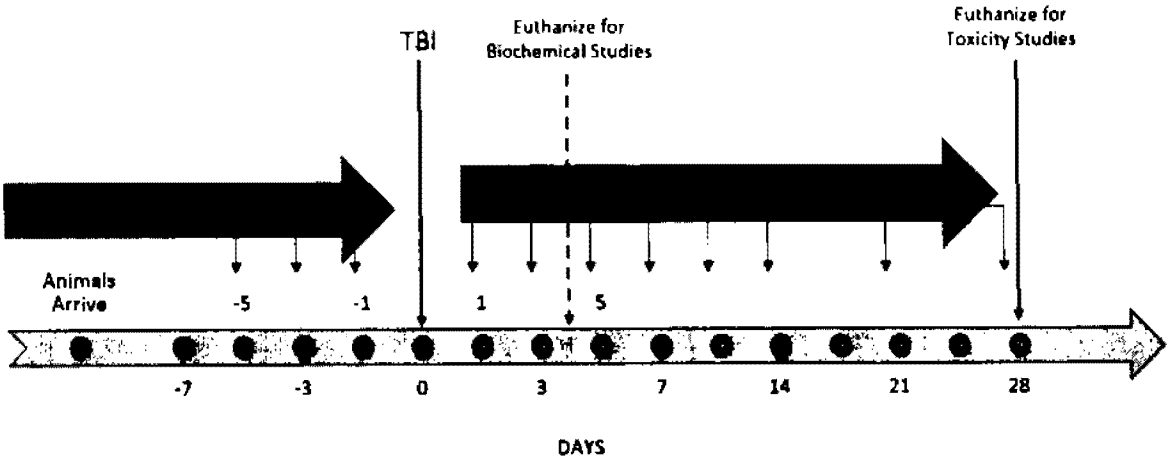

Figure 1: Timeline summary chart for the TBI experiments. See text for details.

In general, the methods we have followed in the case of GTA in the previous protocol will be followed to determine neuroprotective effectiveness with all four ketoesters and melatonin. Two biochemical and three behavioral end points will be followed to assess effectiveness. The biochemical end points will include 1) changes in NAA content in the ipsilateral side and 2) changes in ATP content on the ipsilateral side. The behavioral end points will include 1) neurological score 2) Rotarod balance test and 3) locomotion based parameters. We have found that we cannot perform craniotomy surgery and impact injury on more than 25 animals per day. Therefore we plan to limit each experiment to a total of 25 animals. These will include 5 groups of five animals: 1) sham control (surgery but no CCI injury), 2) injured-untreated and 3-5) three groups of injured and treated animals with the 3 doses selected from the initial dose-response

study. The experiments will be repeated for each of the 4 ketoesters listed above and melatonin. Also, the experiments will be repeated three times to increase the number of animals in each group (5 animals for biochemical end points and 10 animals for behavior end points); these animal numbers are based on our previous experience with the use of GTA as a neuroprotectant in TBI (5). **Our plan is to administer the ketone esters intragastrically, melatonin intraperitoneally, all once daily during the first week and once on alternate days during the next three weeks.** The animals will be tested for neurological score, Rotarod balancing and locomotion activity on alternate days starting from the second day. For measurement of biochemical end points, the studies will be repeated as described above except that the animals will be euthanized on the fourth day following the behavioral testing done on days 1 and 3. We have designed the experimental plan based on our recent experience with testing the neuroprotective effect of GTA on TBI (16). The methods given in our recent publication will be used for determination of NAA and ATP. The biochemical analyses will be done using fresh or frozen tissues obtained from animals euthanized by decapitation under anesthesia. The methods for the neurological scoring, Rotarod and locomotion behavioral tests are given below.

Traumatic brain injury in the rat using controlled cortical impact: Adult male Sprague-Dawley rats (200-300 g wt) will be housed on a 12-h light/dark cycle (06:00-18:00) at 22-23°C with free access to food and water. Rats will be allowed to acclimate for 5-7 days prior to experimental procedures. Rats will be initially anesthetized with 4-5% isoflurane in 100% O_2 in a vented anesthesia chamber. Following endotracheal intubation, rats are mechanically ventilated with a 1-3% isoflurane mixture. The rats are then mounted in the injury device stereotaxic frame in a prone position secured by blunt ear bars and incisor bar. The head is held in a horizontal plane with respect to the interaural line. An incision and an 8-mm craniotomy is made over the right primary and secondary motor cortex. Injury parameters will include using a 6mm flat steel impact tip, with injuries localized around motor cortex at 0.5 mm posterior to the coronal suture and 3 mm lateral to the sagittal suture (5m/s, 2.5mm deformation). Core body temperature will be monitored by a rectal thermometer and maintained at 36 to 37°C. After removal of the bone flap, injury will be produced with the CCI device (Pittsburg Instruments, Inc.) that was recently acquired and calibrated. Target insult parameters will be a penetration depth of 2.5 mm (for moderate injury) and 3.5 mm (for severe injury) and a constant velocity of 4 m/s. These conditions produce a reliable lesion that progresses over 1 week. Impact velocity will be measured for reproducibility. The bone scalp will be replaced and sealed with dental cement, and the scalp incision will be closed with stainless steel clips. The animals will be placed in a heated cage (35-37°C) to maintain body temperature while recovering from anesthesia. Sham-operated controls will receive craniotomy as described above but no impact injury. The impact tip will be placed lightly on the dura, and then removed before sealing the wound. We have has extensive experience in this model of TBI based on our recent work with GTA, as described in the previous protocol and our recent publication of this work (16).

Neurological score: The neurological score for each CCI injured animal will be assessed on the behavioral testing days according to the method of Garcia et al. (36).

Briefly the 6 part scoring is done as described below by investigators blinded to the treatment groups.

1. Spontaneous Activity: The animal will be observed for 5 minutes in its cage. Scores indicate the following: 3, rat moved around and approached at least three walls of the cage; 2, slightly affected rat moved about in the cage but did not approach all sides and hesitated to move, although it eventually reached at least one upper rim of the cage; 1, severely affected rat did not rise up and barely moved in the cage; and 0, rat did not move at all.

2. Symmetry in the Movement of Four Limbs: The rat is held in the air by the tail to observe symmetry in the movement of the four limbs. Scores indicate the following: 3, all four limbs extended symmetrically; 2, limbs on left side extended less or more slowly than those on the right; 1, limbs on left side showed minimal movement; and 0, forelimb on left side did not move at all.

3. Forepaw Outstretching: The rat is brought up to the edge of the table and made to walk on forelimbs while being held by the tail. Symmetry in the outstretching of both forelimbs is observed while the rat reached the table with the hindlimbs kept in the air. Scores indicate the following: 3, both forelimbs were outstretched, and the rat walked symmetrically on forepaws; 2, left side outstretched less than the right, and forepaw walking was impaired; 1, left forelimb moved minimally; and 0, left forelimb did not move.

4. Climbing: The rat is placed on the wall of a wire cage. Normally the rat uses all four limbs to climb up the wall. When the rat was removed from the wire cage by pulling it off by the tail, the strength of gripping is noted. Scores indicate the following: 3, rat climbed easily and gripped tightly to the wire; 2, left side was impaired while climbing or did not grip as hard as the right side; and 1, rat failed to climb or tended to circle instead of climbing.

5. Body Proprioception: The rat is touched with a blunt stick on each side of the body, and the reaction to the stimulus is observed. Scores indicate the following: 3, rat reacted by turning head and was equally startled by the stimulus on both sides; 2, rat reacted slowly to stimulus on left side; and 1, rat did not respond to the stimulus placed on the left side.

6. Response to Vibrissae Touch: A blunt stick is brushed against the vibrissae on each side; the stick is moved toward the whiskers from the rear of the animal to avoid entering the visual fields. Scores indicate the following: 3, rat reacted by turning head or was equally startled by the stimulus on both sides; 2, rat reacted slowly to stimulus on left side; and 1, rat did not respond to stimulus on the left side.

The score given to each rat at the completion of the evaluation is the summation of all six individual test scores. The minimum neurological score is 3 and the maximum is 18.

Rotarod balance test: See section V. 4.4.6

Locomotor activity and Habituation experiments: See section V.4.4.6

Safety/Toxicity Analyses: At the end of the 1 month chronic study, the animals will be euthanized by transcardial perfusion with 10% neutral buffered formalin under anesthesia. While the animal is under anesthesia, about 1 ml blood will be removed

from the heart before the start of the formalin perfusion. Subsequently, the fixed tissue will be removed and used for histological and immunohistochemical analyses. Serum obtained from blood will be used for the biochemical analysis to determine safety/toxicity due to the treatment conditions.

Studies involving treatment combinations: Following successful completion of the ketoester and melatonin studies, we plan to test different combinations for possible additive or synergistic effects. Also, we plan to test effect of grapefruit juice on ketone body ester/GTA effectiveness in view of the fact that grapefruit juice contains potent anti-esterase flavenoids that will prevent premature hydrolysis of the esters in the gut, liver and plasma, possibly increasing delivery of the esters to the brain and enhancing their effectiveness. Our plan is to mix the esters with grapefruit juice in equal proportion and administer them via oral or intragastric routes. We have no reason to think that the effect, if any, will be specific to a particular ketone ester or ketone ester/GTA combination because of the nonspecific nature of these esterases. Therefore, we plan to perform this study with the most effective ketone ester or ketone ester/GTA combination. All other aspects of the study will be as described for individual ketone esters.

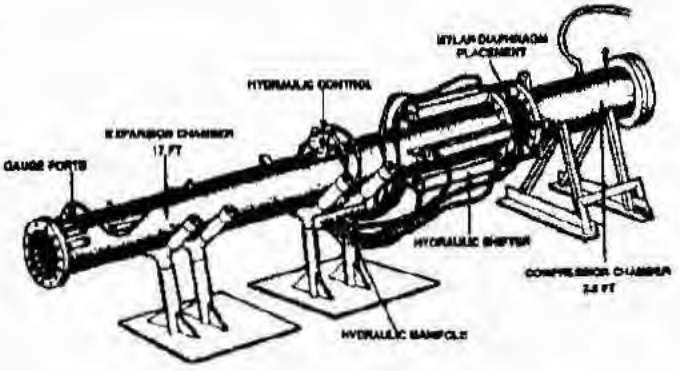

Figure 2. Shock tube used to generate BOP injuries in rats

Blast over pressure injury: The blast injury will be performed at WRAIR (see Figure 2) under the supervision of Dr. (b)(6) a collaborator in the proposed studies (WRAIR animal protocol # 11-PN-23S). Groups of male Sprague Dawley rats (250-300 g) will be anesthetized with isoflurane, placed in a prone position in a holder that is transversely secured across the mouth of the shock tube (Figure 2) and exposed to sham treatment (i.e. anesthesia and positioning without BOP) or moderate BOP (126kPa) using a compression driven shock tube, in which air blast is triggered by mylar membranes rupturing at predetermined pressure (60). Duration of apnea and latency to recover righting reflex after BOP will be recorded for subjects in all the injury groups. Categorization of a 126kPa BOP intensity as moderate is based upon the observations Dr. (b)(6) research group have made with rats exposed to this pressure in experiments conducted to date (37). Higher pressure injury will be tested in subsequent studies based on the results with the moderate injury. Detailed studies using this brain injury model will be planned in the future if the initial results are encouraging.

As mentioned above, the BOP injury will be carried out at WRAIR under the supervision of Dr. (b)(6) We understand that LAM has developed a procedure to

transport animals to and from WRAIR for this purpose. This currently approved procedure at LAM will be followed.

Calculation of the number of animals required

Experiment 1: Initial Dose Response and Time Course Studies using Control Rats.

Dose Response = 120 (5 animals/group x 4 ketoesters x 6 doses (0 dose + 5 doses around the one used for GTA in the earlier studies)

Time Course = 120 [5 animals/group x 4 ketoesters x 6 time points (0, 2h, 4h, 8h and 16h and 32h)]

Total = 240 (120 + 120): **Pain Category: C =240 , D =0 , E =0 .**

Experiment 2: TBI Study with Individual Neuroprotectants.

Animals required for each TBI experiment = 25 (5 animals /group x five groups[sham control --surgery but no CCI injury, injured-untreated, three groups of injured and treated animals with the 3 doses selected from the initial dose-response study].

Repetition of the study = 3

Number of Neuroprotectants = 5 (four ketoesters and melatonin)

Total = 375 (25 x 3 x5). **Pain Category: C =0 , D =0 , E =375**

All the animals will undergo surgery under anesthesia, but the subsequent pain will not be alleviated. So all the animals are listed under category E.

Experiment 3: TBI study with combination of Neuroprotectants

Animals required for each TBI experiment = 25 (5 animals /group x five groups[sham control--surgery but no CCI injury, injured-untreated, three groups of injured animals treated with three optimal doses].

Repetition of the study = 3

Combinations = 4 (ketone body ester + melatonin, GTA + melatonin, ketone body ester + GTA and ketone body ester + grape fruit juice)

Total = 300 (25 x 3 x 4). **Pain Category: C =0 , D =0 , E = 300.**

All the animals will undergo surgery under anesthesia, but the subsequent pain will not be alleviated. So all the animals are listed under category E.

Experiment 4: Blast Over Pressure (BOP) Injury Model

Animals required for each BOP experiment = 25 (5 animals /group x five groups[sham control (surgery but no BOP injury, injured-untreated, three groups of injured and treated animals with 1- optimal ketone body ester, 2-GTA and 3-melatonin]

Repetition = 3

Total = 75 (3 x 25). **Pain Category: C = 15 , D =0 , E = 60 .**

The control animals will be given anesthesia, but no surgery or exposure to BOP. So they are listed under category C. All the other animals will be subjected to BOP under anesthesia, but the subsequent pain will not be alleviated. So these animals are listed under category E.

B. N-Acetylaspartate: A novel biomarker for traumatic brain injury in body fluids:

The goal is to analyze the time course of NAA concentration changes in serum samples after brain injury using the controlled cortical impact model of TBI in rats. Serum samples will be analyzed to determine the time course of the rise and decline of NAA levels after injury and to compare the changes in NAA levels using three levels of injury severity; mild, moderate and severe.

Controlled cortical impact (CCI) model: We will follow our ongoing CCI protocol described in the previous section with minor modifications. The surgery will be conducted on a clean lab bench surface with restricted traffic flow through the area during surgery. The lab bench will be disinfected with 70% alcohol before and after each animal surgery. Briefly, male Sprague-Dawley rats (200-300 g) are anesthetized with 3% isoflurane vaporized in O2 (flow rate = 0.8 L/min) and then maintained with 2% isoflurane during surgery. When the animal is anesthetized, its head will be shaved with a hair clipper. The head will be cleaned with betadine and alcohol, each repeated 3 times alternately. Then the animal will be placed into a stereotaxic frame (appropriate frame size will be used for rats) after treating its pressure points such as ears and nose with lidocaine jelly. A heated pad with thermister-controlled feedback will be put between the animal's body and the base of the stereotaxic frame. Following fixing the head in a stereotaxic frame, and after retracting the scalp, a dental drill-trephine is used to make a 5 mm craniotomy over the left motor cortex, 0.5 mm posterior to the coronal suture and 3 mm lateral to the sagittal suture. Care will be taken to avoid injury to the dura, which will be continuously bathed in sterile physiological saline during the procedure. Body temperature is monitored throughout the surgery by a rectal probe and

maintained at 37.0 ± 0.5°C by using a heated pad, as indicated above. After removal of the bone flap injury is produced with a CCI device (Pittsburg Instruments, Inc.) that is pre-calibrated. Immediately after the injury, the bone flap is replaced and sealed with dental acrylic cement and the scalp is sutured and skin closed. The animals are then placed in a heated cage (35-37°C to maintain body temperature while recovering from anesthesia. Sham-operated controls will receive craniotomy as described above but no impact injury.

This injury model will be used in order to analyze increases in serum NAA levels at several post injury time points and with 3 levels of impact injury severity. The three injury severity models are defined as mild (2mm CCI impact tip with 1mm of cortical deformation0, moderate(6mm impact tip with 1mm deformation) and severe (6mm impact tip with 3mm of cortical deformation). Impact parameters for each of the 3 levels of injury severity will be measured for reproducibility. Injury severity will be confirmed for each rat by monitoring pre and post-injury NAA levels in the brain at several time points using magnetic resonance spectroscopy (MRS) using the small animal MRS system at USUHS. Greater NAA reductions in the left hemisphere as detected by MRS will be indicative of more severe injuries.

Rat blood sample collection: Blood samples will be taken from each rat at multiple post-injury time points via a temporary vein cannula that will be implanted during CCI surgery. Blood samples (0.1 - 0.2 ml) will be taken at 20 min., 40 min., 1hr, 2hr, 4hr, 24hr, 48 h, 1 week, 2 weeks, 3 weeks and 4 weeks. Blood will be centrifuged, and serum samples will be frozen rapidly on dry ice for later analysis.

Noninvasive analysis of brain NAA by MRS: As an independent measure of injury severity and NAA loss from the brain, NAA levels in the left frontal pole will be monitored by MRS prior to injury, and at several post-injury time points including 1 hour, 24 hours, 48 hours, 1 week and 4 weeks post-injury.

In vivo MRS will be performed on the BrukerBioSpec system (Bruker NMR, Inc., Billerica, MA) in the Translational Imaging Facility at CNRM (Center for Neuroscience and Regenerative Medicine). This system consists of a 7-Tesla (T), 20-cm horizontal bore, superconducting magnet (Magnex Scientific, Abingdon UK) with a Biospec 70/20 console and computer workstation with latest Paravision software for imaging and spectroscopy. An Autopac mouse and rat positioning and physiological monitoring system, an 86 mm quadrature transmit coil and a phase array mouse head coil (4 channels) will be used for MRS acquisition. The rats will be anesthetized with isoflurane (1-2%) during positioning and scanning. Localized single voxel PRESS MRS will be applied with parameters of TR=2500ms, TE=25ms, number of average = 256, and voxel size of 3-5 mm3. We have used this system to monitor the levels of NAA in Asp-NAT knockout heterozygote mice with voxels localized to the hippocampus. The system is highly sensitive and can detect even modest changes in brain NAA levels associated with mild CCI injuries.

Total number of rats for the study = 10 animals/group X four groups (control, mild, moderate and severe) X 2 (1 + 1 confirmation study) = 80. **Pain Category C = 0**

; D = 0 ; E = 80.

All the animals will undergo surgery under anesthesia, but the subsequent pain will not be alleviated. So all the animals are listed under category E.

Calculation of total number of animals

A. Neuroprotection Strategies for TBI

Experiment 1 + Experiment 2 + Experiment 3 + Experiment 4 = 240 + 375 + 300 + 75 = 990. **Pain Category C = 255, D = 0 , E = 735**

B. N-Acetylaspartate: A novel biomarker for traumatic brain injury in body fluids.

Experiment 1 = 80. **Pain Category C = 0, D = 0, E = 80**

C. Extra animals for training procedures and anesthesia failures.

10% of the animals in category E = 82

Grand Total = 990 + 80 + 82 = 1152.
Pain Category C =255 ; D = 0 ; E = 897

V.2. Data Analysis: Data will be presented as mean ± SEM. Parametric data (NAA, ATP etc.) will be analyzed using a paired 2-tailed t test for the paired data or a 1-way analysis of variance (ANOVA) followed by (b)(6) multiple comparisons post test. Nonparametric data (histology data, behavioral data) will be analyzed using a Wilcoxon signed rank test for paired data or a Kruskal-Wallis test (nonparametric ANOVA). An associated probability value of $P < 0.05$ will be considered significant.

Power analysis using the quantitative data obtained from our earlier studies have indicated that for behavioral studies a sample size of 10 animals per group will have 80% power to detect a difference of 1.5 standard deviations between groups assuming a 5% significance level, and should be sufficient for the outcomes we intend to measure; power calculations were performed using nQuery Advisor® 4.0 software. Similar calculations have indicated that 5-6 animals/group is sufficient for biochemical and immunohistochemical studies. The total number of animals for each experiment has been calculated based on the above considerations.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: The specific nature of the project does not permit use of non-animal alternatives. The global physiological changes occurring in different cell types in the *in-vivo* brain injury models cannot be reproduced in single cell culture system, or any alternatives.

V.3.2. Animal Model and Species Justification: Controlled cortical impact (CCI) in the rat is one of the most extensively used models of TBI in the literature and this allows us to compare our results with those from other established laboratories.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	Rattus Norvegicus	
V.3.3.2. Strain/Stock:	Sprague-Dawley	
V.3.3.3. Source/Vendor:		
V.3.3.4. Age:		
V.3.3.5. Weight:	200-300g	
V.3.3.6. Sex:	Male	
V.3.3.7. Special Considerations:		
V.3.4. Number of Animals Required (by Species):	1152	

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:

In the initial studies conducted to test the effect of GTA on traumatic brain injury using the CCI model, we have focused on the obvious behavioral, biochemical and histological parameters which are known to be well associated with TBI. We have found that most of the animals recover fast and start running around in apparent normal way in a matter of hours. Occasionally, some (less than 10%) are found to show a greater impairment. As a refinement, we plan to look for such animals and euthanize them since they are likely to be outliers in the study as well. Also, animals will be acclimated before the behavioral studies.

V.3.5.2. Reduction:

We have considered reducing the total number of control animals by avoiding separate control groups. However, in view of the variations between animals, especially in behavioral parameters, we do not feel comfortable in comparing with a control group done on another day. Therefore, we are unable to reduce the number of animals in the control group. Also, we have tried to use the minimum number of animals for each experiment based on our prior experience with GTA (earlier protocol).

V.3.5.3. Replacement:

In silico methods and tissue culture approaches cannot be used for evaluation of the questions studied here, since these actions can only be observed in an intact living animal. At the present time, animals lower than rodents in the phylogenetic scale cannot be used to model brain injury in human, because of wide anatomic and physiological differences.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	255	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	897	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

For CCI model of TBI, rats will be initially anesthetized with 3-5 % isoflurane in 100% O_2 in a vented anesthesia chamber. Rats under anesthesia will be placed on a stereotaxic instrument and continuous isofluorane anesthesia (2%) will be given using a nose cone.

For blast overpressure exposure, rats will be initially anesthetized as described above. However, continuous anesthesia will not be given because of the short duration of the blast exposure. That rats are under anesthesia will be determined by their lack of response to pinching the toes.

We do not plan to use analgesia for these studies. One of the main purpose of these studies is to evaluate the anti-inflammatory and healing effects of different neuroprotectants or combinations on CCI-induced or BOP-induced brain injuries and the associated pain to be evaluated by behavioral/neurological tests. Thus, any additional pain relief efforts in experiment will interfere with the goal of this study. The same situation applies to the other goal of testing NAA as marker for TBI in body fluids. However, pain will be alleviated to the extent possible without compromising the goals of the project. Carprofen (2-5 mg/kg, SC) or meloxicam (1-2 mg/kg, SC) will be given to rats as an analgesic, if animal show signs of pain or stress such as a hunched posture, not eating/drinking, reluctance to move during the recovery period.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

Animals will be dually housed and adapted to their environment for at least 5 days prior to the experiments. During this time, the animals will be adapted to the behavioral studies by two trial runs on the behavioral study set ups.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PubMed, Agricola:

V.4.1.3.2. Date of Search: 3/28/2013

V.4.1.3.3. Period of Search: 1966-2012

V.4.1.3.4. Key Words of Search: animal, rat, mouse, pain, analgesia, transcardial perfusion, controlled cortical impact model.

V.4.1.3.5. Results of Search: PUBMED: A total of 152 publications were obtained when all key words except transcardial perfusion were used. However, none appeared when transcardial perfusion was included in the search. All of the 152 publications involved studies on different aspects of pain, including neural mechanisms and newer pain relieving agents. When key words rat and transcardial perfusion were combined, 47 publications describing studies involving the use of transcardial perfusion were obtained. When key words mouse and transcardial perfusion were combined, 13 publications describing studies involving the use of transcardial perfusion were obtained. None of them provided any alternatives to the proposed painful procedures. Agricola: None of the publications provided an alternative to the painful procedure used in this protocol.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

One of the main purpose of this study is to evaluate the anti-inflammatory and healing effects of different neuroprotectants or combinations on CCI-induced or BOP-induced *brain injuries and the associated pain* to be evaluated by behavioral/Neurological tests. Thus, any additional pain relief efforts in experiment will interfere with the goal of this study. The same situation applies to the other goal of testing NAA as marker for TBI in body fluids. However, pain will be alleviated to the extent possible without compromising the goals of the project. Carprofen (2-5 mg/kg, SC) or meloxicam (1-2 mg/kg, SC) will be given to rats as an analgesic, if animal show signs of pain or stress such as a hunched posture, not eating/drinking, reluctance to move during the recovery period.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions:

The surgical procedures will be performed aseptically following the LAM guidelines. Anesthesia will be induced by isoflurane vapor in 100% oxygen in a commercially available induction chamber. Once anesthesia is induced, animals will be transferred to the designated surgery area and anesthesia will be maintained via nose cone. See Section V.4.1.2.1 Anesthesia/Analgesia/Tranquilization for details.

When the animal is anesthetized, its head will be shaved with a hair clipper. The head will be cleaned with betadine and alcohol, each repeated 3 times alternately. Then the animal will be placed into a stereotaxic frame (appropriate frame size will be used

for rats) after treating its pressure points such as ears and nose with lidocaine jelly. A heated pad with thermister-controlled feedback will be put between the animal's body and the base of the stereotaxic frame. Ophthalmic ointment will be applied to the eyes to prevent dryness. The body will be covered with a sterile drape with an opening to expose the head.

V.4.3.2. Procedure: *Animal surgery, anesthesia and CCI-TBI:* The surgery will be conducted at the Surgery room, Center of LAM on a clean bench surface under strict aseptic precautions with restricted traffic flow through the area during surgery. The lab bench will be disinfected before and after each animal surgery. Adult male Sprague-Dawley rats (200-300g wt) will be initially anesthetized with 3-5% isoflurane in a 100%O_2 mixture in a vented anesthesia chamber. The rats are then mounted in the injury device stereotaxic frame in a prone position secured by ear bars and incisor bar and are mechanically ventilated with a 1-3% isoflurane in 100% O_2. The rat head will be held in a horizontal plane with respect to the interaural line. An incision and a 6mm craniotomy is made over the left parietal cortex (Bregma 3.70mm, interaural 12.70mm). The core body temperature will be monitored by a rectal thermistor probe and maintained at 36 to 37°C. After removal of the bone flap, injury will be produced with the CCI device. A penetration depth of 2.5mm for moderate injury and 3.5 mm for severe injury with a velocity of 4m/s will be used. Impact velocity will be measured. The bone scalp is replaced and sealed with dental cement, and the scalp incision is closed with surgical clips.

Temporary vein cannula implantation: We plan to implant a temporary cannula on the lateral tail vein since repeated blood samples are required over a relatively short period of time. This will be done during the surgery associated with the CCI. An intravenous catheter is inserted into the vein by puncture of the skin and taped in situ . A heparin flush is used (0.1 ml) after placement and between samples to prevent clotting. An access port is inserted into the exteriorized end of the cannula, which stops the blood from flowing, and the catheter is taped into place. Aseptic technique will be used and a local anaesthetic cream (e.g. EMLA cream) will be applied to the site 30 minutes prior to insertion of the catheter. The tail will be washed with diluted Hibiscrub (1%) in order to see the blood vessel. The lateral tail vein will be accessed approximately one-third along the length of the tail from the tail tip.

V.4.3.3. Post-surgical Provisions: After CCI-TBI surgery, animals will be kept in warm condition by a heated blanket or light until they recover from anesthesia. The surgical area will be observed, and Carprofen (2-5 mg/kg, SC) or Meloxicam (1-2 mg/kg, SC) will be given to rats as an analgesic, if animal show signs of pain or stress such as a hunched posture, not eating/drinking, reluctance to move during the recovery period.The animals will be observed twice daily after surgery and recovery.

V.4.3.4. Location: (b)(6) facilities at USUHS

V.4.3.5. Surgeon: Dr (b)(6)

V.4.3.6. **Multiple Major Survival Operative Procedures**: N/A

V.4.3.6.1. **Procedures**: N/A

V.4.3.6.2. **Scientific Justification**: N/A

V.4.4. **Animal Manipulations**:

V.4.4.1. **Injections**:

Drug name (concentration)	Dose	Frequency	Route	Site	Volume	Needle size
Neuroprotectant keto esters (1 gm/ml)	5-8 g/kg	Once or once daily	intragastric	Stomach via mouth	Up to 2.0 mL	Gavage needle for rat
Melatonin (10 mg/ml)	20 mg/kg	Once daily	Intraperitoneal	Lower abdomen	Up to 0.5 ml	25 ga 1 in
Fatal Plus or equivalent (50 mg/ml)	85-100 mg/kg	Once	Intraperitoneal	Lower abdomen	Up to 0.5 ml	25 ga 1 in
Carprofen (7.5 mg/ml)	2-6 mg/kg	Once	subcutaneous	Interscapular	Up to 0.2 ml	25 ga 1 In
Meloxicam (2.5 mg/ml)	1-2 mg/kg	Once	subcutaneous	Interscapular	Up to 0.2 ml	25 ga 1 in

V.4.4.2. **Biosamples**: Blood will be collected from live animals via temporary vein cannula. Tissues such as brain, lung, liver, kidney, heart and blood will be collected post mortem.

V.4.4.3. **Adjuvants**: N/A

V.4.4.4. **Monoclonal Antibody (MAbs) Production**: N/A

V.4.4.5. **Animal Identification**: The animals will be identified by cage card. However, individual animals after procedure will be given a particular identifying tattoo on their pinna of the ear, tail, arms or legs.

V.4.4.6. **Behavioral Studies**: The behavioral studies will be performed daily starting at 24h following TBI. Given the wide spread nature of the brain damage in focal brain injuries such as in the proposed TBI model, we expect major and multiple behavioral changes in these animals . Our collaborator, Dr (b)(6) has extensive

(Endevco) incorporated into a low profile aluminum holder are used to record the static (i.e. side-on pressure) and dynamic pressure (i.e. blast wind) to which each rat is exposed. Piezoresistive gauges (Endevco) incorporated into a low profile aluminum holder are used to record the static (i.e. side-on pressure) and dynamic pressure (i.e. blast wind) to which each rat is exposed. For whole body exposures, anesthetized rats are placed in a transverse prone position in a mesh pouch that is positioned 2.5 ft within the mouth of the shock tube. The mesh pouch is suspended and tightly secured to the 4 arms of the holder.

V.4.4.8. Tissue Sharing: Tissues may be shared with other investigators upon request.

V.4.5. Study Endpoint: The animals will be euthanized for tissue collections and also for histological analysis at the end of each study. ~~For histological analysis, transcardial formalin perfusion will be performed following injection of a 85-100 mg/kg dose of Fatal Plus or another pentobarbital based euthanasia solution~~.

Early Endpoint: Any of the following will represent a reason for early euthanasia: inability to ambulate or reach food or water, self mutilation, continuous circling, or persistent labored respiration or gasping, loss of 20% of weight below baseline, prostration/inactivity for up to 24 hours, or a significant decline in vital signs. These signs, if observed by animal facility personnel and no study personnel can be reached, may be taken by the veterinarian as cause for euthanasia.

V.4.6. Euthanasia: Some animals in each group will be euthanized by decapitation following injection of a 100 mg/kg dose of pentobarbital based euthanasia solution. Others will be euthanized by transcardial perfusion following euthanasia solution administration (Fatal Plus, 85-100mg/kg or other pentobarbital based euthanasia solution, Intraperitoneal, 400-500µl volume, 25 gauge needle).

Transcardial perfusion will be done as described (16,17). Briefly, animals will be deeply anesthetized with Fatal Plus (85-100 mg/kg), and the heart will be exposed by opening up the thoracic cavity after confirming that the animal is fully under anesthesia. To monitor the depth of anesthesia we will test whether the animal has lost its toe pinch (pedal withdrawal) reflex. The animals will be perfused with either 10% neutral buffered formalin or 4% freshly depolymerized paraformaldehyde through the left ventricle using a peristaltic pump. The right atrium will be cut and approximately 200 ml of fixative solution will be passed through the circulatory system. Following the perfusion, tissues will be removed to a container of fresh fixative solution prior to histopathological analysis.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for all rats in this protocol.

V.5.1.1. **Study Room:** CCI-TBI will be conducted in LAM's surgery room or CNRM facilities.

Part of the experiments in V.1.A will be conducted at WRAIR. For this purpose, we will follow the guidelines given in the LAM SOP No 22-425. Briefly, the rats will be transported in a government van and a temperature of 68-79°F will be maintained inside the van. The van will be thoroughly cleaned before and after use with MB-10. The cages will be restrained using the seat belts to avoid sliding around.

The rats will be at WRAIR for 4-5 hours to give enough recovery time following the anesthesia and blast exposure. The rats will be monitored at 30 min intervals during this period by the PI or an associate and should a rat experience unexpected severe injury, the rat will be euthanized using the procedure given in the USUHS protocol. Also, the transport of rats to and from WRAIR will be coordinated with the veterinarians of both WRAIR and USUHS.

Building(s) (b)(6) Room Number(s) (b)(6) (b)(6)

V.5.1.2.
Special Husbandry Provisions: No separate housing required after surgery

Food Restriction:	Yes		No	X
Fluid Restriction:	Yes		No	X

V.5.1.3. **Exceptions:** _____ _____

V.5.2. **Veterinary Medical Care:**

V.5.2.1. **Routine Veterinary Medical Care:** The animals will be observed daily according to LAM SOPs.

V.5.2.2. **Emergency Veterinary Medical Care:** All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. **Environmental Enrichment:**

V.5.3.1. **Enrichment Strategy:** All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. **Enrichment Restrictions:**

VI. **STUDY PERSONNEL QUALIFICATIONS AND TRAINING:**

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Intragastric feeding, euthanasia, decapitation, perfusion, CCI-TBI.	(b)(6)	Ph.D. 8 yers exp in TBI models	Investigator training, 2003, Rodent handling class, 2003
Consulting		Ph.D., 20+ years exp.	Investigator training, 1998, Rodent handling class, 1998
Behavioral studies	(b)(6)	Res. Assistant, 3-6 months experience in rodent handling	Investigator training, 2012, Rodent handling class,2012
Consulting		Ph.D., 15+ years experience	Investigator training, 2000, Rodent handling class, 2000
Supervision of transport of rats, euthanasia		Res. Associate, 1 month experience in rodent handling.	Investigator training, 2013, Rodent handling class, 2013

VII. BIOHAZARDS/SAFETY: Gowns, Gloves, and masks will be used for personal protection and sharp items will be disposed off in separate containers as per safety rules. The volatile anesthetic agents will be properly closed and removed from the procedure area. Chemicals and hazardous waste used in this protocol will be handled in accordance with all applicable state and federal guidelines, regulations, and standing operating procedures. Waste anesthetic gas (isoflurane) will be scavenged and exposure to investigators will be minimized using the set up used in the LAM operating room since the surgeries will be done in one of those operating rooms.

VIII. ENCLOSURES: References

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course. (b)(6) 6/18/2013

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

_____ _____

Principal Investigator Signature (b)(6) Date 6/18/2013

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) 6/18/2013

Principal Investigator Signature Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number: (b)(6)

B. Animal Protocol Title: Development of Neuroprotection strategies for Brain injury using Rat models.

C. Principal Investigator: (b)(6) Ph.D.

D. Performing Organization: USUHS

E. Funding: BAA-MRMC

F. Objective and Approach:. .

a: Neuroprotection Strategies for TBI. The central hypothesis is that melatonin and esters of the ketone bodies are very useful as neuroprotectants for clinical use because of their effectiveness, lack of toxicity and lipophilic properties that help to overcome the blood brain barrier. We propose the following specific aims. First, we will synthesize esters of β-OHB and acetoacetate using glycerol as well as 1,3-butane-diol separately, and test their relative effectiveness as well as that of melatonin alone as well in combinations employing our ongoing CCI model of TBI in the rat. Second, we will determine whether or not these esters and melatonin exhibit acute or chronic toxicity by analyzing blood chemistry and tissue pathology. Subsequently, we will determine whether the effectiveness can be increased by combining with 1) GTA, the acetate ester we have used previously and 2) grapefruit juice, which contains anti-esterase flavenoids that will reduce premature hydrolysis of the esters in the gut and liver, thus increasing delivery of intact esters to the brain. Based on the outcome from the CCI model of TBI, we plan to test selected strategies in the blast over pressure model of brain injury as well.

b. N-Acetylaspartate: A novel biomarker for traumatic brain injury in body fluids:.NAA is present in the human brain at a concentration of approximately 10 mM, and an approximately 1000-fold gradient exists between the intracellular and extracellular compartments. NAA has been found to be a reliable biomarker in ALS patients with an approximately 5 fold higher serum concentration than in healthy age matched controls .Our central hypothesis is that brain injury causes cellular damage and NAA efflux from neurons to the extracellular space and subsequently to the bloodstream. The NAA efflux can be directly measured in the cerebrospinal fluid and will be reflected in body fluids such as blood and urine. Changes in NAA levels in one or both of these body fluids can be used to monitor brain injury and recovery after TBI to aid in diagnosis as well as prognosis. Our immediate goal is to develop a simple and reliable method for analyzing NAA in serum and urine after TBI for diagnostic purposes,validating its specificity to TBI and demonstrating that the NAA measurement can be converted to a point-of-care(POC) test. The long term goal of this proposal is to adapt the NAA biomarker method to a simplified hand held biosensor device for use on injured soldiers in combat support hospitals as well as for use in civilian hospital emergency rooms

G. **Indexing Terms (Descriptors)**: . Glyceryltriacetate, traumatic brain injury, blast overpressure injury, rat, mouse, animal model, ketone body ester, melatonin, N-acetylaspartate, biomarker, controlled cortical impact model.

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July 28, 2013

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on July 28, 2013:

Animal Protocol Title: "Development of Normative Data for Behavior Testing in the CNRM Mouse Behavioral Assessment Core"

USUHS Protocol Number: (b)(6)

Expiration Date: July 27, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6). This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

Revised

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Development of normative data for behavior testing in the CNRM Mouse Behavioral Assessment Core

GRANT TITLE (if different from above): CNRM Pre-Clinical Studies Core

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Center for Neuroscience and Regenerative Medicine

EARLIEST ANTICIPATED FUNDING START DATE: Ongoing

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6)	APG	(b)(6)	6/26/13
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	Professor & Chair	(b)(6)	06.26.2013
Research Un Typed Name	Signature M.D., Ph.D.	Title	Telephone	Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)	APG	(b)(6)	6/26/13
Statistician Signature	Department	Telephone	Date

Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)	LAM	(b)(6)	26 Jun 13
an Signature DVM	Department	Telephone	Date

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

ANIMAL PROTOCOL TITLE: Development of normative data for behavior testing in the CNRM Mouse Behavioral Assessment Core

GRANT TITLE (If different from above): CNRM Pre-Clinical Studies Core

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S):

TECHNICIANS(S): (b)(6) M.S.N., (b)(6) M.D., (b)(6) Ph.D, (b)(6) B.A., (b)(6) M.S.

I. NON-TECHNICAL SYNOPSIS:

This research will develop new behavioral tests for use in brain injury and PTSD research in the Pre-Clinical Studies Core of the Center for Neuroscience and Regenerative Medicine (CNRM). The CNRM Pre-Clinical Studies Core is a shared facility used by many scientists to assess the behavior of laboratory mice after various experimental manipulations. Behavioral testing is used as a measure of nervous system function after experimental manipulations, including other behavioral training experiences, brain injury, and after treatments that alter the impact of those experiences or injuries. The tests currently available to researchers are designed to measure behavior across many functional domains such as motor function, sensory abilities, exploration, learning and memory, anxiety, and depression. There are multiple tests available to test a single functional domain such as "memory", and some tests have been shown to be more sensitive in detecting mild functional abnormalities after experimental manipulations. We aim to find more sensitive cognitive tests that distinguish mild injuries resulting from trauma and/or stress. In addition, measures of other behavioral domains such as circadian rhythms, ingestive behaviors, and neuropsychiatric traits such as irritation and aggression have been successfully modeled in rodents by other investigators, and we will develop these assays and provide baseline and post-injury data through the experiments described in this protocol. These studies will validate and determine the effects of brain injury on these additional tests that will be made available to all CNRM researchers.

II. BACKGROUND:

II.1. Background:

The Pre-Clinical Studies Core was established at the University approximately four years ago and is a facility shared by CNRM researchers (and other investigators) to evaluate the functional performance of mice after experimental manipulations that cause traumatic brain injury (**TBI**), psychological stress or other changes, and the impact of experiential or genetic factors, or pharmacological or cell replacement treatments. The Core currently offers over 20 behavioral tests (Table 1) and several thousand behavioral tests have been performed in the Core facilities by CNRM investigators over the past three years, resulting in numerous publications and scientific abstracts/presentations that have contributed to pre-clinical drug development and translational research.

Table 1. Behavioral tests available in the CNRM Pre-Clinical Studies Core	
TEST	**FUNCTION**
Open field	Exploration
Hole-board test	Exploration/anxiety
Rotarod	Motor coordination
Beam walk	Motor coordination
Grip strength meter	Muscular strength
Hanging wire test	Muscular strength
Fear conditioning	Classical conditioning
Barnes maze	Spatial learning and memory
Morris water maze	Spatial learning and memory/working memory
Novel object recognition test	Episodic memory
Y-maze	Episodic/working memory
Zero maze	Anxiety
Elevated plus maze	Anxiety
Forced-swim test	Depression/learned helplessness
Sucrose preference test	Depression/hedonic motivation
Female urine sniffing test	Depression/hedonic motivation
Intruder test	Aggression
Sociability test	Social interaction
24-hour activity chambers	Circadian rhythms
Acoustic Startle Response	Sensory/anxiety
Hot-plate	Sensory/pain

Despite great success of the tests offered by the Core to date in studies of TBI, several needs of CNRM investigators have arisen: 1. Investigators using transgenic animals have expressed the need for basic sensory testing, 2. As milder, closed-head (no craniotomy) models of brain injury have become increasingly popular, the need for more sensitive behavioral tests has become apparent. In this protocol we propose to develop several new tests that will address these two needs; sensory testing will allow investigators to assess transgenic animals to determine if they have sensory deficits that will interfere with (or alter interpretations of) subsequent cognitive and neuropsychiatric testing, and exploration of alternative protocols for cognitive testing may reveal testing strategies that are sensitive enough to detect behavioral deficits after mild injuries.

The goal of this research is to integrate new behavioral tests into the CNRM Pre-Clinical Studies Core's current offering of functional tests for scientific investigators. Under protocols (b)(6) (b)(6) expired) and (b)(6) the majority of the current tests have been validated in control animals and used in TBI research, and the results are available to all investigators interested in employing behavioral testing in their experiments. This research will expand the offerings to include more sensitive cognitive tests, tests of sensory systems including vision and olfaction, and an assay of irritation, a neuropsychiatric complaint of brain-injured human patients. A database of "normative" information on how uninjured C57BL/6J adult male mice will first be collected to validate testing equipment and data-collecting procedures. We will then use controlled cortical impact (CCI) to induce TBI at mild and severe levels to determine the impact

of this brain injury on functional performance and to identify behavioral tests that are able to discriminate mild injuries.

This protocol first outlines a plan for *pilot studies* to address the above-described needs using new equipment and procedures. This will permit the staff of the Core to gain familiarity with each apparatus, formalize the specific steps in handling the mice and executing the tests, and develop the skill sets required to obtain reliable experimental data. The Core employees, in turn, will have experience that allows them to instruct investigators in how to perform the Core testing protocols. The *pilot studies* serve a second purpose. Although there are literally thousands of published reports concerning mouse behavior testing, to establish an efficient Core facility requires that CNRM investigators are skilled in using the specific equipment in the Core, and that there exist an established data base of information about mouse performance on each apparatus. "Local" data derived from this protocol is critical since the tests will be performed on the instrumentation specific to the Core, which previous studies have shown is an important consideration in attempting to derive comparable data across research groups (Brown, 2007; Wahlsten *et al*, 2003).

Many behavioral testing apparatuses measure a variety of parameters. For example, a single behavioral test may measure the time an animal engages in a certain response, the number of those responses in a testing session, how an animal's response changes over time or repeated trials. Basic information of these parameters can guide investigators to what measures they should record and what measures could be most relevant to the aspect of behavior they wish to model. Thus, statistical data obtained from the second, *full-scale studies* of this protocol can be utilized by investigators when they prepare their own experimental plans where consideration should be given to sample size and statistical power.

Once the *full-scale studies* have resulted in a full set of data on the new behavioral tests, *TBI studies* will commence to identify behaviors that are affected by TBI. Ideally, gross deficits/differences on each of the behavioral tests described in this protocol will result from severe CCI. However, the greater challenge will be to identify the tests that can discriminate between animals with a mild CCI and uninjured mice. The results obtained from the *TBI studies* will be made available to all investigators using the CNRM Pre-Clinical Studies Core. From this work, the Core staff will be more knowledgeable concerning the utility and limitations of each test, and experienced in the precise conditions of each test protocol, helping to guide investigators during the planning and execution of experiments.

II.2. Literature Search for Duplication:
II.2.1. Literature Source(s) Searched: BRD
II.2.2. Date of Search: July 24 19, 2013
II.2.3. Period of Search: 1998-2009
II.2.4. Key Words and Search Strategy:
Mouse, behavior, brain injury and Animal, mouse, behavior, standardization, and traumatic brain injury and active avoidance, Morris water maze, Cliff Test, Olfactory Avoidance Test, Novel Object Recognition Test, Bottle Brush Test

II.2.5. Results of Search:
The BRD database includes thousands of records of previous research programs. The searches derived a total of 14 unique hits. None of these programs pertained to establishing a mouse behavioral core facility or validating the use of specific behavioral tests in pre-clinical brain injury research, and the individual addition of the terms "active avoidance, Morris water maze, Cliff Test, Olfactory Avoidance Test, Novel Object Recognition Test, or Bottle Brush Test" derived 0 hits. **Conclusion**: No related hits indicated there is no overlap with previous research programs in the DoD.

II.2.6. Literature Source(s) Searched: CRISP and NIH RePORTER
II.2.7. Date of Search: July 24, 2013
II.2.8. Period of Search: active projects through fiscal year 2013
II.2.9. Key Words and Search Strategy:
Animal, mouse, behavior, standardization, active avoidance, Morris water maze, Cliff Test, Olfactory Avoidance Test, Novel Object Recognition Test, Bottle Brush Test

II.2.10. Results of Search:
CRISP is no longer an active search engine/web site. The suggested substitution is NIH RePORTER, and this site was used. The keywords animal, mouse, behavior, standardization produced five independent hits. Two of the hits were related to development of animal behavior cores, but were aimed at research on substance abuse. Another hit was for support for molecular phenotyping of mutant mouse lines, and another for development of an animal model of pre-term births. Another hit was for development of a pharmacology core. Search with the terms of specific behavioral tests resulted in 0 hits. Finally, a search was performed with the words, "blast brain injury mouse," and this derived 8 hits, but none of these pertained to developing standardized data. **Conclusion**: No related hits indicated there is no overlap with previous research federally sponsored research programs.

II.2.11. Literature Source(s) Searched: PubMed
I.2.7. Date of Search: July 24, 2013
II.2.12. Period of Search: >22 million references; 1949-2013.
II.2.13. Key Words and Search Strategy:

A. ("behavior, animal"[mesh] OR "stereotyped behavior"[mesh] OR "social behavior"[mesh]) AND "muridae"[mesh] AND ("animal experimentation"[mesh] AND "animals, laboratory"[mesh] AND "models, animal"[mesh])

B. (("muridae"[mesh] AND "brain injuries"[majr] AND "behavior, animal" [majr]) AND ("memory, episodic"[mesh] OR "memory, short-term"[mesh] OR "depth perception"[mesh] OR "avoidance learning"[mesh] OR "circadian rhythm"[mesh] OR "irritable mood"[mesh]))

C. ("behavior, animal"[mesh] OR "stereotyped behavior"[mesh] OR "social behavior"[mesh]) AND "muridae"[mesh] AND ("animal experimentation"[mesh] AND "animals, laboratory"[mesh] AND "models, animal"[mesh]) AND (active avoidance OR Morris water maze OR Cliff Test OR Olfactory Avoidance Test OR Novel Object Recognition Test OR Bottle Brush Test)

II.2.14. Results of Search:

A. PubMed was used with the keywords listed. A total of 39 hits were obtained. A search is each of these hits indicated four relevant publications (Bohannon, 2002; Moy *et al*, 2004; Paylor *et al*, 2006; Schneider *et al*, 2006). These articles related the importance of standardization of housing conditions, vendor source, and the criticality of behavioral testing conditions including the time interval between tests as important mediators of behavioral responses. The P.I. is also aware of the broader literature and has a comprehensive EndNote file system (obtained from searches over the years) of relevant publications. Importantly, there is an interaction between the genotype of a mouse and the environmental housing and testing conditions (Crabbe *et al*, 1999; Tucci *et al*, 2006), and consistency in housing, feeding, handling, behavioral testing procedures, testing sequence, and reducing overall distress are all important variables. Perhaps more relevant to a search are the "how-to" books, which the P.I. has extensively consulted (Buccafusco, 2009; Chen, 2009; Crawley, 2007; Jones and Mormède, 1999). These publications outline many of the same caveats related to animal testing, and the need to strive for consistency and standardization of housing and testing conditions. **Conclusion**: Numerous publications emphasize the need for standardization of housing and testing conditions, and requirements for "local" establishment of procedures.

B. Pubmed was used with the keywords listed to find literature pertaining to TBI and the specific behavioral tests we are proposing to use; nineteen hits were obtained. Of the 19 hits, five of those specifically related to behavioral testing after TBI (Baratz *et al*, 2011; Schwarzbold *et al*, 2010; Tweedie *et al*, 2007; Vonder Haar *et al*, 2011; Zohar *et al*, 2011). Although these papers describe behavioral deficits after traumatic brain injury in mice, none use the specific tests we plan to employ. We have an extensive database of articles describing prior research on TBI in rodents, and have considered these sets of data as we have planned our future TBI experiments.

C. Search "C" above that included the specific names of behavioral tests resulted in 0 hits for active avoidance, 7 hits for Morris water maze, but none of these references pertained to TBI or blast brain injury, zero hits for the Cliff Test, 0 hits for the Olfactory Avoidance Test, 1 hit for the Novel Object Recognition Test, but this reference pertained to study of the Fragile X syndrome mouse model and not TBI or blast brain injury, and 0 hits for the Bottle Brush Test.

III. OBJECTIVE\HYPOTHESIS:

The *objective* of this project is to provide investigators with novel behavioral tests in the CNRM Pre-Clinical Studies Core for use in studies on TBI and post-traumatic stress disorder (PTSD). We will perform pilot studies to formalize precise testing procedures and to acquire the necessary skills to consistently apply the procedures in an attempt to normalize the data-collection procedures used across laboratories. Once standard procedures are determined, we will collect full-scale normative data from control mice on each of the new tests, and determine the impact of mild or severe brain injury on behavioral performance on each of these tests. It is *hypothesized* that the establishment of formal testing procedures and the acquisition of skills in the consistent application of procedures will enhance the reliability and validity of behavioral testing data generated by investigators who use the Core facility in their research. In the short term no set criteria will be established to directly evaluate this hypothesis in terms of predicted quantitative outcome. However, the P.I. and staff have experience in behavioral testing, and will

use the data to formalize detailed experimental protocols for test execution and assess the overall consistency of results over the course of the protocol. We further *hypothesize* that a relatively severe brain injury induced by CCI will alter performance on the majority of the tests that we plan to add to the Core's offerings. However, identifying functional differences after a mild injury is a greater challenge, and we hope and expect that these additional tests will be sensitive enough to provide opportunities for measurement of significant differences between mildly-injured mice and control animals.

IV. MILITARY RELEVANCE:

The *relevance* of this protocol harkens to the major stated objective of the CNRM and for establishing a mouse behavior testing core facility at the University: "to address the current needs of the medical community to better diagnose and intervene for the prevention of the long term consequences resulting from traumatic brain injury, particularly in the context experienced by service members in Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF)." According to a respected RAND Corporation report (Tanielian and Jaycox, 2008), as many as 18.5% of the service members returning from these conflicts meet the criteria for a diagnosis of either PTSD or depression, and 19.5% of service members have reported experiencing a probable TBI during deployment. These estimates translate to approximately 320,000 cases of "probable TBI" and 300,000 cases of reported symptoms for depression or PTSD. Investigators with projects related to developing a better understanding of the biological processes and treatment for TBI and/or psychological trauma is necessary for the care of our service members. The laboratory mouse is used as an animal model for symptoms resulting from TBI and PTSD; many of the behavioral tests described in this protocol model symptoms reported in veterans such as irritability, sleep disturbances and memory loss. Basic research using this model will advance our knowledge of biological mechanisms of brain injury and psychological maladies and may lead to more effective treatments.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

General Approach: The CNRM Pre-Clinical Studies Core has eight novel behavioral tests (described below) it will make available to CNRM researchers. In addition, one of the behavioral tests from prior protocols has not been fully developed (Novel Object Recognition) and we would like to continue development of this assay, resulting in a total of nine behavioral tests described in this protocol. The plan for this protocol is to employ separate groups of mice in six testing modules, labeled "Experiments" (see below).

Overall experimental design. The work for establishing procedures for all behavioral testing will be conducted in three phases. First, *pilot studies* are considered an important first step to gain familiarity with the apparatuses, develop specific details of testing procedures, streamline practical matters in the sequence of testing, and for practice in data collection (Festing and Altman, 2002). Following completion of pilot studies, *full-scale studies* will be performed to collect statistical data on the performance of mice on each test. Following completion of the full-scale studies, *TBI studies* will begin and determine the effects of mild and severe levels of controlled cortical impact (CCI) on functional performance on each of the described tests.

Separate groups of mice will be evaluated on one of the six behavioral experiments. The rationale for not testing an individual mouse on all nine tests is based upon findings that repeated testing of mice alters response patterns due to general stress, fatigue, "testing experience," and

learning (McIlwain *et al*, 2001; Võikar *et al*, 2005). The testing schemes have been designed to try and avoid exposing mice to more than one behavioral testing "domain." For example, the Morris water maze, modified hole-board, and novel object recognition test all assess memory and cognitive performance. An attempt is also made to avoid testing animals on tests that assess similar neurological functions. Nevertheless, many of the tests outlined below assess overlapping psychological dimensions or challenge several neurological systems at once. Separate measurements obtained by a single testing apparatus can assess distinct neurological systems (Wahlsten *et al*, 2003) and, when possible, multiple measures are recorded so that these separate systems are evaluated. For example, the Any-Cage isolation chambers (cf. V.1.2. Experiment 2) are home-cages that allow multiple measurements of mice in home-cage environments: wheel-running activity and general exploration in the cage, diurnal rhythms and ingestive behaviors among many others.

Coordination of behavioral testing with CNRM investigators. In May, 2009, CNRM investigators assented that, when feasible, their mice will be assessed using the tests described in CNRM#2 SOP "Pre- and Post-TBI Behavioral Assessment in Mice" (Anon., 2009). Investigators may wish to evaluate their experimental subjects on additional behavioral tests depending upon the animal model and hypotheses of their work. Core staff will coordinate behavioral testing with investigators in how best to validate the findings of their work with respect to additional behavioral testing, including how the testing best fits with the experimental treatments of their research.

Sequence of testing. Logistical constraints in testing procedures, animal distress and fatigue (both physical and psychological) require that each animal is employed on 1-3 tests. In addition, when an Experiment (described below) involves more than one test the more distressful or aversive task is scheduled last; an attempt to reduce confounding of test results from previous distress.

Strain choice. The strain choice for this protocol is the male C57Bl/6J strain (b)(4) (b)(4) The C57Bl/6J mouse was selected since this strain generally performs "average" on many behavioral tests (Crawley, 2008). The data set derived from the use of this strain is also perhaps most useful to CNRM investigators. The Bl/6 mouse is an inbred strain, providing a very high degree of genetic homogeneity (Crabbe, 1999)and it is one of the traditional strains used for studies that will eventually employ this strain as the "control" or "wild type" condition for a related mouse strain that harbors a transgene or gene "knockout" (Crawley *et al*, 1997; Mishina and Sakimura, 2007).

V.1.1. Experiment 1: *Development of an assay for active and passive avoidance in naïve and brain-injured mice.*

Experiment 1a will collect pilot and full-scale data regarding the performance of 39 mice (**Table 2a**) on a passive and active avoidance place task (c.f. Experiment 1, V.4.4.6). In avoidance tests, an animal learns to avoid an environment in which an aversive stimulus (i.e, shock) was previously experienced. For individual cohorts of mice, the experiment will take place over four days. Fifteen of the mice will be used in pilot work to train Core staff in the operation of the newly-acquired equipment. Most likely, the mice for the pilot studies will be purchased in groups of five, allowing investigators three independent opportunities to test the equipment and experimental protocol. When all pilot work is completed, another 24 mice will be used in a

Table 2a		
Experiment	**No. of Mice**	**Pain Category**
Pilot	15	E
Full-Scale	24	E
Total	**39**	

formal, *full-scale study* to collect "normative" data that will be summarized and provided as a guide for expected mouse performance on the behavioral tests.

Table 2b		
Group	**No. of Mice**	**Pain Category**
Naive	20	E
Sham	20	E
Mild	20	E
Severe	20	E
Total	**80**	

After completion of the full-scale study, *TBI studies* (Experiment 1b) will commence in which performance on the active and passive avoidance behavioral tests will be compared between naïve, sham-operated, mildly-injured and severely-injured (mild vs. severe determined by depth of impact) mice. Twenty mice will be included in each surgical group (**Table 2b**), and behavioral testing will begin seven days after sham or TBI surgery. Because foot-shock is a necessary component of the avoidance training trials, for pain/distress (Section V.4.1.1. APHIS Form 7023 Information), all mice in Experiment 1 are in Category E.

V.1.2. Experiment 2: *Evaluation of 24-hour home cage phenotypic behaviors and circadian rhythms.*

The goal of Experiment 2 is to evaluate 24 hour activity of mice. The apparatus (Any-Maze Cage (AMc; Stoelting CO, Wood Dale, IL) is a home cage encased within an isolation chamber that limits external influences from the housing facilities and other animal activities and odors. Mice will remain in the isolated home cages for up to four weeks (c.f. Experiment 2, V.4.4.6). The chamber is equipped with an infrared sensitive light and LEDs that provide continuous tracking of the animal via a built-in camera linked to Any-Maze tracking software. The chambers collect continuous data related to behaviors, including but not limited to, movements and spontaneous activity, wheel-running activity, diurnal activities, rearing behaviors, and food and water consumption. Monitoring activity cycles in rodents is a challenge. Rodents are highly responsive to environmental cues. In the AMc system, the isolation reduces disturbances from lights and sounds generated by human activities in the animal facility and it reduces access to the smells and movement of neighboring animals in adjacent home cages. The chambers, then, may reduce variability in behavioral responses from fluctuations in external activities in the animal facility, while allowing investigators and technicians to enter the room to monitor temperature and humidity and to ensure the chambers are operating properly. The Core is in the process of obtaining eight of these cages and will utilize the system twice for pilot studies (thus, a need for 16 mice for the *pilot studies*). Twenty-four additional mice will be used in a *formal study* for the acquisition of "normative" data that will be summarized and provided as a guide for expected mouse performance (wheel running, amount of food and water consumption, inactivity, etc.; **Table 3a**). For pain/distress (Section V.4.1.1. APHIS Form 7023 Information), the 40 mice are in Category C.

Table 3a		
Experiment	**No. of Mice**	**Pain Category**
Pilot	16	C
Full-Scale	24	C
Total	40	

After completion of the full-study (normative data collection), *TBI studies* will begin to determine the effects of cortical brain injury on home cage activities including locomotion, rearing, eating and drinking, and diurnal rhythms. Twenty mice will be included in each surgical group (**Table 3b**); mice will be housed in the AMc beginning immediately after surgery and will remain in the cages for up to four weeks. For pain/distress (Section V.4.1.1. APHIS Form 7023

Table 3b		
Group	**No. of Mice**	**Pain Category**
Naive	20	C
Sham	20	D
Mild	20	D
Severe	20	D
Total	**80**	

Information), naïve mice (n=20) utilized in Experiment 1b are in Category C; the remaining 60 mice are in Category D.

V.1.3. Experiment 3: *Assessment of spatial learning and memory and visual acuity in mice.*
In Experiment 3 (c.f. Experiment 3, V4.4.6), we will use a modified and extended version of the Morris water maze (MWM) to evaluate learning and memory in mice. This procedure has been demonstrated to be effective in detecting cognitive differences in mice with mild TBI (Zhao *et al*, 2012). Finally, two or more days after completion of the MWM, a final vision assessment will be performed with the *visual cliff test*, which provides a simple and gross measure of visual abilities by evaluating the animal's ability to detect a false drop-off at the edge of a surface. We are combining the MWM and visual cliff test into one experiment as the visual cliff tests is relatively simple and can be performed in combination with the MWM test without affecting results. Additionally, tests of visual acuity are often performed after the MWM test to ensure that any deficits on the test can be attributed to true learning and memory deficits rather than to a loss of visual acuity as a result of experimental manipulations or genetic modifications.

As the basic apparatus and testing procedures for the MWM have been perfected under prior protocols (b)(6) and the visual cliff test is relatively simple and does not require specialized equipment, we have reduced the number of animals requested for pilot testing in this experiment. A total of 95 mice are requested for both normative data collection and TBI studies (**Table 4**). Fifteen of the mice will be used in pilot work and we believe that these data in combination with subsequent data from control animals in the TBI studies can be combined and used as "normative" data that will be summarized and provided as a guide for expected mouse performance in these behavioral tests. The 15 pilot animals will be tested first, after which TBI studies will take place. For pain/distress (Section V.4.1.1. APHIS Form 7023 Information), the 35 mice in the pilot and naïve categories were deemed as appropriate for Category C; the remaining mice (n=60) are in Category D.

Table 4		
Experiment	**No. of Mice**	**Pain Category**
Pilot	15	C
Naive	20	C
Sham	20	D
Mild CCI	20	D
Severe CCI	20	D
Total	95	

V.1.4. Experiment 4: *Mouse performance on a working memory MWM task and olfactory-avoidance test.*
Mice will be used to assess group performance on a modified version of the MWM designed to evaluate short-term (working) memory. This version of the test requires only two trials per day with a short inter-trial interval and tests the ability of a mouse to use information gained in the first "sample" trial to locate the platform in the second trial. In addition, prior to MWM testing, basic olfactory function will be assessed with an olfactory avoidance test in which the average distance from a piece of filter paper infused with an aversive odor is measured (c.f. Experiment 4, V.4.4.6 for details of both testing procedures). As basic testing procedures were acquired successfully under prior protocols, pilot testing will be limited to 15 mice and the data from those animals will be combined with the results from control animals in subsequent TBI studies

and used as a normative data set. Eighty mice are requested for the TBI studies (**Table 5**), which will begin once the pilot studies are completed successfully.

Table 5		
Experiment	**No. of Mice**	**Pain Category**
Pilot	15	C
Naive	20	C
Sham	20	D
Mild	20	D
Severe	20	D
Total	95	

For pain/distress (Section V.4.1.1. APHIS Form 7023 Information), the 35 mice in the pilot and naïve categories were deemed as appropriate for Category C; the remaining mice (n=60) are in Category D.

V.1.5. Experiment 5: *Exploration, anxiety and spatial memory in a modified hole-board environment.*

The goal of Experiment 5 is to evaluate multiple dimensions of mouse behavior in a modified hole-board test (c.f. Experiment 5, V.4.4.6). Under prior protocols (b)(6) we collected baseline data and evaluated the effects of TBI on simple exploratory behavior in the hole-board. In the current protocol we propose to elaborate on the experimental design to allow measurement of exploration, anxiety and spatial memory all in a single test. We will collect pilot data and subsequently conduct TBI studies (**Table 6**); the results from the pilot experiment and control animals in the TBI studies will be combined to create a normative data set. The mice are habituated to the hole-board environment for two consecutive days, and each of the 16 holes in the apparatus is baited with a small piece (~ .03g) of almond to encourage hole-exploration. On Days 3-6, four of the holes are baited with almond and the mice must use spatial cues to learn the pattern of distribution of the food rewards. The number of hole pokes and distance traveled in the habituation trials provide measures of exploration, risk-assessment (visits to center holes, stretch-attenuated postures) assess anxiety-levels, and the latency to acquire food reward during the training trials measure spatial learning and memory. For four days prior and for the duration of the experiment, mice will be fed a restricted diet such that body weights are reduced to approximately 85% of free-feeding weight.

Table 6		
Experiment	**No. of Mice**	**Pain Category**
Pilot	15	C
Naive	20	C
Sham	20	D
Mild CCI	20	D
Severe CCI	20	D
Total	95	

For pain/distress (Section V.4.1.1. APHIS Form 7023 Information), the 35 mice in the pilot and naïve categories were deemed as appropriate for Category C; the remaining mice (n=60) are in Category D.

V.1.6. Experiment 6: *Evaluation of episodic learning and memory with the Novel Object Recognition test.*

The goal of Experiment 6 is to evaluate a mouse's ability to remember objects they previously had explored. This memory test has the benefit of not requiring extensive training trials and the ability to be completed in three short trials over two days. The mouse is placed in the test arena on the first day for 10 minutes to acclimate to the testing environment. On the second day, two identical objects are placed in the arena and Any-Maze tracking software records the amount of time the mouse spends exploring the two objects.

Table 7a		
Experiment	**No. of Mice**	**Pain category**
Pilot	15	C
Full-Scale	24	C
Total	39	

During the second trial, four hours later, one of the objects is replaced with a new object and the animal is returned to the arena. Tracking software records the amount of time the animal spends exploring the "new" object vs the "old" object. As we had difficulty fine-tuning the precise testing conditions under prior protocol APG-10-754, we have simplified the testing procedure and propose complete pilot (n=15) and full-scale (n=24) experiments (**Table 7a**) to perfect this assay. After successful completion of the full-scale studies, TBI studies (**Table 7b**) will begin in which the effects of brain injury on episodic memory will be evaluated.

Table 7b		
Group	**No. of Mice**	**Pain category**
Naive	20	C
Sham	20	D
Mild CCI	20	D
Severe CCI	20	D
Total	**80**	

After completion of the Novel Object Recognition test, mice will be individually housed for five days to assess irritability using the "bottle brush test" (c.f. V.4.4.6, Experiment 6) (Riittinen *et al*, 1986).

For pain/distress (Section V.4.1.1. APHIS Form 7023 Information), the 39 mice in the pilot and full-scale studies of Experiment 6a (n=39) are in Category C; naïve mice of Experiment 6b (n=20) are also in Category C and the remaining surgical animals (n=60) are in Category D. The "bottle brush" test has been categorized as Category "C," since the distress is momentary and the mouse does not sustain any physical harm or long-lasting damage (Anon., 2012).

V.2. Data Analysis:

To estimate the number of animals needed, the scheme outlined by Wahlsten was used (Wahlsten, 1999). Wahlsten suggests that *effect size*, δ, between two experimental groups for mouse experiments can be generally classified as *small*, *moderate*, or *large*, with δ values of 0.5, 0.75, and 1, respectively, where $\delta = \frac{(\mu_1 - \mu_2)}{\sigma}$. For an estimation of the number of mice for the *full-scale studies*, it is assumed that a 30% difference between the average behavioral scores for two groups could represent a moderate effect. The formula above was transformed to $\sigma = \frac{(\mu_1 - \mu_2)}{\delta}$ to compute an estimate of the expected standard deviation of the residuals (σ) when there is a 30% change in behavioral score (e.g., $\mu_1 = 100$, $\mu_2 = 130$) and an expected "moderate effect" ($\delta = 0.75$). This suggests $\sigma \approx 40$ as an estimate of the standard deviation of the residuals.

The SigmaStat computer program, *Determination of Computing Power and Sample Size* was then used to estimate the number of animals needed for the *full-scale* experiments. This program calculates the minimum number of data units (mice) per group that is needed to achieve a certain level of *power* (sensitivity) of a statistical test based upon certain probability estimates. To achieve a power ≈ 0.80, in this case using $\mu_1 = 100$, $\mu_2 = 130$, and $\sigma \approx 40$, about 28 mice/group would be required. Since this is an estimate, we propose that for the *full-scale* studies outlined in Experiments 1-5, 24 mice may be sufficient and more convenient for the progression of testing procedures in the Core. For many of the tests, 8 mice can be tested simultaneously, allowing all 24 mice to be tested in three rounds of testing. In this case, if the same assumptions hold ($\sigma \approx 40$ with n=24 animals, $\delta = 0.84$), and there would be a possible 33% difference between groups compared to 30%.

From previous experiences a sample size of 20-24 mice/full-scale experiment appears more than ample for behavioral testing and for what is usually reported in publications of mouse behavior testing (number of subjects per group is usually in the mid-teens). However, this estimate is justifiable since the statistical data obtained from this work will be used by multiple

groups as a "standard" of comparison, and the resulting data is vital in seeking to strive for comparability across data obtained by many research teams. In addition, larger sample sizes have been used by previous groups attempting to initially validate their testing procedures. Rogers and colleagues (Rogers *et al*, 1997), for instance, used 37-89 mice/group to validate the "SHIRPA protocol" in one study, and two cohorts of 10 mice/group for further validation and statistical reporting (Rogers *et al*, 1999). Likewise, this estimate appears to be in line with suggestions by internationally recognized experts. For example, Crawley suggests that "$N = 20$ mice or more per genotype are often needed to complete the first set of behavioral tests in a new line of mutant mice" (Crawley, 2007). Finally, it is likely that some of the mice will be "uncooperative" (Wahlsten *et al*, 2003). Data from these animals may be eliminated from the statistical calculations—though the percentage of "non-responders" on specific tests will be recorded and reported—since extreme scores can alter summary descriptions of the response pattern. A larger sample size will allow us to better estimate the percentage of "non-responders" on each behavior measure, and maintain adequate sample sizes for statistical estimation should the percentage be high on some measures.

Statistical Analyses: The SigmaStat 2.03 (SPSS) statistical analysis program will be used to compute the arithmetic mean and standard deviation of mouse behavior on each test. In addition, a repeated measures single-factor analysis of variance (ANOVA) will be used to assess whether or not performance on each test is the same when comparisons are made of repeated testing results. If the data suggests there is variation between the testing of each cohort of mice, a nested ANOVA can be used to assess whether or not there are differences between testing events on repeated tests. Finally, to assess some degree of the relationship of performance on tests within an experiment, correlation coefficients will be computed for each animal's score on each subtask.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

The need to employ animals for this research is required in order to assess *in vivo* behavioral performance. These data will provide a means for relating behavioral responses to central nervous system function *in vivo*. Non-animal alternatives do not replicate the three dimensional structure or temporal changes that occur as a function of complex behavioral responses to tasks presented to a live animal. Further, computer modeling and *in vitro* methods will not address experimental questions of the complex, real-time changes and responses that occur during experience.

V.3.2. Animal Model and Species Justification:

By consensus of members of the CNRM, male C57Bl/6J mice [redacted] Stock 000664) have been chosen as a suitable species and animal model for this research (Anon., 2009). This is based upon the previous use of this strain in innumerable studies and their suitability as biomedical models for understanding central nervous system functions in normal and pathological states in humans and in other animals. The mouse is used for these studies because of their well-understood anatomy and endocrine responses, and the very extensive database of prior physiological research that can be integrated with this new information. It is necessary to use mice instead of a more-derived species since the research results should be directly associated with intense efforts in other laboratories throughout the world to try and further our understanding and eventual treatment effectiveness for psychological stress and brain injury.

Mice are a commonly used small animal model for the study of neurobiological genetics research, including the impact of physical injury and environmental stress upon neuron connectivity, experiential effects on synaptic plasticity, signal transduction, and for assessing treatments to reduce neurological damage and improve quality of life. In transgenic and gene knockout models in neuroscience and behavioral research, "wildtype" C57Bl/6 mice often serve as the genetic "background" or control for comparison of genetic and genetic/environmental effects (Crabbe, 1999; Crawley *et al*, 1997; Mishina *et al*, 2007). The employment of only male mice is to minimize the experimental design variation by eliminating the hormone cycling effects of female mice.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	*Mus musculus*	
V.3.3.2. Strain/Stock:	C57Bl/6	
V.3.3.3. Source/Vendor:	(b)(4)	
V.3.3.4. Age:	5-12 weeks	
V.3.3.5. Weight:	18-25 grams	
V.3.3.6. Sex:	Males	
V.3.3.7. Special Considerations:	None	

V.3.4. Number of Animals Required (by Species): Mice: 643

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:

Two refinements will be performed or tested in our protocol. 1. Mice will be acclimated to investigator handling before the commencement of behavioral testing, to relieve distress from the potential combination of handling distress and the testing procedures, 2. Pilot studies will be employed to enhance the "quality" of the data acquired in the full-scale and TBI studies. The pilot studies serve as an opportunity to train lab personnel to gain skills in consistency of animal handling. The pilot studies permit "refinement" of the particular parameters of testing procedures and the use of the testing equipment. Finally, 3. Analgesics and anesthetics will be used to decrease possible pain and distress resulting from surgery.

We are using a rodent model (mouse) versus a nonhuman primate model. The mouse model is the least sentient and smallest animal model we could use for this study and still obtain acceptable results. In addition, we are attempting to perform a series of measurements of mouse behavior that

requires making assurances that there is sufficient number of animals to derive valid measures of statistical data.

V.3.5.2. Reduction:

Power calculations have been performed to determine the minimum number of animals needed to reach statistical significance. When possible, mice will be tested in multiple assays (Experiments 3 and 6) and the mice from pilot and full-scale studies will be available for use on other protocols after study completion. Data derived from this protocol will be available to other investigators, allowing them to make better estimates of the minimal number of animals required for their experiments.

V.3.5.3. Replacement:

Biological response to central nervous system injury, psychological stress, and experimental treatments is highly complex; no alternative to *in vivo* experimentation is available or practical.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	224	
V.4.1.1.1.2. Column D:	300	
V.4.1.1.1.3. Column E:	119*	

*These animals are used in Experiment 1.

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

TBI and sham procedures: Mice undergoing CCI or sham procedures will be anesthetized with isoflurane + 100% oxygen (0.5-4% isoflurane, induction; 0.5-3% isoflurane, maintenance). Isoflurane will be delivered using a precision vaporizer via a rodent anesthesia machine. Induction will occur in an appropriately-sized clear viewing chamber. Following induction, rodents will be moved to the stereotaxic device and anesthesia will be maintained via a nose cone. Waste anesthetic gases will be passively scavenged using a charcoal filter. Lack of paw-pinch reflex will be used to ensure adequate depth of anesthesia prior to beginning the procedure. Close attention will be given to ensure the mice remain unconscious and are unresponsive to pain and, if needed, supplemental administration of anesthetic will be given.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:
Mice are will be returned to the animal facility after behavioral testing. They will be monitored in their cages for complications, such as lethargy, reluctance to ambulate, inability to obtain feed, and seizures. It is expected that complications will not be encountered. Any mouse that experiences problems will be evaluated and treated or euthanized as determined by research staff or PI and Veterinarian.

V.4.1.2.3. Paralytics: N.A.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PubMed
V.4.1.3.2. Date of Search: June 20, 2013
V.4.1.3.3. Period of Search: 1986-2013.
V.4.1.3.4. Key Words of Search: Keywords: ("Models, Theoretical"[Mesh] OR "In Vitro"[Publication Type] OR "cell line"[mh] OR "Cells, Cultured"[mh] OR "virtual cell"[All] OR "Computer Simulation"[Mesh] OR "Cadaver"[mh] OR "ex vivo"[All]) AND ("Laboratory Animal Science"[mh] OR "Animal Rights"[All] OR "Animal Care Committees"[All] OR "Animal Use Alternatives"[mh] OR "Animal Testing Alternatives"[mh] OR "Animal Welfare"[mh] OR "Animal Experimentation"[mh]) AND ("reduction"[All] AND "refinement"[All] AND "replacement"[All]) OR "3 Rs"[All] OR "Stress, Psychological"[mh] OR ("Pain"[mh] AND ("Analgesics"[mh] OR "Analgesia"[mh] OR "Anesthesia and Analgesia"[mh])) AND ("Brain Injuries"[mh] OR "Euthanasia, Animal"[mh]) AND ("Mice"[mh] OR "Rodentia"[mh])

V.4.1.3.5. Results of Search: A total of 19 references were identified with this search. None of the results were relevant to alternative methods for biomedical studies in mouse core facilities or alternatives to behavioral testing.

V.4.1.3.6. Sources Searched: Agricola
V.4.1.3.7. Date of Search: June 20, 2013
V.4.1.3.8. Period of Search: No date limits.
V.4.1.3.9. Key Words of Search: mouse AND behavior AND injury
V.4.1.3.10. Results of Search: Two references were returned by this search. One study used cultured neurons to investigate inhibition of fungus-produced toxins (Merrill *et al*, 1993) and the other reported gene expression patterns during early myogenesis in mice (Shoji *et al*, 2009). Neither paper provides alternatives to animal models of traumatic brain injury or *in vivo* behavioral testing.

V.4.1.3.11. Sources Searched: ALTWEB; CAAT (Johns Hopkins Center for Alternative for Animal Testing); AWIC
V.4.1.3.12. Date of Search: June 20, 2013
V.4.1.3.13. Period of Search: No date limits.
V.4.1.3.14. Key Words of Search: Mouse/murine, behavior, injury, standardization
V.4.1.3.15. Results of Search: AWIC returned 90 references from the query listed. All references related to appropriate care, treatment, reduction of pain and euthanasia in laboratory animals.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: We request that some animals (cf. V.4.1.1.1.3) be utilized for experiences considered Category E. Category E mice will be used in testing paradigms that inherently involve some discomfort/distress as a means of "motivating" the animal to respond. We wish to test the mice without treatment with sedatives or analgesics. With the exception of administration of brief and mild shock during the active and passive avoidance tasks (V.1.1 Experiment 1), none of these behavioral tests involve pain or any physical injury; thus an analgesic is contraindicated. While some tests evoke mild distress, treatment with a sedative would significantly alter their level of "motivation" and change their responses to the task.

V.4.2. Prolonged Restraint: N.A.

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions:
Mice will be individually inspected and weighed before all procedures to assure they are in good health for the procedures.

V.4.3.2. Procedure:
All surgical procedures will be performed as described in CRNM SOP#1 and mice will be selected at random for individual procedures. All mice will first be anesthetized as described in Section V.4.1.2.1. All TBI or sham procedures are conducted in a designated area of the CNRM core facilities (Room (b)(6) on a clean table surface that is covered with clean absorbent cloth. The table will be disinfected before and after animal surgeries.

CCI or sham surgery: Two groups of mice will receive a TBI of differing severity level by controlled cortical impact. The mice will be weighed, head hair clipped, and mounted in a stereotaxic frame in a prone position, secured using atraumatic ear bars and an incisor bar. The mouse calvarium will be held in the horizontal plane with respect to the interaural line. To assess anesthetic depth during surgery, the corneal reflex will be evaluated by lightly touching the cornea with a cotton swab to elicit an eye blink. The assessment of the paw and ear pinch reflexes consists of the gradual application of pressure on the hind paw or ear. Lack of eye blink or paw or ear withdrawal is an indication of deep anesthesia. Disposable sterile surgery gloves will be worn by investigators during the surgery. Surgery utensils will be autoclaved before use and cleaned with 70% ethanol after each animal's surgery and a draping will be used around the surgical area. After aseptic treatment with 3 cycles of povidone-Iodine solution alternative with alcohol, an incision and a 6mm (diameter) craniotomy will be made over the left cortex. The bone flap will be removed, and for the two groups of mice undergoing CCI, injury will be produced with the CCI device (Clark *et al*, 1997; Dixon and Kline, 2009), using an impact tip of 3.0 mm in diameter, a penetration depth of 2.0 mm (severe CCI group) or 1.0 mm (mild CCI group) and a velocity of 5 m/s as insult parameters to the dural surface. The location of the injury will be in the parietal region of the brain (2.5 mm posterior to Bregma, 1.5 mm lateral). Following CCI, the scalp incision is sutured. The CCI impactor will be disinfected by 70% ethanol after each use. Surgical instruments will be rinsed in saline and alcohol and disinfected for 10 seconds in a hot-bead sterilizer. Animals will be returned to their home cages following surgery and monitored until they regain consciousness. Mice undergoing sham surgery will receive all procedures described here except the impact with the CCI device.

V.4.3.3. Post-surgical Provisions:
Mice will be monitored at least once per day following TBI or sham procedures. Animals are not expected to experience pain or discomfort from TBI, but if needed will be provided with acetaminophen (Tylenol) in their drinking water (1 mg/ml; ~200 mg/kg b.w.) as an anti-inflammatory precaution. Animals will be observed again one hour after home cage placement to ensure there are no signs of discomfort. If needed, the DLAM veterinarian will be consulted should there be signs of continued discomfort or distress.

V.4.3.4. Location: Housing in DLAM (b)(6) Any-Maze Cage housing (b)(6) Surgery and behavioral testing in the CNRM Pre-Clinical Studies Core facilities (b)(6)

V.4.3.5. Surgeon: (b)(6)

V.4.3.6. Multiple Major Survival Operative Procedures:
V.4.3.6.1. Procedures: N/A
V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:
V.4.4.1. Injections: N/A**V.4.4.2.Biosamples:**
Following behavioral testing, the animals will be euthanized and mice used in pilot and full-scale behavioral experiments and made available for tissue sharing by investigators in the CNRM (an e-mail announcing tissue availability will be sent to CNRM PIs), or the live animals will be transferred to the Department of Laboratory Medicine (DLAM). DLAM will use the animals either for training purposes or as 'sentinels' as appropriate. In cases where animals are euthanized by individuals on this protocol, the tissues will be used for validating and/or acquisition of training and data for a variety of assays. For example, for western blotting biosamples the mouse brain from animals will be removed from the calvarium to obtain samples of the cerebral cortex (fronto-occipital region and entorhinal region), hippocampus, diencephalon, and brain stem. Samples are stored in liquid nitrogen before preparation for western blotting. Other mice may be used for transcardial perfusion for immunohistochemistry biosamples. In this case, the mice will be anesthetized as described in Section V.4.1.2.1. Once the mice are deeply anesthetized and unresponsive to paw pinch, a midline thoracotomy (or abdominal incision) is made to expose the heart, a 20-22 gauge blunt tip needle connected to a tube (¼ in ID and 1/16 in wall) inserted into the left ventricle through the apex and the right atrium incised. After an initial washout with 20-30 ml of cold 100 mM phosphate buffered saline (PBS), transcardial perfusion with 50-75 ml cold 4% paraformaldehyde in PBS will be delivered through a gravity perfusion system until adequate perfusion reached. The brain will then be removed from the calvarium and post-fixed before tissue sectioning for immunohistochemical staining.
V.4.4.3. Adjuvants: N/A
V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A
V.4.4.5. Animal Identification:
All animals will be identified with cage cards. Within cages, tail marks with permanent marker and/or ear punches (performed during surgery while animals are under anesthesia) will be used to identify individual mice.

V.4.4.6. Behavioral Studies:

Experiment 1: Development of an assay for active and passive avoidance in naïve and brain-injured mice. A commercial *place-avoidance system* (Bio-Signal Group, Brooklyn, NY) will be used to collect and analyze data from naïve and brain-injured mice. The apparatus consists of a ~50cm (diameter) metal disk with 40 cm high transparent walls that allows the mice to view distal room cues; the apparatus and testing room will remain unchanged throughout the testing protocol. The procedure will be conducted as previously described (Abdel Baki *et al*, 2009), and testing will take place in four phases over four days:

Phase 1 (habituation/open field): No less than three days after CCI or sham surgery, the mice will be habituated to the testing environment in a single ten-minute open field test in which the animals are free to explore the entire arena. The data from this phase of the experiment will be analyzed to assess exploratory and anxiety-like behaviors in a circular open field environment.

Phase 2 (passive avoidance; PA): Phase 2 takes place 24 hours after habituation. Each mouse will receive four 10-min PA training trials in a single day, with a 10-12 min inter-trial interval (ITI). During these trials, a 60° region of the circular arena is electrified by a grid underneath the floor, and the mouse receives a single .3 mA, 500 ms shock each time the electrified region is entered. Additional shocks are administered every 1.5s until the animal leaves the shock zone. Olfactory cues (urine and feces deposited on the arena floor) and visual cues around the room are used by mice in this phase to learn place avoidance and stop entering the shock-region of the apparatus. An overhead camera and video-tracking system are used to track movements of the mouse and calculate the distance traveled and number of shock zone entrances.

Phase 3 (active avoidance; AA): The day after PA trials, each mouse undergoes six 10-min AA trials with a 10-12 min ITI. During these trials, the same 60° shock region of the arena is stationary while the arena rotates. In these trials, although the electrified region of the arena remains in the same spatial location, the arena rotates and the mouse must keep moving to avoid being shocked. In these trials, only visual room cues can be used by the animal to locate the electrified area as the olfactory cues rotate with the arena. Movements of the mouse are tracked and multiple measures are calculated: the latency to first entrance of the shock zone measures memory of the zone location between trials, number of shocks administered assays the motivation to escape shock; distance traveled, average speed and linearity of movement (average each 2s of the ratio dist(linear)/dist(integrated) provide measures of motor function.

Phase 4: (AA; conflict learning (CL): In the CL trials, the shock zone location is shifted 180° from the original location for six 10-min trials (10-12 min ITI). These trials test proactive interference, or whether the avoidance memory obtained in Phase 3 AA trials conflict with avoidance memory currently being learned in Phase 4. Measures calculated from the camera-tracked movements of the mouse include the distance traveled and the number of entrances into the shock zone.

Experiment 2: Evaluation of 24-hour home cage phenotypic behaviors and circadian rhythms.

Twenty-four hour activity will be evaluated in the Stoelting Company's new mouse *Any-Maze Cages (AMc)*. The AMc is a wooden box measuring 15"H x 20"W x13"D that houses a single mouse and acts as an isolation chamber. It contains food and water, as well as a running wheel, and has the capability to monitor the amount of food and water intake 24 hours a day. In

addition, the chamber has a built-in infra-red-sensitive camera and infra-red and ambient light LEDs. Software controls the light period (ambient light LEDs ON) and dark period (infra-red light LEDs ON/ambient light LEDs OFF). Using Any-Maze software, the camera records the animal's movements and circadian activities "24/7." The chambers are also furnished with a ventilation fan and a temperature sensor. The latter allows the temperature to remain constant and stable within the chamber.

Following a five-day habituation period after arrival, or immediately after CCI or sham surgery, individual mice will be placed in the AMc system to monitor 24-hour activity and food/water intake for up to four weeks after surgery. The plan would be to open the chamber doors once per day to visually inspect the cage and animal and provide fresh food and water, if needed.

Experiment 3: Assessment of spatial learning and memory and visual acuity in mice.
Spatial learning and memory will be initially assessed in the *Morris Water Maze (MWM)* using the procedure described for prior CNRM studies (Budinich *et al*, 2013; Yu *et al*, 2012). A Stoelting Morris water maze (diameter 4.0 m) tank is filled with tap water (25°C) to a depth of 20-30 cm. A clear plastic platform is placed in one of the "quadrants" of the tank floor and visual cues (solid geometric shapes) are placed on the room walls in locations that can be viewed by animals in the pool. On day 1 (no less than seven days after CCI or sham surgery), a single pre-training trial is first used where the mouse is gently placed on the platform in the tank and the mouse remains there for 15 seconds. If the mouse jumps into the water during this time, it is allowed a brief swim (< 60 s) before being guided back to the platform. On day 2, training trials begin where the mouse is placed near the side of the tank in a location away from the platform, and is allotted 90 seconds to swim to the platform. Upon finding the platform the mouse remains on the platform for 15 seconds. If the mouse does not reach the platform in the allotted 90 seconds, they are gently guided to the platform and allowed to remain for 15 seconds. A total of four trials, separated by 1-2 min, are performed each day. Swimming movements of the mouse are tracked, and swim speed, distance before reaching the platform and latency to find the platform are calculated by Any-Maze software. Finally, on the fourth day of training (Day 5) approximately one hour after the final training trial, a single trial is performed with the platform removed from the tank. The time spent in the pool quadrant where the platform was located is measured, compared to time spent in the other three quadrants. After all trials, mice are placed in a clean cage underneath a heating lamp until thoroughly dried (approximately five minutes) to maintain thermoregulation.

Beginning the next day, *reversal training trials* will take place in which the location of the hidden platform is moved to the opposite side of the tank (Zhao *et al*, 2012). The pre-training phase is omitted, and each mouse receives four 90-sec trials for four days. A second probe trial will take place approximately one hour after the final reversal training trial. Finally, immediately after the reversal probe trial, a single visible platform trial will be conducted. A pipette with a very visible "flag" will be secured to the platform. The latency of the mouse to navigate to the cued platform is recorded (maximum of 90 sec).

No less than two days later, a final vision test will be performed with the *visual cliff test (VC)* as described previously (Salinger *et al*, 2003). The VC evaluates the ability of a mouse to see a supposed drop-off on the edge of a horizontal surface. A plexiglas box with a horizontal surface connects to a vertical drop (~ .5 m), connected to a second horizontal plane at a lower level. The horizontal surfaces and vertical drop are covered with brightly patterned adhesive to accentuate

the drop-off. A piece of Plexiglas extends from the higher horizontal plane, extending across the drop-off and providing a solid horizontal surface. The mouse is placed close to the edge of the upper horizontal surface and the movements of the mouse are tracked by Any-Maze software. A mouse that "sees" the drop-off will retreat and move away from the false edge; a mouse with reduced visual abilities may move across the "cliff" without pausing (Crawley, 2007). C57BL/6 mice with eyesight intact will show 80-90% safe responses, meaning they step back toward the higher horizontal surface (Fox, 1965).

As this test is often confounded by other sensory abilities (Crawley, 2007), a blind animal may rely successfully on non-visual senses including tactile feedback from the whiskers. We are requesting that we be allowed to shave off the whiskers prior to this test. As this is the last test performed in this experiment, animals will be euthanized within 24-hr after whisker-removal.

Experiment 4: Mouse performance on a working memory MWM task and olfactory-avoidance test.

Mice typically show avoidance behaviors toward spoiled smells including aliphatic acids (Hebb *et al*, 2002). The *olfactory-avoidance test* assesses the ability to detect an aversive scent (Siopi *et al*, 2012) and is performed in a clean open field arena (40 cm x 40 cm) no less than three days after sham or CCI surgery. The mouse is first habituated in the test environment for 10 min, after which two pieces of filter paper (each 5 cm x 5 cm), one sprayed with water and the other with acetic acid (a short aliphatic acid) diluted to 5% are introduced in successive 3-min trials with one minute between each trial. Movements of the mouse during the trials are tracked by an overhead camera linked to Any-Maze software, and the software calculates the average distance of the mouse from the filter paper. Additional scoring performed by an investigator blinded to the surgical condition of the mouse may include the number of investigations of the water- and acid- soaked papers and the comparative amount of time each mouse spends investigating each piece of filter paper. The arenas will be thoroughly cleaned with 70% ethanol and dried after each use to remove all olfactory cues.

No less than three days later, the same mice will be tested in a *modified version of the MWM designed to test short-term (or working) memory* (Steele and Morris, 1999). The water tank and basic procedure used is the same as Experiment 3, but the platform is moved to a new location each training day, creating what is called 'delayed matching to place.' In this modified procedure the animal cannot know the location of the platform on the first trial of each day, but once this information is gained, the information is 'stored' for subsequent trials on that day. Each mouse will receive four trials per day, and acquisition is expected to take 8-10 days. The inter-trial interval can be modified to control difficulty of the task; we will begin with a 1-2 s interval as described in Experiment 3, but may increase that interval to make the test more challenging.

Experiment 5: Exploration, anxiety and spatial memory in a modified hole-board environment.

The modified hole-board test has the advantage and being able to measure exploration, anxiety and memory in a single test (van der Kooij *et al*, 2010). The hole-boards are 40 cm x 40 cm inserts that fit within our current open field chambers, and they contain 16 holes evenly-spaced (~ 7cm), each with a diameter of 3 cm and depth of 1 cm. Several printed geometric shapes

located on the walls of the room serve as spatial cues to the animals in the apparatus. The body-weight of mice will be reduced to approximately 85% of their free-feeding weight for four days prior to the beginning of the experiment; food restriction will begin no less than three days after TBI or sham surgery (Anon.; Rowland, 2007; Toth and Gardiner, 2000). Body weight will be taken each morning to monitor weight loss and adjust food as needed, and animals will be physically inspected to ensure there are no signs of ill health (Anon., 2011). For two consecutive days, the mice are given a single three minute acclimation to the test environment. During habituation, all 16 holes will be baited with a small food reward (.01-.02 g almond) to encourage visits to the holes. Video tracking reports results on locomotion, time spent in the center of the apparatus and the frequency of head-pokes into a hole. On the third day, only four of the holes will be baited with food and the pattern of the location of food rewards will remain consistent for the remainder of the experiment. Each mouse then undergoes spatial learning trials, four trials per day for three days. Each trial lasts until the mouse finds all four food rewards, and the latency to complete the trial is recorded for each mouse. Olfactory cues will be eliminated by placing a small drop of almond extract in the holes that do not contain the food reward. On the sixth day (the day immediately following the final spatial learning trial) a reversal-task will begin. During this part of the test, the locations of the food rewards will be scrambled. Mice will be trained for three days with four trials per day. Each trial lasts until the mouse finds all four food rewards.

Experiment 6: Evaluation of episodic learning and memory with the Novel Object Recognition test, and assessment of irritability in mice

The Novel Object Recognition (NOR) test was first developed by Ennaceur and Delacour (Ennaceur and Delacour, 1988) and adapted for use in mice by Dodart and colleagues (Dodart *et al*, 1997). The NOR test assesses the ability of an animal to remember a prior object in the environment. The task will begin no less than three days after sham or TBI surgery and consists of three phases: a) Habituation – the mouse is allowed to freely explore the environment in the absence of objects for one hour. B) Acquisition – 24 hours after habituation – the mouse is placed back into the familiar arena containing two identical objects for ten minutes. C) Test – one hour after Acquisition – for ten minutes the mouse is returned to the arena containing two objects: one identical to the same from the acquisition phase, the other is novel. During the acquisition and test phases the two objects are located in opposite and symmetrical corners, and the location of novel vs. familiar is counterbalanced between subjects. The amount of time exploring each object in the Test phase is compared; a mouse that remembers the objects from the Acquisition phase will spend a larger proportion of time exploring the novel object of the Test phase.

No less than three days after completion of the NOR test, *irritability* will be assessed in mice employing the "bottle brush" method (Riittinen *et al*, 1986). Mice will be housed individually for the five days of the test. The mouse will be gently prodded, or what is also called, "nudged," in the home cage with a clean white bottle brush moving against it, and each mouse is nudged 20 times daily, consecutively, with 10-15 sec between each stage. Each prodding consists of five stages: a) Rotating brush approaching the mouse from the opposite side of the cage, b) Rotating brush touching whiskers of the mouse, c) Rotating brush returning to starting position on opposite side of the cage, d) Rotating brush in starting position, e) Brush (not rotating) at starting position. Each stage of the prodding is about 1.5-2 sec, except for the last stage which can be prolonged waiting for the mouse to return to its end of the cage.

The following responses will be observed and recorded during the prodding: a) Escape, b) Biting, c) Digging, d) Jumping, e) Boxing, f) Grooming, g) Climbing, h) Following, i) Exploration, j) Tail rattling. Behaviors of the mouse are never to be interrupted unless the mouse climbs onto the edge of the cage (at which point the investigator's hand should gently nudge the mouse back down) or if the mouse jumps out of the cage (an unlikely event since the cage walls are ~25 cm height), it will be immediately captured and returned to the appropriate end of the cage. The tests occur in a small room with a closed door that has a sweep guard, so there is a low chance of losing a mouse. If the mouse "follows" the brush to the opposite side of the cage, the mouse is gently nudged by hand to its side of the cage.

V.4.4.7. Other Procedures:

Transfer of mice (after testing) to DLAM. If possible, some of the mice used in this protocol will be transferred to DLAM. DLAM will be notified of the pending availability of mice, which may be used for other training purposes by DLAM or for sentinel purposes.

Decapitation for tissue sharing or for protein analyses by western blotting and immunohistochemistry. At the study endpoint, the mice will be anesthetized as described in Section V.4.1.2.1. Once the mice are deeply anesthetized and unresponsive to paw pinch, mice are decapitated using a guillotine designed for rodents (cf., **V.4.6. Euthanasia**) for western blotting. As indicated under Sections V.4.4.2 and V.4.6. for immunohistochemistry the animals must be perfused with fixative before removal of the brain.

V.4.4.8. Tissue Sharing:

Excess mouse tissue will be made available to other investigators in our or other labs.

V.4.5. Study Endpoint:

The study endpoint is survival for the previously described durations (10-16 weeks of age), at which time mice will be euthanized. Euthanasia will be performed as stated.

V.4.6. Euthanasia:

Euthanasia will be performed in accordance with the guidelines of the "2013 Report of the AVMA Panel on Euthanasia." Methods of euthanasia on this protocol include decapitation and perfusion. Mice are first anesthetized for these procedures.

Perfusion: Following anesthesia per Section V.4.1.2.1 we will ensure mice are deeply anesthetized and unresponsive to paw pinch then a midline thoracotomy is made to expose the heart, a 20-22 gauge blunt tip needle connected to a tube (¼ in ID and 1/16 in wall) inserted into the left ventricle through the apex and the right atrium incised. After an initial washout with 20-30 ml of cold 100 mM phosphate buffered saline (PBS), transcardial perfusion with 50-75 ml cold 4% paraformaldehyde in PBS will be delivered through a gravity perfusion system until adequate perfusion reached. The brain will then be removed from the calvarium and post-fixed before tissue sectioning for immunohistochemical staining.

Decapitation: Following anesthesia per Section V.4.1.2.1 we will ensure mice are deeply anesthetized and unresponsive to paw pinch then use a rodent guillotine to decapitate the anesthetized mice. The decapitation equipment will be cleaned and maintained to ensure the proper function by routinely checking to ensure it is not rusted or blunt. It will be either sharpened or replaced if it does not function properly. A log book will be kept of the maintenance of the guillotine.

In the event that LAM personnel must euthanize an animal for humane reasons, cylinderized carbon dioxide will be used in the LAM necropsy room. Using a clean cage carbon dioxide will be administered at a rate to replace greater than 20% of the cage volume per minute. Once the mice have not moved or breathed for at least one minute they will be removed from the chamber. Euthanasia may be ensured by cervical dislocation or opening of the thorax.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for the mice used in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s (b)(6)

Building(s) Room Number(s

Building(s) Room Number(s

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes X No

Fluid Restriction: Yes No X

V.5.1.3. Exceptions: Mice tested in the modified hole-board experiment (V.1.5. Experiment 5; n=85) will be restricted to approximately 85% of free-feeding body-weight beginning four days before the experiment. This restriction is necessary for the mice to be motivated during the experiment which requires them to remember the location of small food rewards. To ensure proper weight loss, mice will be weighed every morning. The diet provided will be altered to meet the ~85% weight loss. Mice from the same cohort will be used to monitor body weight change (Anon., 2011).

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:

The PI or research staff will observe the mice following all procedural manipulations. DLAM personnel will observe the mice at least once a day for general health and husbandry condition. Any mouse observed to be lethargic, losing weight, exhibiting gross neurological deficits (such as inability to eat or drink, continual circling, seizures, etc.) or any other signs of distress or illness will be evaluated by the PI and/or veterinary staff then euthanized as previously described.

V.5.2.2. Emergency Veterinary Medical Care:

All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies except as described below. All mice will be group-housed, and standard nestlets provided by LAM will be provided in each cage.

V.5.3.2. Enrichment Restrictions: After completion of the NOR test in Experiment 6, we are requesting these animals be individually housed for the duration of the irritability part of the experiment (five days).

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Surgery (controlled cortical impact)	(b)(6)	(b)(6) has several years of experience performing this procedure in mice. She has trained (b)(6) under prior protocol (b)(6) (b)(6) and will instruct him further as necessary.	Prior TBI studies under (b)(6) will be trained by (b)(6) (b)(6)
Rodent Behavioral Testing		(b)(6) has > 15 years of experience in animal handling and behavioral testing and an M.S. in Behavioral Neuroscience. (b)(6) has two years of experience with testing, and (b)(6) has been trained under prior protocol (b)(6)	Personal laboratory experience (b)(6) will be trained by (b)(6) (b)(6)
Anesthetic injections		(b)(6) and (b)(6) all have many years of experience in animal handling and drug administration..	All listed investigators have taken the Rodent Handling and Procedural Techniques.*
Euthanasia by decapitation		(b)(6) experience with rodent decapitation >30 years. He will instruct listed colleagues on this procedure.	Performed similar procedure for recent Protocols (b)(6) (b)(6)
Intracardial perfusions		(b)(6) & (b)(6) have many years of experience with perfusions.	Personal laboratory experience (b)(6) (b)(6) and (b)(6) (b)(6) will receive training from the approved personnel on this protocol.

*All listed investigators have attended the USUHS Investigator Training Course: Dr (b)(6) on August 17, 2005, (b)(6) completed several online USUHS Investigator Training Courses on October 28, 2009 and attended the Rodent Handling and Procedural Techniques on November 19th, 2009. She is previously approved to perform the above procedures under (b)(6) Dr. (b)(6) has completed the online USUHS Investigator Training Course on September 9, 2008 and the Rodent Handling and Procedural Techniques on September 24, 2009. She is approved to perform the above procedures under protocol (b)(6) completed several USUHS online investigator training courses including "Working with the IACUC" (non-VA) on April 23, 2013 and the USUHS Investigator Training Courses on June 6, 2013 (b)(6) completed USUHS online training on February 7, 2013 and attended the USUHS Investigator Training Course on August 16, 2012.

(b)(6)

VII. BIOHAZARDS/SAFETY:

To minimize exposure to animal allergens when working with lab animals, lab personnel will wear lab coats and/or scrubs, gloves and masks. We will always to wear lab coat and gloves when we contact toxic chemicals such as phenol/chloroform during RNA isolation and perform some steps in a fume hood to minimize risk of exposure to these teratogens. Potential fumes

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. **Training:** I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) 6/24/13

Prin Date

G. **Training:** The following personnel will attend the next USUHS Investigator/Animal User Training Course:

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6)

Princi _____ 6/25/13
Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL** or **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Prin _____ 6/26/13
Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number: (b)(6)

D. **Animal Protocol Title:** Development of normative data for behavior testing in the CNRM Mouse Behavioral Assessment Core

C. Principal Investigator: (b)(6) Ph.D.

D. Performing Organization: Uniformed Services University of the Health Sciences

E. Funding: $491,383

F. Objective and Approach:

The goal of this research is to integrate new behavioral tests into the CNRM Pre-Clinical Studies Core's current offering of functional tests for scientific investigators. In the past three years the Core has offered over 20 behavioral tests for investigators to use for behavioral assessment in their experiments. The majority of these tests have been validated in control animals and used in traumatic brain injury (TBI) research, and the results are available to all investigators interested in employing behavioral testing in their studies on brain injury. This research will expand the offerings to include more sensitive cognitive tests, tests of sensory systems including vision and olfaction, and an assay of irritation, a neuropsychiatric complaint of brain-injured human patients. A database of "normative" information on uninjured C57BL/6 adult male mice will first be collected to validate testing equipment and data-collecting procedures. We will then use controlled cortical impact (CCI) to induce TBI at mild and severe levels to determine the impact of this brain injury on functional performance and to identify behavioral tests that are able to discriminate mild injuries.

To approach this problem we will use laboratory mice. Mice will be used in a series of behavior tests to assess their performance and gather basic statistical information about the scores obtained by a "typical" mouse and by a mouse with brain injury. The data collected will then be summarized using conventional statistical approaches (mean, standard deviation, analysis of variance, correlation and regression analyses). We will use as few mice as possible while still using enough to make valid conclusions. We must undertake this work in live mice to determine behavioral testing scores to "standardize" the procedures for testing mice and to provide other experimenters with "normative" data about what they can expect will be the typical behavioral patterns of their mice when they use the core facility. To do this initial research we must use animals since cells or tissues will not reflect the actual biology of a live organism. We hope that this basic research and use of animals will aid in understanding some of the causes for the long-term effects stress and brain injury has upon warfighters returning home to their towns, work, and families, and that this research may allow us to provide better treatments to help suffering soldiers.

G. Indexing Terms (Descriptors): Animal, mice, traumatic brain injury, behavioral testing

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4712
http://www.usuhs.mil

June 2[illegible]

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on June 20, 2013:

Animal Protocol Title: "Nanotechnology-based Intranasal Brain Delivery of Neuroprotectants in Rat Models"

USUHS Protocol Number: (b)(6)

Expiration Date: June 19, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6) Ph.D.

(b)(6) Ph.D.
Vice-Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

Learning to Care for Those in Harm's Way

Revised

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET
IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Nanotechnology-based Intranasal Brain Delivery of Neuroprotectants in Rat Models.

GRANT TITLE (if different from above): 1) Intranasal CNS delivery of drugs to protect against organophosphorous threat agents (NIH, active).

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: NIH (active)

EARLIEST ANTICIPATED FUNDING START DATE: Ongoing

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6)	APG	(b)(6)	4/29/2013
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	[illegible]	(b)(6)	04/29/2013
Research Unit Chief / Dept. Head Signature Typed Name	Title	Telephone	Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

		~~Telephone~~	~~Date~~
Statistician Signature Typed Name	Department		

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

LAM (b)(6)

(b)(6) LAM (b)(6) 29 Apr 2013

Attending/Consulting Veterinarian Signature — Department — Telephone — Date

Typed Name: (b)(6) DVM

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

ANIMAL PROTOCOL TITLE: Nanotechnology-based Intranasal Brain Delivery of Neuroprotectants in Rat Models.

GRANT TITLE (if different from above): 1) Intranasal CNS delivery of drugs to protect against organophosphorous threat agents (NIH, active).

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): Drs (b)(6) Ph.D., (b)(6) Ph.D., (b)(6) Ph.D.

TECHNICIANS(S): Ms. (b)(6) B.S., (b)(6) M.S

I. **NON-TECHNICAL SYNOPSIS:** Nose to brain delivery bypasses the blood brain barrier (BBB) to rapidly deliver drugs to the central nervous system (CNS) via multiple neural pathways wherein drugs are observed in the brain within 10 minutes of application in humans. The major advantage of this approach is that charged molecules or even high molecular weight drugs, which cannot bypass the BBB from the general circulation, can be rapidly delivered to the CNS. Efficiency of nose-to-brain delivery can be significantly improved by adjusting the physicochemical properties of the formulations using membrane-adhesive nanoemulsions or biodegradable nanoparticles. Our long term objective is to establish and optimize for human use the efficacy of intranasal brain delivery systems for use with chemical threat agent-related therapeutics, which can be of rapid and practical use in preventing and treating chemical threat agent mediated casualties. The immediate goal is to develop an intranasal nanoemulsion approach for this purpose and optimize effectiveness in a preclinical model system in the rat. Use of nanoemulsion technology for intranasal brain delivery of drugs is an innovative approach to bypass the BBB and efforts along these lines are underway in many laboratories. The successful use of an intranasal brain delivery technology against chemical threat agents will open up its wider application in numerous other areas of clinical medicine and research.

II. BACKGROUND:

II.1. Background:

Exposure to organophosphorous chemical threat agents (CTAs) is a potential risk for military and the civilian populations alike. CTAs exert their toxic effects by inhibiting acetylcholinesterase (AChE) leading to the accumulation of acetylcholine at synaptic and neuromuscular junctions resulting in symptoms of acute CTA poisoning including salivation, lacrimation, defecation, muscular twitching, seizures, status epilepticus and ultimately death due to respiratory failure. Available treatment for acute CTA poisoning includes combinations of: 1) oxime to reactivate the inhibited AChE; 2) atropine to

antagonize the action of excess acetylcholine formed at muscarinic receptors and 3) diazepam or midazolam to allosterically potentiate action of the inhibitory neurotransmitter γ-amino butyric acid (GABA) at GABAA receptors. The two major limitations of the current therapeutic strategies are: 1) poor blood-brain barrier (BBB) permeability of oximes and 2) resistance to reactivation of the CTA-inhibited AChE due to its rapid 'aging' [1]. Quick delivery of oximes to the brain is critical to reactivate the rapidly 'aging' AChE in the brain and protect the brain from acute and subsequent chronic injuries.

Non-invasive intranasal nose-to-brain delivery of therapeutics was first invented by William H. Frey II in 1989 and patented in 1997. This method bypasses the BBB to rapidly deliver drugs to the CNS extracellularly and paracellularly along the olfactory and trigeminal neural pathways [2] wherein drugs are observed in the CSF within 10 minutes of application in humans . Dr. Frey's patented intranasal insulin treatment has been found to improve memory, attention and functioning in patients with Alzheimer's disease in clinical trials performed by Dr. Suzanne Craft and colleagues [3,4] and to improve memory in normal human adults as well [5]. Intranasal treatment has also been used for treating stroke [6], brain tumors [7], seizure and many other neurological disorders in animal models [8-13]. The major advantage of this approach is that charged molecules or even high molecular weight drugs, which cannot bypass the BBB from the general circulation, can be rapidly delivered to the CNS. Efficiency of nose-to-brain delivery can be significantly improved by adjusting the physicochemical properties of the formulations using mucoadhesive nanoemulsions or biodegradable nanoparticles [44, 14-23]. Our central hypothesis is that intranasal administration of therapeutic formulations of oxime, atropine and midazolam in a nanoemulsion formulation can rapidly deliver significant amounts of these drugs to the brain and small amounts to lungs and the blood stream to protect against CTAs up to 30 minutes after exposure. Our long term objective is to establish and optimize for human use the efficacy of intranasal brain delivery systems for use with chemical threat-related therapeutics, which can be of rapid and practical use in preventing and treating CTA casualties. The immediate goal is to develop an intranasal mucoadhesive nanoemulsion approach for this purpose and optimize effectiveness in a preclinical model system.

Use of nanoemulsion technology for intranasal brain delivery of drugs is an innovative approach to bypass the BBB and efforts along these lines are underway in many laboratories. The proposed intranasal brain delivery technique using nanoemulsion needs to be investigated for protection against CTAs or any other situations involving civilian mass casualty. The major advantages of the successful use of this non-invasive technology against CTAs are: 1) it can rapidly deliver oximes (which cannot cross the BBB under physiological conditions) to the brain in addition to the lungs and blood to reactivate the inhibited AChE; and 2) it can be self-administered immediately after exposure to prevent 'aging' of the inhibited AChE and its resistance for reactivation. The successful use of an intranasal brain delivery technology against chemical threat agents will open up its wider application in numerous other areas of clinical medicine and research.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s)

Literature Sources Searched: PubMed, BRD, FEDRIP

II.2.2. Date of Search: 2-19-2013

II.2.3. Period of Search: 1970-present

II.2.4. Key Words and Search Strategy: Intranasal brain delivery, nanoemulsion, nanoparticle, nerve agent, paraoxon, rat.

II.2.5. Results of Search: PUBMED: Six articles were found when keywords intranasal brain delivery and nanoemulsion were combined. None of them involves the proposed work involving nerve agents. BRD: None. FEDRIP: None.

Therefore, the proposed research and the goals do not duplicate earlier research in these areas of research.

OBJECTIVE\HYPOTHESIS: Our central hypothesis is that intranasal administration of therapeutic formulations of oxime, atropine and midazolam in a nanoemulsion formulation can rapidly deliver significant amounts of these drugs to the brain and small amounts to lungs and the blood stream to protect against CTAs at 30 min or later time points. Our long term objective is to establish and optimize for human use the efficacy of intranasal brain delivery systems for use with chemical threat-related therapeutics, which can be of rapid and practical use in preventing and treating civilian mass casualties. The immediate goal is to develop the intranasal mucoadhesive nanoemulsion approach for this purpose and test them for effectiveness in a preclinical model system.

III. MILITARY RELEVANCE:

Exposure to organophosphorous chemical threat agents (CTAs) is a potential risk for military and the civilian populations alike. Use of nanoemulsion technology for intranasal brain delivery of drugs is a highly innovative approach to bypass BBB and efforts along these lines are underway in many laboratories. The proposed intranasal brain delivery technique using nanoemulsion needs to be investigated for protection against CTAs or any other situations involving civilian mass casualty. The major advantages of the successful use of this non-invasive technology against CTAs are: 1) it can rapidly deliver oximes (which cannot cross the BBB under physiological conditions) to the brain in addition to lungs and blood to reactivate the inhibited AChE; and 2) it can be self administered immediately after exposure to prevent 'aging' of the inhibited AChE and its resistance for reactivation. The successful use of an intranasal brain delivery technology against chemical threat agents will open up its wider application in numerous other areas of clinical medicine and research

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

1. Animal studies to determine the optimal mucoadhesive nanoemulsion for the proposed neuroprotection studies: Male Sprague Dawley rats (220 to 250 gm) will be used for these studies. Rats will be anesthetized by exposure to isoflurane (3%). Animals will be given intranasal administration of 2-PAM made in different nanoemulsions (3 different particle sizes of chitosan as well as hyaluronate based mucoadhesive nanoemulsions) as well as nanoparticle forms up to a maximum volume of 50 µl in the supine position with the head tilted back so that the formulation can drain to the olfactory epithelium at the roof of the nasal cavity under anesthesia. Changes in blood pressure or mucous secretions are not expected to affect the uptake of the neuroprotectants under this condition. Also, only one nasal passage way will be used at a time and the viscosity of the emulsion will be adjusted in such a way that it will not have any adverse effect on the epithelial layer. The animals will be euthanized at 30 min and 2 hrs after intranasal administration. Brain, lungs and blood will be collected for measuring the levels of 2-PAM using HPLC/MS. In the case of brain, different regions (olfactory bulb, cortex, hippocampus, cerebellum and brain stem) will be dissected for analysis. Spinal cord also will be collected for determining the delivery to the whole CNS. Group 1: Animals given intranasal administration of 2-PAM made in chitosan based nanoemulsions –30 rats (5 animals each for three different particle sizes and 2 time points). Group 2: Animals given intranasal administration of 2-PAM made in hyaluronate based nanoemulsions –30 rats (5 animals each for three different particle sizes and 2 time points). Group 3: Animals given intranasal administration of 2-PAM made in nanoparticle preparations –30 rats (5 animals each for three different particle sizes and 2 time points). **Total number of rats required for this part is 90. Pain Category C = 0, D = 90 , E = 0**

2. Determination of the optimum doses of drugs for combination in the optimal nanoemulsion or nanoparticle preparation: Three different concentrations of atropine, 2-PAM and midazolam (up to the doses used for intramuscular route) will be made using the optimum mucoadhesive formulation obtained from the above study for determining the optimum doses of drug combination for the neuroprotection studies. The doses used for intramuscular route for atropine, 2-PAM and midazolam are 2 mg/kg, 25 mg/kg and 5 mg/kg respectively. Rats will be anesthetized by isoflurane as mentioned above. Three serial dilutions of the drug combinations will be made in the optimal nanoemulsion/nanoparticle preparation and will be given intranasally as described above and the animals euthanized at 30 min and 2 hrs. Brain, lungs and blood will be collected for measuring the levels of drugs using HPLC/MS. In the case of brain, different regions of the brain (olfactory bulb, cortex, hippocampus, cerebellum and brain stem) will be dissected for analysis. The optimal doses of each drug will be selected based on the highest uptake into the brain. Total number of animals required for this part of the study = 5 animals/group x 3 doses x 2 time points = 30. **Pain Category : C = 0 ; D = 30 ; E = 0 .**

3. To test efficacy of the intranasal brain delivery method of CTA

therapeutic formulation for protection against the insecticide (CTA) paraoxon

The protective efficacy of the optimal therapeutic formulation containing the drug combination obtained from the above study will be tested against the model CTA, the insecticide paraoxon in radiotelemetry probe implanted rats. About one week after the radiotelemetry probe implantation, therapeutic formulation containing the mixture of all the three drugs will be given intranasally 30 min after exposure to paraoxon (1.0 mg/kg s.c). **This dose was selected based on the observation that it will cause status epilepticus in 75% of the animals (24).**The protective efficacy will be compared to that of the traditional intramuscular route. The protective efficacy will be determined using mortality, EEG measurement using radiotelemetry and neuropathological examination using hematoxylin/eosin staining in addition to measuring the activity of AChE and butyrylcholinesterase (BChE) in the blood and different tissues including different regions of the brain. **Group 1: (control, 16 rats)**; Group 2: (Paroxon exposed, 16 rats) – rats given paraoxon; Group 3: (intranasal treatment group, 16 rats) - rats given paraoxon + intranasal treatment 30 min after the paraoxon injection; Group 4: (intramuscular treatment group, 16 rats) – rats given paraoxon + traditional intramuscular treatment with atropine (2 mg/kg), 2-PAM (25 mg/kg) and midazolam (5 mg/kg) 30 min after paraoxon injection. This experiment will be repeated once to confirm it. Total number of rats implanted with radiotelemetry probe is **128. Pain Category C = 0 , D = 32 , E = 96.**

Pilot dose response study: As recommend by the reviewer, a pilot dose response study will be carried out using three doses around the proposed dose given above. The final dose to be used will be determined based on this pilot study. Pain category C = 0, D =16, E = 48. Total = 64.

Mortality and EEG measurements will be carried out up to 24 hrs post paraoxon exposure. At the proposed dose level, the animals are expected to experience prolonged seizures starting in about 5 min of treatment with about 75% of animals exhibiting status epilepticus. Also, the animals are expected to display a combination of chewing, head-bobbing, single and bilateral limb clonus and rearing leading to constant full body tremors. Animals will be monitored at 30 min intervals during the first 2 hours and every 8 hrs intervals thereafter including the evening hours. Half the number of survived animals after 24 hrs will be euthanized and blood/tissues collected for AChE and BChE assay. In the case of brain, different regions of the brain (olfactory bulb, cortex, hippocampus, cerebellum and brain stem) will be dissected for the enzyme analysis.

For histopathological analysis, 24 hrs post-parathion/paraoxon exposure, the other half of rats will be perfused transcardially with 4% paraformaldehyde under anesthesia. Brain and lung will be collected for histopathological analysis using hematoxylin/eosin staining. The histopathological analysis will be carried out by the board certified pathologist available in the Laboratory Animal Medicine at AFFRI. EEG recording and analysis will be carried out with help from our departmental colleague, Dr. (b)(6) using the Stellate (Montreal, Quebeck, Canada) EEG monitoring system. EEG recordings will be visualized offline with filter settings at 0.3 Hz low frequency filter, 60

Hz notch filter, and 70 Hz high frequency filter, using the Harmonie Viewer 6.1c from Stellate. An electrographic seizure will be defined as a period of EEG changes marked by an abrupt starting and termination that includes a minimum of 10 s of consistent, repetitive discharges at least double the amplitude of the background activity and not less than 1Hz in frequency. A single graphoelement will be classified as sharp wave when it is clearly distinguishable from movement artifacts, has twice the amplitude of the background EEG and is less than 1 ms in duration.

Calculation of animal numbers:

Experiment 1 + Experment 2 + Experiment 3 + **Pilot dose response study** +10% extra for training procedures and anesthesia failures = **90 + 30 + 128 + 64 + 31 = 343**; **Pain Category C = 0 , D = 185 , E = 158**

V.2. Data Analysis: Data will be presented as mean ± SEM. Parametric data will be analyzed using a paired 2-tailed t test for the paired data or a 1-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons post test. Nonparametric data (histology data, behavioral data) will be analyzed using a Wilcoxon signed rank test for paired data or a Kruskal-Wallis test (nonparametric ANOVA). An associated probability value of $P < 0.05$ will be considered significant.

Power analysis using the quantitative data obtained from our earlier studies have indicated that for most of our behavior type studies a sample size of 10 animals per group will have 80% power to detect a difference of 1.5 standard deviations between groups assuming a 5% significance level, and should be sufficient for the outcomes we intend to measure; power calculations were performed using nQuery Advisor® 4.0 software. Similar calculations have indicated that 5-6 animals/group is sufficient for biochemical and immunohistochemical studies. The total number of animals for each experiment has been calculated based on the above considerations. Sixteen animals/group are for the EEG studies involving paraoxon treatment in view of the expected mortality in this study.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: The specific nature of the project does not permit use of non-animal alternatives. The global physiological changes occurring in different cell types in the *in-vivo* brain injury models cannot be reproduced in single cell culture system, or any alternatives.

V.3.2. Animal Model and Species Justification: Male Sprague-Dawley (SD) rats (Rattus norvegicus) will be used for these experiments. SD rats have been chosen as a suitable species and animal model for this research because they have been extensively used in nerve agent research to evaluate new medical countermeasures.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	Rattus Norvegicus	
V.3.3.2. Strain/Stock:	Sprague-Dawley	
V.3.3.3. Source/Vendor:		
V.3.3.4. Age:		
V.3.3.5. Weight:	220-250g	
V.3.3.6. Sex:	Male	
V.3.3.7. Special Considerations:		

V.3.4. Number of Animals Required (by Species): 343

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1.Refinement:

Rats will be anesthetized with isoflurane and reach a surgical plane of anesthesia for restraint purposes before decapitation for collection of brain tissues. All EEG surgery will be performed under appropriate anesthesia and pre-and post-surgical analgesics will be used to relieve pain. For example, Lidocaine jelly will be used at ear pressure points and Bupivicaine used at EEG electrode placement. Any rat exhibiting signs of pain or impairment beyond that from this model will be euthanized at an early endpoint.

V.3.5.2. Reduction:

We have considered reducing the total number of control animals by avoiding separate control groups. However, in view of the variations between animals, especially in behavioral parameters, we do not feel comfortable in comparing with a control group done on another day. Therefore, we are unable to reduce the number of animals in the control group. Also, we have tried to use the minimum number of animals for each experiment based on our prior experience and experience of other investigators in the field.

V.3.5.3. Replacement:

In silico methods and tissue culture approaches cannot be used for evaluation of the intranasal transit of drugs to the brain, or to assess their effects on paraoxan toxicity, since these actions can only be observed in an intact living animal. Also, animals lower than rodents in the phylogenetic scale cannot be used to model brain injury studies in human, because of wide anatomic and physiological differences.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:		
V.4.1.1.1.2. Column D:	185	
V.4.1.1.1.3. Column E:	158	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

For surgical implantation of cortical electrodes for EEG recordings, animals will receive one dose of buprenorphine (0.05 mg/kg s.c.) before any incisions are made. The animals will be anesthetized with isoflurane (3% for induction, 2.0-2.5% for maintenance; with oxygen). Anesthesia will be induced by isoflurane vapor in 100% oxygen in a commercially available induction chamber. Once anesthesia is induced, animals will be transferred to the designated surgery area and anesthesia will be maintained via nose cone. To monitor the depth of anesthesia (including during surgery time) we will test whether the animal has lost its toe pinch (pedal withdrawal) reflex.

Prior to incision, animals will receive bupivicaine (0.25% solution, up to 0.5 ml) at electrode placement sites (infusion of surrounding tissue), at a maximum of 0.2 ml within any one animal, and buprenorphine HCl (0.05 mg/kg, s.c.) to assist in post-operative pain management. If animals continue to show signs of pain or stress such as a hunched posture, not eating/drinking, reluctance to move during the recovery period, we plan to give additional doses at 8 hour intervals (see below).

Following surgery for implantation of cortical electrodes, animals will receive buprenorphine HCl (0.05 mg/kg, s.c.), which will be given at a minimum one time at the end of the day of surgery, just before close of business. This is in addition to the preemptive dose given prior to surgery. All surgeries will be performed in the AM. Additional doses will be given every 8-12 hours, up to 3 days after the surgery as needed, in consultation with the attending veterinarian.

The buprenorphine dose may be increased on consultation with a LAM veterinarian, up to a dose of 0.25 mg/kg i.m. or s.c. at each buprenorphine dosing time point, if animals continue to show signs of pain or stress such as a hunched posture, not eating/drinking, reluctance to move.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:.

For the EEG studies, following paraoxon exposure the animals will be closely monitored for signs of seizure onset or to determine if they have developed serious complications that may require early euthanasia (see V.4.4.7 and V.4.6 sections below). As mentioned earlier, the animals are expected to experience prolonged seizures starting in about 5 min of treatment with about 75% of animals exhibiting status epilepticus.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PubMed, Agricola:

V.4.1.3.2. Date of Search: 3/28/13

V.4.1.3.3. Period of Search: 1966-2012

V.4.1.3.4. Key Words of Search: animal, rat, pain, analgesia, transcardial perfusion, seizure.

V.4.1.3.5. Results of Search: PUBMED: A total of 139 publications were obtained when all key words except transcardial perfusion were used. However, none appeared when transcardial perfusion was included in the search. All of the 139 publications involved studies on different aspects of pain, including neural mechanisms and newer pain relieving agents. When key words rat and transcardial perfusion were combined, 47 publications describing studies involving the use of transcardial perfusion were obtained. None of them provided any alternatives to the proposed painful procedures. Agricola: None of the publications provided an alternative to the painful procedure used in this protocol.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

The goal is to develop intranasal brain delivery methods for protection from nerve agents. There is no alternative for inducing brain damage in rats that mimics the damage caused by nerve agents to determine whether a compound is neuroprotective. The only way to determine the neuroprotective efficacy of a putative drug is to cause actual status epilepticus in an animal model to induce damage prior to administration of the neuroprotective compound and to determine the number of surviving neurons and improvements in other CNS pathologies.

Without exposing the animals to nerve agents or model compounds, it is not possible for us to conduct these studies. These studies are justified because the use of nerve agents against military personnel in international conflicts, and also against civilians, is a major threat at the present time. Thus, the knowledge obtained from the proposed investigations may benefit many active duty personnel, as well as civilians.

Paraoxon exposure is thought to cause some pain and/or distress due to the intense physiological changes produced by these toxicants. Anesthetics and analgesics for relief of pain or distress are known to have profound effects on brain function and may also interact with chemical agents, thereby complicating the interpretation of the results. The goal of the study is test the effect of 2-PAM to block the seizure activity in the brain. Therefore, the dose of paraoxon that causes seizure activity in the maximum number of animals was selected for the proposed study.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions:

A week prior to the experiments involving EEG recordings, the animals will be implanted with cortical electrodes for electroencephalogram (EEG) recordings. These EEG recordings will be used to confirm the onset and intensity of behavioral convulsions observed in the animals. The surgical procedures will be performed aseptically following the LAM guidelines. Animals will receive one dose of buprenorphine (0.05 mg/kg s.c.) before any incisions are made. Anesthesia will be induced by isoflurane vapor in 100% oxygen in a commercially available induction chamber. Once anesthesia is induced, animals will be transferred to the designated surgery area and anesthesia will be maintained via nose cone. See Section V.4.1.2.1 Anesthesia/Analgesia/Tranquilization for details.

When the animal is anesthetized, its head will be shaved with a hair clipper. The head will be cleaned with betadine and alcohol, each repeated 3 times alternately. Then the animal will be placed into a stereotaxic frame (appropriate frame size will be used for rats) after treating its pressure points such as ears and nose with lidocaine jelly. A heated pad with thermister-controlled feedback will be put between the animal's body and the base of the stereotaxic frame. Ophthalmic ointment will be applied to the eyes to prevent dryness. The body will be covered with a sterile drape with an opening to expose the head. Prior to incision, animals will receive bupivicaine at electrode placement sites (infusion of surrounding tissue) to assist in pain management. The injection volume will be limited to the minimum required for pain control, to minimize health risks. Care will be taken to avoid systemic effects.

V.4.3.2. Procedure:

Surgery to implant cortical electrodes: The skin will be incised longitudinally along the midline from eye level to the neck level and retracted to the sides with hemostats. The periosteum will be scraped off from the skull. Five stainless steel machine screws (the smallest possible size that provide adequate anchoring) will be used as electrode contact and simultaneous anchoring points for the headpiece that will be built from acrylic cement to secure the female electrode connector in place. The screws will be manually positioned in the frontal or parietal bone plates and one over the cerebellum. The screws will be partially screwed in manually without penetrating the dura mater.

V.4.3.3. Post-surgical Provisions:

In the case of the EEG electrode impantation procedure, excess cement will be removed from the headpiece to make its edges smooth. If necessary, skin will be sutured at both ends of the incision line. The animal will be released from the

stereotaxic frame and placed in a recovery cage on a chemical pocket warmer wrapped in cloth or paper until the animal fully recovers from anesthesia. Alternatively, the animals may be placed in a forced-air recovery chamber warmed to 34 °C. Once fully awake and performing purposeful movements, the animal will be moved back to the holding area. At least 7 days will be allowed for recovery after surgery before any experimentation.

We expect that about 90% of the animals will be in good health to be used in the study. This decision will be jointly made by the animal-care personnel and the investigators. All efforts will be made to provide all animals with the best care possible. Each animal will be individually monitored throughout the study by experienced personnel from our laboratory. We will also closely work with the veterinarian and animal-care staff of the animal facility to ensure that adequate treatment is provided in a timely manner should it be deemed necessary.

After surgery, the animals will be placed in a clean cage provided with a recirculating warm-water veterinary heating pad or in a forced-air recovery chamber warmed to 34 °C to maintain proper body temperature. Following surgery for implantation of cortical electrodes, animals will receive buprenorphine HCl (0.05 mg/kg, s.c.), which will be given at a minimum one time at the end of the day of surgery, just before close of business. This is in addition to the preemptive dose given prior to surgery. All surgeries will be performed in the AM. The medication will be administered by the by qualified PI staff. If necessary, based on consultation with the attending veterinarian, additional buprenorphine may be given as needed.

Additional doses of buprenorphine will be given if required, based on assessment of PI and attending veterinarian of animal's condition, which will include assessment of the presence of the following signs of pain/distress: hunching, abnormal gait, abnormal attitude (increased or decreased aggression), inability to move to food/water, excessive porphyrin staining of eyes/nose, shuddering or twitching, unkempt appearance, and vocalization when handled.

Following at least 1 hour on the warming pad or in a forced-air recovery chamber, upon full recovery in which the animal can maintain an upright posture and is ambulatory, the animal will be returned to its holding room cage. Laboratory personnel will provide the care of the animal until full recovery if it has not already occurred. All animals will be checked at least once 30 minutes after return to home cage, to ensure recovery is continuing, and at least once again just prior to the end of the work day to ensure animals are recovering normally. Animals will be examined at least twice daily by the PI staff for one week post-surgery to ensure that no complications occur. Observations will be recorded for each animal in a place that is accessible to the animal care staff (post-surgery cage cards may be used for this purpose). PI staff observations will be separate from routine observations conducted by the animal care staff

V.4.3.4. Location:

(b)(6) facilities at USUHS

V.4.3.5. Surgeon: Dr (b)(6)

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

Drug name (concentration)	Dose	Frequency	Route	Site	Volume	Needle size
Bupivicaine (2.5 mg/ml)	5.0 mg/kg	Once	subcutaneous	Surgery site	Up to 0.5 mL	25 ga 1 in
Buprenorphine (0.025 mg/ml)	0.05 mg/kg	Q8-12H	subcutaneous	Interscapular	Up to 0.5 ml	25 ga 1 in
Fatal Plus or equivalent (50 mg/ml)	85-100 mg/kg	Once	intraperitoneal	Lower abdomen	Up to 0.5 ml	25 ga 1 in
Paraoxon (0.5 mg/ml)	1.0 mg/kg	Once	subcutaneous	Interscapular	Up to 0.5 ml	25 ga 1 in
Atropine (1 mg/ml)	2.0 mg/kg	Once	Intramuscular	Caudal thigh	Up to 0.5 ml	25 ga 1 in
Midazolam (2.5 mg/ml)	5.0 mg/kg	Once	Intramuscular	Caudal thigh	Up to 0.5 ml	25 ga 1 in
2-PAM (12.5 mg/ml)	25 mg/kg	Once	Intramuscular	Caudal thigh	Up to 0.5 ml	25 ga 1 in

V.4.4.2. Biosamples: Tissues such as brain, lung, liver, kidney, heart and blood will be collected post mortem.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: The animals will be identified by cage card. However, individual animals after procedure will be given a particular identifying tattoo on their pinna of the ear, tail, arms or legs.

V.4.4.6. Behavioral Studies:

V.4.4.7. Other Procedures: On the day of exposure to the nerve agent, Paraoxon will be administered intraperitoneally and the animals will be connected to recording leads via the connection plug on their heads. Brain EEG activity will be recorded while they are in cylindrical plastic recording chambers, 16" in diameter and 26" high with Iso-pad bedding on the floor, where they are free to move about. The period of confinement in the recording chambers will not exceed 3 hours on the experimental day or 1 hour on the day after, if needed.

V.4.4.8. Tissue Sharing: Tissues may be shared with other investigators upon request.

V.4.5. Study Endpoint: All rats will be euthanized within 24 hrs of receiving the proposed dose of paraoxon. The animals will be euthanized for tissue collections and also for histological analysis at the end of each study. For histological analysis, transcardial formalin perfusion will be performed following injection of a 85-100 mg/kg dose of Fatal Plus or another pentobarbital based euthanasia solution.

Early Endpoint: Any of the following will represent a reason for early euthanasia: inability to ambulate or reach food or water, self mutilation, continuous circling, or persistent labored respiration or gasping, loss of 20% of weight below baseline, prostration/inactivity for up to 24 hours, or a significant decline in vital signs. These signs, if observed by animal facility personnel and no study personnel can be reached, may be taken by the veterinarian as cause for euthanasia.

V.4.6. Euthanasia: Some animals in each group will be euthanized by decapitation following injection of a 100 mg/kg dose of pentobarbital based euthanasia solution. Others will be euthanized by transcardial perfusion following euthanasia solution administration (Fatal Plus, 85-100mg/kg or other pentobarbital based euthanasia solution, Intraperitoneal, 400-500µl volume, 25 gauge needle).

Transcardial perfusion will be done as described (16,17). Briefly, animals will be deeply anesthetized with Fatal Plus (85-100 mg/kg), and the heart will be exposed by opening up the thoracic cavity after confirming that the animal is fully under anesthesia. To monitor the depth of anesthesia we will test whether the animal has lost its toe pinch (pedal withdrawal) reflex. The animals will be perfused with either 10% neutral buffered formalin or 4% freshly depolymerized paraformaldehyde through the left ventricle using a peristaltic pump. The right atrium will be cut and approximately 200 ml of fixative solution will be passed through the circulatory system. Following the perfusion, tissues will be removed to a container of fresh fixative solution prior to histopathological analysis.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for all rats in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes	_____	No	X
Fluid Restriction:	Yes		No	X

V.5.1.3. Exceptions: _____ _____

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: The animals will be observed daily according to LAM SOPs.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions:

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Intragastric feeding, euthanasia, decapitation, perfusion, EEG lead implantation	(b)(6)	Ph.D. 8 yers exp in TBI models	Investigator training, 2003, Rodent handling class, 2003
Consulting		Ph.D., 20+ years	Investigator training

		exp.	1998, Rodent handling class, 1998
Consulting	(b)(6)	Ph.D., more than 10 years experience in EEG studies.	Investigator training 2003. Rodent handling class, 2003
Behavioral studies		Res. Assistant, 3-6 months experience in rodent handling	Investigator training 2012, Rodent handling class,2012
Consulting		Ph.D., 15+ years experience	Investigator training,2000, Rodent handling class, 2000
Euthanasia		Research Associate, M.S.	Investigator training,June, 2013, Rodent handling class, June, 2013

VII. **BIOHAZARDS/SAFETY**: Gowns, Gloves, and masks will be used for personal protection and sharp items will be disposed off in separate containers as per safety rules. The volatile anesthetic agents will be properly closed and removed from the procedure area. Chemicals and hazardous waste used in this protocol will be handled in accordance with all applicable state and federal guidelines, regulations, and standing operating procedures. Waste anesthetic gas (isoflurane) will be scavenged and exposure to investigators will be minimized using the set up used in the LAM operating room since the surgeries will be done in one of those operating rooms. Double gloves will be worn while preparing solutions of Paraoxon to avoid any body contact with the insecticide.

VIII. **ENCLOSURES**: References

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) 6/18/2013

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) 6/18/2013

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) 6/18/2013

Principal Investigator Signature Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: Nanotechnology-based intranasal Brain Delivery of Neuroprotectants in Rat Models.

C. Principal Investigator: (b)(6) Ph.D.

D. Performing Organization: USUHS

E. Funding: NIH

F. Objective and Approach:. .

Intranasal CNS delivery of drugs to protect against organophosphorous threat agents: Our central hypothesis is that intranasal administration of therapeutic formulations of oxime, atropine and midazolam in a nanoemulsion formulation can rapidly deliver significant amounts of these drugs to the brain and small amounts to lungs and the blood stream to protect against CTAs at 30 min or later time points. Our long term objective is to establish and optimize for human use the efficacy of intranasal brain delivery systems for use with chemical threat-related therapeutics, which can be of rapid and practical use in preventing and treating civilian mass casualties. The immediate goal is to develop the intranasal mucoadhesive nanoemulsion approach for this purpose and test them for effectiveness in a preclinical model system.

G. Indexing Terms (Descriptors): . intranasal brain delivery, nanoemulsion. nanoparticles, seizure, insecticide, paraoxon, nerve agent.

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Institutional Animal Care and Use Committee
Phone (b)(6)
Fax (b)(6)

November 7, 2014

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on June 5, 2014:

Animal Protocol Title: "The Effect of Traf2 and Nck Interacting Kinase (Tnik) Knockout on Freezing Behavior in Fear Conditioning (Mouse)"

USUHS Protocol Number: (b)(6)

Expiration Date: June 4, 2017

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

Learning to Care for Those in Harm's Way

Revised

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE:
The effect of Traf2 and Nck Interacting Kinase (Tnik) knockout on freezing behavior in fear conditioning (mouse)

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)
FUNDING AGENCY: USUHS
EARLIEST ANTICIPATED FUNDING START DATE: Current

PRINCIPAL INVESTIGATORS: Dr. (b)(6) PhD

(b)(6)	APG	(b)(6)	4/29/14
	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)		vice chair	(b)(6)	4/29/14
Typed name:	Signature	Title	Telephone	Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)

Statistician Signature

Typed Name: (b)(6)

PSY
Department

(b)(6)

4/22/14
Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)

Att
Typ
ure

LAM
Department

(b)(6)
Telephone

4/25/14
Date

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: Dr. (b)(6) PhD

ANIMAL PROTOCOL TITLE:
The effect of Traf2 and Nck Interacting Kinase (Tnik) knockout on freezing behavior in fear conditioning (mouse)

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): Dr. (b)(6)

TECHNICIANS(S):
(b)(6) USU Neuroscience PhD Candidate

I. NON-TECHNICAL SYNOPSIS:

Post traumatic stress disorder (PTSD) is a major public health concern in both military and civilian populations. According to recent research as many as 8.6% of military personnel returning from Iraq and Afghanistan show symptoms of PTSD lasting at least 30 days [1]. With so many soldiers returning from combat there will be a high emotional and financial burden on families to support and care for these veterans. To improve current treatments and develop new ones it is important to determine what leads to individual differences between humans that lead to either enhanced risk or resilience in

developing PTSD. This leads to studying genes that lead to the development of PTSD to target for future treatment. To study the genetics of fear, an animal model was developed in which mice were selectively bred over several generations to show high or low levels of fear. Looking for different levels of activity in the brains of these rodents one brain region in particular was found to be more active in the animals more prone to fear. This brain region was the hippocampus which has long been recognized to be important in learning and memory. We identified a gene that is highly expressed in the hippocampus of these fear susceptible animals which is caused Traf2 and Nck Interacting Kinase (Tnik). This gene has been shown in a different animal model to be important to memory as well.

We propose to study several aspects of this gene to determine how it influences fear memory. We propose to determine how Tnik is regulated in brain cells in the hippocampus, how Tnik affects the size, shape and composition of brain cells and how Tnik affects fear memory formation in mice.

II. BACKGROUND:

II.1. Background:

Post traumatic stress disorder (PTSD) is a major health concern in both military and civilian populations with a serious impact on quality of life as well as a large financial burden on individuals experiencing PTSD as well as their families. One of the major aspects of PTSD is the onset of a fearful response to situations involving a context similar to the traumatic event which led to the development of PTSD. Several brain regions have been implicated in PTSD including the amygdala, specifically the lateral amygdala, frontal brain regions including the cingulate cortex, the thalamus and the hippocampus [2, 3]. The hippocampus has also been more generally shown to be an important brain region in learning and memory [4-6]. With many of the symptoms of PTSD being related to memory, including flashbacks, intrusive memories, nightmares and amnesia the importance of the hippocampus in the symptoms of PTSD is apparent [2].

To investigate fear memory on a molecular level animal models of fear memory have been created. One major method for studying fear memory is fear conditioning which can be either cued or contextual. Contextual fear conditioning involves placing an animal in a context for a training session and exposing them to a foot shock. For the test phase animals are placed back in the same context and freezing behavior, which is indicative of fear in animals, is measured. In cued fear conditioning an animal is placed into a training chamber and exposed to a cue, usually a tone, prior to receiving a foot shock. The animal is then placed into a

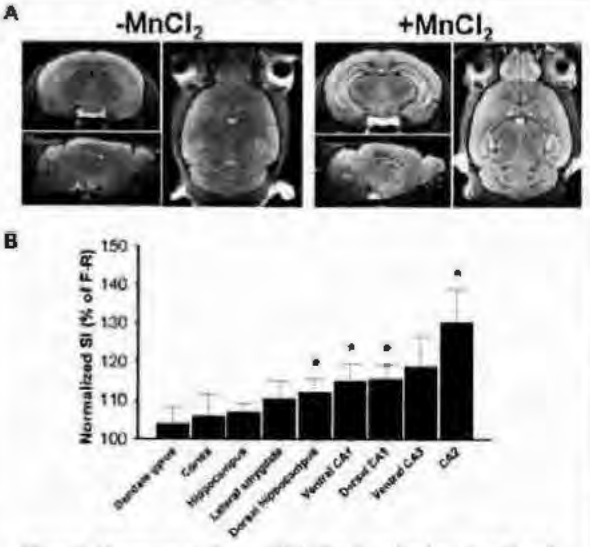

Figure 1. Manganese enhanced MRI data from the brains of FR and FS mice. There were significant differences found in the dorsal hippocampus, dorsal and ventral CA1 as well as CA2 showing heightened activity in those regions in the FS mice. These were the only regions showing significant differences in activity though no work has yet been done investigating the mechanisms underlying these differences [1].

novel context and exposed to the cue and freezing behavior is again measured. These paradigms are similar to symptoms of PTSD where a specific context or cue sets off anxiety like behavior in a person with PTSD.

A recent animal model has been developed in which an intercross line of mice are selectively bred for several generations based on their performance on a fear conditioning task [7]. This led to both fear susceptible (FS) and fear resilient (FR) animals who display high or low levels of freezing respectively. Utilizing these FR and FS mice researchers have identified several quantitative trait loci (QTL) specific to the different lines believed to underlie the observed phenotypes [7]. These include genes in both the prefrontal cortex as well as the amygdala [8, 9]. Functional imaging work done with these mice in the lab of Dr. (b)(6) at USU have displayed functional differences between the FS and FR mice [10]. Interestingly while the researchers anticipated differences in the amygdala the only significant differences found were actually in several regions of the hippocampus including the dorsal hippocampus, dorsal and ventral CA1 as well as CA2 (Figure 1). Since differences in activity have been observed in the hippocampus work into understanding the molecular mechanisms underlying these functional differences are important in determining what drives the behavioral differences between the FS and FR mice.

Recent work in our lab utilizing hippocampal tissue from FS and FR mice and RNA sequencing has revealed a pattern of genes showing differential expression. Among these genes differentially expressed in the FS and FR mice we identified a group of genes which have been recently shown to affect neuronal morphology, synaptic activity and memory performance. Two of these genes, Traf and Nck interacting kinase 2 (Tnik) and cytoplasmic linker associated protein 2, (Clasp2) are more highly expressed in the FS relative to the FR mice. These genes were identified by RNA sequencing and confirmed by qPCR.

Recently Tnik has been identified as an important signaling molecule in hippocampal neurons through research conducted on a Tnik knockout animal [11]. Work with this knockout animal line has shown that Tnik is essential for the incorporation of AMPA receptors into the post synaptic density and absence of Tnik leads to a decrease in synaptic plasticity. The knockout mice also display deficits in spatial learning and memory which have been shown to be dependent on hippocampal neuronal function [11]. Tnik expression has been shown to be highly regionally specific to regions CA1 and the molecular cell layer of the dentate gyrus in the hippocampus (Figure 2, Allen Brain Atlas). These regions have been shown to be important in the formation of fear memory as false fear memories to a novel context have been created

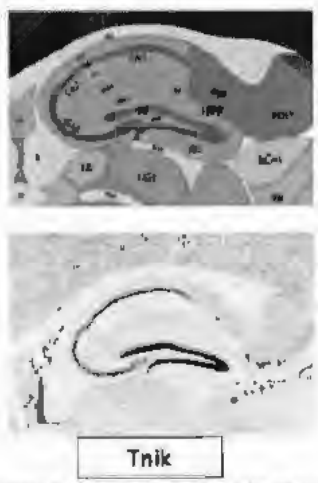

Figure 2. These images are from the Allen Brain Atlas resources. Top- Atlas. Middle- ISH image of mouse brain labeled with Clasp2 specific antibody. Bottom-ISH image from mouse brain slice labeled with Tnik specific antibody. These images show highly regionally specific expression of Tnik restricted to the CA1 area of the hippocampus and the granule cell layer of the dentate gyrus which are two areas important to memory formation and consolidation.

via activation of neurons of the CA1 region of the hippocampus during fear conditioning [12].

Currently we propose performing fear conditioning experiments in a Tnik knockout animal to determine what role Tnik plays in the formation of fear memories. We will be performing both cued and contextual fear conditioning. Using these same knockout animals we intend to analyze slices for synaptic composition and neuronal morphology.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:
Biomedical Research Database (BRD)
Federal Research in Progress (FEDRIP)
PubMed

II.2.2. Date of Search:
3/14/2014

II.2.3. Period of Search:
1950 to present

II.2.4. Key Words and Search Strategy:
We did advanced searches of all databases with the following keywords:
Traf2 and Nck Interacting Kinase (Tnik) fear conditioning
Traf2 and Nck Interacting Kinase (Tnik) hippocampus
Traf2 and Nck Interacting Kinase (Tnik) memory
Traf2 and Nck Interacting Kinase (Tnik) knockout

II.2.5. Results of Search:
There is only one article published pertaining to the Tnik knockout animal. It found a spatial memory deficit but did not do any research with fear memory. Tnik has been shown to associate with proteins in the post synaptic density including DISC1 which has been found to be related to schizophrenia [13]. Tnik has also been found to drive AMPA receptor trafficking in hippocampal neurons which is also related to fear memory [11].
Traf2 and Nck Interacting Kinase (Tnik) fear conditioning- 0 results
Traf2 and Nck Interacting Kinase (Tnik) hippocampus- 2 results
Traf2 and Nck Interacting Kinase (Tnik) memory- 1 result
Traf2 and Nck Interacting Kinase (Tnik) knockout- 2 results

III. OBJECTIVE\HYPOTHESIS:
We believe that Tnik knockout animals will display learning deficits in contextual fear conditioning experiments as shown by decreases in freezing behavior. We also anticipate that neurons in Tnik knockout animals will have fewer projections and decreased cell body size when compared to control.

IV. MILITARY RELEVANCE:

PTSD affects approximately 8.6% of individuals returning from combat missions in Iraq and Afghanistan [14]. New treatments for these returning soldiers with greater efficacy need to be created to aid in their transition back to civilian life and to improve their quality of life post deployment. To aid in the creation of new treatments the molecular mechanisms leading to increased risk or resilience of developing PTSD symptoms is being investigated. Fear memory in animals is used to mimic symptoms of PTSD in humans for research purposes. To study the genetics underlying fear memory an animal model of fear susceptible and fear resilient animals was developed. This gene was identified using deep sequencing technology using hippocampal tissue from these animals. By studying the genetics of fear memory researchers hope to identify new molecular targets for treating soldiers returning home from combat with symptoms of PTSD. This gene serves as a viable candidate for treatment if it is confirmed to be involved in the molecular processes leading to increased susceptibility to fear. Our current proposed research aims to determine what effect this gene has on hippocampal structure and function and how this affects the formation of fear memories in rodents. Confirmation that this gene is important in fear memory development will help determine if it is a viable candidate for human PTSD treatment.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1: Experimental Design and General Procedures:

In experiment 1 we will determine the behavioral differences between Tnik knockout animals and their wild type littermate controls. We will do this by assessing the differences in freezing behavior of these 2 groups in contextual and cued fear conditioning experiments. We will also test basic locomotion and anxiety prior to fear conditioning.

Experimental Timeline. Male mutant and wild type (WT) adult mice (approximately 4 months) will be tested on the behavioral paradigms (open field, elevated plus maze, contextual and cued fear conditioning) according to the details below. Animals will receive 3 days of rest between the open field and elevated plus maze tasks. Fear conditioning will begin 3 days following the elevated plus maze.

Open Field. Open field locomotor activity will be measured using an Accuscan Electronics Digiscan infrared photocell system. Animals are placed singly in a 20 x 20 x 30 cm clear Plexiglas arena and a Plexiglas lid with multiple ventilation holes placed on top of the arena. Data are automatically gathered and transmitted to a computer operating the Fusion 3.4 software system for data collection and analyses. The interfaced software measures two dozen activity variables, including total distance and horizontal and vertical activity. Chambers are cleaned between subjects. The duration of locomotor activity testing is 120 min per session.

Elevated Plus Maze. The EPM is a widely used a measure of anxiety. Mice will be placed individually on the center platform facing a closed arm and allowed to explore the maze for 5 min. Behavior will be videotaped via closed circuit TV camera for scoring. Behaviors scored include: percent time spent in the open arms [(time spent in open arms/total time) X100], percentage of open arm entries [(open arm entries/total

arm entries) X100], and percentage of closed arm entries [(closed arm entries/total arm entries) X 100]. Percent time spent in the open arms and percentage of open arm entries are chosen to index anxiety.

Fear Conditioning. Animals will be habituated for 10 minutes in each context the day prior to conditioning. Approximately 15 animals will be used per condition (n=15, N=30, Power Analysis explanation under heading V.2). The intensity and duration of foot shocks was piloted previously in Dr. (b)(6) lab at the Uniformed Services University. This paradigm has been shown to reliably induce a lasting fear response in mice.

Day 1: Habituation 10 minutes in each context	Day 2: Conditioning Context A- 3 pairings of tone/shock (0.8mA)	Day 3: Contextual Fear Test Context A- 15 minute trial (freezing measured)	Day 4: Cued Fear Test Context B- 20 minute trial 10 30 second tones (freezing measured)

Contextual Fear Conditioning. Mice will be placed in a conditioning chamber (context A) for training for 2 minutes prior to onset of the conditioned stimulus (tone). The conditioned stimulus will last for 30 seconds prior to the onset of the unconditioned stimulus (foot shock, 0.8 mA) which will coincide with the last 2 seconds of the conditioned stimulus. The mice will then remain in the chamber for an additional 30 seconds prior to onset of the next trial. Three training trials will be performed. Mice will then be tested 24 hours later for freezing behavior (lack of movement for at least 5 seconds) in a 15 minute trial in the conditioning chamber (context A) [15].

Cued Fear Conditioning. Following conditioning and 24 hours after the contextual fear testing mice will be placed in a novel context (context B) and presented with the cue (tone) from the conditioning phase. Ten presentations of the tone will be presented at random intervals following a 3 minute habituation period. Freezing behavior will be assessed in this 20 minute test phase as well.

V.1.2. Experiment 2:

In experiment 2 we will perform RNA sequencing analysis of hippocampal tissue from knockout animals and wild type littermates before and after conditioning experiments from experiment 1. This will lead to the comparison of transcriptomes from four different groups: knockout before conditioning, knockout after conditioning, wild type before conditioning and wild type after conditioning.

Experimental Timeline. cDNA libraries will be prepared from hippocampal tissue of each animal from experiment 1 (approximately 4 months). Separately a group of 30 animals (15 KO and 15 WT) that have not undergone behavioral experiments will also be selected for RNA sequencing. This will lead to 60 total animals, 30 from the behavioral experiments (all of which will also undergo RNA sequencing of hippocampal tissue) and an additional 30 animals that have not been fear conditioned for sequencing experiments.

RNA Sequencing. Hippocampi of mice will be isolated 24 hours following either training or the test phase of fear conditioning. Briefly, mice will be anesthetized, decapitated and their hippocampi will be isolated by micro dissection. Brains will be placed in Qiazol (Qiagen) and homogenized prior to RNA isolation using the RNAeasy Mini kit. cDNA will be generated from RNA samples using reverse transcriptase PCR. cDNA libraries for sequencing will be prepared using the Illumina library preparation kit

and clustered to a flow cell for sequencing on the cBot system. Following clustering paired end reads will be obtained on the Illumina deep sequencing platform.

V.2. Data Analysis:

Open Field. Total distance, horizontal and vertical activity and percent time spent in center will be compared between the 2 groups.

EPM. Behaviors to be compared between the 2 groups include: percent time spent in the open arms [(time spent in open arms/total time) X100], percentage of open arm entries [(open arm entries/total arm entries) X100], and percentage of closed arm entries [(closed arm entries/total arm entries) X 100].

Fear Conditioning Analysis. The freezing behavior in the 2 conditions will be compared with a t-test comparing total time freezing.

RNA Sequencing Analysis. Base calls will be made using CASAVA. All subsequent analyses will be performed using tools in Galaxy (Tophat2 and Cufflinks). Differential gene expression analysis and visualization will be performed using GenePattern. Differential analysis will be performed comparing both the effects of conditioning on gene expression (conditioned animals versus naïve) as well as the comparison of Tnik -/- and WT littermates both before and after fear conditioning.

Group Size Calculation. Group numbers were calculated using G Power software (α=0.05, β=0.95) [16]. Several different effect sizes were derived from the literature for calculations (d=1.2-1.5) [17-19]. These effects seizes were drawn from literature performing contextual fear conditioning in animals with genetic knockouts shown to have effects on hippocampal function. This range of effect sizes gave us a range of 11-16 animals required to give sufficient power for our fear conditioning experiments. If significance in our results is achieved before all animals are utilized remaining animals will not be required for our experiments.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

These experiments are part of a larger project in which cell culture experiments will be utilized whenever possible to reduce the number of animals used. For the behavioral experiments animals must be utilized as there are no alternatives. The RNA sequencing experiments are being proposed to make further use of the animals that have already been used for the behavioral experiments.

V.3.2. Animal Model and Species Justification:

This is the only commercially available animal with a genetic deletion of Tnik. This is why we have selected this animal to determine the role that Tnik plays in the formation of different types of fear memory.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	*Mus Musculus*	

V.3.3.2. Strain/Stock: B6NTac;B6N-A^{tm1Brd} Tnik$^{tm3a(EUCOMM)Wtsi}$/WtsiBiat

V.3.3.3. Source/Vendor: Colony to be maintained at USU

V.3.3.4. Age: 8-10 weeks

V.3.3.5. Weight: 25-40 g

V.3.3.6. Sex: Male

V.3.3.7. Special Considerations: N/A

V.3.4. Number of Animals Required (by Species):
We will utilize 60 male mice total for experiments #1 and #2.

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:
Fear conditioning is well established in mice and we anticipate that behavioral differences will be easiest to attain in this model. Also the only available genetic knockout is a mouse so this model is the only animal available for our experiments. Measures cannot be taken to relieve the temporary distress of animals during fear conditioning because this will interfere with the behavioral paradigm we have selected. The foot shock and associated minor and temporary pain associated with it are required for the induction of fear memory.

V.3.5.2. Reduction:
The number of animals we anticipate using in these experiments is derived from literature on fear conditioning in mice. We will also be using overlapping animals for both contextual and cued fear conditioning as well as sequencing to reduce the number of animals used.

V.3.5.3. Replacement:
All the parts of this project in which cell line work could replace or pilot test animal work we have already done so. The remaining portion proposed here cannot be done without an animal model. This mouse model is the only genetic knockout for Tnik available.

V.4. Technical Methods: Experiment #1 –
Open Field. Open field locomotor activity will be measured using an Accuscan Electronics Digiscan infrared photocell system. Animals are placed singly in a 20 x 20 x 30 cm clear Plexiglas arena and a Plexiglas lid with multiple ventilation holes placed on

top of the arena. Data are automatically gathered and transmitted to a computer operating the Fusion 3.4 software system for data collection and analyses. The interfaced software measures two dozen activity variables, including total distance and horizontal and vertical activity. Chambers are cleaned between subjects. The duration of locomotor activity testing is 120 min per session.

Elevated Plus Maze. The EPM is a widely used a measure of anxiety. Mice will be placed individually on the center platform facing a closed arm and allowed to explore the maze for 5 min. Behavior will be videotaped via closed circuit TV camera for scoring. Behaviors scored include: percent time spent in the open arms [(time spent in open arms/total time) X100], percentage of open arm entries [(open arm entries/total arm entries) X100], and percentage of closed arm entries [(closed arm entries/total arm entries) X 100]. Percent time spent in the open arms and percentage of open arm entries are chosen to index anxiety.

Contextual Fear Conditioning. Mice will be placed in a conditioning chamber (context A) for training for 2 minutes prior to onset of the conditioned stimulus (tone). The conditioned stimulus will last for 30 seconds prior to the onset of the unconditioned stimulus (foot shock, 0.8 mA) which will coincide with the last 2 seconds of the conditioned stimulus. The mice will then remain in the chamber for an additional 30 seconds prior to onset of the next trial. Three training trials will be performed. Mice will then be tested 24 hours later for freezing behavior (lack of movement for at least 5 seconds) in a 15 minute trial in the conditioning chamber (context A) [15].

Cued Fear Conditioning. Following conditioning and 24 hours after the contextual fear testing mice will be placed in a novel context (context B) and presented with the cue (tone) from the conditioning phase. Following a 3 minute habituation period animals will be presented the tone 10 times at random intervals. Freezing behavior will be assessed in this 20 minute test phase as well.

Experiment #2 – Subjects will be given ketamine and xylazine as the sedative and anesthetic. Animals will be injected (i.p.) with 80 mg/kg of Ketamine and 10 mg/kg Xylazine using a 26-gauge needle. Once an adequate depth of anesthesia has been confirmed by lack of response to toe pinch, the mice will be decapitated. The brain will be removed from the cranium and freshly frozen under dry ice before sectioning and micropunching hippocampal tissue for isolating total RNA using Trizol and Qiagen columns.

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	30	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	30	

V.4.1.2. Pain Relief / Prevention:
No measures can be taken to alleviate pain because the painful stimulus is required for the induction of fear memory.

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:
Prior to brain removal and hippocampal dissection, ketamine and xylazine will be used as the sedative and anesthetic. Animals will be injected (i.p.) with 80 mg/kg of Ketamine and 10 mg/kg Xylazine using a 26-gauge needle. The total volume of injected anesthetic depends on the weight of the animal. For example, a 25 gram mouse will receive a single i.p. injection of 20 μl ketamine (from a 100 mg/ml stock) plus 250 μl xylazine (from a 1 mg/ml stock).

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: N/A

V.4.1.2.3. Paralytics:
N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched:
PubMed

V.4.1.3.2. Date of Search:
3/18/14

V.4.1.3.3. Period of Search:
1950-Present

V.4.1.3.4. Key Words of Search:
Fear conditioning
Foot shock
Predator Odor
Air Puff

V.4.1.3.5. Results of Search:
While there are alternatives to foot shocking as the unconditioned stimulus for fear conditioning including air puffs and predator odor these also lead to similar stress on the animal as foot shocks. In addition fear conditioning utilizing foot shocks is the most established and commonly used form of conditioning used to date. No measures can be taken to alleviate pain because the painful stimulus is required for the induction of fear memory.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
Fear conditioning is commonly used as a model of fear memory. The foot shocks do not cause any physical damage to the animals and the pain is necessary for inducing a fear memory.

V.4.2. Prolonged Restraint:
N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions:
N/A

V.4.3.2. Procedure:
N/A

V.4.3.3. Post-surgical Provisions:
N/A

V.4.3.4. Location:
N/A

V.4.3.5. Surgeon:
N/A

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures:
N/A

V.4.3.6.2. Scientific Justification:
N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:
Ketamine (80mg/kg) and xylazine (10mg/kg) will be injected IP using a 26-gauge needle. The total volume of injected anesthetic depends on the weight of the animal. For example, a 25 gram mouse will receive a single i.p. injection of 20 μl ketamine (from a 100 mg/ml stock) plus 250 μl xylazine (from a 1 mg/ml stock).

V.4.4.2. Biosamples:
Total RNA from hippocampal brain regions. Hippocampi of mice will be isolated 24 hours following either training or the test phase of fear conditioning. Briefly, mice will be anesthetized, decapitated and their hippocampi will be isolated by micro dissection. Brain tissue will be placed in Qiazol (Qiagen) and homogenized prior to RNA isolation using the RNAeasy Mini kit.

V.4.4.3. Adjuvants:
N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production:

N/A

V.4.4.5. Animal Identification:
Animals will be identified with cage cards throughout this study.

V.4.4.6. Behavioral Studies: See section V.1.1.

V.4.4.7. Other Procedures: N/A

V.4.4.8. Tissue Sharing:
No tissue sharing will take place on this project.

V.4.5. Study Endpoint:
All animals will be euthanized and tissue will be isolated following completion of the behavioral portion of the experiments. If animals display any signs such as dramatic weight loss (>20% compared to cagemates), lethargy, squinting, ruffled fur they will be removed from the study immediately and euthanized by CO2 inhalation in the LAM.

V.4.6. Euthanasia:
Subjects will be given ketamine and xylazine as the sedative and anesthetic. Animals will be injected (i.p.) with 80 mg/kg of Ketamine and 10 mg/kg Xylazine using a 26-gauge needle. Once an adequate depth of anesthesia has been confirmed by lack of response to toe pinch, the mice will be decapitated.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:
Building(s) _____ Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No X

Fluid Restriction: Yes _____ No X

V.5.1.3. Exceptions:
N/A

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
LAM personnel will provide routine checks for health and general husbandry at least once/day. A LAM veterinarian will evaluate any animals exhibiting signs of illness (such as ruffled coat, hunched, squinting, loss of >20% weight loss...etc). Animals will typically be monitored for changes in body weight, behavior, appetite, excretion, hair

coat, etc. After consulting with the PI (if available), moribund animals will be euthanized as described. Animals will also be checked on by the PI or a member of the lab on a daily basis and any issues concerning animal health will be brought to the veterinarians' attention.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions:
N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Open Field Testing Elevated Plus Maze Fear conditioning	(b)(6)	graduate student (4 years)	USU Investigator Training Course 2011
		PI, 12 years experience	Training from Dr. (b)(6) lab, 2000; USU Investigator Training Course 2012
		17 years of experience in animal research Conducted animal research at University of Alberta, Canada, and UTSW Medical Center at	Attended all the training classes required by USU in order to be approved to conduct animal research on Oct 19, 2010.

		Dallas, TX.	
Euthanasia/Hippocampal Dissection	(b)(6)	graduate student (4 years)	USU Investigator Training Course 2011
		PI, 12 years experience	Training from Dr. (b)(6) lab, 2000; USU Investigator Training Course 2012

VII. BIOHAZARDS/SAFETY:

All personnel handling animals will use protective gloves, lab coat, and masks at all times to minimize exposure to allergens and agents used during experimental procedures.

VIII. ENCLOSURES:

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 4/30/14

P _____ Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

(b)(6) _____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) [redacted] 4/30/14

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) [redacted] 4/30/14

Pr Date

X. PROTOCOL ABSTRACT:

Abstract. Post traumatic stress disorder (PTSD) is a major health concern with high economic and quality of life concerns for both military and civilian populations. Intrusive fearful thoughts and memories are one of the hallmarks of PTSD. Accordingly, animal models of fear memory are used to investigate this aspect of PTSD in a controlled setting. In a recent high throughput transcript sequencing study of the hippocampus of an animal model displaying high and low fear several genes were identified which are differentially expressed. Of these genes one candidate, Traf2 and NcK interacting kinase (Tnik) has been discovered with regionally specific expression in the hippocampus and known functions related to synaptic plasticity and neuronal morphology. We propose a series of experiments investigating how this gene affects fear memory formation. We propose experiments in a mutant mouse lacking Tnik expression where performance on fear memory tasks will be assessed. We will also examine the transcriptomes of Tnik -/- and WT animals to determine what genes are altered by Tnik activity. The proposed experiments will lead to a greater understanding of the underlying cellular mechanisms affecting fear memory formation which will further our understanding of inter individual differences determining risk of developing PTSD.

A. Animal Protocol Number:

B. Animal Protocol Title:

The effect of Traf2 and Nck Interacting Kinase (Tnik) knockout on freezing behavior in fear conditioning (mouse)

C. Principal Investigator:

Dr (b)(6) PhD (Anatomy, Physiology and Genetics-USUHS)

D. Performing Organization:

Uniformed Services University of the Health Sciences

E. Funding:

USUHS

F. Objective and Approach:

Post traumatic stress disorder (PTSD) is a major public health concern in both military and civilian populations with as many as 8.6% of military personnel returning from Iraq and Afghanistan with symptoms of PTSD [1]. To improve current treatments and develop new ones it is important to determine what leads to individual differences between humans that lead to either enhanced risk or resilience in developing PTSD. To study the genetics of fear, an animal model was developed in which mice were selectively bred over several generations to show high or low levels of fear. Looking for different levels of activity in the brains of these rodents one brain region in particular was found to be more active in the animals more prone to fear. This brain region was the hippocampus which has long been recognized to be important in learning and memory. We identified a gene that is highly expressed in the hippocampus of these fear susceptible animals which is caused Traf2 and Nck Interacting Kinase (Tnik). This gene has been shown in a different animal model to be important to memory as well.

We propose to study several aspects of this gene to determine how it influences fear memory. We propose to determine how Tnik affects the genetic composition of brain cells and how Tnik affects fear memory formation in mice. We will do this by assessing performance on fear conditioning tasks in a mutant line of mice that does not express Tnik. We will compare the level of fear shown by these mutant animals to normal controls. We will also perform RNA sequencing to determine how the absence of Tnik signaling affects the expression of other genes in neurons.

G. Indexing Terms (Descriptors):

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

October 16, 2014

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on October 16, 2014:

Animal Protocol Title: "Evaluation of the Effects of Multiple Blast- or Concussive-Brain Injuries on Neuropathological and Behavioral Outcomes in Male and Female Mice (Mus musculus)"

USUHS Protocol Number: (b)(6)

Expiration Date: October 15, 2017

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6) Ph.D.

(b)(6) Ph.D.
Vice-Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

Revised

IACUC Date Stamp

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Evaluation of the effects of multiple blast- or concussive-brain injuries on neuropathological and behavioral outcomes in male and female mice (*Mus musculus*)

GRANT TITLE (if different from above): CNRM Pre-Clinical Studies Core

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Center for Neuroscience and Regenerative Medicine

EARLIEST ANTICIPATED FUNDING START DATE: Ongoing

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6) | APG (Department) | (b)(6) (Office/Lab Telephone) | 9-3-14 (Date)

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with research practice.

(b)(6) | APG (Title) | (b)(6) (Telephone) | 09.08.2014 (Date)

Research Unit Typed Name: (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) | APG (Department) | (b)(6) (Telephone) | 9-3-14 (Date)

Statistician Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) | LAM (Department) | (b)(6) (Telephone) | 9-4-14 (Date)

Attending/Consulting Veterinarian Signature
Typed Name (b)(6) VMD

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

ANIMAL PROTOCOL TITLE: Evaluation of the effects of multiple blast- or concussive-brain injuries on neuropathological and behavioral outcomes in male and female mice (*Mus musculus*)

GRANT TITLE (if different from above): CNRM Pre-Clinical Studies Core

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S):

TECHNICIANS(S): (b)(6) M.S., (b)(6) M.D., (b)(6) Ph.D., (b)(6) M.D., (b)(6) Ph.D.

I. NON-TECHNICAL SYNOPSIS:

The goal of this research is to evaluate and compare the brain and behavioral effects of traumatic brain injury (**TBI**) in male and female mice. TBI will be produced using one of two models related to TBI from explosions (no explosions are actually used), or using one of two related to concussions caused by a head injury. Each mouse will be exposed to one of these injury methods one, three or five times. Performance on behavioral tests will be evaluated after TBI, as will brain changes using magnetic resonance imaging (**MRI**), microscope "histology" and molecular biology methods to evaluate pathological changes. This work is critical for standardizing multiple TBI and behavioral procedures used within the Center for Neuroscience and Regenerative Medicine (**CNRM**) Pre-Clinical Studies Core; shared facilities that are used by many scientists to study the effects of TBI. The data collected from this research will be made available to CNRM researchers as a guide to assist them in their choices of which TBI model they will use in their experiments, and which behavioral tests will be appropriate for their experiments. In addition, Core personnel will be better trained in all of the procedures that researchers use within the facilities, and will be better prepared to help investigators perform TBI procedures and behavioral tests. This is an important step for establishing the surgical and behavioral testing procedures that all investigators should follow in their experiments and for establishing standardized procedures so that data collected by different research laboratories can be compared.

II. BACKGROUND:

II.1. Background:

Traumatic brain injury (**TBI**) is defined as the loss of brain function as the result of a penetrating head injury or a blow to the head. Each year over one million people in the United States sustain a TBI [1] and TBI accounts for a significant proportion of casualties in modern day war, with an estimated 15-20% of soldiers sustaining a brain injury during their deployment [2].

Within the field of neurotrauma, there are currently numerous animal models described for inducing brain injury [3-5]. The focus of this protocol is on the effects of single and multiple concussive (closed-head) and blast-like brain injuries. The CNRM Pre-Clinical Studies Core currently has two concussive injury models available to investigators for their experiments: **CBI** (closed-brain injury) and **WDI** (weight-drop injury). CBI [6, 7] is a procedure in which a metal

rod mechanically strikes the surface of the skull and results in damage to the underlying cortex. The severity of the injury is controlled by the depth of the penetration and the velocity with which the impactor strikes the surface. CHI induced by a weight-drop device [8] is a simple and fast procedure in which the skull is left intact and a free-falling weight produces a blunt focal injury. The height from which the weight is dropped and the mass of the weight determine the severity of the resulting brain injury.

In addition to the concussive brain injury models described above, we will also collect data on the effects of single or repeated exposure to the two blast-related injury models available in the core facilities: **HIFU** (high-intensity focused ultrasound) and ABS (advanced blast simulator). HIFU [9] is a model of TBI that was developed in collaboration between USUHS and the FDA, and is a non-surgical procedure that aims to simulate a blast-induced TBI; HIFU employs a high-pressure wave that passes through the scalp, skull, and brain to induce a more diffuse and global brain injury than those resulting from contusive or concussive (CBI and WDI) injuries. Additionally, the Core has a recently-installed Advanced Blast Simulator (**ABS**) for which procedures need to be formalized and anatomical and behavioral effects defined. The ABS produces an atmospheric overpressure shock similar to the shock wave generated by blasts of explosives in combat situations.

The ultimate goal of this research is to formalize the procedures and compare the anatomical and behavioral effects of the four described methods of inducing TBI (CBI, WDI, HIFU, ABS) in C57Bl/6J adult male and female mice. These injury methods are available to CNRM investigators using the Pre-Clinical Studies Core, and the data that result from the experiments described in this protocol will create a database of information about the anatomical effects of TBI and the performance of C57Bl/6J adult mice on a series of behavioral tests within the CNRM Pre-Clinical Studies Core. This will aid CNRM investigators in deciding which procedure for inducing TBI (and which behavioral tests) will be best in their experimental questions. In addition, this protocol will formalize the specific steps in handling the mice, injury procedures, and executing the subsequent behavioral tests. The Core employees, in turn, will gain experience that will allow them to instruct investigators in how to perform the Core protocols.

This protocol serves a second purpose. Although there are hundreds of published reports describing behavioral testing in mice after TBI, establishing an efficient Core facility requires CNRM investigators to be skilled in using the specific equipment in the Core. An established database of information about surgical procedures and performance on behavioral tests after TBI is also necessary. The "local" data set that will be derived from this protocol is critical since previous studies have shown that it is an important step in the attempt to derive comparable data across research groups [10, 11]. Furthermore, a "local" data base affords an opportunity for statistical power calculations, providing better estimation of sample size for investigators' research plans with mice.

Finally, the results of this protocol will be made available to the Core staff and to all investigators using the Core to affirm consistent use in the facility of equipment employed for modeling brain injury and subsequent behavioral testing. These data will be used as an initial confirmation that investigators are properly using the equipment and testing mice in a consistent manner that derives data that is comparable over time, across investigators, and within individual studies [12], as well as comparison of responses in male and female mice [13].

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: BRD

II.2.2. Date of Search: August 28, 2014

II.2.3. Period of Search: 1998-2009

II.2.4. Key Words and Search Strategy: All fields: Rodent + behavior ± blast ± concussion

II.2.5. Results of Search:

The BRD database includes thousands of records of previous research programs. The search derived a total of eight unique hits.

1. *Understanding the Impact of Repeated Blast Exposures on Brain Function*, supported by M6: Combat Casualty Care, performance site: Duke University, Durham, NC.
2. *Safety and Efficacy of Recombinant Factor VIIa in the Treatment of Traumatic Brain Injury in Rats*, supported by M6: Combat Casualty Care, performance site: Walter Reed Army Institute of Research. Silver Spring, MD.
3. *CIP#S-06-053 The Efficacy of Pharmacological Treatment of Blast Injury in a Rodent Model*, supported by M6: Combat Casualty Care, performance site: Naval Medical Center San Diego, CA.
4. *Zinc Supplementation and Neurologic Recovery Rate in Rats with Mild Traumatic Brain Injury (mTBI)*, supported by M5: Human Systems Technology, performance site: U.S. Army Research Institute of Environmental Medicine, Natick, MA.
5. *Blockage of Nociceptin Signaling Reduces Biochemical, Structural, and cognitive Deficits after Traumatic Brain Injury*, supported by M6: Combat Casualty Care, performance site: University of Oklahoma, College of Pharmacy
6. *Treatment of TBI with hormonal and Pharmacological Support, Preclinical Validation using Diffuse and Mechanical TBI Animal Models*, supported by M6: Combat Casualty Care, performance site: University of Alabama at Birmingham
7. (b)(6)
8. [redacted]

Please note grants 7 and 8 were held by the P.I., supported by approved IACUC Protocols, and the work has been completed.

Conclusion: No related hits indicated there is no overlap with previous research programs in the DoD None of these programs pertained to validating the newly-designed ABS system for use in TBI research.

II.2.6. Literature Source(s) Searched: NIH RePORTER

II.2.7. Date of Search: July 17, 2014

II.2.8. Period of Search: active projects through fiscal year 2014

II.2.9. Key Words and Search Strategy: Rodent, behavior, blast, concussion

II.2.10. Results of Search:

CRISP is no longer an active search engine/web site. The suggested substitution is NIH RePORTER, and this site was used. The keywords rodent, behavior, blast and concussion produced one hit (Projects NIH 1I01RX000380-01 and 5I01RX000380-02, *Blast and blunt mechanisms for mild-TBI induce differing outcomes*). This hit was for a project studying the

behavioral consequences of a single rotational injury or blast in rats. However, the project employed rats and was not as comprehensive as the studies outlined here. **Conclusion**: No related hits indicated there is no overlap with previous research federally sponsored research programs.

II.2.11. Literature Source(s) Searched: PubMed
I.2.7. Date of Search: August 22, 2014
II.2.12. Period of Search: >22 million references; 1949-2014.
II.2.13. Key Words and Search Strategy: ("behavior, animal"[mesh]) AND "rodentia"[mesh] AND "blast injuries"[majr] AND ("animals, laboratory"[mesh] OR "models, animal"[mesh])
II.2.14. Results of Search:
PubMed was used with the keywords listed, and two hits were obtained. One of the hits described chronic traumatic encephalopathy after blast injury in veterans and also in a mouse model of blast-induced TBI [14] , and the other used behavioral and imaging assays to assess blast-induced hearing loss in rats [15]. The P.I. is aware of the broader literature describing pre-clinical animal models, testing procedures, testing sequence, and reducing overall distress (e.g., [16-20]) and has an extensive EndNote bibliography (obtained from searches over the years) of relevant publications. Conclusion: Neither of these experiments used the injury methods or conducted the extensive behavioral analyses and brain imaging that we describe in this protocol.

III. OBJECTIVE\HYPOTHESIS:

The objective of this project is to provide empirical data about the neuropathological and behavioral consequences of different procedures of inducing multiple blast- or concussive-brain injuries in adult male and female C57Bl/6J strain mice. This project also allows investigators working in the Core to formalize the precise injury methods and behavioral testing procedures that will be employed and provide an opportunity for investigators to develop the skills for undertaking the procedures. It is *hypothesized* that different methods of inducing TBI in mice will result in different patterns of behavioral and neuropathological changes. Furthermore, single injuries may incur little to no functional or neuropathological consequences with more severe changes seen only after multiple exposures to a concussive or blast injury.

We also expect that the establishment of formal procedures and the acquisition of skills in the consistent application of procedures will enhance the reliability and validity of data generated by investigators who use the Core facility in their research. In the short term no set criteria will be established to directly evaluate this hypothesis in terms of predicted quantitative outcome. However, the P.I. and staff already have extensive experience in rodent TBI models and behavioral testing, and will use the data to formalize detailed experimental protocols.

IV. MILITARY RELEVANCE:

The military relevance of this protocol complies with the major stated objective of the CNRM and the establishment of a CNRM rodent core facility at the University: "to address the current needs of the medical community to better diagnose and intervene for the prevention of the long term consequences resulting from traumatic brain injury, particularly in the context experienced by service members in Operation Iraqi Freedom (**OIF**) and Operation Enduring Freedom (**OEF**)." Due to the use of improvised explosive devices, the nature of injuries resulting from the wars in Afghanistan and Iraq differ from previous wars with the signature injury being blast injury; it is estimated that about 75% of the casualties from recent conflicts in the Middle

East result from explosions [21], and nearly 10,000 soldiers have been wounded in action from IEDs [22, 23]. According to a respected RAND Corporation report [24], as many as 18.5% of the service members returning from these conflicts meet the criteria for a diagnosis of either post-traumatic stress disorder (**PTSD**) or depression, and 19.5% of service members have reported experiencing a probable TBI during deployment. These estimates translate to approximately 320,000 cases of "probable TBI" and 300,000 cases of reported symptoms for depression or PTSD. Most cases of TBI in OIF and OEF arise from blast injury associated with the detonation of explosive devices in the form of non-contact closed head injury with concussive (impact) and primary blast wave exposure. Although there is no doubt, based on anecdotal clinical observation, that blast can induce cognitive deficits and TBI in military personnel, the exact mechanisms by which blast results in injury to the central nervous system are unknown. Furthermore, the actual components of the blast wave, direct or indirect, that result in brain injury are also unclear. Scientific research related to developing a better understanding of the biological processes and treatment for blast-induced TBI and/or psychological trauma is necessary for the care of our service members. The laboratory mouse, using these injury models, is expected to advance our knowledge of biological mechanisms of brain injury and psychological maladies and may lead to more effective treatment.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

General Approach: The CNRM Pre-Clinical Studies Core presently has two concussive-related TBI models available for investigators to use in experiments with mice: closed-brain injury (**CBI;** performed by having a piston strike the exposed skull at a pre-determined location, velocity and depth [6, 7]) and weight-drop injury (**WDI;** a free-falling weight hits the exposed skull [8]). Two blast-related injury models are also available: high-intensity focused ultrasound (**HIFU**; similar to a primary blast injury where a high-pressure wave passes through the brain causing tensile distortion of brain structures, without an acceleration injury [9]) and advanced blast simulator (**ABS**; exposure to an atmospheric overpressure shock similar to the shock wave generated by blasts of explosives in combat situations (in development). TBI will be induced in male and female mice using one of those four methods. Each mouse will receive one, three or five injuries (multiple injuries will be separated by 24 hours), and compared to mice that receive one, three or five sham procedures. Following TBI, mice will either be evaluated on an extensive range of behavioral tests, sacrificed within one week to assess short-term histopathological changes or changes in brain anatomy will be assessed with magnetic resonance imaging (**MRI**). The plan for this protocol is to employ separate groups of mice in four behavioral testing modules, labeled "Experiments 1a-1d" (see below). In addition, separate groups of mice will be imaged with MRI at three different time points after the final induction of TBI ("Experiment 2") or sacrificed within one week of final TBI for assessment of short-term neuropathology following injury ("Experiment 3").

Overall experimental design. The basic design is to compare four injury models with three numbers of exposures, appropriate sham-operated animals, and two genders of mice. Including male and female mice and the appropriate sham control groups, the final number of experimental groups is 42. Statistical analyses of these groups will be limited to meaningful comparisons, e.g., comparing male and female behavioral performance after 1, 3 or 5 exposure to HIFU or sham procedures, or, comparing the effects of number of concussive-like brain injuries (CBI or WDI)

on behavioral and immunohistochemical measures. Full-scale studies will be performed to assess the brain anatomy at multiple time points, and to collect statistical data on the performance of mice on at least 10 behavioral tests after single or multiple TBI induced by blast-related methods: ABS or HIFU, or concussive-type procedures: CBI or WDI. We are aware that this is a very ambitious protocol that will likely not be completed in three years; we anticipate that this protocol will be re-submitted in three years to continue the studies.

Separate groups of mice will be evaluated in one of the four groups of behavioral experiments (Experiments 1a-1d). The rationale for not testing an individual mouse on all the behavioral tests is that repeated testing of mice alters response patterns due to general stress, fatigue, "testing experience," and learning [25, 26]. Many of the tests employed in this protocol have the potential to cause stress to the animals (e.g., fear conditioning and Porsolt forced-swim test) and the experiments have been designed to limit the number of potentially stressful tests to which each animal is exposed to one. This minimizes animal distress and also results in data for which stress is not a potential confounding variable.

Additionally, the testing schemes have been designed to try to avoid exposing mice to more than one behavioral testing "domain." For example, the Morris water maze, Barnes maze, and passive avoidance tests all assess memory and cognitive performance. An attempt is made to avoid using the same animals on tests that assess similar neurological functions, as prior experience on one test within a particular "domain" may affect later performance on tests that assess similar functions. Nevertheless, many of the tests outlined below assess overlapping psychological dimensions or challenge several neurological systems at once. Separate behavioral measurements obtained during a single behavioral test can assess distinct neurological systems [10] and, when possible, multiple measures are recorded so that these separate neurological systems are evaluated. For example, the open field test is one of the most common behavioral tests. A mouse is placed in a closed arena and a software program records a number of measures during the test. Measures of *activity* and *motor function* include distance traveled, changes in activity at half the total duration of testing time, and turning rate (all parameters captured by the software), while grooming, freezing, fecal boli, and thigmotaxis (remaining near area walls and not exploring the center of the arena) reflect *anxiety* (www.jax.org/phenome, Open Field Test).

Sequence of behavioral testing. Logistical constraints in testing procedures, animal distress and fatigue (both physical and psychological) require that each animal is employed on no more than three behavioral tests. Experiment 1a is an exception to this guideline since the majority of the tests are very brief, non-stressful, occur over a period of about four weeks, and have been proven in our prior work to be very robust in detecting the effects of more severe contusive brain injuries such as controlled cortical impact (CCI). In addition, when a sub-study of Experiment 1 (described below) involves more than one test, the more distressful or aversive task is scheduled last, in an attempt to reduce confounding of test results from previous distress.

Strain choice. The strain choice for this protocol is the C57Bl/6J strain. The C57Bl/6J mouse was selected since this strain generally performs "average" on many behavioral tests [27]. The data derived from the use of this strain is also perhaps most useful to CNRM investigators. The C57Bl/6J mouse is an inbred strain, providing a very high degree of genetic homogeneity [28] and it is one of the traditional strains used for studies that will eventually employ this strain as the "control" or "wild type" condition for a related mouse strain that harbors a transgene or gene "knockout" [29, 30]. Due to the recent mandate by the NIH that both male and female mice be

included in pre-clinical animal studies [13], both male and female C57BL/6J mice will employed in these experiments.

V.1.1. Experiment 1: *Neurobehavioral assessment of male and female mice after single or repeated traumatic brain injury.*

Experiment 1 will employ sub-groups of behavioral tests (Experiments 1a-1d) to assess functional deficits after TBI. **Table 1** lists the number of animals needed for the entirety of Experiment 1. (Each sub-study of Experiment 1 will employ 1,008 mice, for a grand total of 4,032 mice in Experiment 1.) Fewer animals will be needed for the evaluation of neuropathological and anatomical changes at multiple time points after TBI using immunohistochemistry and magnetic resonance imaging; the number of animals needed for these studies (Experiments 2 and 3) are listed in Tables 2 and 3, respectively. Note that the procedures used for sham controls for WDI and CBI injuries are identical; only one sham control group is needed for both injury types.

Table 1. Experiments 1a-1d: Short-term neuropathology following single or multiple TBI

TBI procedure	Male C57BL/6J			Female C57BL/6J		
	1X	3X	5X	1X	3X	5X
CBI	96	96	96	96	96	96
WDI	96	96	96	96	96	96
CBI/WDI Sham	96	96	96	96	96	96
HIFU	96	96	96	96	96	96
HIFU Sham	96	96	96	96	96	96
ABS	96	96	96	96	96	96
ABS Sham	96	96	96	96	96	96

Table 1 includes mice for all behavioral studies, Experiments 1-4. Twenty-four mice per group will be included in each of the Experiments, resulting in a total of 96 animals in each group or 4,032 animals for all behavioral studies.

Experiment 1a: *Assessment of the effects of blast and concussive-brain injuries on exploration, motor skills, anxiety, learning and memory and behavioral flexibility.*

Post-TBI behavioral performance will be evaluated in mice on several well-known and widely-used behavioral tests in TBI research. Testing will begin the day after the final injury, and will continue for approximately four weeks.

A total of 1,008 mice (**Table 1**) are requested to measure exploration in an open field environment (Stoelting Open Field Arena with Any-maze Behavioral Tracking Software [31-33]), motor skills on an Ugo Basile Rota-Rod [34], anxiety in an elevated zero maze [35, 36], episodic memory in a Y-Maze [37], and for spatial learning and memory and subsequent behavioral flexibility in standard Morris water maze protocol [32, 38, 39] and reversal tasks [40]. Details of behavioral procedures are found in V.4.4.6. Behavioral Studies.

For pain/distress (Section V.4.1.1. APHIS Form 7023 Information), all mice for Experiment 1a are in Category D.

Experiment 1b: *The effects of repeated concussive or blast TBI on home cage circadian rhythms and on performance in an active place avoidance task.*
Experiment 1b assesses the performance of 1,008 mice (**Table 1**) in specialized home cages (Any-Cages, Stoelting) and on an active place avoidance test (BioSignal Group Interactive Tracking System Active Avoidance System, [41, 42]). Within 1-2 hours after regaining consciousness after the final TBI, mice will be placed in Any-Cages to assess levels of mobility, wheel running, eating and drinking among many other measures. The mice will be left in these cages for no more than four weeks. No sooner than 24 hours prior to removal from the Any-Cages, training on the active avoidance place system will begin (cf V.4.4.6. Behavioral Studies).

For pain/distress (Section V.4.1.1. APHIS Form 7023 Information), all mice for Experiment 1b are in Category D. Please note the description of the experimental procedure for active avoidance, under V.4.4.6. Behavioral Studies, the paragraphs under subheading 2b of Experiment 1b (page 20). Animals in the active avoidance study were categorized as Category D, since mice quickly learn to avoid the momentary exposure to the mild, escapable shock.

Experiment 1c: *Assessment of sociability and classical fear conditioning after repeated concussive or blast TBI.*
In Experiment 1c, (cf. Behavioral Studies, Section V.4.4.6) we will assess behavioral responses to the presence of two unfamiliar mice (the three-chambered sociability test [43, 44] 10 days after TBI. Two days later, mice will be tested for conditioned fear responses and extinction [45].

A total of 1,008 mice are requested (**Table 1**) for Experiment 3. (The "unfamiliar mice" of the sociability test described in Section V.4.4.6, Experiment 3, will be weight-matched sham-treated mice from other experiments described in this protocol. They are not enumerated in Table 1.)

For pain/distress (Section V.4.1.1. APHIS Form 7023 Information), all mice for Experiment 1c are in Category E as the fear conditioning procedures require the use of an unescapable shock.

Experiment 1d: *Performance on a Barnes maze spatial memory test, acoustic startle reflex (ASR) and the Porsolt forced-swim test.*
Details of behavioral testing in Experiment 1d are found in section V.4.4.6. Behavioral Studies). Ten days after the final TBI or sham procedure, spatial learning and memory will be assessed in the Barnes maze [46, 47]. The acoustic startle test [48], and the Porsolt forced-swim test [49] will be conducted, respectively, 20 days and 28 days after surgery. In Experiment 1d, a total of 1,008 mice are requested (**Table 1**).

Since the Porsolt forced-swim test is distressing (animals must tread water to stay afloat), all 1,008 mice that will be used are in Category E (Section V.4.1.1. APHIS Form 7023 Information).

V.1.2. Experiment 2: *Brain imaging after repeat blast- or concussive TBI.*
The goal of Experiment 2 is to assess changes in brain anatomy after TBI using magnetic resonance imaging (MRI). Mice will be anesthetized and imaged with the Bruker 7T MRI system in the Center for Neuroscience and Regenerative Medicine (CNRM) Translational Imaging Facility (TIF), Room G046. This procedure is described in greater detail in V.4.4.7.

Imaging will be performed once before surgical procedures and again at four time points after the final TBI: 24 hours, 72 hours, 30 days and six months. As both imaging and behavioral testing will take place at similar times post-injury, and imaging requires anesthesia, the same mice cannot be used in both Experiments 1 and 2. For this experiment, 210 mice are requested (**Table 2**).

Table 2. Magnetic resonance imaging after single or multiple TBI						
TBI procedure	**Male C57BL/6J**			**Female C57BL/6J**		
	1X	**3X**	**5X**	**1X**	**3X**	**5X**
CBI	5	5	5	5	5	5
WDI	5	5	5	5	5	5
CBI/WDI Sham	5	5	5	5	5	5
HIFU	5	5	5	5	5	5
HIFU Sham	5	5	5	5	5	5
ABS	5	5	5	5	5	5
ABS Sham	5	5	5	5	5	5

Table 2. Number of animals requested for imaging after TBI. (Experiment 2; Total n = 210)

For pain/distress (Section V.4.1.1. APHIS Form 7023 Information), all of the mice in this experiment are in Category D.

V.1.3. Experiment 3: *Neuropathological effects of single and repeat concussive- and blast-injuries.*

The goal of Experiment 3 is to thoroughly describe and quantify the anatomical and immunohistochemical changes that occur following single or repeated traumatic brain injury. *For longer-term neuropathology, brain tissue from mice in Experiment 1 will be used.* The purpose for the additional mice in this experiment is to assess *short-term neuropathological changes* following injury. The mice in this experiment (n = 420; **Table 3**) will be assigned to one of the injury groups, then sacrificed at either 24 or 48 hours, or one week, following injury. No behavioral testing or imaging will take place on these animals. Euthanasia and tissue collection will be performed as described in V.4.4.2. Biosamples. Brain tissue will be processed for the analysis of biomarkers including (but not limited to) MAP-2, APP, GFAP, Iba-1, iNOS, tau, and F4/80.

Table 3. Short-term neuropathology following single or multiple TBI						
TBI procedure	**Male C57BL/6J**			**Female C57BL/6J**		
	1X	**3X**	**5X**	**1X**	**3X**	**5X**
CBI	10	10	10	10	10	10
WDI	10	10	10	10	10	10
CBI/WDI Sham	10	10	10	10	10	10
HIFU	10	10	10	10	10	10
HIFU Sham	10	10	10	10	10	10
ABS	10	10	10	10	10	10
ABS Sham	10	10	10	10	10	10

Table 3. Number of animals requested for short-term neuropathology after TBI. (Experiment 3; Total n = 420)

For pain/distress (Section V.4.1.1. APHIS Form 7023 Information), the 420 mice that undergo sham or TBI procedures are in Category D.

V.2. Data Analysis:

To estimate the number of animals needed for the behavioral experiments (Experiments 1a-1d), the scheme outlined by Wahlsten was used [50]. Wahlsten suggests that *effect size*, δ, between two experimental groups for mouse experiments can be generally classified as *small*, *moderate*, or *large*, with δ values of 0.5, 0.75, and 1, respectively, where $\delta = \frac{(\mu_1 - \mu_2)}{\sigma}$. For an estimation of the number of mice for the behavioral experiments described in this protocol, it is assumed that a 30% difference between the average behavioral scores for two groups could represent a moderate effect. The formula above was transformed to $\sigma = \frac{(\mu_1 - \mu_2)}{\delta}$ to compute an estimate of the expected standard deviation of the residuals (σ) when there is a 30% change in behavioral score (e.g., $\mu_1 = 100$, $\mu_2 = 130$) and an expected "moderate effect" ($\delta = 0.75$). This suggests $\sigma \approx 40$ as an estimate of the standard deviation of the residuals.

The SigmaStat computer program, *Determination of Computing Power and Sample Size* was then used to estimate the number of animals needed for these experiments. This program calculates the minimum number of data units (mice) per group that is needed to achieve a certain level of *power* (sensitivity) of a statistical test based upon certain probability estimates. To achieve a power ≈ 0.80, in this case using $\mu_1 = 100$, $\mu_2 = 130$, and $\sigma \approx 40$, about 28 mice/group would be required. Since this is an estimation, we propose that for the *full-scale* studies outlined in Experiments 1-8, 24 mice may be sufficient and more convenient for the progression of testing procedures in the Core. In this case, if the same assumptions hold ($\sigma \approx 40$ with n=24 animals, $\delta = 0.84$), there would be a possible 33% difference between groups compared to 30%.

From previous experiences a sample size of 24 mice/full-scale experiment appears more than ample for behavioral testing and for what is usually reported in publications of mouse behavior testing (number of subjects per group is usually in the mid-teens). However, this estimate is justifiable since the statistical data obtained from this work will be used by multiple groups as a "standard" of comparison, and the resulting data are vital in seeking to strive for comparability across data obtained by many research teams. In addition, larger sample sizes have been used by previous groups attempting to initially validate their testing procedures. Rogers and colleagues [51], for instance, used 37-89 mice/group to validate the "SHIRPA protocol" in one study, and two cohorts of 10 mice/group for further validation and statistical reporting [52]. Likewise, this estimate appears to be in line with suggestions by internationally recognized experts. For example, Dr. (b)(6) (p. 32) suggests that "*N* = 20 mice or more per genotype are often needed to complete the first set of behavioral tests in a new line of mutant mice" [53]. Finally, it is likely that some of the mice will be "uncooperative" [10]. Data from these animals may be eliminated from the statistical calculations—though the percentage of "non-responders" on specific tests will be recorded and reported—since extreme scores can alter summary descriptions of the response pattern. A larger sample size will allow us to better estimate the percentage of "non-responders" on each behavior measure, and maintain adequate sample sizes for statistical estimation should the percentage be high on some measures.

Experiments 1a – 1d will be run in two phases. After completion of testing of half the animals requested for each group (n=12), we will analyze the data, determine statistical power, and decide if more animals will need to be added to the groups. It is very unlikely that we will need to include 24 mice in each group as described in this protocol, but the "extra" mice included

in this protocol will give us the flexibility to further refine the TBI and the behavioral testing procedures in an attempt to help investigators in the Core over the next three years. Any change in procedure will be submitted as a request to the IACUC using the guidelines of the descriptions for "minor" and "major" modifications.

For the experiments that do not include a need for behavioral testing (Experiments 2 and 3), we believe we require fewer animals. We estimated that 10 mice/group may be sufficient for the short-term neuropathology studies (Experiment 3) and 5 mice/group adequate for imaging (Experiment 2). This estimation is based upon prior experience that we often obtain reliable data with this number of animals per treatment group.

Statistical Analyses: The SigmaStat 2.03 (SPSS) statistical analysis program will be used to compute the arithmetic mean and standard deviation of surgical groups on each test measurement. A 3-way analysis of variance (ANOVA) can be performed on the factors, Injury (4 levels for CBI, WDI, HIFU, ABS) x Injury Treatment (2 levels for TBI or Sham treatment) x Gender (2 levels), or 4-way mixed model ANOVA, with the same mentioned factors and a "repeated measures" factor when animals receive more than one experience with the same test on different days. *Post* hoc comparisons can then be used to further explore effects. However, experience from previous work indicates, particularly with behavioral data, that the data does not always meet the assumptions of normality and homogeneity of variance. In this case, simpler comparisons can be undertaken or data transformations can be performance (e.g., ln). Non-parametric comparisons may also be employed. Finally, for some comparisons between behavioral test results and histopathology, or mRNA or protein levels, changes to assess the degree of the relationship within an experiment can be determined by computing correlation coefficients.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

Employing animals in this research is necessary to assess the effects of brain injury on brain anatomy and on *in vivo* behavioral performance. These data will provide a means for relating behavioral responses to central nervous system structure and function *in vivo*. Non-animal alternatives do not replicate the three dimensional structure or temporal changes that occur as a function of complex behavioral responses to tasks presented to a live animal. Further, computer modeling and *in vitro* methods will not address experimental questions of the complex, real-time changes and responses that occur during experience.

V.3.2. Animal Model and Species Justification:

By consensus of members of the CNRM, C57Bl/6 mice have been chosen as a suitable species and animal model for this research [54]. This is based upon the previous use of this strain in innumerable studies and their suitability as biomedical models for understanding central nervous system functions in normal and pathological states in humans and in other animals. The mouse is used for these studies because of their well-understood anatomy and endocrine responses, and the very extensive database of prior physiological research that can be integrated with this new information. It is necessary to use mice instead of a "lower species" since the research results should be directly associated with intense efforts in other laboratories throughout the world to try and further our understanding and eventual treatment effectiveness for psychological stress and brain injury. Mice are a commonly used small animal model for the study of neurobiological genetics research, including the impact of physical injury and environmental stress upon neuron

connectivity, experiential effects on synaptic plasticity, signal transduction, and for assessing treatments to reduce neurological damage and improve quality of life. In transgenic and gene knockout models in neuroscience and behavioral research, "wildtype" C57Bl/6J mice often serve as the genetic "background" or control for comparison of genetic and genetic/environmental effects [28, 30, 53]. As a result of the recent directive by the NIH to include female animals in pre-clinical research [13], this work proposes the use of both male and female mice.

V.3.3. Laboratory Animals

		Species #1	Species #2
V.3.3.1.	**Genus & Species:**	*Mus musculus*	
V.3.3.2.	**Strain/Stock:**	C57Bl/6J	
V.3.3.3.	**Source/Vendor:**	(b)(4)	
V.3.3.4.	**Age:**	8-12 weeks	
V.3.3.5.	**Weight:**	20-25 grams	
V.3.3.6.	**Sex:**	Males and Females	
V.3.3.7.	**Special Considerations:**	None	

V.3.4. Number of Animals Required (by Species): Mice: 4,662

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:
Two refinements are included in our protocol. **1.** Mice will be acclimated to investigator handling before behavioral testing to relieve distress from the potential combination of handling distress, surgical procedures and behavioral testing, and **2.** Analgesics and anesthetics will be used to decrease possible pain and distress resulting from surgery.

V.3.5.2. Reduction:
Power calculations have been performed to determine the minimum number of animals needed to reach statistical significance. Data derived from this protocol will be available to other investigators, allowing them to make better estimates of the minimal number of animals required for their experiments. For the sociability test (V.4.4.6 Behavioral Studies, Experiment 1c), mice to be used for Experiments 2 and 3 will be the "unknown mice" placed in the small cylindrical cages. That is, mice from Experiments 2 and 3 will be used in the sociability test prior to injury procedures. We believe their use for the sociability test will not have an impact upon other measures described in this protocol.

Additionally, the same group of animals will be used appropriate sham controls for both CBI and WDI injuries, as the sham procedures for each injury are identical. This reduces the number of sham controls needed for the full experiments.

V.3.5.3. Replacement:
Biological response to central nervous system injury, psychological stress, and experimental treatments is highly complex; no alternative to *in vivo* experimentation is available or practical. We are using a rodent model (mouse) versus a nonhuman primate model. The mouse model is the least sentient and derived animal model we could use for this study and still obtain acceptable results. In addition, we are attempting to perform a series of measurements of mouse behavior that requires making assurances that there is sufficient number of animals to derive valid measures of statistical data.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

		Species #1	Species #2
V.4.1.1.1.1.	Column C:		
V.4.1.1.1.2.	Column D:	2,646	
V.4.1.1.1.3.	Column E:	2,016	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

CBI, WDI, HIFU and sham procedures: Mice undergoing CBI, WDI, HIFU or sham procedures for the above-listed injuries will be anesthetized with isoflurane + 100% oxygen (0.5-4% isoflurane, induction; 0.5-3% isoflurane, maintenance). Isoflurane will be delivered using a precision vaporizer via a rodent anesthesia machine. Induction will occur in an appropriately-sized clear viewing chamber. Following induction, rodents will be moved to the stereotaxic device and anesthesia will be maintained via a nose cone. Waste anesthetic gases will be passively scavenged using a charcoal filter. Lack of paw-pinch reflex will be used to ensure adequate depth of anesthesia prior to beginning the procedure. Close attention will be given to ensure the mice remain unconscious and are unresponsive to pain and, if needed, supplemental administration of anesthetic will be given.

ABS and sham procedures: Mice receiving ABS exposure or appropriate sham procedures will receive an intraperitoneal injection of sterile ketamine (80 mg/kg) and xylazine (10 mg/kg) in the ventral caudal abdomen with a 26-28 gauge needle. Isoflurane will be used as described above in

future studies if it becomes available in ABS facilities. Lack of paw-pinch reflex will be used to ensure adequate depth of anesthesia prior to beginning the procedure. Close attention will be given to ensure the mice remain unconscious and are unresponsive to pain and, if needed, supplemental administration of anesthetic will be given.

MRI: Prior to imaging, mice will be anesthetized with isoflurane + 100% oxygen (2-4% isoflurane) in a clear induction chamber [55]. During the imaging procedure, animals will be monitored constantly and anesthesia will be maintained with isoflurane (1-3%) via a flow-through nose cone. A facility vacuum will actively scavenge all waste anesthetic gases.

Analgesia: Animals are not expected to experience pain or discomfort from any TBI procedures, but if needed they will be provided with acetaminophen (Tylenol) in their drinking water (1 mg/ml; ~200 mg/kg b.w.) as a precaution. Animals will be observed again one hour after home cage placement to ensure there are no signs of discomfort (difficulty breathing, hunched posture, trembling, lack of reaction to gentle touching (lethargy), or seizure activity). If needed, the LAM veterinarian will be consulted should there be signs of continued discomfort or distress.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:
Mice will be returned to the animal facility after TBI or sham procedures, or imaging. They will be monitored in their cages (and during behavioral testing) on Days 1 and 2 for complications, such as lethargy, reluctance to ambulate, inability to obtain feed, and seizures. Complications are not expected. Mice that will be returned to the animal facility after ABS procedures will be monitored in their cages for evidence of peritonitis or other complications following intraperitoneal injection of ketamine and xylazine. It is expected that complications will not be encountered. Any mouse that experiences problems will be evaluated and treated or euthanized as determined by research staff, PI or veterinarian.

V.4.1.2.3. Paralytics: N.A.

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PubMed
V.4.1.3.2. Date of Search: July 17, 2014
V.4.1.3.3. Period of Search: 1949-2014.
V.4.1.3.4. Key Words of Search: Keywords: ("Models, Theoretical"[Mesh] OR "In Vitro"[Publication Type] OR "cell line"[mh] OR "Cells, Cultured"[mh] OR "virtual cell"[All] OR "Computer Simulation"[Mesh] OR "Cadaver"[mh] OR "ex vivo"[All]) AND ("Laboratory Animal Science"[mh] OR "Animal Rights"[All] OR "Animal Care Committees"[All] OR "Animal Use Alternatives"[mh] OR "Animal Testing Alternatives"[mh] OR "Animal Welfare"[mh] OR "Animal Experimentation"[mh]) AND ("reduction"[All] AND "refinement"[All] AND "replacement"[All]) OR "3 Rs"[All] OR "Stress, Psychological"[mh] OR ("Pain"[mh] AND ("Analgesics"[mh] OR "Analgesia"[mh] OR "Anesthesia and Analgesia"[mh])) AND ("Brain Injuries"[mh] OR "Euthanasia, Animal"[mh]) AND ("Mouse"[mh] OR "Rodentia"[mh])
V.4.1.3.5. Results of Search: A total of 21 references were identified with this search. None of the searches were relevant to alternative methods for investigations of mouse core facilities or alternatives to animal models of brain injury or behavioral testing.

V.4.1.3.6. Sources Searched: Agricola
V.4.1.3.7. Date of Search: July 17, 2014
V.4.1.3.8. Period of Search: No date limits.
V.4.1.3.9. Key Words of Search: Mouse/murine, behavior, model/standard/control, brain injuries
V.4.1.3.10. Results of Search: No references were returned by this search.

V.4.1.3.11. Sources Searched: ALTBIB
V.4.1.3.12. Date of Search: July 17, 2014
V.4.1.3.13. Period of Search: Any year up to 2000
V.4.1.3.14. Key Words of Search: Mouse, behavior, model, brain injury
V.4.1.3.15. Results of Search: One hit was returned from the search of the ALTBIB database. The paper describes the neurotoxic role of microglia following excitotoxicity in the CNS, and does not provide any alternatives to the surgical, behavioral and anatomical procedures proposed here for comparing mouse models of TBI.

V.4.1.3.16. Sources Searched: DoD Biomedical Research Database (BRD)
V.4.1.3.17. Date of Search: August 28, 2014
V.4.1.3.18. Period of Search: 1998-2009
V.4.1.3.19. Key Words of Search: All fields: Rodent + behavior + Alternatives ± blast ± concussion
V.4.1.3.20. Results of Search: A search for alternatives indicated no prior funded projects addressed this area of research

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: We request that some animals (cf. V.4.1.1.1.3) be utilized for experiences considered Category E. Category E mice will be used in testing paradigms that inherently involve some inescapable discomfort/distress as a means of "motivating" the animal to respond. We wish to test the mice without treatment with sedatives or analgesics. With the exception of the fear conditioning test (Experiment 1D) that causes momentary discomfort, none of these behavioral tests involve pain or any physical injury; thus an analgesic is contraindicated. While some tests evoke mild distress, treatment with a sedative would significantly alter their level of "motivation" and change their responses to the task.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: N/A

V.4.3.1. Pre-surgical Provisions: N/A
V.4.3.2. Procedure: N/A
V.4.3.3. Post-surgical Provisions: N/A
V.4.3.4. Location: N/A
V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: N/A
V.4.3.6.1. Procedures: N/A
V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:
Anesthetic injections will be administered as described in V.4.1.2.1. Anesthesia/Analgesia/ Tranquilization and analgesic as described in Section V.4.3.3. Post-surgical Provisions.

V.4.4.2. Biosamples:
Following behavioral testing and imaging, the mice will be euthanized and available for tissue sharing by investigators in the CNRM, or the live animals will be transferred to the Center for Laboratory Medicine (LAM). In cases where animals are euthanized by individuals on this protocol, the tissues will be used for validating and/or acquisition of training and data for a variety of assays. For example, for western blotting biosamples the brains will be removed from the calvarium to obtain samples of the cerebral cortex (fronto-occipital region and entorhinal region), hippocampus, diencephalon, and brain stem. Samples are stored in liquid nitrogen before preparation for western blotting.

Because of the value of these mice, at the time of euthanasia, animals that are planned for cardiac perfusion and receive a thoracotomy ((c.f., "Perfusion" under V.4.6. Euthanasia) plasma will be obtained from a cardiac puncture blood sample [56] to begin a plasma repository in coordination with the CNRM BioMarkers Group for possible future analyses (plasma stored at -80°C in potassium-EDTA tubes). There presently is no plan for these samples, by my laboratory will keep the samples for possible future use.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification:
All animals will be identified with cage cards.

V.4.4.6. Behavioral Studies:

Reflex Suppression: Several laboratories using concussive brain injury models and shock tubes have developed procedures that permit the employment of isoflurane as anesthetic [57-62]. All animals in the behavioral experiments of the current study (Experiments 1-4) that are anesthetized with isoflurane will be evaluated for "reflex suppression" [63, 64]. Following sham, blast or concussive brain-injury exposure, the duration of the suppression of three reflexes is determined. *Corneal reflex* is evaluated by lightly touching the rodent's cornea and determining when a blink response appears. *Paw flexion* is evaluated by lightly pinching the hind paw until a withdrawal reflex is elicited. A *righting reflex* is determined by gently placing the rodent on its back and determining the time when the animal can right itself to the correct anatomical position.

Behavioral tests specific to Experiments 1a-1d are described in detail below:

1. **Experiment 1a:** Performance on a standard post-TBI battery of behavioral tests: Open field, rotarod, elevated zero maze, Y-Maze and Morris water maze (standard and reversal).

   The following set of behavioral tests was developed under protocol APG-10-789 as a standard assessment of the neurobehavioral effects of TBI on a range of behavioral domains. With the exception of the Morris water maze which will be performed last and just prior to

euthanasia, all tests are non-stressful and no more than two tests are performed on a given day.

a. **Open field testing**: Animals will be placed in an open field environment (40 cm x 40 cm) and allowed to move freely for up to one hour [31]. A camera over the apparatus is paired to Any-Maze video tracking software (Stoelting, Co, Wood Dale, IL) and the position of the animal is tracked for the duration of the test session. The software is able to provide a wealth of information regarding the behavior of the animal in the open field environment, including but not limited to the total distance traveled, movement speed, time spent immobile, and time/distance in user-defined physical zones of the open field arena. In addition, photobeam arrays allow measurement of movement in the z-plane (rearing). These measures allow conclusions regarding exploration, locomotion and anxiety-like states of the rodents. All mice will be tested on days 1, 7, 14, 30, and 60 following final TBI.

b. **Rotarod**: All animals will be evaluated for motor performance using the Ugo Basile rotarod apparatus [34, 65]. Briefly, the animal is placed upon the apparatus, which has a rotating rod. The rod is slowly accelerated from 4 rpm to 40 rpm over two minutes. The amount of time the animal remains on the rod is recorded as the motor performance score. Each animal undergoes three trials, and the average of those trials is calculated and used as the final score. All mice will be trained on this test for three days prior to the first TBI or sham procedures: the animal is first placed on the slowly-rotating (4 rpm) for one minute, after which the rod accelerates to 40 rpm as described above. The score from the third training day is used as a pre-TBI baseline measurement for the test(s) following TBI. After the final TBI, mice will be tested on the rotarod on post-injury days 1, 3, 7, 10, 14 and 30.

c. **Elevated zero maze** – The elevated zero maze will be used to evaluate anxiety-like behaviors following single or repeated TBI [35, 36]. It is a circular apparatus (diameter 50 cm) divided into four equal quadrants. Two of the quadrants (located on opposite sides of the maze) are enclosed by high walls; the other two quadrants are open with 1 cm walls). The test consists of a single trial. Mice are individually placed at the edge of one of the enclosed sections of the maze, facing the inside of the closed quadrant. Each mouse is allowed to explore the maze for five minutes before being returned to the home cage. The time spent in each quadrant is registered from video recordings by the Any-Maze tracking software, and the time spent in the enclosed quadrants is compared to the time spent in the open quadrants, with an increased amount of time in the enclosed quadrants indicating a higher level of anxiety. Typical baseline values are 15-25% time in open quadrants. Following single- or repeated-TBI, mice will be tested in this apparatus on post-injury days 5, 15 and 40.

d. **Y-Maze** – The Y-Maze is a test of visuo-spatial and working memory, and is effective in discriminating between sham-controls and mice with brain injury sustained by controlled cortical impact in the CNRM [37, 66]. The Y-Maze consists of three enclosed arms, 50 cm long, 11 cm wide, and 10 cm high, made of black Plexiglas, set at an angle of 120° to each other, in the shape of a Y. The floor of the maze is covered with the mouse's home cage wood shavings; mixed between trials to eliminate olfactory cues. Visual cues are placed around the maze in the testing room and kept constant throughout the testing sessions. The test

consists of two trials separated by four hours. Briefly, in the first trial (acquisition trial), mice are placed at the end of a pseudo-randomly chosen arm (start arm) and allowed to explore the maze for 5 min with one of the arms closed. Mice are returned to their home cages in a separate room during the inter-trial interval. In the second trial (retention trial), the mice are allowed to explore freely all three arms of the maze for 5 min. The time spent in each arm is registered from video recordings by an observer blind to the treatment condition of the mice. The time spent in the novel arm (previously closed in the first trial) will be calculated as a percentage of the total time in all three arms during the first 2 min of the retention trial. This time corresponds to the maximal exploratory activity in the novel arm, which typically declines in the subsequent 2 min. Values are compared with a random level (chance level) for time spent exploring the three arms. Mice will be tested in the Y-Maze on Day 10 following the final TBI or sham procedure.

e. **Morris water maze** – The Morris water maze is a highly-employed test of reference and spatial memory and has been used successfully in TBI experiments performed by many investigators in the CNRM [37, 67, 68]. A Stoelting Morris water maze (diameter 4.0 m) tank is filled with tap water (25°C) to a depth of 20-30 cm. A clear plastic platform is placed in one of the "quadrants" of the tank floor and visual cues (solid geometric shapes) are placed on the room walls in locations that can be viewed by animals in the pool. On Day 1 (no less than seven days after CCI or sham surgery), a single pre-training trial is first used where the mouse is gently placed on the platform in the tank and the mouse remains there for 15 seconds. If the mouse jumps into the water during this time, it is allowed a brief swim (< 60 s) before being guided back to the platform. On Day 2, training trials begin where the mouse is placed near the side of the tank in a location away from the platform, and is allotted 90 seconds to swim to the platform. Upon finding the platform the mouse remains on the platform for 15 seconds. If the mouse does not reach the platform in the allotted 90 seconds, they are gently guided to the platform and allowed to remain for 15 seconds. A total of four trials, separated by 1-2 min, are performed each day. Swimming movements of the mouse are tracked, and swim speed, distance before reaching the platform and latency to find the platform are calculated by Any-Maze software. Finally, on the fourth day of training (Day 5) approximately one hour after the final training trial, a single trial is performed with the platform removed from the tank. The time spent in the pool quadrant where the platform was located is measured, compared to time spent in the other three quadrants. After all trials, mice are placed in a clean cage underneath a heating lamp until thoroughly dried (approximately five minutes) to maintain thermoregulation.

Beginning the next day, *reversal training trials* will take place in which the location of the hidden platform is moved to the opposite side of the tank [69]. The pre-training phase is omitted, and each mouse receives four 90-sec trials for four days. A second probe trial will take place approximately one hour after the final reversal training trial. Finally, immediately after the reversal probe trial, a single visible platform trial will be conducted. A pipette with a very visible "flag" will be secured to the platform. The latency of the mouse to navigate to the cued platform is recorded (maximum of 90 sec).

Morris water maze training will begin approximately 60 days following final TBI or sham procedures.

2. **Experiment 1b: Home cage circadian activity and active place avoidance**

a. Twenty-four activity will be evaluated in the Stoelting Company's new mouse *Any-Maze Cages (AMc)*. The AMc is a wooden box measuring 15"H x 20"W x13"D that houses a single mouse and acts as an isolation chamber. It contains food and water, as well as a running wheel, and has the capability to monitor the amount of food and water intake 24 hours a day. In addition, the chamber has a built-in infra-red-sensitive camera and infra-red and ambient light LEDs. Software controls the light period (ambient light LEDs ON) and dark period (infra-red light LEDs ON/ambient light LEDs OFF). Using Any-Maze software, the camera records the animal's movements and circadian activities "24/7." The chambers are also furnished with a ventilation fan and a temperature sensor. The latter allows the temperature to remain constant and stable within the chamber.

Immediately after the final TBI or sham procedure, individual mice will be placed in the AMc system to monitor 24-hour activity and food/water intake for up to four weeks after surgery. Visual inspections of the animals will be made by research staff daily via a computer monitor, and scales within the cages provide information regarding amounts of food and water available to each mouse, eliminating disruptive door openings. Check sheets will be filled daily with amounts of food and water remaining in each cage, temperature of each cage, and a check indicating the animal was visually seen on the computer screen. LAM personnel will ensure that daily recordings are made by research staff including weekends and holidays.

b. No sooner than 24 hours after removal from Any-Cages, mice will be tested in an active place avoidance task (Biosignal; [41]). The apparatus consists of a ~50cm (diameter) metal disk with 40 cm high transparent walls that allows the mice to view distal room cues; the apparatus and testing room will remain unchanged throughout the testing protocol. Testing will take place in four phases over four days:

Phase 1 (habituation/open field): No less than three days after CCI or sham surgery, the mice will be habituated to the testing environment in a single ten-minute open field test in which the animals are free to explore the entire arena. The data from this phase of the experiment will be analyzed to assess exploratory and anxiety-like behaviors in a circular open field environment.

Phase 2 (passive avoidance; PA): Phase 2 takes place 24 hours after habituation. Each mouse will receive four 10-min PA training trials in a single day, with a 10-12 min inter-trial interval (ITI). During these trials, a 60° region of the circular arena is electrified by a grid underneath the floor, and the mouse receives a single .3 mA, 500 ms shock each time the electrified region is entered. Additional shocks are administered every 1.5s until the animal leaves the shock zone. Olfactory cues (urine and feces deposited on the arena floor) and visual cues around the room are used by mice in this phase to learn place avoidance and stop entering the shock-region of the apparatus. An overhead camera and video-tracking system are used to track movements of the mouse and calculate the distance traveled and number of shock zone entrances.

Phase 3 (active avoidance; AA): The day after PA trials, each mouse undergoes six 10-min AA trials with a 10-12 min ITI. During these trials, the same 60° shock region of the arena is stationary while the arena rotates. In these trials,

although the electrified region of the arena remains in the same spatial location, the arena rotates and the mouse must keep moving to avoid being shocked. In these trials, only visual room cues can be used by the animal to locate the electrified area as the olfactory cues rotate with the arena. Movements of the mouse are tracked and multiple measures are calculated: the latency to first entrance of the shock zone measures memory of the zone location between trials, number of shocks administered assays the motivation to escape shock; distance traveled, average speed and linearity of movement (average each 2s of the ratio dist(linear)/dist(integrated)) provide measures of motor function.
Phase 4: (AA; conflict learning (CL): In the CL trials, the shock zone location is shifted 180° from the original location for six 10-min trials (10-12 min ITI). These trials test proactive interference, or whether the avoidance memory obtained in Phase 3 AA trials conflict with avoidance memory currently being learned in Phase 4. Measures calculated from the camera-tracked movements of the mouse include the distance traveled and the number of entrances into the shock zone.

3. **Experiment 1c: The three-chambered sociability test and the fear conditioning test**
   a. The Sociability Test [43, 70] allows the mouse to freely visit three connected chambers. No less than 10 days after final TBI or sham exposure, each mouse is placed individually into the middle chamber (with the doors to the side chambers closed) for five minutes to allow the animal to acclimate to the apparatus. After the acclimation period, a small cylindrical wire cage containing a second (unfamiliar) mouse is placed into one of the side chambers. The other side chamber also contains a wire cage, but it is empty. The test mouse is placed in the middle chamber and the video system measures the amount of time (in a 10 minute test period) that the test mouse spends in the chamber containing the second mouse, as well as the time in the middle chamber and the third chamber. The amount of time the test mouse spends in the chamber with the unfamiliar mouse is an indicator of "sociability." This test session is followed immediately by a second session where the wire cage in the third chamber is occupied by a second unfamiliar mouse. Again, measures are taken to assess interactions of the test mouse with the first and second stranger mice, where the latter assesses "social novelty." The test mice placed in the cylindrical chambers are habituated to the wire cages for two days before the sociability test by placing them into the cages for five minutes each day. After their use, they will be returned to the animal facility. Two days after the sociability test, classic fear learning will be assessed in the fear conditioning test. We do not expect their experience in the sociability test to affect results obtained during fear conditioning.
   b. The Ugo Basile Fear Conditioning System is employed to assess contextual fear, where the mouse is initially placed in the test chamber and receives a mild electric shock that induces a fear response to an initially neutral [71]. On the first day of testing, at least 12 days after TBI or sham procedures, the mouse is placed in a sound-proof test chamber that presents a tone (800Hz, 80dB) for 30 seconds. A mild foot shock (2 seconds, 0.5mA) is then delivered 2½ seconds later. This process is repeated four times. The next day, the mouse is placed in a different, novel test chamber and allowed to explore. Freezing is measured over a 6 minute period, where the tone is presented during the last 4 minutes of the trial. Finally,

on the third day the mouse is placed back into the test chamber, for 5 minutes, where they had experienced the electric shock (*no tone or electric shock is given at this time*). Measures of freezing and ambulation are recorded in each test condition.

4. **Experiment 1d: <u>The Barnes maze, acoustic startle test, and Porsolt forced-swim test</u>**
   a. The <u>Barnes maze</u> [46] will employ the method described by Holmes and colleagues [71]. Over trials, mice learn to locate a hidden box located in one of the 20 holes located near the periphery of a round, brightly-lit maze platform. Mice are given three trials/day (trial duration of 180s) over eight days (Days 10-17 after the TBI or sham procedures). The Any-Maze video software records the number of holes visited, distance traveled, and time to reach the escape box for each trial.
   b. Three days after completion of Barnes maze testing, mice will be tested in the San Diego Instruments <u>Startle Reflex System</u> using the protocol described by Curzon and colleagues [72] and the one that was described and approved in protocol numbers (b)(6) In brief: five minutes before testing begins, individual mice are acclimated to the test chamber. The audio system in the test chamber emits "background noise" of 65 dB. After the acclimation period, the mouse is presented with four 120 dB sound bursts (40 msec apart), and the "acoustic startle reflex" is then assessed after these initial tests by presenting three sound pressure levels of stimuli (90, 105, 120 dB; each 40 msec duration; random order; average 15 sec between sounds, but in a range of 5-30 sec). A total of 10 trials per stimulus level are presented to each mouse. On the following day, a second test is performed to measure "pre-pulse inhibition." For these trials, five stimuli combinations are presented: a randomly chosen period to measure startle after no auditory stimulus, a 120-dB pulse alone trial with no pre-pulse stimulus, then three pre-pulse + pulse trials of 70, 75, and 80 dB pulses (20 msec duration) followed 100 msec later by a 120-dB pulse (40 msec duration), respectively. Twelve trials of these five conditions are presented with an average of 20 sec (range 5-25 sec) between trials.
   c. No less than one week after testing in the acoustic startle chambers, mice will be tested in the <u>Porsolt forced swim test</u> which is used to assess "behavioral despair" [29, 49]. The mouse is placed in a cylindrical container of water (10 cm deep, 25±1°C) and the amount of time the mouse swims is recorded. A pre-exposure session of two minutes immediately precedes the test trial, which is four minutes in duration (the total duration of the exposure is six minutes). Mice tend to float with their heads above water if they stop swimming; however, they will be continuously monitored during the test and any animal that appears to be in danger of sinking will be immediately removed from the water by the experimenter.

V.4.4.7. <u>Other Procedures</u>:

All TBI procedures will be performed as described below, and mice will be selected at random for individual procedures. All mice will first be anesthetized as described in Section V.4.1.2.1.

CBI, WDI, HIFU and relevant sham procedures are conducted in a designated area of the CNRM core facilities (Room (b)(6) on a clean table surface that is covered with clean absorbent cloth. The table will be disinfected before and after procedures. ABS procedures will be conducted in the designated area for the advanced blast simulator (b)(6)

CBI or sham procedures: The CBI procedure [6, 7] utilizes the CNRM's Pre-Clinical Core Facility's Impact One™ Sterotaxic Impactor to produce a blow to the surface of the mouse head. For the CBI procedure, mice will be weighed, anesthetized, head hair clipped, mounted in a stereotaxic frame in a prone position, and secured using atraumatic ear bars and an incisor bar. The ear bars are reversed in the stereotaxic device, so that the pointed ends face away from the mouse skull. The flat end of the ear bars are capped with a black stopper from a 5 ml syringe to hold the mouse calvarium in the horizontal plane with respect to the interaural line. To assess anesthetic depth during the procedure, the corneal reflex will be evaluated by lightly touching the cornea with a cotton swab to elicit an eye blink. The assessment of the paw and ear pinch reflexes consists of the gradual application of pressure on the hind paw or ear. Lack of eye blink or paw or ear withdrawal is an indication of deep anesthesia. The impactor tip is then centered over the parietal region of the head/skull (coordinates, -2.5 AP, 2.5 ML, 0 DV; note that no skin incision is made). The tip is then raised 1.2 mm from the surface of the head and the impactor is set to deliver a 5 mm tip extension at a velocity of 5 m/s and dwell time of 100 ms. Just before impact, the isoflurane is turned off to minimize the risk of apnea, the impact is delivered, and the mouse is immediately released from the stereotaxic device. The mouse is then placed in a clean cage on a heating pad for recovery and monitored. Once they regain consciousness, animals will be returned to their home cages and monitored. Following CBI, the impactor tip will be disinfected with 70% ethanol. Mice undergoing sham surgery will receive all procedures described here except the impact with the CBI device.

Mice that undergo multiple injuries (3x or 5x) will be given 24 hours of recovery between each injury. After 24 hours, the mouse is re-anesthetized and the TBI (or sham) procedures are performed exactly as before.

WDI: A closed-head TBI will be induced by a weight-drop method in which a 333g weight on a rod (tip of 3mm) falls from a 3cm height. First, mice will be anesthetized as described in Section V.4.1.2.1 and weighed. Throughout the procedure, anesthetic depth will be monitored by testing corneal, paw and ear pinch reflexes. After head hair is clipped, each mouse will be moved to the platform of the CHI which will be maneuvered to a position underneath the impact rod such that the desired location of impact will be achieved. The rod will then be dropped from a height of 3 cm to result in a moderate to severe TBI. The rod will be retracted immediately to prevent a "rebound" injury that could result in secondary trauma. Mice will be returned to their home cages after the procedure and monitored until they regain consciousness. As sham-controls for this procedure are identical to those from the CBI procedure, no specific sham group will be used for WDI and the CBI sham-operated animals will be used for comparison.

Mice that undergo multiple injuries (3x or 5x) will be given 24 hours of recovery between each injury. After 24 hours, the mouse is re-anesthetized and the TBI procedures are performed exactly as before.

ABS: Mice will receive TBI by an overpressure shock wave generated by the advanced blast simulator (ABS). All mice/rats are first anesthetized as described in Section V.4.1.2.1 and weighed. Mice are individually secured in a holder that is inserted inside the distal end of the shock tube. After sustaining a short duration of shock wave stimulation (<10 msec), animals will

be returned to their home cages after the procedure and monitored until they regain consciousness. A shock of "moderate intensity" as determined in behavioral and neuropathological validation experiments currently approved in protocol (b)(6) will be used for all ABS exposures in this protocol. Sham-treated animals will receive anesthesia and be placed in the holder at the distal end of the shock tube, but no shock wave will be produced. If a mouse is to receive more than one exposure to the ABS, 24 hours will elapse between individual exposures. All animals will be monitored at least once per day following TBI or sham procedures.

HIFU: HIFU procedures will be performed as described in CNRM SOP#1 [73]. In brief, mice are first anesthetized as described in Section V.4.1.2.1 and weighed. The scalp is shaved and the hair-removal product, Nair®, is placed on the scalp using sterile gauze and allowed to set for 4-5 minutes. The product is then wiped off the scalp and the scalp is rinsed with sterile gauze soaked with water and patted dry, then coated with a conduction medium (Aquasonic-100® lotion; to enhance wave transmission into the skull). The HIFU transducer is then gently placed on the scalp over the region of the sagittal suture of the skull and a high pressure wave (1-10 ms) is generated. Mice will be returned to their home cages after the procedure and monitored until they regain consciousness. Sham-treated animals will be anesthetized and scalp hair removed with Nair®, but will not be exposed to the high-pressure wave. Twenty-four hours will be allowed between HIFU or sham exposures for mice that receive more than one injury.

Model characterization: Pre-clinical models have great utility for determination of the biological and behavioral consequences of TBI, and what therapeutic treatments may hold promise for the human condition. However, one difficulty with this research relates to having an idea of the actual stimulus that was employed to cause injury. The usual approach by experimenters is to report general injury treatment conditions. There has been recent commentary, including a guide from the National Institute of Neurodegenerative Disorders and Stroke, that investigators should improve reportage of experimental treatment conditions as a way to improve the quality of pre-clinical data [12, 74-77].

For some experiments, a high speed camera (Motion Pro Y4, IDT) and image acquisition software (Motion Studio, IDT) will be used to capture motion at a rate of 4,500 frames per second. Each video "clip" is approximately 2 seconds in duration. Subsequently, the acquired videos are imported into motion analysis software (Proanalyst Professional Edition, Xcitex) to determine device, and/or head and body movement. This will be performed following the procedures outlined in USUHS IACUC Policy #25 [78]. Video images will be stored on a dedicated laptop that is password protected. In addition, to ensure availability of the data should the hard drive in the laptop fail, a backup external drive will be employed and also stored in the cabinet. The laptop and external drive are located in a secured area of the LAM facility in Room (b)(6) which has a locking door, and is stored in a locked cabinet. The videotaping will be performed by Dr. (b)(6) in the mouse surgery suite (Room (b)(6) or ABS testing suite (Room (b)(6) and viewed in Room (b)(6) Only Dr. (b)(6) and (b)(6) will have access to the videotapes as they analyze and summarize the findings. The data analysis phase is labor-intensive and will require retaining the video recordings until all data analysis is completed, but we request retaining the images for three years after the completion of this protocol (e.g., October 1, 2016 if this is the date for initial approval of this protocol + 3 years to September 30, 2019). At that time the images will be erased from the laptop's hard drive and the external hard drive. Please note the measures are converted to numerical values in a spreadsheet and this information will be retained. Actual video images will never be utilized in a presentation at USU

or outside the University. Should there be any need for a depiction of a rodent in a publication or a lecture, drawings will be created in place of actual images. It is expected that the data presented in graphical format will be adequate for readers or lecture attendees to visualize motion parameters.

Magnetic resonance imaging (Experiment 2). Imaging will be performed on the Bruker 7T MRI system in the Center for Neuroscience and Regenerative Medicine (CNRM) Translational Imaging Facility (TIF), Room (b)(6) before surgical procedures and again at four different time points after TBI: one day, three days, 30 days and six months [55]. Mice will be anesthetized with isoflurane (2-4%) in a clear induction chamber and anesthesia will be maintained via a "flow-through" tube that is used during imaging. T1 and T2-weighted images (1.0 mm thick) will be acquired with gradient echo sequence, and hyper-intense areas (indicating high water content) will be measured to assess acute edema. Hypo-intense regions will be measured to evaluate the extent of hemorrhage. The imaging procedure will last approximately two hours per animal at each time point.

V.4.4.8. Tissue Sharing:
Excess mouse tissue will be made available to other investigators in our or other labs.

V.4.5. Study Endpoint:
The study endpoint is survival for the previously described durations (**Table 4**), at which time mice will be euthanized. Euthanasia will be performed as stated.

Table 4. Summary of Animal Testing and Procedure Days and End Points for Euthanasia

Experiment	Behavior Test	Test Day(s)	Pain Category*	No. Mice	End Point (Day)†
Behavioral Studies					
1a	Open Field	1, 4, 14, 30, 60	D	≤4,032	61-64
	Rotorod	1, 3, 7, 10, 14, 30	D		
	Zero Maze	5, 15, 40	D		
	Y-Maze	10	D		
	Water Maze	20-24	D		
1b	Any-Cage	0-28	D	≤1,008	31-34
	Active Avoidance	29 or 30	D		
1c	Sociability Test	10-11	D	≤1,008	15-18
	Conditioned Fear	12-14	E		
1d	Barnes Maze	10-17	D	≤1,008	29-32
	Acoustic Startle Test	20	D		
	Porsolt Swim Test	27 or 28	E		
Neuropathology		**Euthanasia Days**			
2	–	1, 2, 7	D	210	1, 2, 7
MRI		**Imaging Days**			
3	–	1, 3, 30, 180	D	420	181-185

*Pain **Category D** refers to mice sustaining a TBI or sham procedure that will be alleviated with analgesia treatment. The behavioral tests in this case elicit little or very mild distress and no pain. The behavioral tests in **Category E** refer to animals that will receive alleviation from discomfort by the administration of analgesic following TBI or Sham treatment, but that the behavioral task (Conditioned Fear or the Porsolt Swim Test) involves brief inescapable discomfort. Since all animals in Experiment 1c and 1d receive these behavioral tests, they are classified as Category E.

†*End Point* refers to time after final TBI or Sham treatment. A range of days is indicated to allow for flexibility for investigator absence due to weekends.

Animals will be observed again one hour after home cage placement to ensure there are no signs of discomfort (difficulty breathing, hunched posture, trembling, lack of reaction to gentle touching (lethargy), or seizure activity). If needed, the LAM veterinarian will be consulted should there be signs of continued discomfort or distress.

V.4.6. Euthanasia:
Euthanasia will be performed in accordance with "AVMA Guidelines for the Euthanasia of Animals: 2013 Edition." Methods of euthanasia on this protocol include decapitation and perfusion. Mice are anesthetized before these procedures. Euthanasia will be performed by the PI or the staff in the CNRM Pre-Clinical Studies Core.

Perfusion: Some animals will be used to obtain brain samples for immunocytochemistry. Following anesthesia per Section V.4.1.2.1. , we will ensure mice are deeply anesthetized and unresponsive to paw pinch. Once the mice are deeply anesthetized and unresponsive to paw pinch, a midline thoracotomy is made to expose the heart. A 20-22 gauge tip needle is inserted into the left ventricle to aspirate 1 ml of circulating blood, which will be used to obtain plasma (see V.4.4.2. Biosamples). Next, a 20-22 gauge blunt tip needle connected to a tube (¼ in ID and 1/16 in wall) is inserted into the left ventricle through the apex, and the right atrium is incised. After an initial washout with 50 -100 ml of cold 100 mM phosphate buffered saline (PBS) for approximately 2 min, transcardial perfusion with 100-250 ml cold 4% paraformaldehyde in PBS will be delivered through the pulsatile perfusion system (BRL, CP-600) until adequate perfusion reached. The brain will then be removed from the calvarium and post-fixed before tissue sectioning for immunohistochemical staining.

Decapitation: Some mice will be used to obtain brain mRNA and protein samples. Following anesthesia per Section V.4.1.2.1, we will ensure mice are deeply anesthetized and unresponsive to paw pinch then use a rodent guillotine to decapitate the anesthetized mice. The decapitation equipment will be cleaned and maintained to ensure the proper function by routinely checking to ensure it is not rusted or blunt. It will be either sharpened or replaced if it does not function properly. A log book will be kept of the maintenance of the guillotine.

In the event that LAM personnel must euthanize an animal for humane reasons, cylinderized carbon dioxide will be used in the LAM necropsy room. Using a clean cage, carbon dioxide will be administered at a rate to replace greater than 20% of the cage volume per minute. Once the mice have not moved or breathed for at least one minute they will be removed from the chamber. Euthanasia may be ensured by cervical dislocation or opening of the thorax.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for the mice used in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6)

Building(s) Room Number(s)

Building(s) (b)(6) Room Number(s) (b)(6)

V.5.1.2.
Special Husbandry Provisions:

Food Restriction: Yes _____ No X

Fluid Restriction: Yes _____ No X

V.5.1.3. **Exceptions:** None.

V.5.2. **Veterinary Medical Care:**

V.5.2.1. **Routine Veterinary Medical Care:**
The PI or research staff will observe the mice following all experimental procedures. LAM personnel will observe the mice at least once a day for general health and husbandry condition. Any mouse observed to be lethargic, losing weight, exhibiting gross neurological deficits (such as inability to eat or drink, continual circling, seizures, etc.) or any other signs of distress or illness will be evaluated by the PI and/or veterinary staff then euthanized as previously described.

V.5.2.2. **Emergency Veterinary Medical Care:**
All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. **Environmental Enrichment:**

V.5.3.1. **Enrichment Strategy:** Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies.. Animals are grouped-housed. Standard nestlets, tunnels, or toys provided by LAM will be provided in each home cage.

V.5.3.2. **Enrichment Restrictions:** N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING*

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Concussive brain injuries (**CBI and WDI**)	(b)(6)	All personnel listed have many years of experience with general rodent surgeries.	Dr [redacted] has four years of experience with rodent surgeries related to her clinical practice as a microsurgeon, and has assisted with TBI procedures in the core facilities for 4 years. All other personnel have many years of personal laboratory experience with aseptic rodent surgeries. (b)(6)
High intensity focused ultrasound (**HIFU**)		All personnel listed have many years of experience with general rodent surgeries.	HIFU has been regularly performed by Core staff for approximately four years.
Advanced blast simulator (**ABS**)		All individuals have experience with animal models of TBI.	All individuals have received extensive on-site training from the manufacturer of the ABS.
Rodent Behavioral Testing		Ms. (b)(6) has 15 years of experience in animal handling and behavioral testing and an M.S. in Behavioral Neuroscience.	(b)(6) has many years of personal laboratory experience (b)(6) has received training from (b)(6) (b)(6)
Magnetic resonance imaging (**MRI**)		Drs (b)(6) will instruct all other personnel.	Drs (b)(6) has many years of experience in animal imaging and will direct this aspect of the work.
IP injections		All listed personnel have many years of experience in rodent handling and drug administration.	All listed investigators have taken the Rodent Handling and Procedural Techniques* and have experience with injections.
Euthanasia by decapitation		Dr (b)(6) experience with rodent decapitation >35 years. He will instruct listed colleagues on this procedure.	Performed similar procedures for recent Protocols (b)(6) (b)(6)
Intracardial perfusions		All listed personnel have many years of experience performing perfusions in rodents.	Performed similar procedures for recent protocols (b)(6) (b)(6)

*Most investigators have attended the USUHS Investigator Training Course: Dr. (b)(6) on August 17, 2005, Ms. (b)(6) completed several online USUHS Investigator Training Courses on October 28, 2009 and attended the Rodent Handling and Procedural Techniques on November 19th, 2009. Ms. (b)(6) is previously approved to perform the above procedures under (b)(6) and (b)(6) Dr [redacted] has completed the online USUHS Investigator Training Course on September 9, 2008 and the Rodent Handling and Procedural Techniques on September 24, 2009. She is approved to perform TBI procedures, drug administration, including drugs for anesthesia, and euthanasia for decapitation under Protocol (b)(6) completed several USUHS online investigator training courses including "Working with the IACUC" (non-VA) on April 23, 2013 and the USUHS Investigator Training Courses on June 6, 2013. In addition, the investigators have recently completed the new required training with the Collaborative Institutional Training Initiative (**CITI**) Program, "Investigators, Staff and Students – Lab Animal Research." Drs (b)(6) and Ms. (b)(6) completed the (b)(6)

(b)(6) course on May 9, 2014, Dr. [redacted] on May 13 & 15, 2014, Dr [redacted] on May 14, 2014, Dr. [redacted] on August 22, 2014.

VII. BIOHAZARDS/SAFETY:

To minimize exposure to animal allergens when working with lab animals, lab personnel will wear lab coats and/or scrubs, gloves and masks. We will always wear lab coats and gloves when we contact toxic chemicals and perform some steps in a fume hood to minimize risk of exposure to these teratogens. Potential fumes from fixative are minimized by allowing the fixative to be constantly washed down the sink drain.

VIII. ENCLOSURES:

Literature search results for Sections II.2 (Searches for unnecessary duplication) are available upon request.

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 9-3-14
Princi Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____
_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 9-3-14
Prin Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress (WILL) or **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Princi _____ 9-3-14

Date

X. PROTOCOL ABSTRACT:

A. **Animal Protocol Number:** (b)(6)

B. **Animal Protocol Title:** Evaluation of the effects of multiple blast- or concussive-brain injuries on neuropathological and behavioral outcomes in male and female mice

C. **Principal Investigator:** (b)(6) Ph.D.

D. **Performing Organization:** Uniformed Services University of the Health Sciences

E. **Funding:** Center for Neuroscience and Regenerative Medicine

F. **Objective and Approach:** ·

The goal of this research is to measure and compare the anatomical and behavioral effects of widely-used methods of inducing traumatic brain injury in mice. There are many animal models of TBI in use in the field of neurotrauma, and there are no studies comprehensively describing and comparing the anatomical damage and behavioral changes that result after the induction of TBI using various procedures. We will use four different methods of inducing TBI and subsequently assess the animals using at least 10 behavioral tests. In addition, mice will undergo magnetic resonance imaging at three different time points after injury to measure anatomical changes and the extent of edema and hemorrhage that results from TBI. All injury and behavioral procedures described in this protocol will be performed within the CNRM Pre-Clinical Studies Core, shared facilities used by many investigators studying TBI. As a result of the experiments described in this protocol, Core personnel aiding investigators will become better trained and more proficient in performing the procedures, and thus be better able to help investigators plan and carry out their studies. This is an important initial step for establishing the experimental procedures that all investigators should follow when they use the Core and for establishing procedures so that data collected by various research programs can be compared across laboratories. In addition, the data collected from this research will be made available to researchers as a "brain injury guide" to help them decide which method of TBI and subsequent behavioral tests will be best for their individual experiments.

To approach this problem we will use laboratory mice. Mice will receive TBI and be imaged and used in a series of behavior tests to assess the consequences of brain injury. The data collected will then be summarized using conventional statistical approaches (mean, standard deviation, analysis of variance, correlation and regression analyses). We will use as few mice as possible while still using enough to make valid conclusions. To do this initial research we must use animals since cells or tissues will not reflect the actual biology and behavioral responses of a live organism. We hope that this basic research and use of animals will aid in understanding some of the causes for the long-term effects stress and brain injury has upon the war fighter returning home to their town, work, and families, and that this research may allow us to provide better treatments to help suffering soldiers.

G. **Indexing Terms (Descriptors):** Animal, mice, brain injury, behavioral testing, imaging, anatomy

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November 3, 2015

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS

SUBJECT: IACUC Approval of Protocol - Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via designated member review on April 17, 2015.

This study was revised and approved by DMR on November 3, 2015.

Animal Protocol Title: (b)(4)
(b)(4)

USUHS Protocol Number: (b)(6)

Expiration Date: April 16, 2018

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

Revised

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: (b)(4) (b)(4)

GRANT TITLE (if different from above): (b)(4) (b)(4)

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: (b)(4)

EARLIEST ANTICIPATED FUNDING START DATE: 10/16/2011

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6) _____ APC (b)(6) 03/03/2015

Principal Investigator Signature | Department | Office/Lab Telephone | Date

SCIENTIFIC REVIEW: *This animal use proposal received appropriate peer scientific* review and is consistent with good scientific research practice.

(b)(6) _____ _____ (b)(6) _____

Research/Unit Chief / Dept. Head Signature | *Title* | *Telephone* | *Date*

Typed Name (b)(6) MD PhD

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient *data and/or is not excessive. and the statistical design is appropriate for the intent of the* study.

(b)(6) _____ 3/3/2015

Statistician Signature | Department | Telephone | Date

Typed Name (b)(6) Ph.D.

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and *manipulations that may cause more than slight or momentary pain or distress, even if* relieved by anesthetics or analgesics.

_____ LAM _____ _____

Attending/Consulting Veterinarian Signature | Department | Telephone | Date

Typed Name

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER:

PROTOCOL TITLE: (b)(4) (b)(4)

GRANT TITLE (if different from above): (b)(4) (b)(4)

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: (b)(4)

EARLIEST ANTICIPATED FUNDING START DATE: 10/16/2011

PRINCIPAL INVESTIGATOR: (b)(6)

Principal Investigator Signature | Department | Office/Lab Telephone | Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice

Research Unit Chief / Dept. Head Signature | Title | Telephone | Date
Typed Name: (b)(6) MD, PhD

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

Statistician Signature | Department | Telephone | Date
Typed Name: (b)(6) Ph.D.

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)

Attending/Consulting Veterinarian Signature | LAM Department | (b)(6) Telephone | 4/7/2015 Date
Typed Name: (b)(6) VMD

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6)

ANIMAL PROTOCOL TITLE: (b)(4)
(b)(4)

GRANT TITLE (if different from above): (b)(4)
(b)(4)

USUHS PROJECT NUMBER: (b)(6), (b)(4) funding is continuing up to 10/15/16)

CO-INVESTIGATOR(S): (b)(6)

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS: (b)(4)
(b)(4)

II. BACKGROUND:

II.1. Background:

The effects (b)(4)

The possibility of a terrorist attack against civilians, or military troops deployed overseas is at present in the minds of both citizens and government officials. Mass casualties can be inflicted with chemical or biological toxins/weapons. Nerve agents are lethal chemical weapons that have been used in war and in terrorist attacks, with devastating consequences. Organophosphate nerve agents display a very high affinity for brain acetylcholinesterase (AChE) (9, 10, 11, 63). Inhibition of AChE by OPs and the resulting accumulation of acetylcholine can initiate seizures by activating nicotinic and muscarinic receptors, which are widely distributed throughout the brain. These receptors are present not only at postsynaptic sites in cholinergic synapses, but also on presynaptic terminals of both excitatory and inhibitory synapses, regulating the release of glutamate and gamma-aminobutyric acid (GABA) (37, 108). Therefore, it is conceivable that OP-induced inhibition of AChE can tilt the balance between the two major excitatory and inhibitory neurotransmitter systems—glutamatergic and GABAergic, respectively (38, 55). One of the clinical manifestations of exposure to nerve agents is seizure activity and status epilepticus (SE), which can lead to death or brain damage, with long-term cognitive and other behavioral consequences (8, 35, 56, 91, 99). (b)(4)
(b)(4)

(b)(4) Current medical countermeasures against nerve agent poisoning are not adequately effective in preventing seizure-induced brain damage (57, 89, 96). (b)(4)

(b)(4)

(b)(4) This is very important because in an emergency situation there is no opportunity for pretreatment. Our ultimate goal is to develop a medical countermeasure against nerve agents that will effectively stop seizures and protect from brain damage and the resulting behavioral deficits, and do so without significant acute and/or long-term adverse effects. (b)(4)

(b)(4)

The necessity to study immature, aged, and female rats

Having demonstrated the effectiveness of the (b)(4) (b)(4) we now propose to test if these compounds are equally effective in young and aged rats, as well as in female rats. Are there reasons to expect age- and/or gender-related differences in the (b)(4) (b)(4) First, let us examine if the existing literature suggests differential propensity for seizure generation, and, thereby, differential susceptibility to seizurogenic agents between genders and in different ages. It is well documented that young animals and humans are more prone to generating seizures than adults, but the neuropathology induced by seizure activity is less severe and more transient in the developing brains (41, 42, 67). It is particularly relevant to mention that pilocarpine-induced SE causes inflammation and epileptogenesis in rats, but not in infant rats (64). Aged humans (45, 58) and animals (68,110) are more susceptible to both seizures and the associated neuropathology compared to adults. Gender-related differences in seizure susceptibility exist in animals and humans (47), but more research is needed to clarify and categorize these differences. For example, in humans, males have a greater susceptibility to SE, but females have a higher incidence of idiopathic epilepsies (23, 74). In animals, male rats also are more susceptible to limbic seizures and SE than female rats (94, 83), but aged female mice are more sensitive to kainic acid-induced seizures (113). There are only few studies on the age- and gender-dependency of the susceptibility to nerve agent toxicity. In guinea pigs, the lethal potencies of VX and sarin are significantly higher in the adult males than in any other age or in females (30). In rats, the lethal potency of soman is greater in 5 day-old rats than in 30 day-old rats (106), and in the young (up to 30 days) and the aged males, compared to the adults (101,106), suggesting that the toxicity of soman during an animal's life span approximates a U-shaped curve (101). Our proposed studies will yield data on the age- and gender-related susceptibility to soman toxicity and lethality. Based on the limited information available, as reviewed above, our working hypothesis is that

the LD50 will be lower in the immature and aged rats (greater susceptibility/lethality to soman), greater in female rats or similar to the LD50 in males, but the soman-induced neuropathology –which will be examined at multiple points, 24 hours, 1 week, 1 month, and 3 months after soman exposure– will be lower in the immature rats, and the recovery of the damage will be faster (the reverse will probably be true in the aged rats). It is also possible, however, that the soman-induced SE in the early postnatal rats will produce disruption in the normal brain development, which will become evident in the behavioral studies proposed here.

(b)(4)

(b)(4) On an acute basis, GABA agonists in developing rats can facilitate instead of suppressing seizures (82). In response to metabotropic glutamate receptor-anticonvulsants, different responses have been observed even in a narrow range of young ages (7 to 25 day-old rats) (66), and, in general, young rats are more responsive to NMDA and non-NMDA receptor antagonists used as anticonvulsants compared to adult rats (67). Aged animals and humans also differ from other age groups in their responses to anticonvulsants, one reason being the different pharmacokinetics in the aged organism (45, 58, 68, 110).(b)(4)

(b)(4)

Significance of studying the protection of the amygdala, the hippocampus, and the prefrontal cortex

(b)(4)

(b)(4) There are two reasons that we choose to study these structures. First, the amygdala and the hippocampus are seizure-prone structures that play a central role in temporal lobe epilepsy, which is the most common form of epilepsy, the (5, 6, 7, 76, 85). It is not surprising therefore that the amygdala and the hippocampus also appear to play a central role in the generation of seizures induced by nerve agents (4), as suggested by the rapid increases in extracellular glutamate in these brain regions after nerve agent exposure (52, 53, 54), and the profound damage these structures suffer by exposure to nerve agents (2, 4, 12, 35, 40, 46, 80, 102, 103, 104, 111).(b)(4)

(b)(4)

(b)(4)

(b)(4) The second reason is that the amygdala, the hippocampus and the prefrontal cortex are the most important brain regions in affective and cognitive functions. The amygdala is best known for its central role in emotional behavior, being a key component of the brain's neuronal network that determines the emotional significance of external events (26, 29, 61). Many emotional/psychiatric disorders are associated with pathophysiological changes in the amygdala (21, 28, 88, 109). In addition, both the amygdala and the hippocampus play a pivotal role in mediating or modulating cognitive functions (26, 61). The amygdala modulates memory acquisition and consolidation, decision-making, and interpretation of the emotional significance of external and internal events via extensive, reciprocal connections with the prefrontal cortex (36, 84) (the brain area considered to be the center for cognitive functions) and the hippocampus (76). Long-term memory storage is thought to occur in neocortical areas, but the amygdala appears to be the site for storage of fear-memory (29, 65), whereas the hippocampus is the storage site of short-term memory and the gateway for consolidation of long-term memory in neocortex (20, 33, 60). Therefore, damage to these regions is likely to be significantly responsible for the long-term cognitive and behavioral deficits that follow nerve agent exposure, in both animals (9, 50, 51, 69, 70, 71, 72, 73) and humans (18, 27, 77, 81, 97). For these reasons it is important to determine the neuropathological and pathophysiological changes induced in the amygdala, hippocampus and prefrontal cortex by (b)(4)

(b)(4)

Progress from the previous protocol

(b)(4)

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: Databases searched: BRD (Biomedical Research Database), DOAC (DTIC Online Access Controlled)* Technical Reports, DOAC Research in Progress, FEDRIP, PubMed, Web of Science

II.2.2. Date of Search: 18 February 2015

II.2.3. Period of Search: 1985-2015

II.2.4. Key Words and Search Strategy: (b)(4)

(b)(4)

II.2.5. Results of Search:

Database	Number of Results
PubMed*	42
Web of Science*	29
DOAC	205 (includes 0 in BRD)
FEDRIP	2
Total all databases	278

No citations were found, other than our own work, to use (b)(4)

(b)(4)

III. OBJECTIVE\HYPOTHESIS: (b)(4)

(b)(4)

female rats. The 16-20 month category is designated as "aged" by the supplier, and even 8 month-old male rats have been shown to be more sensitive to soman than immature or adult rats (101). After exposure to soman, the animals will be euthanized at 24 hours, 1 week, 1 month, or 3 months to evaluate nerve agent-induced pathology. Neuronal loss, using design-based stereology, and neurodegeneration, using Fluoro-Jade-C staining will be studied in the amygdala, hippocampus, and prefrontal cortex, at 24 hours, 1 week, 1 month, and 3 months after soman exposure.

In specific aim 3 we will determine whether treatment of immature (7 to 14 days-old) and adult (50 to 70 days-old) male and female rats with (b)(4) protects the animals against the behavioral alterations that are observed after (b)(4) (b)(4) At 1 month and 3 months after soman exposure, we will perform a comprehensive neurobehavioral evaluation in these animals. These tests will evaluate motor, cognitive, mnemonic and emotional abnormalities known to be associated with (b)(4) Motor performance will be evaluated using the roto-rod test: performance on this test has been shown to be reduced in rats following soman exposure (32). Cognitive impairment will be determined using tests for novelty preference in the open field, and mnemonic cognitive processes will be evaluated using the t-maze test, performance in which has also been shown to be affected by soman exposure (32, 78). Finally, emotional behavior will be evaluated by measuring the acoustic startle response/pre-pulse inhibition, and with tests of unconditioned fear (light/dark boxes, elevated plus-maze); increases in these responses have been seen following exposure to soman in rats (25).

In specific aim 3 we will also determine whether treatment of immature (7 to 14 days-old) and adult (50 to 70 days-old) male and female rats with (b)(4) protects the animals against alterations in synaptic plasticity and neuronal excitability that are observed after (b)(4)
(b)(4)

Behavioral and pathophysiological studies will be performed in both the immature and the adult male and female rats, but not in the aged animals, where variability among rats in cognitive/behavioral and physiological functions may be very high even in the control group, making it difficult to determine the deficits resulting from the (b)(4)
(b)(4)

IV. MILITARY RELEVANCE: (b)(4)
(b)(4)

(b)(4)

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures: (b)(4)

(b)(4)

V.1.1. Experiment 1:

To determine the *in vivo* efficacy of post-exposure administration of (b)(4)

(b)(4)

(b)(4) (7 to 14 days old), adult (50 to 70 days old), and aged (16 to 20 months old) **male** rats. (b)(4)

(b)(4)

A week prior to the experiments, 30%-50% of the animals will be implanted with cortical electrodes for electroencephalogram (EEG) recordings. These EEG recordings will be used to confirm the onset and intensity of behavioral convulsions observed in the rest of the animals. For the EEG recordings, along with behavioral observations, the timing (time to onset and duration) and the intensity of seizures will be measured via continuous EEG monitoring. Animals will be placed in individual recording chambers, and a baseline EEG will be obtained before administration of any drug.

(b)(4)

(b)(4)

(b)(4) The determination to classify a seizure as being terminated will be the progressive diminution and eventual elimination of repetitive high amplitude spikes or sharp waves in the EEG and the absence of obvious signs of clonic convulsive behaviors. For a seizure to be considered terminated the EEG will have to be free of repetitive high amplitude spikes or sharp waves within the 3-hour EEG recording period on the day of exposure and during the 30-min EEG sample obtained 24 hours after exposure.

The following dependent measures will be used to establish the effectiveness of the drug treatments: the speed (latency) at which the EEG "normalizes" following drug treatment, the overnight change in body weight and the extent of the neuropathological damage at 24 hours, 1 week, 1 month, and 3 months after soman exposure. With the drug treatment protocol described above, we expect a very high survival rate. However,

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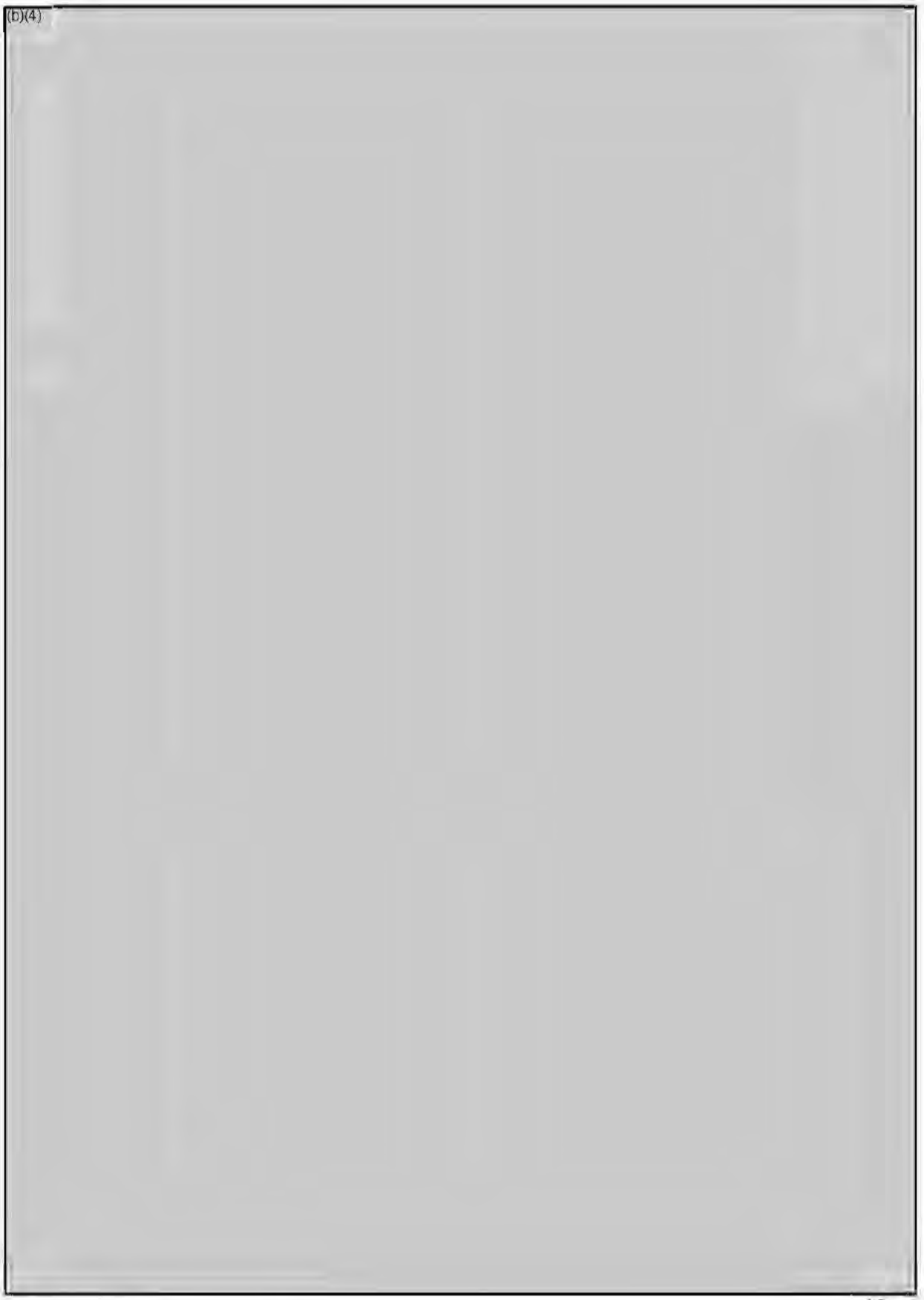
(b)(4)



(b)(4)

Considerations regarding Experiment 1:

1) In a previous protocol we have obtained neuropathology data for adult male rats at 24 hours and 1 week after soman (b)(4) exposure, but not at 1 month and 3 months after exposure. In this study we will perform additional experiments in adult male rats at 24 hours and 1 week after soman, which are necessary for comparisons with other ages and the females.

2) The age we chose for the immature rats (7 to 14 days old at which time they open their eyes) coincides with the rapid developmental changes in the brain. The gender of the immature rats may not be possible to determine at the time of soman exposure, but it will be determined at 1 week, 1 month and 3 months after soman exposure, when neuropathology and behavioral studies will be carried out. The same issue applies to the (nominally female) immature rats in Experiment 2, and it is expected that the numbers of male and female animals will even out between the 2 experiments. If not, additional animals will be ordered.

V.1.2. Experiment 2:

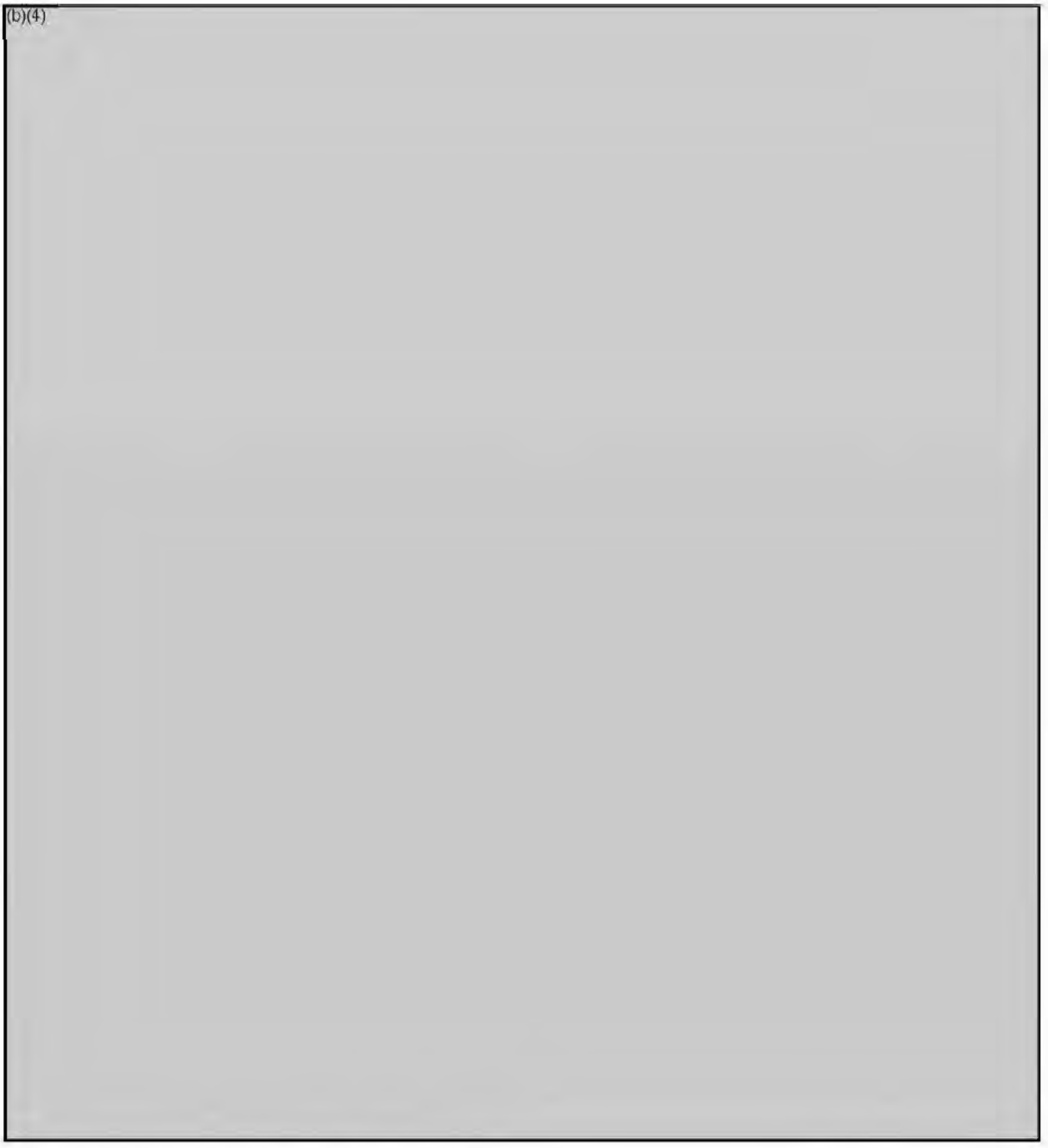
The experimental design of Experiment 2 is exactly the same as described in Experiment 1 with the exception of the use of **female** rats.

(b)(4)

(b)(4)

Immature, adult, and aged **female** rats will be randomly assigned to groups per Tables 4, 5, and 6 below:

(b)(4)



Considerations regarding Experiment 2:

1) The age we chose for the immature rats (7 to 14 days old at which time they open their eyes) coincides with the rapid developmental changes in the brain. The gender of the immature rats may not be possible to determine at the time of soman exposure, but it will be determined at 1 week, 1 month and 3 months after soman exposure, when neuropathology studies will be carried out.

V.1.3. Experiment 3:

In specific aim 3 we will determine whether treatment of immature (7 to 14 days old) and adult (50 to 70 days old) male and female rats with (b)(4) protects the animals against the behavioral alterations that are observed after nerve agent exposure (**Experiment 3A**). At 1 month and 3 months after soman exposure, we will perform a comprehensive neurobehavioral evaluation in these animals. These tests will evaluate motor, cognitive, mnemonic and emotional abnormalities known to be associated with exposure to soman and other organophosphates. Motor performance will be evaluated using the roto-rod test; performance on this test has been shown to be reduced in rats following soman exposure (32). Cognitive impairment will be determined using tests for novelty preference in the open field, and mnemonic cognitive processes will be evaluated using the t-maze test, performance in which has also been shown to be affected by soman exposure (32, 78). Finally, emotional behavior will be evaluated by measuring the acoustic startle response/pre-pulse inhibition, and with tests of unconditioned fear (light/dark boxes, elevated plus-maze); increases in these responses have been seen following exposure to soman in rats (25).

In specific aim 3 we will also perform electrophysiological experiments to determine whether treatment of immature (7 to 14 days old) and adult (50 to 70 days old) male and female rats with (b)(4) protects the animals against alterations in synaptic plasticity and neuronal excitability that are observed after nerve agent exposure (**Experiment 3B**). At 1 month and 3 months after soman exposure, rats will be euthanized, and brain slices containing the amygdala, hippocampus and prefrontal cortex will be prepared. Alterations in neuronal excitability and synaptic plasticity (long-term potentiation) in these three brain regions will be performed using both whole-cell recordings and extracellular, field potential recording techniques.

Behavioral and electrophysiological experiments will be conducted in both immature and adult male and female rats, but not in aged animals. Variability among aged rats in cognitive/behavioral and physiological properties may be very high even in the control group, making it difficult to determine the functional deficits resulting from the exposure to soman and the protection conferred by the (b)(4)

(b)(4)

Experiment 3A: To determine whether treatment of immature (7 to 14 days old) and adult (50 to 70 days old) **male and female** rats with (b)(4) protects the animals against the behavioral alterations that are observed after nerve agent exposure.

(b)(4)

(b)(4)

A week prior to the experiments, 30%-50% of the animals will be implanted with cortical electrodes for electroencephalogram (EEG) recordings. These EEG recordings will be used to confirm the onset and intensity of behavioral convulsions observed in the rest of the animals. For the EEG recordings, along with behavioral observations, the timing (time to onset and duration) and the intensity of seizures will be measured via continuous EEG monitoring (animals will be placed in individual recording chambers, and a baseline EEG will be obtained before administration of any drug).

(b)(4)

(b)(4) The determination to classify a seizure as being terminated will be the progressive diminution and eventual elimination of repetitive high amplitude spikes or sharp waves in the EEG and the absence of obvious signs of clonic convulsive behaviors. For a seizure to be considered terminated the EEG will have to be free of repetitive high amplitude spikes or sharp waves within the 3-hour EEG recording period on the day of exposure and during the 30-min EEG sample obtained 24 hours after exposure.

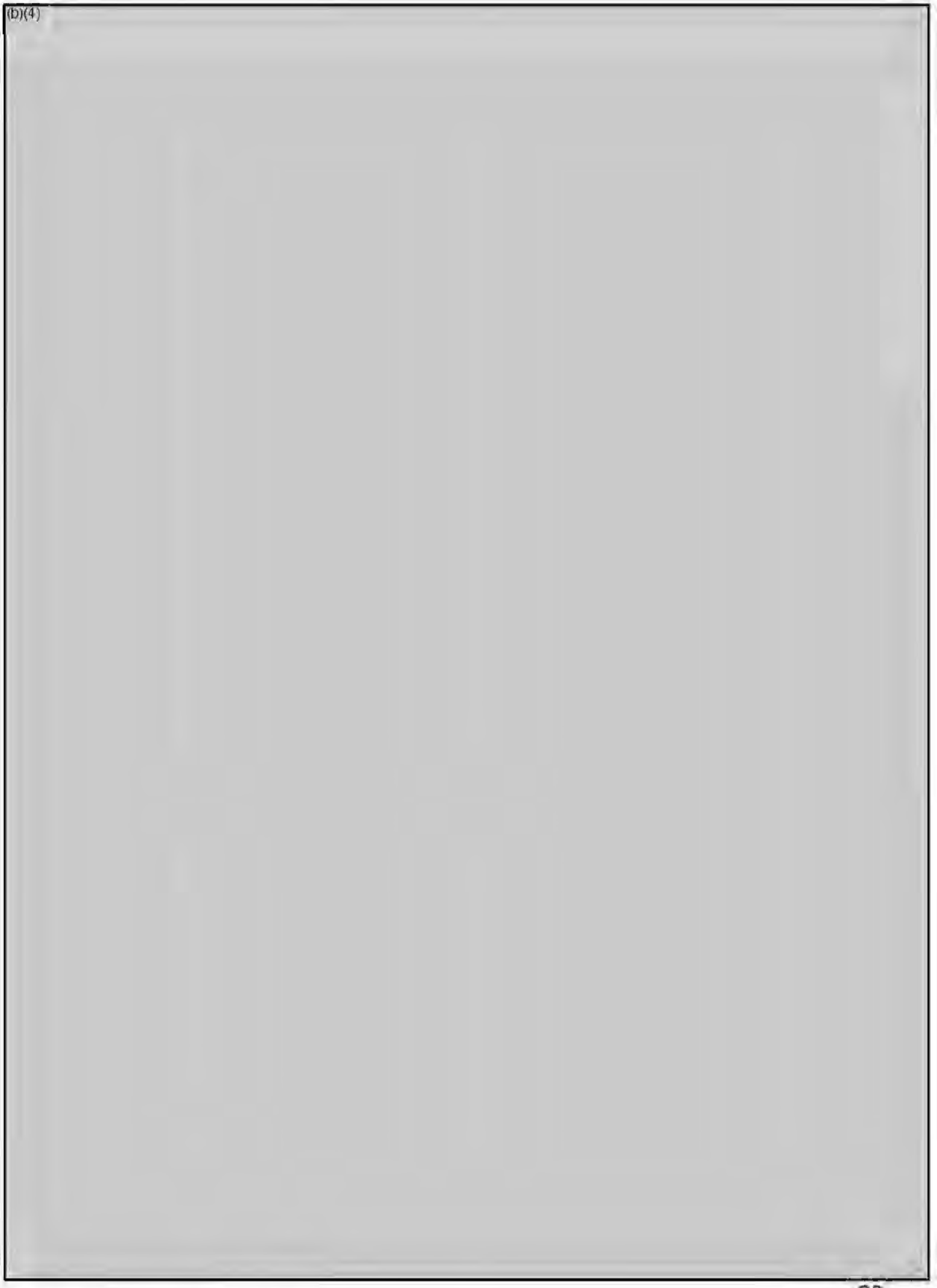
(b)(4)

We will perform a comprehensive battery of behavioral and neurological tests (see Section V.4.4.6. Behavioral Studies) on the (b)(4) rats, the (b)(4) (b)(4) 1 month and 3 months after soman exposure. These tests will evaluate motor, cognitive, mnemonic and emotional abnormalities known to be associated with (b)(4)

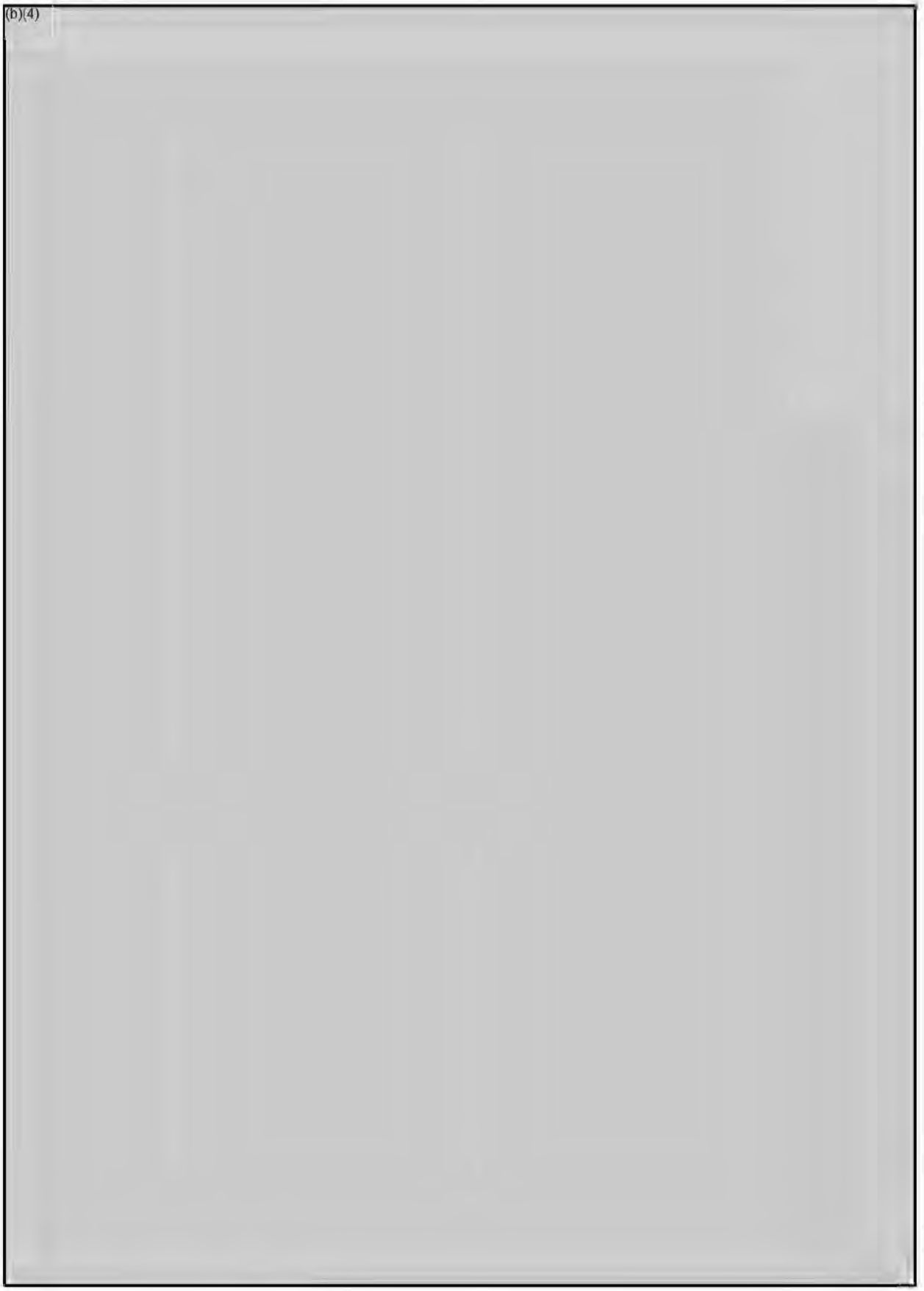
(b)(4) Motor performance will be evaluated using the roto-rod test; performance on this test has been shown to be reduced in rats following (b)(4) (b)(4) Cognitive impairment will be determined using tests for novelty preference in the open field, and mnemonic cognitive processes will be evaluated using the t-maze test, performance in which has also been shown to be affected by soman exposure (32, 78, 79). Finally, emotional behavior will be evaluated by measuring the acoustic startle response/pre-pulse inhibition, and with tests of unconditioned fear (light/dark boxes, elevated plus-maze); increases in these responses have been seen following (b)(4) in rats (25).

(b)(4)

(b)(4)



(b)(4)



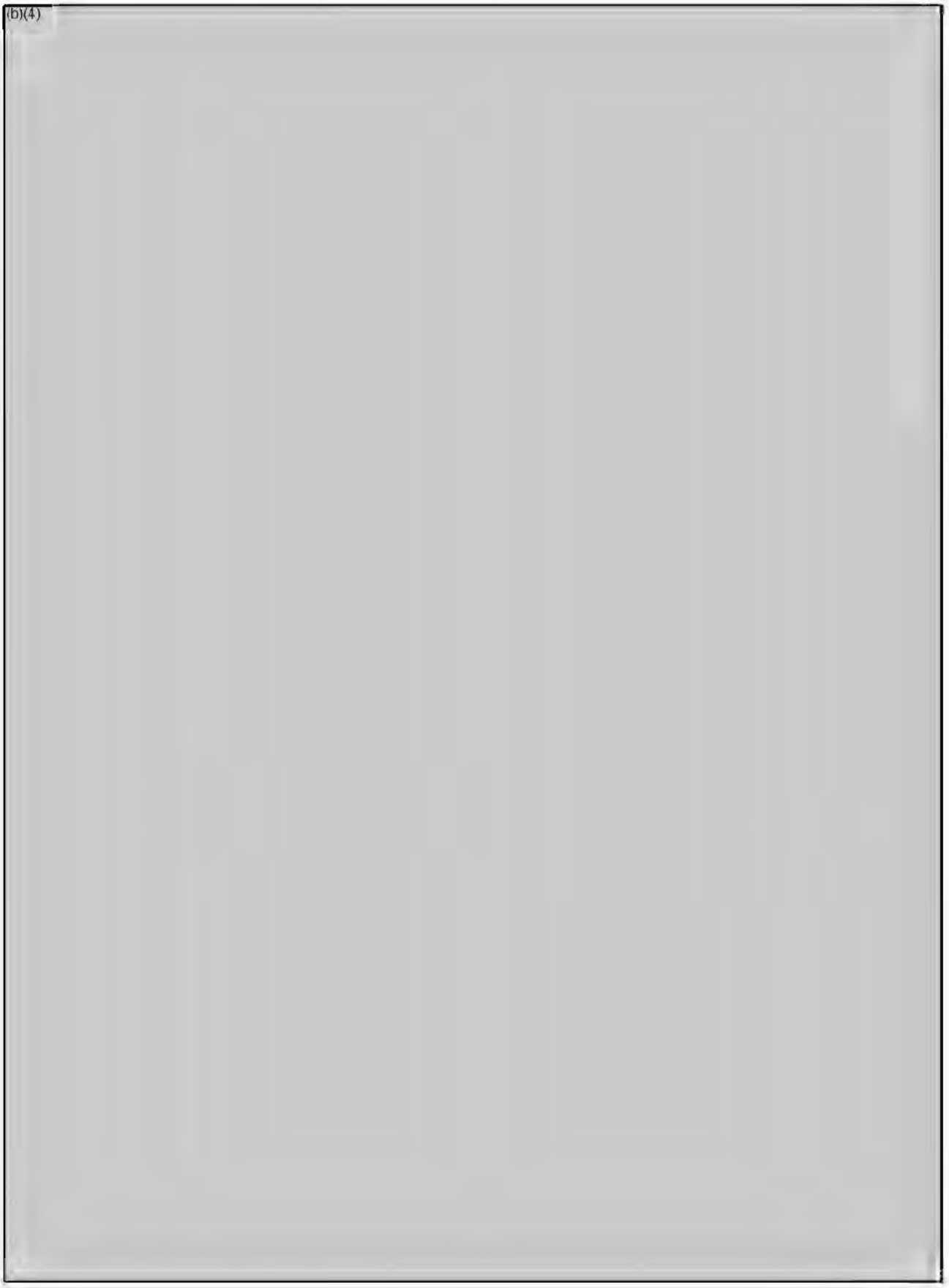
(b)(4)

Immature (7 to 14 days old), and adult (50 to 70 days old) **male and female rats** will be (b)(4) One month or three months after (b)(4) electrophysiological experiments will be conducted in the (b)(4)

Pathophysiological alterations will be studied in *in vitro* brain slices containing the amygdala and hippocampus and in slices from the prefrontal cortex. These studies will be performed using both extracellular, field potential recording techniques and whole-cell recordings.

(b)(4)

(b)(4)



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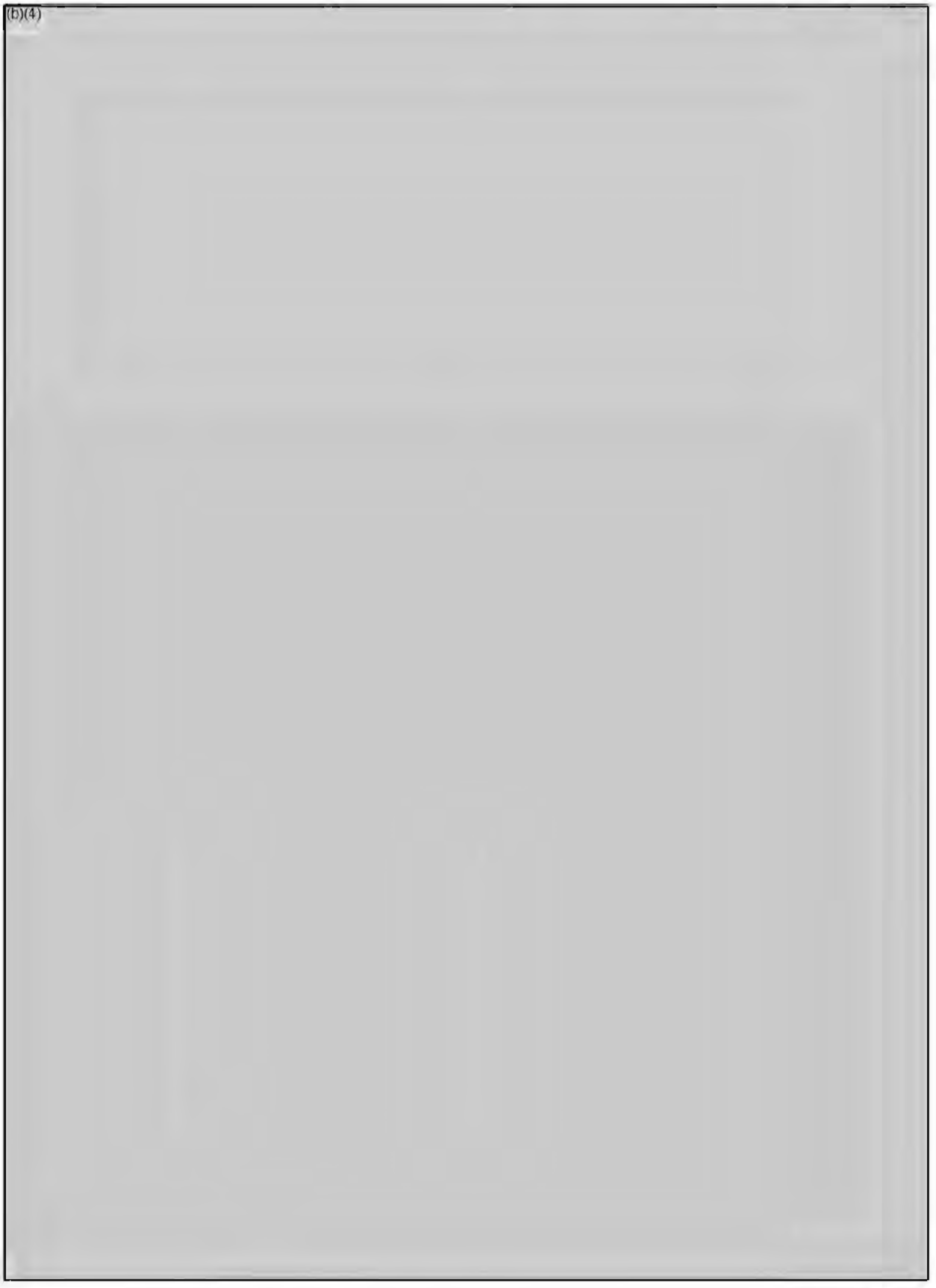
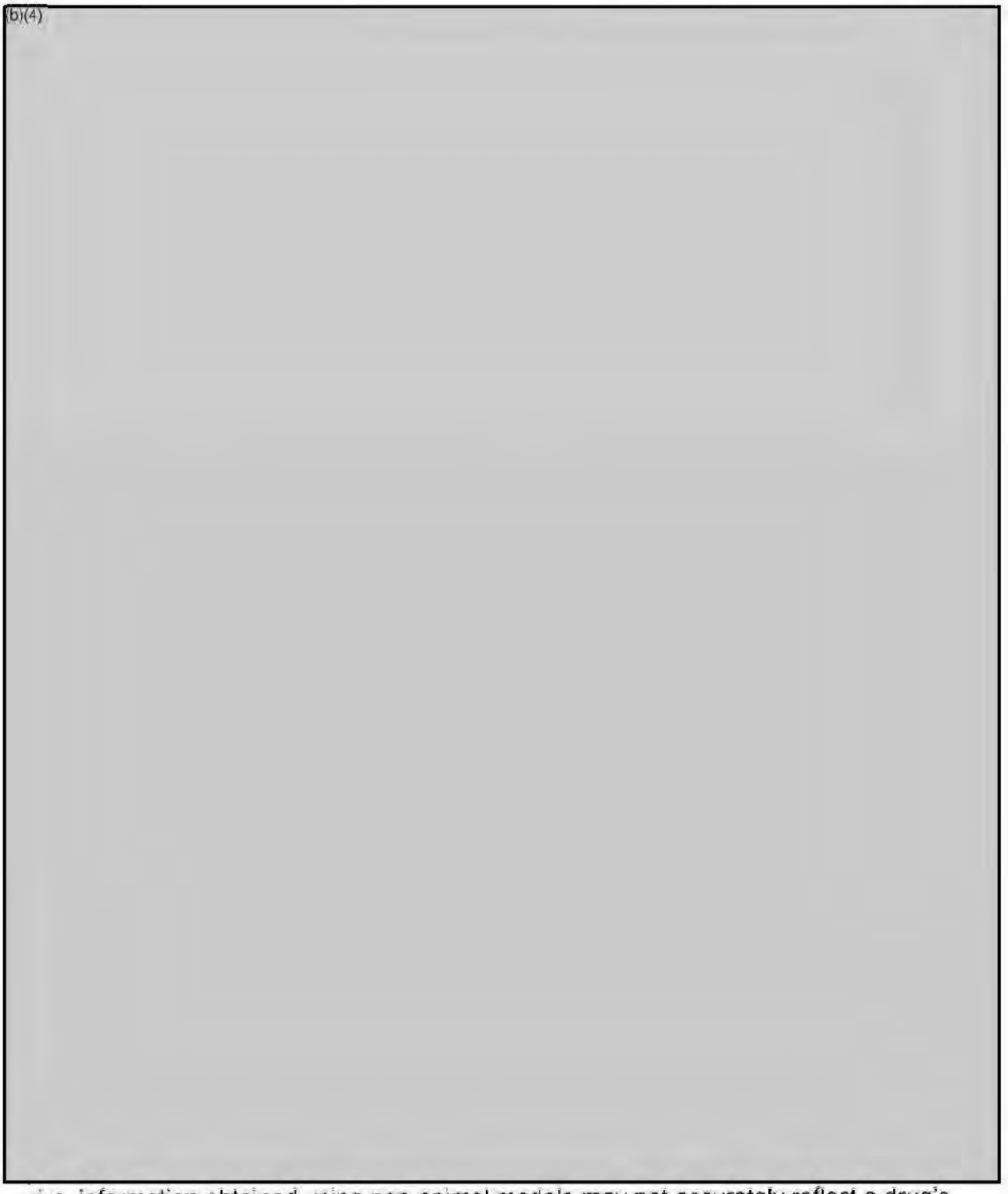


(b)(4)



vivo, information obtained using non-animal models may not accurately reflect a drug's action in a whole animal.

V.3.2. Animal Model and Species Justification: Immature (7 to 14 days old), adult (50 to 70 days old), and aged (16 to 20 months old) Male and Female Sprague-Dawley (SD) rats (*Rattus norvegicus*) will be used for these experiments. SD rats have been chosen as a suitable species and animal model for this research because they

have been extensively used in nerve agent research to evaluate new medical countermeasures. Also, the SD rat is a primary model used in studying the pathophysiology of the amygdala, hippocampus, and prefrontal cortex. Consequently, it is helpful to use the same animal to make comparisons with the literature. Rats can provide up to eight 400-µm thick brain slices per animal. By using the SD rats, we can produce high quality slices and thus facilitate and expedite the acquisition of data. The rat brain slice preparation allows stable whole-cell patch clamping recording and various pharmacological manipulations under controlled conditions. Thinner slices can easily be prepared for morphological staining. Lactating surrogate mothers will be required to accompany the rat pups that have not yet been weaned.

V.3.3. Laboratory Animals: Note that all animals used in Experiments 1, 2 and 3A and B will be purchased and received by the principal investigator (b)(6) at USUHS.

		Species #1	Species #2
V.3.3.1.	**Genus & Species**:	*Rattus norvegicus*	
V.3.3.2.	**Strain/Stock**:	Sprague-Dawley (b)(4)	
V.3.3.3.	**Source/Vendor**:	(b)(4)	
V.3.3.4.	**Age**:	7-14 days (males and females) 50-70 days (males and females) 16-20 months (males and females) Adult lactating retired breeders (age to be determined by vendor)	
V.3.3.5.	**Weight**:	Appropriate for age at time of delivery	
V.3.3.6.	**Sex**:	Male and Females for experimental animals Female for lactating surrogate mothers	
V.3.3.7.	**Special Considerations**:	Lactating surrogate mothers (retired breeders from the supplier) will be required to travel with the immature rats that have not yet been weaned and to be housed with pups at USUHS. Single housing is required after EEG surgery on adult and aged rats to prevent animals from interfering with implant healing.	

Single housing is also required after agent exposure when animals may still be sick or debilitated. Animals may be pair-housed until the day of surgery, after which they must be singly housed.

V.3.4. Number of Animals Required (by Species):

(b)(4)

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:
Rats will be anesthetized with isoflurane and reach a surgical plane of anesthesia for restraint purposes before decapitation for preparation of brain slices. All EEG surgery will be performed under appropriate anesthesia and pre-and post-surgical analgesics will be used to relieve pain. Any rat exhibiting signs of pain or impairment beyond that from this model will be euthanized at an early endpoint per paragraph V.4.5.

V.3.5.2. Reduction:
Use of an *in vitro* brain slice preparation in Experiments 3B allows the harvest of multiple brain slices per rat, thus significantly reducing the number of animals needed to obtain the proposed data. Power calculations have been performed to determine the minimum number of rats needed to reach statistical significance for each experiment. If the first 6 animals in *in vivo* treatment groups (Experiments 1, 2 and 3A and B) survive, the remaining 4 animals will not be ordered.

V.3.5.3. Replacement:
Biological response to drug treatment is highly complex; no alternative to animal experimentation is available or practical. We are using a rodent model (rat) versus a

nonhuman primate model. The rat model is the least sentient and smallest animal model we could use for this study and still obtain acceptable results. In addition, we will perform a series of morphological measurements that require a sufficiently large animal to assure that there is sufficient brain mass for multiple samplings. Mice would not be sufficient.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

In this protocol 288 lactating mothers from Experiments 1, 2, and 3A and B will be available for transfer for training or experimental purposes, or euthanized if they cannot be used (Column B). Six hundred eight (608) male SD rats from Experiment 1 and 3A and B and 608 female SD rats from Experiment 2 and 3A and B will receive an injection of saline instead of nerve agent in combination with (b)(4) and therefore will experience no pain (Column C). Eight hundred (800) male SD rats from Experiment 1 and 3A and B and 800 female SD rats from Experiment 2 and 3A and B will be injected with nerve agent and experience intense seizures. While the drugs/treatments under investigation may reverse some of these signs during certain phases of these experiments, these animals will experience some level of seizure or physical stress and discomfort for some period of time during the experiments and will be included in Column E (Non-alleviated pain).

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	(b)(4)	
V.4.1.1.1.2. Column D:	0	
V.4.1.1.1.3. Column E:	(b)(4)	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:
For surgical implantation of cortical electrodes for EEG recordings, animals will receive one dose of buprenorphine (0.05 mg/kg s.c.) before any incisions are made. The animals will be anesthetized with isoflurane (3% for induction, 2.0-2.5% for maintenance; with oxygen). Anesthesia will be induced by isoflurane vapor in 100% oxygen in a commercially available induction chamber. Once anesthesia is induced, animals will be transferred to the designated surgery area and anesthesia will be maintained via nose cone. To monitor the depth of anesthesia (including during surgery time) we will test whether the animal has lost its toe pinch (pedal withdrawal) reflex. Prior to incision, animals will receive bupivicaine at electrode placement sites (infusion of surrounding tissue), at a maximum of 0.2 ml within any one animal, and buprenorphine HCl (0.05 mg/kg, s.c.) to assist in post-operative pain management.

Following surgery for implantation of cortical electrodes, animals will receive buprenorphine HCl (0.05 mg/kg, s.c.), which will be given at a minimum one time at the end of the day of surgery, just before close of business. This is in addition to the preemptive dose given prior to surgery. All surgeries should be performed in the AM. Additional doses may be given every 8-12 hours, up to 3 days after the surgery as needed, in consultation with the attending veterinarian.

The buprenorphine dose may be increased, up to a dose of 0.25 mg/kg i.m. or s.c. at each buprenorphine dosing time point, depending on the pain status of the animal. Dose will not be adjusted without approval of the attending veterinarian.

For brain pathology studies, the animals will be anesthetized with 75-100 mg/kg, i.p., sodium pentobarbital prior to euthanasia.

For amygdalo-hippocampal and prefrontal cortex slice preparations, rats will be anesthetized by isoflurane inhalation under a hood until they are non-responsive to toe-pinch, (b)(4)

(b)(4)

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

(b)(4)

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: : BRD (Biomedical Research Database), DOAC (DTIC Online Access Controlled)* Technical Reports, DOAC Research in Progress, FEDRIP, PubMed, Web of Science

V.4.1.3.2. Date of Search: 18 February 2015

V.4.1.3.3. Period of Search: 1985-2015

V.4.1.3.4. Key Words of Search: (b)(4)

(b)(4)

V.4.1.3.5. Results of Search:

Database	Number of Results
PubMed*	5
Web of Science*	27
FEDRIP	8
Total all databases	40

*Duplicate citations removed in PubMed/Web of Science bibliography

The references found in this search cited the use of several *in vitro* models, including primary neuronal cultures and acute and organotypic hippocampal slices. The cited studies investigated protection from neurotoxicity, release or uptake of neurotransmitters, gene expression, and electrophysiological effects of ACh stimulation. We will use hippocampal, amygdala, and prefrontal cortex slices obtained from rats in this protocol. None of the other models offer any advantage over the slice preparations we propose to use. None are able to duplicate the behavioral convulsions, electrographic seizures, or brain damage produced in an *in vivo* animal model, which are necessary to evaluate anticonvulsant and neuroprotective drugs.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
There is no alternative for inducing brain damage in rats that mimics the damage caused by nerve agents to determine whether a compound is neuroprotective. The only way to determine the neuroprotective efficacy of a putative drug is to cause actual status epilepticus in an animal model to induce damage prior to administration of the neuroprotective compound and to determine the number of surviving neurons.

(b)(4)

(b)(4)

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions:

A week prior to the experiments described in Experiment 1, 2 and 3A and B, 30%-50% of the animals will be implanted with cortical electrodes for electroencephalogram (EEG) recordings. These EEG recordings will be used to confirm the onset and intensity of behavioral convulsions observed in the rest of the animals. Animals will receive one dose of buprenorphine (0.05 mg/kg s.c.) before any incisions are made. Anesthesia will be induced by isoflurane vapor in 100% oxygen in a commercially available induction chamber. Once anesthesia is induced, animals will be transferred to the designated surgery area and anesthesia will be maintained via nose cone. See Section V.4.1.2.1 Anesthesia/Analgesia/Tranquilization for details.

When the animal is anesthetized, its head will be shaved with a hair clipper. The head will be cleaned with betadine and alcohol, each repeated 3 times alternately. Then the animal will be placed into a stereotaxic frame (appropriate frame size will be used for rats) after treating its pressure points such as ears and nose with lidocaine jelly. A heated pad with thermister-controlled feedback will be put between the animal's body and the base of the stereotaxic frame. Ophthalmic ointment will be applied to the eyes to prevent dryness. The body will be covered with a sterile drape with an opening to expose the head. Prior to incision, animals will receive bupivicaine at electrode placement sites (infusion of surrounding tissue) to assist in pain management. The injection volume will be limited to the minimum required for pain control, to minimize health risks. Care will be taken to avoid systemic effects.

V.4.3.2. Procedure:

The skin will be incised longitudinally along the midline from eye level to the neck level and retracted to the sides with hemostats. The periosteum will be scraped off from the skull. Five stainless steel machine screws (the smallest possible size that provide adequate anchoring) will be used as electrode contact and simultaneous anchoring points for the headpiece that will be built from acrylic cement to secure the female electrode connector in place. The screws will be manually positioned in the frontal or parietal bone plates and one over the cerebellum. The screws will be partially screwed in manually without penetrating the dura mater.

V.4.3.3. Post-surgical Provisions:

Excess cement will be removed from the headpiece to make its edges smooth. If necessary, skin will be sutured at both ends of the incision line. The animal will be released from the stereotaxic frame and placed in a recovery cage on a chemical pocket warmer wrapped in cloth or paper until the animal fully recovers from anesthesia. Alternatively, the animals may be placed in a forced-air recovery chamber warmed to 34 °C. Once fully awake and performing purposeful movements, the animal will be moved back to the holding area. At least 7 days will be allowed for recovery after surgery before any experimentation.

Only the rats that maintain good health following the surgery will be used for experiments. This decision will be jointly made by the animal-care personnel and the investigators. All efforts will be made to provide all animals with the best care possible. Each animal will be individually monitored throughout the study by experienced personnel from our laboratory. We will also closely work with the veterinarian and animal-care staff of the animal facility to ensure that adequate treatment is provided in a timely manner should it be deemed necessary.
After surgery, the animals will be placed in a clean cage provided with a recirculating warm-water veterinary heating pad or in a forced-air recovery chamber warmed to 34 °C to maintain proper body temperature. After recovery from anesthesia (evidence of righting reflex and purposeful movements) the animal will be given at least one dose of an analgesic, buprenorphine HCl (0.05 mg/kg s.c.), for post-operative analgesia, which will be given every 8-12 hours up to 3 days after the surgery. The medication will be administered by the by PI or by qualified PI staff. If necessary, based on consultation with the attending veterinarian, additional buprenorphine may be given as needed. Additional doses of buprenorphine will be given if required, based on assessment of PI and attending veterinarian of animal's condition, which will include assessment of the presence of the following signs of pain/distress: hunching, abnormal gait, abnormal attitude (increased or decreased aggression), inability to move to food/water, excessive porphyrin staining of eyes/nose, shuddering or twitching, unkempt appearance, and vocalization when handled.

Following at least 1 hour on the warming pad or in a forced-air recovery chamber, upon full recovery in which the animal can maintain an upright posture and is ambulatory, the animal will be returned to its holding room cage. Laboratory personnel will provide the care of the animal until full recovery if it has not already occurred. All animals will be checked at least once 30 minutes after return to home cage, to ensure recovery is continuing, and at least once again just prior to the end of the work day to ensure animals are recovering normally. Animals will be examined at least once daily by the PI

staff for one week post-surgery to ensure that no complications occur. Observations will be recorded for each animal in a place that is accessible to the animal care staff (post-surgery cage cards may be used for this purpose). PI staff observations will be separate from routine observations conducted by the animal care staff.

After surgery the animals will be monitored daily and, in case of signs of infection, neomycin will be applied topically.

V.4.3.4. Location:
Surgeries (implantation of electrodes) will be performed at USUHS in LAM, Room (b)(6) Euthanasia and perfusions will be performed in room (b)(6) at USUHS. Euthanization will occur in chemical hoods in (b)(6)

It is anticipated that all of the EEG surgery will be performed at USUHS (LAM).

V.4.3.5. Surgeon:
(b)(6)

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:
All drug injections will be performed using 1-ml syringes fitted with 25-26 gauge, 1/2"or 5/8" needles. Injections on (b)(4)
(b)(4)
(b)(4) All these compounds will be prepared at concentrations to deliver volumes of 0.5 ml/kg. Vehicle solutions for (b)(4) is saline and for (b)(4) The control animals for the (b)(4) compound-treated groups will receive just the appropriate vehicle. Anesthesia and analgesia will be administered as described in Section V.4.1.2.1 Anesthesia/Analgesia/Tranquilization.

(b)(4)

(b)(4)

V.4.4.2. Biosamples:

(b)(4)

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification:
Cage cards, headpieces numbered with nontoxic ink. This will be the responsibility of the USUHS research team.

V.4.4.6. Behavioral Studies:
The following procedures will be performed at the USUHS:

Rotarod test: It measures an animal's ability to maintain balance by coordinating movement and making postural adjustments. It also measures the ability to improve these skills with practice. Each rat rotarod treadmill consists of a motor-driven drum with constant speed or accelerating speed modes of operation. The drum (7.0 cm diameter) allows each animal to maintain a suitable grip. It is divided into four test zones (8.9 cm wide) so that up to four animals may be tested at the same time. The device consists of a smooth hard plastic cylinder with concentric circular plastic sides (39.7 cm diameter) to prevent the rat from laterally climbing off the cylinder. Rats are placed on the device facing the wall with the rod rotating in the direction opposite from the animal (so that the rat uses its paws to pace forward and avoid falling off the rod). When a rat falls off the rotating drum (height of 26.7 cm), it breaks a photobeam, stopping the timer associated with that zone. In each trial, the speed of rotation is increased from 0 revolutions per minute (rpm) to 35 rpm for a maximum of 3 or 5 min. The mean duration on the device (based on 3 consecutive trials) is recorded.

Preference for Novelty (Open Field): Testing will be performed in a Plexiglas cage (56cm x 34cm x 20cm) with the floor divided in 18 equal squares. Three identical aluminum cubes will be evenly distributed in the cage in fixed positions. Three other cubes will make up the novel objects. One novel object will be similar to the neutral cubes in that its top will be uneven with tracks making up a square pattern (tactile stimuli). One novel object will be identical with the neutral objects except that a slight smear of cheese will be placed on the side facing the experimenter (olfactory stimuli). The third object will be similar to the neutral blocks except that it will be slightly smaller with uneven sides (visual stimuli). Locomotor activity will be measured using an Omnitech Electronics Digiscan infrared photocell system. One-hour activity measurements are obtained during animals' active or dark cycle.

T-maze: The task is based on the premise that animals have evolved an optimal strategy to explore their environment and obtain food with a minimum amount of effort. The T-maze apparatus is made of white Perspex. The runway of the maze is 80 cm long and its side arms are 30 cm long. The width of the maze is 10 cm and the walls are 10 cm high. A guillotine door is used to open the start box and two additional doors are used to block access to the arms, as necessary. Distinctive extra-maze cues are placed in the surrounding walls. Habituation sessions will be performed during 2 consecutive days, allowing free exploration of the maze for 15 min each day. Each rat will receive 10 precision pellets of food, scattered evenly throughout the maze, on each of the habituation sessions. During training each trial consists of a sample run and a test run. In the sample run, rats will be placed at the start position and required to make a forced choice by blocking the access to one of the arms. Two pellets will be positioned at the end of the open arm. The rats will be allowed to eat the pellets and will be immediately placed in a holding cage for 10 s before being returned to the maze for the test run. In the matching-to-place (MTP) and the non-matching-to-place (NMTP) versions of the task the reinforced choice in the test run is either the previously visited arm (MTP) or the arm opposite (NMTP). Each rat will perform 1 block of 10 trials each day. The sequence of arm visits (sample runs) will be randomized for each experimental subject.

Acoustic Startle Response: ASR testing will be conducted with a Startle Response Acoustic Test System (Coulbourn Instruments, PA). This system consists of a weight-sensitive platform in a sound-attenuated chamber. The animals' movements in response to stimuli will be measured as a voltage change by a strain gauge inside each platform and will be converted to grams of body-weight change following analog to digital conversion. These changes will be recorded by an interfaced computer as the maximum response occurring within 200 ms of the onset of the startle-eliciting stimulus. All acoustic stimuli will be administered by an amplified speaker mounted 24 cm above the test cage. During testing, animals will be individually placed in holding cages (14.5 × 7 × 6.5 cm) that are small enough to restrict extensive locomotion, but large enough to allow the subjects to turn around and make other small movements. These will then be placed on the weight-sensitive platform. Following placement of the animal into the chamber, the chamber lid will be closed, leaving the subject in darkness. A three-minute adaptation period occurs in which no startle stimuli is presented. Startle stimuli consist of 120 dB Sound Pressure Level (unweighted scale; re: 0.0002 dynes/cm2) noise bursts of 20 msec duration. In addition to the 120 dB stimuli, the 110 dB level will also be used for comparison to insure that the 120 dB stimulus level not only will meet a response

threshold but also will not reach a leveling out of the maximum response. Thus confirmed, results will be given for the 120 dB level only. Decibel levels will be verified by a Radio Shack Sound Pressure Machine Model 2800 (Provo, UT). Each stimulus will have a 2-ms rise and decay time such that onset and offset will be abrupt, which is a primary criterion for startle. Each trial type (120 dB, 110 dB, or no stimulus) will be presented eight times. Trial types will be presented at random to avoid order effects and habituation. Inter-trial intervals will range randomly from 15 to 25 s.

First day:
Rats will be tested on the rotorod and light-dark task/open field behavior test in the morning of the first day and they will be tested in the T-Maze test in the afternoon of the first day.

Second day:
On the second day they will be acclimated to the acoustic startle response equipment.

Third day:
On the third day rats will be acclimated to the Open Field equipment and 30 minutes after that they will have a second acclimation to the Acoustic Startle response.

Forth day:
On the fourth day they will be tested on the Open Field test and 30 minutes after that they will be tested on the Acoustic Startle Response.

Each rat in Experiment 3 will be given two rounds of 4 days of behavioral testing as described on page 32.

V.4.4.7. Other Procedures:
On the day of exposure to the nerve agent, the animals will be connected to recording leads via the connection plug on their heads. Brain EEG activity will be recorded while they are in cylindrical plastic recording chambers, 16" in diameter and 26" high with Iso-pad bedding on the floor, where they are free to move about. The period of confinement in the recording chambers will not exceed 3 hours on the experimental day or 1 hour on the day after.

The following 5 procedures will be performed at the USUHS:

Histology: Twenty-four hours, 1 week, 1 month, or 3 months after soman exposure, the animals will be deeply anesthetized using 75-100 mg/kg, i.p., sodium pentobarbital, and transcardially perfused with PBS (100 mL) followed by 4% paraformaldehyde (250 mL) in PBS. The brains will be removed and post-fixed overnight at 4° C, then transferred to a solution of 30% sucrose in PBS for 72 hours, and frozen with dry ice before storage at –80° C until sectioning. Sections containing the rostro-caudal extent of the BLA, the hippocampus, and the prefrontal cortex will be cut at 40 µm on a sliding microtome. One series of sections will be mounted on slides (Superfrost Plus; Daigger, Vernon Hills, IL) in PBS for Nissl staining with cresyl violet. An adjacent series of sections will also be mounted on slides for Fluoro-Jade C staining.

Fluoro-Jade C staining (Histo-Chem, Jefferson, AR) will be used to identify irreversibly degenerating neurons in the amygdala, the hippocampus, and the prefrontal cortex. Mounted sections will be air-dried overnight and then immersed in a solution of 1% sodium hydroxide in 80% ethanol for 5 min. The slides will then be rinsed for 2 min in 70% ethanol and 2 min in deionized water (dH20), and incubated in 0.06% potassium permanganate solution for 10 min. After a 2-min rinse in dH20, the slides will be transferred to a 0.0001% solution of Fluoro-Jade C dissolved in 0.1% acetic acid for 10 min. Following three 1-min rinses in dH20, the slides will be dried on a slide warmer, cleared in xylene for at least 1 min and coverslipped with DPX (Sigma). Fluoro-Jade C stained sections undergo tissue shrinkage, so they cannot be used for stereological quantification.

Stereological quantification: Design-based stereology will be used to quantify the total number of neurons on Nissl-stained and GAD67-stained sections. Sections will be viewed with a Zeiss Axioplan 2ie (Oberkochen, Germany) fluorescent microscope with a motorized stage, interfaced with a computer running StereoInvestigator 7.5 (MicroBrightField, Williston, VT). Estimated totals will be determined using the fractionator probe, and all sampling will be done under a 63× oil immersion objective.

Glutamic acid decarboxylase-67 (GAD67) immunohistochemistry: Our previous studies have demonstrated delayed loss of GABAergic interneurons in the BLA (31, 86). To determine what proportion of the lost neurons are GABAergic interneurons, we will combine stereological quantification with GAD67 immunohistochemistry. To label GAD67-immunoreactive neurons, a 1-in-5 series of free-floating sections will be collected from the cryoprotectant solution, washed three times for 5 min each in 0.1 M phosphate buffered saline (PBS), then incubated in a blocking solution containing 10% normal goat serum (NGS; Chemicon, Billerica, MA) and 0.5% Triton X-100 in PBS for one hour at room temperature. The sections will then be incubated with mouse anti-GAD67 serum (1:1000, MAB5406; Chemicon), 5% NGS, 0.3% Triton X-100, and 1% bovine serum albumin, overnight at 4°C. After rinsing three times in 0.1% Triton X-100 in PBS, the sections will be incubated with Cy3-conjugated goat anti-mouse antibody (1:1000; Jackson ImmunoResearch, West Grove, PA) and 0.0001% DAPI (Sigma-Aldrich, St. Louis, MO) in PBS for one hour at room temperature. After a final rinse in PBS, sections will be mounted on slides, air dried for 30 min, then coverslipped with ProLong Gold antifade reagent (Invitrogen, Grand Island, NY).

Brain slice electrophysiology: Coronal slices containing the amygdala and the hippocampus, and slices containing the prefrontal cortex will be prepared from experimental and control rats, 1 month and 3 months after soman exposure. The rats will be anesthetized with isoflurane and then decapitated. The brain will be rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM) 125 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, and 22 glucose, bubbled with 95% O_2 and 5% CO_2 to maintain a pH of 7.4. Coronal slices containing both the hippocampus and the amygdala, and coronal slices containing the prefrontal cortex will be cut at 400 µm with a Vibratome (Series 1000; Leica Microsystems, Bannockburn, IL). Slices will be kept in a holding chamber containing oxygenated ACSF at room temperature, and recordings will be initiated ≥1 hr after slice preparation. For whole-cell recordings, slices will be transferred to a submersion-type

recording chamber, where they will be continuously perfused with oxygenated ACSF, at a rate of 3–4 ml/min. Neurons will be visualized with an upright microscope (Nikon Eclipse E600fn; Nikon, Tokyo, Japan) using Nomarski-type differential interference optics through a 60× water immersion objective. Tight-seal (>1 GΩ) whole-cell recordings will be obtained from the cell body of pyramidal-shaped neurons in the BLA region, the pyramidal layer in the CA1 hippocampal area, and the layer III and V pyramidal cells of the prefrontal cortex. Patch electrodes will be fabricated from borosilicate glass to have a resistance of 1.5–5.0 MΩ when filled with a solution containing (in mM) 135 Cs-gluconate, 10 MgCl2, 0.1 CaCl2, 1 EGTA, 10 HEPES, 2 Na-ATP, 0.2 Na3GTP, pH 7.3 (285–290 mOsm). Neurons will be voltage-clamped using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA); some recordings will be obtained in the current-clamp mode. Access resistance (5–24 MΩ) will be regularly monitored during recordings, and cells will be rejected if resistance changes by >15% during the experiment. The signals will be filtered at 2 kHz, digitized (Digidata 1322A; Molecular Devices), and stored on a computer using pClamp9 software (Molecular Devices). Analysis of recorded currents will be performed using pClamp9 software and the Mini Analysis Program (Synaptosoft, Inc., Leonia, NJ). Extracellular field potentials will be obtained from the BLA, while stimulus pulses are applied to the external capsule, the CA1 hippocampal area, while stimulation is applied to the Schaffer collaterals, and layer III and V of the prefrontal cortex slices, while stimulation is applied to the subcortical white matter. Recording electrodes will be filled with ACSF (resistance: 4 to 7 MΩ). To determine alterations in neuronal and network excitability, whole-cell recordings will be performed in the current-clamp mode to determine changes in the intrinsic excitability parameters of pyramidal cells in the BLA region of the amygdala, the CA1 hippocampal area, and layers III and V of the prefrontal cortex, in slices obtained from the experimental and control groups, at 1 and 3 months following exposure to soman (or saline, for the controls). The following parameters will be measured: resting membrane potential, neuronal input resistance, action potential threshold, action potential characteristics, spontaneous firing, and firing characteristics in response to depolarizing current pulses passed via the recording electrode. Whole-cell recordings in the voltage-clamp mode will also be performed to determine differences, among experimental and control groups, in spontaneous GABAergic and glutamatergic activity in the BLA region of the amygdala, the CA1 hippocampal area, and layers III and V of the prefrontal cortex. These experiments will provide an insight into soman-induced alterations in the intrinsic excitability of pyramidal neurons in the BLA, the CA1 region, and the prefrontal cortex, as well as alterations in the basal, spontaneous activity of the two major neurotransmitter systems that control neuronal network excitability (the GABAergic and the glutamatergic system), and the extent to which the [(b)(4)] [(b)(4)] administered 30 or 60 min after soman exposure prevent these alterations. For alterations in synaptic plasticity, we will test the hypothesis that soman-induced seizures and the resulting neuronal damage alter synaptic plasticity in the amygdala, the hippocampus, and the prefrontal cortex, and that termination of the seizures by administration of the [(b)(4)] will minimize these alterations. Synaptic plasticity will be studied by investigating the characteristics of long-term potentiation (LTP) of synaptic transmission, as we have done previously in both amygdala slices (3) and cerebral cortex slices (2a). In brain slices obtained from rats from experimental and control groups, sacrificed 1 or 3 months after exposure to soman (or saline, for the control groups), field potentials and compound EPSCs will be recorded in the BLA, the

CA1 area, and in the prefrontal cortex in response to electric stimulation of the external capsule, the Schaffer collateral/commisural pathway, and the subcortical white matter, respectively. Because LTP is considered to be the cellular mechanism underlying learning and memory, these experiments may provide an explanation for the memory impairment and other cognitive deficits that result from exposure to nerve agents, and will determine if the (b)(4) can prevent alterations in synaptic plasticity by soman.

Animal monitoring: Animals will be examined after return to their home cage, approximately 30 minutes after return, and once again that day near end of the work day by the USUHS research team. Animals will be checked at least once daily for one week post-exposure by the USUHS PI and staff. Observations will be recorded somewhere accessible to animal care staff. USUHS PI and staff observations for specific signs related to surgery will be in addition to routine observations conducted by the animal care staff.

(b)(4)

(b)(4),(b)(6)

V.4.4.8. Tissue Sharing:
Tissues not used in this study may be made available to other investigators as required.

V.4.5. Study Endpoint:
For **Experiments 1, 2, and 3A and B** the endpoint is euthanasia of the male and female rats for neuropathological studies, preparation of brain slices, or at the end of the behavioral experiments, within 4 months of receipt at USUHS. The study endpoint for the surrogate mothers is the time when the pups are weaned or removed for experiments.

(b)(4)

Early Endpoint: Any of the following will represent a reason for early euthanasia: inability to ambulate or reach food or water, persistent seizures, persistent tremors, self mutilation, continuous circling, or persistent labored respiration or gasping, loss of 20% of weight below baseline, prostration/inactivity for up to 24 hours, or a significant decline in vital signs. These signs, if observed by animal facility personnel and no study personnel can be reached, may be taken by the veterinarian as cause for euthanasia. Some behavioral changes may be caused by nerve agent-induced seizures and will not represent early endpoints: lethargy, weakness, inability to walk straight and uncertain

ambulation (as long as it does not interfere with the animal's ability to eat and drink), intermittent abnormal breathing pattern.

V.4.6. Euthanasia:
If any of the experimental animals develop serious complications due to the agent exposure and/or associated treatments while at USAMRICD, the animal may be euthanized early with an overdose of pentobarbital (>75 mg/kg, IP) or carbon dioxide administered from a cylinder upon consultation between the PI and the attending veterinarian. Cervical dislocation will be used to confirm death by CO2.

For brain pathology studies, at USUHS the animals will be anesthetized with 75-100 mg/kg, i.p., sodium pentobarbital, until a surgical plane of anesthesia is reached (non-response to strong toe-pinch) and then exsanguinated via perfusion with normal saline followed by 4% paraformaldehyde.

For brain slice preparations, rats will be anesthetized with isoflurane inhalation under a chemical fume hood and euthanized by decapitation. Each animal will be placed in a transparent chamber containing a gauze pad saturated with 1 mL of isoflurane. A false bottom will be placed over the gauze pad to prevent the animal from contacting the isoflurane. An airtight lid will be placed on the cage and when the animal has lost its toe pinch (pedal withdrawal) reflex and eyelid blink (palpebral) reflex, and is immobilized, it will be removed from the cage and decapitated with a guillotine. Alternatively, the animal may be anesthetized in a commercial induction chamber with isoflurane vapor in 100% oxygen from a vaporizer.

Following the collection of tissues the remaining carcass will be disposed of IAW the most recent version of VMSB SOP VM-08-301, "Animal Euthanasia."

Comments on the use of decapitation: The guillotine will be maintained in a sharpened condition to ensure rapid and clean decapitation. In the past, one guillotine has been effective for approximately 200 rat decapitations without sharpening. Commercial sharpening will be performed after every 200 procedures or sooner if needed to ensure continued sharpness. The PI has utilized guillotines in two other protocols as co-investigator and three as PI and is proficient in their use. Any other personnel on this protocol will not assist until they have been trained in the use and safety considerations by the PI and are proficient. Decapitation is preferred over other euthanasia procedures because it minimizes residual medications that might affect neuronal responses to stimulation.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

The rats will be maintained under an AAALAC accredited animal care and use program. They will be acclimated upon arrival for 5 days. Animals will be housed in an approved animal holding room after release from acclimation. They may be pair-housed but must

be individually housed post-surgery to ensure no interference with the surgical sites. Food and water will be freely available except during the exposure period. The veterinary staff will provide daily checks of general health and provisions of food and water.

(b)(4)

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No X

Fluid Restriction: Yes _____ No X

V.5.1.3. Exceptions: (b)(4)

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:

The PI or research staff will observe the rats at least twice a day following all procedural manipulations. The veterinary staff will observe the rats at least once a day for general health and husbandry condition. Any rat displaying any other signs of pain, distress or illness will be evaluated by the PI and/or attending veterinarian and, if necessary, euthanized as previously described.

(b)(4)

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

If any rat experiences non-study related distress or pain prior to the study, the PI or co-PI will consult the attending veterinarian. If necessary, the rat will be treated or euthanized as directed by the attending veterinarian. Euthanized rats will not be entered into the study and additional rats may be requested as replacements.

(b)(4)

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

(b)(4)

V.5.3.2. Enrichment Restrictions:
Animals must be individually housed post-surgery to ensure no interference with the surgical sites and implanted electrodes. Also, after exposure to soman rats must be individually housed as the animals may be experiencing seizures as a result of the nerve agent exposure.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Animal handling, agent handling, injections,	(b)(6)	PhD in neuroscience. 20+ yr rodent handling,	Investigator training Dr (b)(6) has performed hundreds

anesthesia, EEG surgery, guillotine usage, euthanasia, electrophysiology	(b)(6)	surgery, tissue dissection, electrophysiological experimentation. Over 200 rats euthanized via guillotine. 6 years performing EEG electrode surgery in rodents.	of surgeries, euthanasia, and anesthesia.
Animal handling, agent handling, injections, anesthesia, EEG surgery, guillotine usage, euthanasia, electrophysiology		PhD in physiology 30+ yr animal handling, injections, surgery, tissue dissection, electrophysiological experimentation. Over 200 rats euthanized via guillotine.	Investigator training Small animal handling workshop 30+ years experience and on-the-job training. Dr. (b)(6) was last trained at USAMRICD on November 1990.
Animal handling, anesthesia, EEG surgery, guillotine usage, euthanasia, perfusions, electrophysiology		PhD in neuroscience. 20+ yr rodent handling, surgery, tissue dissection, and electrophysiological experimentation. Over 200 rats euthanized via guillotine	Dr (b)(6) was retrained as an animal investigator at USUHS in 2007. 20+ years experience and on-the-job training, anesthesia, euthanasia, tissue samples, small animal surgery, perfusions, electrophysiological experimentation.
Animal handling, injections, perfusion, histological preparation, neuropathology		PhD in neuroscience More than 15 years of experience in small animal handling, injections, perfusion, histological techniques, and assessment of neuropathological damage	Investigator training in small animal handling and experimentation, including behavioral tests.
Animal handling, agent handling,		PhD in physiology 20+ yr animal	Investigator training Small animal

injections, anesthesia, EEG surgery, guillotine usage, euthanasia, electrophysiology		handling, injections, surgery, tissue dissection, electrophysiological experimentation. Over 200 rats euthanized via guillotine.	handling workshop 20+ years experience and on-the-job training

VII. BIOHAZARDS/SAFETY:

XCSM agent, decontaminated XCSM agent waste, chemicals, and hazardous waste used in this protocol will be handled in accordance with all applicable state and federal guidelines, regulations, and standing operating procedures.

Use of XCSM: The laboratories follow the standard operating procedures for the use and disposal of XCSM agents, as described IAW the latest version of SOP# 87-201-RS-01, SOP# 87-335-VA-12, and APGR 200-60. These include initial and quarterly monitoring, as well as daily checks, of hood safety.

Use of 4% paraformaldehyde: All operators using paraformaldehyde must wear proper personal protection equipment, i.e., gloves, safety glasses, lab coat, and they will also use a portable chemical hood.

Minimal PPE for animal handling includes nitrile gloves, mask worn in the slung position or readily available, safety glasses with sideshields or goggles, and fully buttoned lab coat. Double gloves will be worn during agent exposure. All agent work will be done in a certified chemical fume hood in a laboratory that has been appropriately permitted IAW USAMRICD Memorandum 385-6.

Waste anesthetic gas (isoflurane) will be scavenged either by evacuation into a chemical fume hood or by extraction with a filter system in a recirculating air cleaning device (Extract-All system, VetEquip Inc., Pleasanton, CA).

VIII. ENCLOSURES:

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 02/15/2015

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ _____

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment

(b)(6)

Principal Investigator Signature

02/18/2015
Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: (b)(4)

(b)(4)

C. Principal Investigator: (b)(6)

D. Performing Organization: USUHS and USARRICD

E. Funding: (b)(4)

F. Objective and Approach: The purpose of this work is to develop improved therapies to treat seizures caused by exposure to nerve agents and to prevent the associated neuropathology.

We propose to accomplish our goals by using in vivo exposure of rats to soman. The (b)(4)

G. Indexing Terms (Descriptors): (b)(4)

(b)(6)

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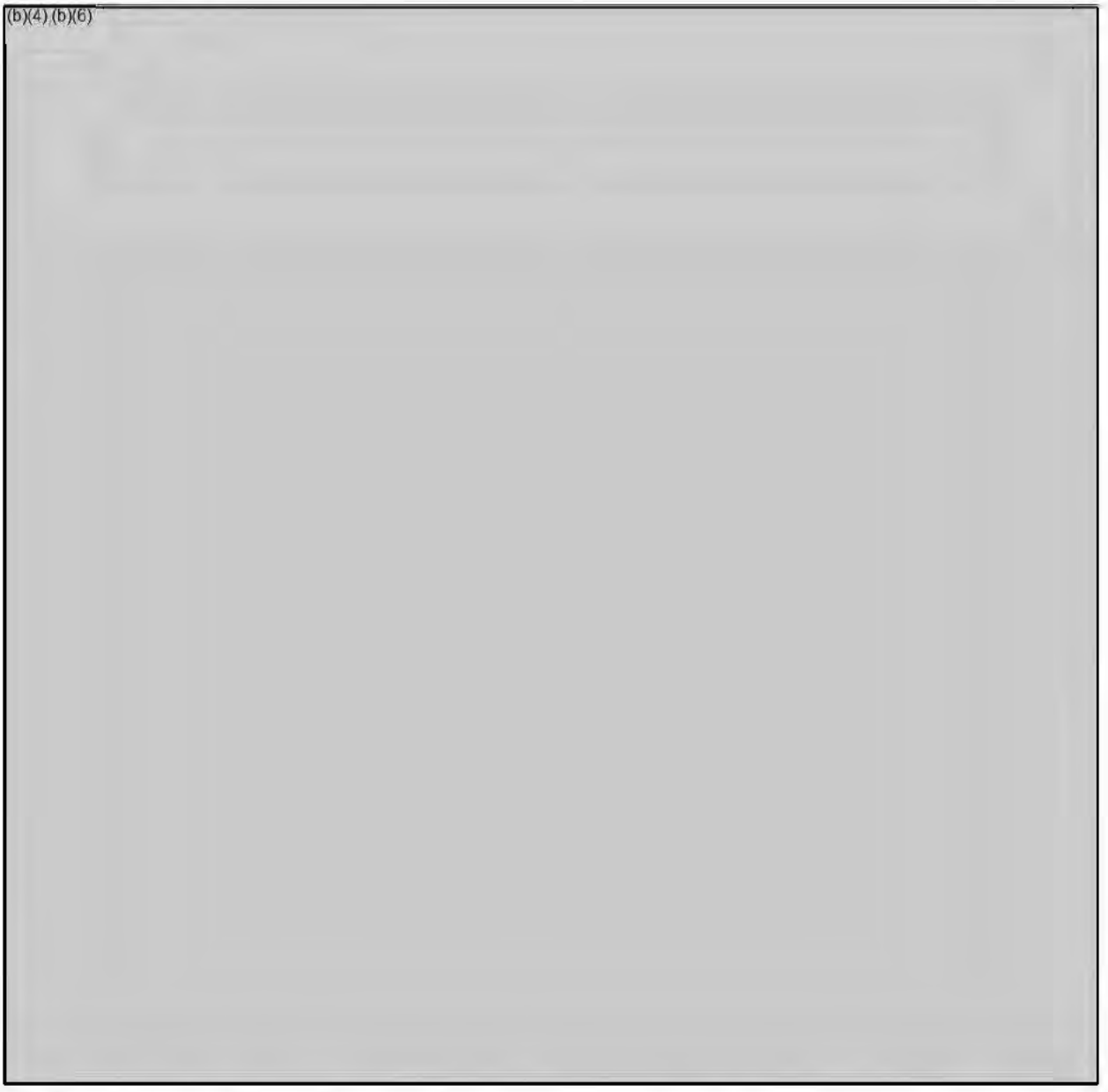
(b)(4),(b)(6)



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Institutional Animal Care and Use Committee
Phone (b)(6)
Fax (b)(6)

September 10, 2015

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on September 10, 2015:

Animal Protocol Title: (b)(4)
(b)(4)

USUHS Protocol Number: (b)(6)

Expiration Date: September 9, 2018

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

Revised

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: (b)(4) (b)(4)

GRANT TITLE (if different from above): (b)(4) (b)(4)

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Center for Neuroscience and Regenerative Medicine

EARLIEST ANTICIPATED FUNDING START DATE: Currently funded until 6/30/16

PRINCIPAL INVESTIGATOR: Dr. (b)(6)

(b)(6) ature — APG Department — (b)(6) Office/Lab Telephone — 8/5/15 Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) Re Signature — APG Vice Chair Title — (b)(6) Telephone — 8/5/15 Date

Typed Name: Dr. (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) Statistician Signature — Preventative Medicine & Biometrics Department — (b)(6) Telephone — 5/6/15 Date

Typed Name: Dr. (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)

Attending/Consulting Veterinarian Signature

LAM
Department

(b)(6)
Telephone

5 Aug 2015
Date

Typed Name: MAJ (b)(6) DVM, MPH &
MAJ (b)(6)

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: Dr. (b)(6) APG

ANIMAL PROTOCOL TITLE: (b)(4) (b)(4)

GRANT TITLE (if different from above): (b)(4) (b)(4)

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S):

TECHNICIANS(S): (b)(6) Graduate Student
(b)(6) Research Associate

I. NON-TECHNICAL SYNOPSIS:

Every year in the U.S., it is estimated that 1.4 million people are hospitalized with a brain injury.[1] Among U.S. military personnel, incidence of depression, PTSD, suicide risk, and TBI symptom severity are all significantly increased as the number of TBIs sustained increases.[2] Alterations in brain energy metabolism, local neuroinflammation, and fluid balance are among the mechanisms implicated for contributing to repeated TBI-related adverse outcomes. Therefore, a subsequent injury after an initial TBI is associated with severe cumulative consequences.

Recently, it has been determined that the adaptive immune system can develop immunological memory to CNS injury and activate production of autoantibodies with pathogenic potential. Studies have detected autoantibodies in biofluids from individuals with CNS injury as well as from animal subjects in TBI preclinical models. However, the contribution of immunological memory responses after repeated TBI events has not been defined. **Our goal in this project is to determine whether TBI establishes immunological memory and whether a repeated injury results in a secondary adaptive immune response.**

T and B lymphocytes are primary components of the adaptive immune system, yet the role of lymphocytes in TBI is not well understood. Evidence in other CNS injuries suggests that T and B cells play significant roles in post-traumatic inflammation, degeneration, or repair. Since T and B lymphocytes have both potential neuroprotective and neuropathogenic roles, elucidating the response of the adaptive immune system to TBI is needed to better understand the mechanisms of repeated TBI. Modulating the immune system after TBI may innovate treatment strategies for improved recovery after TBI. **Our hypothesis is that an initial TBI results in immunological memory and that a subsequent TBI potentiates adaptive immune alterations.** To test this hypothesis, we will complete experiments for the following specific aims:

Aim 1: Determine adaptive immune components that are altered in a murine model of repeated TBI. The changes in adaptive immune system components after TBI are not well defined. We will profile lymphocyte populations by flow cytometry to determine whether memory cell populations are altered. Expression analysis of

lymphocyte populations will also be accomplished to detect transcripts associated with immunological memory responses.

Aim 2: Determine the status of adaptive immune repertoire alteration as a function of autoantibody presence. Studies have detected autoantibodies in subjects after CNS injury and TBI. The level and nature of autoantibodies after repeated TBI has not been defined. We will measure serum autoantibody levels from TBI subjects using 1-dimensional gel and ELISA-based methodologies. Clonotypic enrichment in lymphocyte repertoire will be profiled by sequencing receptor immunoglobulins.

Aim 3: Demonstrate CNS-directed lymphocyte proximity to the brain after repeated TBI. The blood-brain barrier establishes immunoprivilege to mitigate self-reactive responses to host tissue. CNS antigen-mediated responses and lymphocyte chemotaxis to the brain after TBI may initiate autoimmune reactions. We will determine the immune and inflammatory status of the choroid plexus to assess its modulating role of lymphocyte populations in the CNS after TBI. Furthermore, we will analyze T cell receptor repertoire status of the choroid plexus to detect if repeated brain injury results in increased infiltration of CNS-specific lymphocyte populations to the brain.

II. BACKGROUND:

II.1. Background:

It is estimated that 1.4 million people are hospitalized with a brain injury every year in the United States,[1] which does not include an estimated additional 3.8 million untreated concussions.[3] Among U.S. military personnel, incidence of depression, post-traumatic stress disorder (PTSD), suicide risk, and TBI symptom severity are all significantly increased as the number of TBIs sustained increases.[2] In other words, sustaining more than one TBI is associated with severe cumulative consequences. We are interested in better understanding the collective longer-term effects of TBI and repeated TBI by focusing on the adaptive immune system. A central characteristic of the adaptive immune system is the development of immunological memory, or the capacity of the immune system to remember an antigen encounter via activated T and B cells and to rapidly react to the same antigen in a later encounter. Better understanding the role of immunological memory in TBI may help uncover the basis for cumulative damage from repeated TBIs.

Following a moderate or severe brain injury, necrotic cell death causes the release of damage-associated molecular patterns (DAMPs), a.k.a. alarmins, into the extracellualar space. Examples of DAMPs include high-mobility group box-1 (HMGB1) protein, mitochondrial DNA, and N-formyl peptides. It is known that TBI patients often have systemic lung inflammation, and CSF levels of HMGB1 are markedly increased in children after severe TBI.[4] Recently, evidence suggests that TBI causes lung inflammation through the action of HMGB1 protein and the receptor for advanced glycation end products (RAGE).[5] While mice subjected to CCI injury had systemic hypoxia, acute lung injury, pulmonary neutrophilia, and decreased ability of lung expansions compared to control animals, RAGE knockout mice had total attenuation of all these symptoms after TBI.[5] The theory that TBI can cause the release of DAMPs like HMGB1 which can then cause peripheral tissue inflammation and damage is gaining validity. It is not a far leap to also assume that these DAMPs can also cause changes to the adaptive immune system and particularly, changes in immunological memory.

The pathophysiology of TBI varies enormously, but often, TBI induces a metabolic crisis in the CNS to maintain neuronal membrane potentials and ion balances.[6] Alterations in brain energy metabolism, local neuroinflammation, and fluid balance are among the mechanisms implicated for contributing to repeated TBI-related adverse outcomes. Numerous studies have observed immediate activation and recruitment of the innate immune system following TBI, comprised in part by brain microglia, peripheral macrophages, and neutrophils. While early activation of innate immunity is beneficial to injury outcome, late and sustained activation is suggested to be deleterious. Neutrophils can be toxic to vulnerable neurons, and along with monocytes can exacerbate cerebral edema and brain tissue damage in rodent models of stroke and TBI.[7-10] In fact, the magnitude of inflammatory cell recruitment correlates to the loss of neural tissue after TBI.[11] While chronic inflammatory responses are correlated with poor pathology after TBI, it is unclear if adaptive immune components contribute to long-term neuroinflammation after TBI.

Works in other CNS injuries make it clear that T and B lymphocytes play significant roles in post-traumatic inflammation, degeneration, or repair. After spinal cord injury (SCI), lymphocytes may be detrimental to recovery. In recombination-activating gene (rAG) 2-deficient mice that lack both T and B lymphocytes, locomotor recovery was improved following compression SCI, and recovery was associated with more monoaminergic axons caudal to the injury site.[12] In another study of moderate contusion SCI, B-cell knockout mice that lack mature B cells but have functional T cells had improved Basso Mouse Scale locomotor scores, decreased lesion volume, and lower levels of IgM and IgG in CSF.[13] Additionally, in a cerebral ischemia-reperfusion study of male severe combined immunodeficiency (SCID) mice (that lack both T and B cells), the animals developed 40% smaller infarct volume 90 minutes after focal ischemia and 22 hours reperfusion compared with wild type mice.[14] Other studies found around 60% smaller infarct volumes and improved functional outcomes 24-72 hours in $Rag1^{-/-}$ mice that lack T and B lymphocytes.[15,16] Activated B and T cells are also found to infiltrate and accumulate in the injured spinal cord where they form germinal center like clusters of cells that are normally found in the spleen and lymph nodes.[17] While these studies demonstrate a correlation between T & B cell activity and poor outcome, there are other studies that demonstrate a beneficial role of lymphocytes after CNS injury.

Autoimmune T cells specific to myelin basic protein can protect injured CNS neurons from secondary degeneration after a partial crush of the optic nerve.[18] In this study, rats injected with activated anti-myelin basic protein T cells kept 300% more retinal ganglion cells with functionally intact axons compared to rats injected with activated T cells specific for other antigens.[18] In another study, immune-deficient SCID mice manifested cognitive deficits and behavioral abnormalities that were remedied by restoring T cells from age-matched wild-type mice.[19] This study further showed that T cell-based vaccination using glatiramer acetate, a weak agonist of many self-reactive T cells, can overcome the behavioral and cognitive abnormalities that correspond with neurotransmitter imbalance induced by dizocilpine maleate or amphetamine.[19] T and B lymphocytes have both potential neuroprotective and neuropathogenic roles, and understanding TBI mediated immunological memory formation and peripheral and CNS immune alterations can possibly open new avenues for treatment and diagnosis of TBI. Once clonally expanded populations and/or memory-evoking brain antigens are

identified, immunity-related clinically relevant manipulations can be performed to see if outcomes improve after repeated TBI. We can also examine at what point multiple TBIs can confer "protective autoimmunity" or peripheral tolerance. We believe that chronic exposure to CNS antigens will confer peripheral tolerance, like was observed in a study that immunized newborn rats to myelin antigens at birth. When the rats were subsequently subjected to mechanical crush injury to the optic nerve or spinal cord as adults, significantly fewer neurons survived in the tolerance-induced rats than in their matched controls that were immunized at birth with an irrelevant (non-myelin) protein.[25,26] Conversely, in 1999, Schwartz et al. formulated the concept of protective autoimmunity: T-cell mediated adaptive immunity to specific CNS-related autoantigens is needed to combat destructive self-compounds/DAMPs released from the injury.[18] Depending on the nature, severity, and number of brain injuries suffered, autoimmune responses can be modulated to ensure optimal outcomes are achieved. In future studies, we hope further examine the roles protective autoimmunity and peripheral tolerance may play after TBI.

Preliminary Data

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Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:
Biomedical Research Database (BRD)
Federal Research in Progress database (FEDRIP)
Pubmed

II.2.2. Date of Search: July 9, 2015

II.2.3. Period of Search:
We searched keywords for all dates available through each database.

II.2.4. Key Words and Search Strategy:
We did advanced searches for all databases. The key words used were:
"brain injury and lymphocyte": 13 studies (FEDRIP)
"CNS injury and lymphocyte": 10 studies (FEDRIP)
"brain injury and autoantibody": 3 studies (FEDRIP)
"brain injury and lymphocyte": 0 studies (BRD)
"brain injury and autoantibody": 0 studies (BRD)
"brain injury and B cell": 8 studies (BRD)
"CNS injury and B cell" 4 studies (BRD)
"brain injury and lymphocyte and memory B cell": 1 publication (PubMed)
"brain injury and autoantibody and lymphocyte": 26 publications (PubMed)
"brain injury and "memory B cell"": 0 publications (PubMed)
"brain injury and adaptive immune system and lymphocyte": 47 publications (PubMed)

II.2.5. Results of Search:

There were no relevant publications or reported studies about the role of immunological memory cells in repeated TBI. The current literature discusses the role of adaptive immune cells in other forms of CNS injury (e.g. stroke, MS, spinal cord injury) but not its role after traumatic brain injury. There was one study that looked at innate immune responses in the periphery after closed head injury in mice, but the study did not look into the role of lymphocytes (Schwulst; J Trauma Acute Care Surg; 2013).

III. OBJECTIVE\HYPOTHESIS: Our hypothesis is that an initial TBI results in immunological memory and that a subsequent TBI potentiates adaptive immune alterations.

IV. MILITARY RELEVANCE:

It is estimated that 1.4 million people are hospitalized with a brain injury every year in the United States,[1] which does not include an estimated additional 3.8 million untreated concussions.[3] Among U.S. military personnel, incidence of depression, post-traumatic stress disorder (PTSD), suicide risk, and TBI symptom severity are all significantly increased as the number of TBIs sustained increases.[2] In other words, sustaining more than one TBI is associated with severe cumulative consequences. We are interested in better understanding the collective longer-term effects of TBI and repeated TBI by focusing on the adaptive immune system. A central characteristic of the adaptive immune system is the development of immunological memory, or the capacity of the immune system to remember an antigen encounter via activated T and B cells and to rapidly react to the same antigen in a later encounter. Better understanding the role of immunological memory in TBI may help uncover the basis for cumulative damage from repeated TBIs.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1:

The aim of Experiment 1 is to determine adaptive immune components that are altered in a murine model of repeated TBI. There are two sub-tasks in this experiment. Sub-task 1.1 is to determine if immunological memory-associated transcripts are altered in whole blood. Sub-task 1.2 is to determine if lymphocyte populations are altered in the spleen. The outcome measures of aim 2 (measuring autoantibodies) and aim 3 (choroid plexus RNA expression analysis) are folded into sub-task 1.1 and 1.2.

Rationale: Since T and B cells may either promote repair or further tissue injury as shown in other CNS injury models and disease states, it is important to define their roles in TBI. In our study, we want to discern if immunological memory exists and is strengthened in a rodent CCI model of brain injury long after the initial inflammatory episode subsides. The changes in adaptive immune system components after TBI have not been previously well defined. In order to accomplish specific aim 1, we will profile lymphocytes in whole blood to detect the presence of memory- and/or activation-associated gene expression. Characterizing the memory lymphocyte cell signature of TBI may generate a blood-based diagnostic tool to determine if a TBI event is inducing

memory or pathogenic autoimmunity in a military service member. This may provide vital information to determine a return to duty and/or indicate therapeutic intervention. Furthermore, we will identify lymphocyte populations in the spleen to assess adaptive immune system states after repeated TBI.

1.1: Determine if immunological memory-associated transcripts are altered in whole blood.

In order to accomplish specific aim 1, we will profile lymphocyte-related transcripts in whole blood to detect the presence of memory- and activation-associated gene expression after TBI. Whole blood will be collected 14 and 28 days after CCI brain injury. We expect to find greater lymphocyte-related transcript levels in TBI animals compared to controls, and we expect to find greater immunological memory-associated transcripts in repeated TBI animals.

Methods: C57BL/6J male mice between 9 to 10 weeks of age will be subjected to sham, single, or delayed repeated CCI injuries (two injuries spaced 14 days apart). Animals will be anesthetized using 2-4% isoflurane for CCI surgeries. After an incision is made in the scalp, a craniotomy is performed to remove a circular piece of skull over the injury site to permit impactor brain penetration. Using a 3mm impactor tip, the CCI device will strike 2mm lateral of Bregma (in the left hemisphere) at 1.5 m/s at a 15° angle and 100ms dwell time. The impact depth will be relatively mild at 1mm.

A 14-day interval between CCI surgeries for a cohort of animals will allow development of immunological memory and resolution to near baseline levels. At 1-3 days, 14 days, or 28 days after the final surgery, subjects will be euthanized to obtain whole blood, spleen, and choroid plexus material. Animals will be anesthetized prior to the terminal procedure by an intraperitoneal injection of ketamine and xylazine. Once anesthetized, whole blood will be collected via cardiac puncture using a 1 mL syringe with a 25-guage needle. A portion of the whole blood will be placed in a RNAprotect Animal Blood Tube (Qiagen) to stabilize transcript levels at time of collection, and total RNA will be isolated using RNeasy Protect Animal Blood Kit (Qiagen). The remaining portion of the whole blood will be placed in a MiniCollect serum tube (Greiner Bio-One) to collect serum for Western Blots to identify brain-reactive antibodies in serum (Aim 2).

After total RNA is isolated from the blood, globin mRNA is depleted using a GLOBINclear kit (Life Technologies). Total RNA in whole blood has a high expression of globin mRNA (Up to 70%), which is expressed at high levels in red blood cells and reticulocytes. Depleting alpha and beta globin mRNA from the total RNA preparations allows more sensitivity to detect transcripts relevant to our interests. The mRNA portion in total RNA will be converted into a library of template molecules (TruSeq RNA Sample Preparation Kit v2, Illumina). In summary, mRNA molecules will be enriched and fragmented. The RNA fragments will then be copied into first strand cDNA and then second strand cDNA. The cDNA fragments go through an end repair process, the addition of a single 'A' base, and then ligation of the adapters. The final cDNA library is created by purifying and enriching the products with PCR. The library is then quantified using qPCR (KAPA Library Quantification Kit) and library size distribution is determined (Experion DNA analysis kit). Libraries are then diluted and denatured for cBot clustering. After libraries are amplified to produce clonal clusters, they are sequenced using massive parallel synthesis (TruSeq SBS Kit v5, Illumina).

Choroid plexus material will be collected to determine if immunological memory-associated transcripts are altered in a CNS-proximal tissue. Lymphocyte chemotaxis to the brain after TBI may be due to the elevated release of CNS antigens. We will profile complete transcriptomes of the choroid plexus tissue and determine changes in the immune and inflammatory status of the choroid plexus to assess the modulating role of lymphocytes in the CNS after TBI. We will accomplish choroid plexus expression analysis by RNA-seq to interrogate the inflammatory status of the tissue (Aim 3). Since we know cytokine and chemokine transcript markers for pro-inflammation versus anti-inflammation, we can then see which markers are changed after TBI and if there are further changes after repeated TBI. We will then be able to determine changes in the immune and inflammatory status of the choroid plexus to assess the modulating role of lymphocytes in the CNS after TBI. These transcripts may indicate that the lymphocyte activity is either beneficial or deleterious.

Sub-task 1.1 will require 120 mice. There will be 8 mice per group with 5 experimental groups (naïve, sham, single CCI, double sham, and double CCI) and 3 outcomes time points (1-3 days, 14 days or 28 days post surgery). [8 x 5 x 3 = 120 mice]. Naïve mice will not be subjected to any experimental conditions. Sham mice will be anesthetized and given a craniotomy but no brain impact during surgical procedures.

Expected Outcomes and Alternative Approaches: After TBI, we believe that brain antigens are being presented to the immune system to activate lymphocytes and generate immunological memory and plasma cells. With multiple brain injuries, more brain antigens are presented to the adaptive immune system possibly generating clonal expansion of memory and plasma cells. We expect to find increased lymphocyte-related transcripts in TBI animals compared to controls, and we also expect to find greater immunological memory-associated transcripts in repeated TBI animals. Specifically, we anticipate that transcripts like Dock8 will be altered after TBI. Dock8 helps antibody-producing B cells form stable complexes with other immune cells so that B cells can generate more effective antibodies and become memory cells.[27] Our protocols for expression analysis and CCI surgeries have been thoroughly troubleshooted, so we do not anticipate difficulties with these procedures.

1.2: Determine if lymphocyte populations are altered in spleen.

We will profile lymphocyte populations by flow cytometry in a model of repeated TBI to determine whether cell populations are altered. We are utilizing spleen tissue, because it is the largest secondary lymphoid organ that is responsible for initiating immune reactions to blood-borne antigens and contains about ¼ of the body's lymphocytes.

Methods: C57BL/6J male mice between 9 to 10 weeks of age will be used. For this cohort of animals, the same CCI surgery protocol will be used as described in sub-task 1.1. During the terminal procedure 1-3 days, 14 days, or 28 days after the final CCI surgery, spleens will be dissected for flow cytometry analysis. First, a single-cell suspension will be created and cells will be counted and adjusted to 1 x 10^6 cells/mL. After adding an Fc-block (rat IgG_{2b} anti-mouse CD16/CD32 monoclonal antibody, BD Biosciences), cells will be fixed in 1-4% paraformaldehyde. Fluorochrome-conjugated antibodies will then be added to the cells to label various extracellular markers. Sub-task 1.2 will require additional mice, because spleens will be processed differently than

that described for animals in 1.1. In summary, C57BL/6J male mice between 9 to 10 weeks of age will be subjected to single or delayed repeated controlled cortical impact (CCI) injuries (two injuries 14 days apart). At 1-3 days, 14 days or 28 days after the final surgery, subjects will be euthanized to obtain whole blood and spleen. Sub-task 1.2 will require 72 mice. There will be 8 mice per group with 3 experimental groups (naïve, single CCI, and double CCI) and 3 outcome time points (1-3 days, 14 days or 28 days post surgery). [8 x 3 x 3 = 72 mice].

Expected Outcomes and Alternative Approaches: Our preliminary data revealed that 21 days after a single mild closed-skull TBI, TBI animals had an altered percentage distribution of T helper cells (CD4+) and B cells (CD19+) compared to shams. The level of cytotoxic T (CD8a+) cells remained the same between the TBI and sham animals. The percentage of lymphocytes was consistent among all sham and TBI animals at around 40% of all splenocytes, so we are confident that the percentage distribution in the spleen did not change. One hypothesis that can explain these changes in lymphocyte populations is that perhaps certain B cells that are responding to brain antigens are being clonally expanded meaning that the diversity or range of B cells may be decreasing to one proliferating clone. In order to investigate this hypothesis, we will profile antibody genes (Aim 2 & 3).

We anticipate that memory B cell and plasma cell populations will undergo delayed expansion after TBI. In other studies, B and T cell expansion in the spleen and lymph nodes coincides with an increase in circulating IgG antibodies in mice and rats subjected to spinal contusion injury.[17,28] We anticipate making a similar correlation between circulating autoantibody levels and lymphocyte population changes in the spleen. We further anticipate that a TBI will initially expand the T effector population but that levels will decrease at 28 days post injury. We further anticipate that a repeated TBI will change the lymphocyte response as compared to the first injury event. Namely, a dramatic activation of memory T and B cell clonal populations that persist from the first TBI. If successful, and we are able to identify clonally expanded populations, immunity-related clinically relevant manipulations can be done to see if outcomes improve after repeated TBI.

Sub-task	# of animals	Treatment	Euthanization time point after surgery	Pain category
1.1	8	naïve	1-3 days	C
	8	naïve	14 days	C
	8	naïve	28 days	C
	8	sham	1-3 days	E
	8	sham	14 days	E
	8	sham	28 days	E
	8	single CCI	1-3 days	E
	8	single CCI	14 days	E
	8	single CCI	28 days	E
	8	double sham	1-3 days	E
	8	double sham	14 days	E

	8	double sham	28 days	E
	8	double CCI	1-3 days	E
	8	double CCI	14 days	E
	8	double CCI	28 days	E
1.2	8	naïve	1-3 days	C
	8	naïve	14 days	C
	8	naïve	28 days	C
	8	single CCI	1-3 days	E
	8	single CCI	14 days	E
	8	single CCI	28 days	E
	8	double CCI	1-3 days	E
	8	double CCI	14 days	E
	8	double CCI	28 days	E
TOTAL:	192			

V.2. Data Analysis:

We will use analysis of variance (ANOVA) followed by post-hoc t tests to compare blood plasma, chemokine and cytokine data and lymphocyte population data. If data do not meet the assumptions of ANOVA they will be transformed or appropriate nonparametric tests will be used. A sample size of 8 per group will detect differences of 1.5 standard deviations between groups with a 5% two-sided significance level. For histological data, we will quantitate microglia activation by cells per area density and use semi-quantitative descriptive statistics to correlate with the quantitative parameters above.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

We are investigating the role of immunological memory cells after repeated mild TBI. Alternatives to animal models, such as computer models or in vitro cell cultures will not be able to adequately mimic the molecular and cellular events that occur after TBI. In order to learn more about the mechanisms involved after a brain is injured, it is necessary to actually injure a brain in an animal model.

V.3.2. Animal Model and Species Justification:

We will be subjecting the mice to brain injury and then investigating the effects in blood, brain tissue, and spleen, so we need to use an animal with a brain, peripheral lymphoid organs, and blood. We chose to use mice in this study for a variety of reasons: (1) they are mammals with similar cerebral physiology making it possible to study inflammatory and immune-mediated responses; (2) many studies with mice exist where behavior and brain trauma outcomes correlate with human studies making it possible to correlate our findings with previously published data; (3) the relative small size and

short life span of mice will allow us to repeat measures of biochemical and cellular changes after TBI in a feasible manner.

Since we are using this animal model to improve treatment for human TBI patients, we want to do experiments in a laboratory mammal that these techniques have already been worked out in.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	**Mus Musculus**	
V.3.3.2. Strain/Stock:	**C57BL/6**	
V.3.3.3. Source/Vendor:	(b)(4)	
V.3.3.4. Age:	**9-10 weeks old**	
V.3.3.5. Weight:	**23-35g**	
V.3.3.6. Sex:	**Male**	
V.3.3.7. Special Considerations:	**N/A**	
V.3.4. Number of Animals Required (by Species):	192	

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:
The animals used will be anesthetized before the surgical injury procedure (TBI/sham injury) as well before the terminal procedures of tissue and blood collection.

V.3.5.2. Reduction:
We will use material from several tissues sources (peripheral blood, spleen, choroid plexus, and brain) in this experiments thereby reducing the number of animals needed. Since the experimental techniques we are proposing to use have already been characterized and well documented in mice, we will not need to use extra animals to establish the techniques.

V.3.5.3. Replacement:
Replacement alternatives such as cell cultures and the use of less sentient animal species were considered, but we need to use mammals with a brain, peripheral lymphoid organs, and circulating blood to examine the CNS effects of TBI.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:
We will have a total of 192 animals. Here are the treatment groups and number of animals in each group:
Naïve: 48 animals
Sham: 24 animals
Single CCI: 48 animals
Double Sham: 24 animals
Double CCI: 48 animals
These animals are categorized in category C (48 naïve animals) and category E (144 sham (craniotomy), double sham, single CCI, and double CCI animals) shown below.

	Species #1	Species #2
V.4.1.1.1.1. Column C:	48	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	144	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:
Prior to TBI/sham procedures, mice will be administered an inhalational anesthetic. Oxygen will be mixed with isoflurane delivered via precision vaporizer using a rodent anesthesia machine. Mice will be placed in an induction chamber and exposed to a mixture of O2 and isoflurane (2-4%) to induce general anesthesia. Following induction, mice will be removed from the chamber and maintained on 1-2% isoflurane administered by nose cone during surgical procedures. We will monitor the depth of anesthesia by noting a lack of response to toe pinch (pedal withdrawal reflex), mucus membrane color, and rate and quality of respirations. Waste anesthetic gases will be actively scavenged through the central vacuum system or passively scavenged using a charcoal canister.

For terminal tissue dissection procedures (including naïve animals for baseline level measurements), ketamine and xylazine will be used as the sedative and anesthetic. Before collection, they will be injected (i.p.) with 80-100 mg/kg of Ketamine and 10 mg/kg Xylazine intraperitoneally using a 26-gauge needle.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:
Once animals arrive from the vendor (e.g. (b)(4) , they will acclimate in a reverse light-cycle room for 3 days. The reverse light-cycle room in needed to avoid disturbing the sleep cycle (and circadian rhythms) of animals when administering surgical and weighing procedures. LAM will place animals in cages in the same manner

in which they arrived (ie. if 4 animals arrive in three separate boxes, they will similarly place the same 4 animals in 3 separate cages). Animals will receive regular food and water without restriction. After the procedure, if there is significant brain hemorrhaging, we will eliminate the animal from the study and euthanize it. Animals will be placed on a heating pad in a single cage for 30 minutes to recover from the surgery. During this time, animals will be continuously monitored to make sure they come out of anesthesia and are able to right themselves, lay sternal, and lift their heads. Animals will then be monitored daily after surgical procedures to confirm that they recovered and are conscious without any visible signs of distress. Additionally, animals will be monitored and weighed at least every 2 days for significant weight loss (greater than 20% total weight loss relative to initial body weight prior to injury) as an additional sign of distress. If post-procedural pain or distress induces persistent symptoms beyond the first 24 -36 hrs post injury, including hunched posture, labored or gasping breathing, ruffled fur, immobility, failure to feed or to drink, the affected mice will be immediately removed from the study and euthanized..

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PubMed

V.4.1.3.2. Date of Search: July 13, 2015

V.4.1.3.3. Period of Search: 1950-current

V.4.1.3.4. Key Words of Search:
Moderate brain injury model and mice; penetrating brain injury and mice; non-invasive brain injury model and mice

V.4.1.3.5. Results of Search:
In order to investigate peripheral immune changes after brain injury, we need to utilize animals that have a brain, nervous system, circulatory system, and peripheral lymphoid organs. In order to investigate brain injury, we need to systematically and reproducibly injure the brain of animals. There are other forms of brain injury models that can be considered alternatives to the controlled cortical impact injury that we administer in our experiments. Our search results revealed other brain injury models such as weight-drop closed head injury, fluid percussion injury, and middle cerebral artery occlusion). Out of all these options, we chose to utilize a controlled cortical impact model, because it is highly reproducible, injury parameters can be adjusted, and we can compare results with many other labs here at USU. All of our search results included brain injury models that did inflict injury to the brain and while some studies provided analgesia after surgical procedures, none of these injury models can be considered pain-free. Administering brain injury is inherently invasive and painful, and we cou dn't find any experimental studies that administered reproducible brain injury to vertebrates in a pain-free manner (ie. without providing some kind of anesthesia and/or analgesia). In our study, we do provide anesthesia during all surgical procedures, but we do not provide analgesia after surgical procedures, because this will impact our results.

"moderate brain injury model and mice": 91 publications
"penetrating brain injury model and mice": 22 publications
"non-invasive brain injury model and mice": 28 publications

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
Providing pain medication after brain injury will interfere with our ability to interrogate immune-related outcome measures. We can not investigate the role of lymphocytes and immune alterations after TBI if we are providing medication that alters immune function. While no analgesia will be provided after surgeries, all animals will be properly anesthetized before all surgical procedures with isoflurane and before all euthanization procedures with an i.p. injection of ketamine and xylazine

Buprenorphine, an anti-nociceptive partial μ-opioid receptor agonist, along with other opiates and NSAIDs all have effects on the immune system, and thus, will impact our data in this study. Buprenorphine has been shown to dose-dependently suppress splenic natural killer cell activity, lymphocyte proliferation, and IFN-γ production in rats, and these effects were reversed with naltrexone "demonstrating pharmacological specificity of buprenorphine-induced immune alterations."[29] Further, opioid alkaloids and peptides, such as morphine "directly modulate the function of lymphocytes and other cells involved in host defense and immunity."[30] These analgesic compounds modify immune responses to extracellular stimuli like mitogens and antigens, and immune cells express mRNA transcripts encoding various opioid receptors that were originally described in neuronal tissues.[31]

Here are some studies that either show opiates/NSAIDs impact inflammation or can negatively impact TBI outcome:

a) A study looked at altered hyperpolarization-activated cyclic nucleotide-gated (HCN) channel protein expression in trigeminal sensory neurons following inflammation of the dura using application of complete Freund's adjuvant (CFA) to the dura mater in rats. The increased activity of (HCN) channels has been shown in models of nerve injury-induced pain. Buprenorphine was applied following surgery and inflammation-induced increases in HCN1 and HCN2 were not observed. The authors suggest that "if the peak action of buprenorphine is before the peak of the CFA-inducted inflammatory effect then buprenorphine may attenuate the inflammatory response by acting on immune cells."[32]

b) In a study of immature swine (3-5 day-old piglets), premedication with meloxicam, a COX-2 perferential NSAID, prior to brain injury using rapid head rotations without impact produced a significantly higher mortality rate than buprenorphine.[33] No controls were used in this study.

c) Rats treated with Morphine or propofol for 1 hour after CCI were associated with significantly worse motor function on post-TBI day 1-5 compared to animals without anesthesia and shams.[34]

d) In one 5.5-year retrospective study of patients aged 55 years and older with blunt force TBI, patients who were taking anticoagulants and antiplatelet agents (warfarin,

clopidogrel, dipyridamole/aspirin, enoxaparin, and subcutaneous heparin) experienced a higher rate of inpatient mortality and other adverse outcomes caused by the effects of these agents.[35] Although these patients were taking these medications before sustaining a TBI, and we don't know what the specific effects of these agents are on immune system function after TBI, these agents including aspirin negatively affected TBI outcome.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions:
Isoflurane will be administered just prior to and during surgical procedures to provide loss of consciousness. To confirm that animals have a loss of consciousness, the absence of tail, paw reflexes, and corneal reflex will be confirmed.

V.4.3.2. Procedure: Controlled Cortical Impact:
All surgical procedures will take place in the CNRM surgical room for mice. We will follow CNRM's Standard Operating Procedures except for providing post-surgical analgesia (e.g. no Tylenol water will be provided or buprenorphrine). Mice will be anesthetized with isoflurane as described above via inhalation with a nose cose. Corneas will be kept moist during surgery by applying Lubritears ointment. Sterile gloves will be worn for the surgical procedures. The fur on top of the head will be shaved followed by a cleaning of the area with iodine and alcohol three times. All surgical equipment will be thoroughly cleaned, wiped with alcohol, and sterilized using Germinator equipment and allowed to return to room temperature.
Mice will be placed in a stereotaxic apparatus with ear bars. A scalp incision will be made at midline centered approximately at Bregma for 1.5cm incision length. First, the impactor will a 3mm diameter tip will be lined-up directly over Bregma, then moved 2mm to the left. A craniotomy is done (~5mm circular piece of skull is removed) using ahand-held trephine to expose the dura mater. The brain will be impacted at a depth of 1.5mm, velocity 1.5m/s over the exposed area of brain. The burr hole is left open and the incision is closed with absorbable sutures, such as natural chromic gut sutures or polyglycolic acid synthetic sutures. Animals will recover on a circulating heating pad. Once ambulatory, animals will be returned to their cages.

V.4.3.3. Post-surgical Provisions:
If an animal's skull is fractured and there is significant hemorrhaging during the TBI surgical procedure, we will eliminate the animal from the study and euthanize it. After every surgical procedure, animals will be placed on a heating pad in a single cage for 30 minutes to allow recovery from the surgery. During this time, animals will be monitored to make sure they come out of anesthesia and are able to right themselves, lay sternal, and lift their heads. After surgeries, we will monitor animals for any sigs of pain or distress including hunched posture, difficulty breathing, muscle rigidity or lack of muscle tone, twitching, trembling, tremor, self-mutilation, or unkempt appearance (erect, matted, or dull haircoat). Additionally, animals will be monitored for significant weight loss (greater than 20% total weight loss relative to initial body weight prior to injury) as

an additional sign of distress. If post-procedural pain or distress induces persistent symptoms beyond the first 24 -36 hrs post injury, including hunched posture, labored or gasping breathing, ruffled fur, immobility, failure to feed or to drink, the affected mice will be immediately removed from the study and euthanized. No analgesia will be provided after surgeries, because doing so will affect our immune-related outcome measures, which categorizes our animals in CAT E. Additionally, we want to compare our results with those from other labs that do not use any pain relief after TBI surgeries (e.g. Dr. (b)(6) lab).

V.4.3.4. Location: (b)(6)

V.4.3.5. Surgeon: (b)(6)

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:
For terminal procedures, ketamine and xylazine will be used as the sedative and anesthetic. Before animals are euthanized, they will be injected (i.p.) with 80-100 mg/kg of Ketamine and 10 mg/kg Xylazine intraperitoneally using a 26-gauge needle. Again, the total volume of injected anesthetic depends on the weight of the animal. For example, a 25 gram mouse will receive a single i.p. injection of 20-25 µl ketamine (from a 100 mg/ml stock) plus 250 µl xylazine (from a 1 mg/ml stock).

V.4.4.2. Biosamples:
During the terminal procedure, blood samples will be collected via cardiac puncture and 300-800 ul will be collected using a 25 gauge needle attached to a 1mL syringe. Also during the terminal procedure, spleens and choroid plexus material will be collected by surgical removal.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Cage cards will identify animals in each cage. Permanent pen markings will be made on the tails of mice to distinguish them apart.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures:
Saline Perfusion:
For all perfusion procedures, animals will be anesthetized with an i.p. injection of 80-100 mg/kg of Ketamine and 10 mg/kg Xylazine using a 26-gauge needle. We will confirm

that animals are under adequate anesthesia before proceeding with the perfusion (i.e. lack of withdrawal to toe pinch and lack of corneal reflex). The chest cavity will be incised though the abdomen that allows access to the rib cage. Two cuts will be made through the right and left side of the rib cage, and a clamp will be used to open the thoracic cavity and expose the heart. A needle is inserted into the left ventricle up about 5 mm. The right atrium is then cut and the animal is first perfused with PBS + heparin at a flow rate of 20 ml/min. When the blood has been cleared from the body (about 100 ml of PBS used), heads will be decapitated and brain tissue will be dissected so that choroid plexus material can be isolated.

V.4.4.8. Tissue Sharing: There will be no tissue sharing with other labs.

V.4.5. Study Endpoint:

Animals will be euthanized at specified timepoints after TBI/sham injury. If animals die before the completion of the study, they will be removed from the study, and statistical analyses will be performed on the remaining animals. If animals show any visible signs of distress following surgical procedures, such as significant weight loss, they will be euthanized and removed from the study.

V.4.6. Euthanasia:

All animals will be properly anesthetized before terminal procedures with an i.p. injection of 80-100 mg/kg of Ketamine and 10 mg/kg Xylazine using a 26-gauge needle. After animals have been deeply anesthetized, the chest cavity will be incised though the abdomen that allows access to the rib cage. Two cuts will be made through the right and left side of the rib cage, and a clamp will be used to open the thoracic cavity and expose the heart. Blood will be collected via cardiac puncture. Then spleens will be dissected using scissors. Sub-task 1.1 animals will be saline perfused with PBS at a flow rate of 20ml/min, and their brains dissected in order to dissect out choroid plexus material from the ventricles. At the conclusion of every terminal procedure, all animals will be decapitated with sharp scissors to confirm death.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

Animals will be observed everyday to confirm health and viability. Animals will be housed in the same cages/cagemates they arrived to the University with and will have unlimited access to food and water. Animals will be kept in temperature controlled rooms in reverse light cycle.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6)

The experimental procedures will be conducted in the LAM/CNRM facilities in the basement of building (b)(6). After the experimental procedures, and after animals have been allowed to recover on heating pads, they will be taken back to the animal housing room.

V.5.1.2. Special Husbandry Provisions:

We do not anticipate that animals will need nutritional support following TBI. Wet mash food may be placed in the cages of mice 1-3 days after surgeries to make sure that they can reach food.

Food Restriction: Yes _____ No __x__

Fluid Restriction: Yes _____ No x

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
The veterinary technicians in the LAM will observe animals everyday. The criteria used for health evaluation will be weight loss, hair loss/unkempt fur, and decreased locomotor activity. In the event of a debilitating illness or adverse reaction, the animal will be evaluated by the attending veterinarian and possibly euthanized at an earlier timepoint.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Controlled Cortical Impact; isoflurane anesthesia, ketamine/xylazine injections; daily observation of mice after CCI surgeries	(b)(6)	Graduate Student, 6 years experience	Training from (b)(6) 2012 & 2014; USU Investigator Training Course 2012; Rodent Handling Class 2009
Euthanasia/tissue extraction		Graduate Student, 6 years experience	Training from Dr. (b)(6) 2010-2011; USU

	(b)(6)		Investigator Training Course 2012; Rodent Handling Class 2009
Euthanasia/tissue extraction		PI, 14 years experience	Training from Dr. (b)(6) lab, 2000; USU Investigator Training Course 2012
RNA isolation and sequencing		Research Associate, 7 years experience	Blood borne pathogen training 2013; rodent handling course 2012

(b)(6) took the rodent handling training course at USU in 2009.
took the Investigator/animal user training course at USU on March 8, 2012.
Dr. (b)(6) took the rodent handling training course at USU in 2003 and refresher course in 2005.
Dr. (b)(6) took the PI training course at USU on January 17th, 2012.

VII. BIOHAZARDS/SAFETY:

All personnel handling these animals will use protective gloves, lab coat, and covered shoes at all times to minimize exposure to allergens and agents used during experimental procedures. Isoflurane vapors will be scavenged by the waste vapor apparatus.

VIII. ENCLOSURES:

Works Cited

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IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 8/7/15

P Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 8/7/15

29

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) [redacted] 8/7/15

Date

X. PROTOCOL ABSTRACT:

Every year in the U.S., it is estimated that 1.4 million people are hospitalized with a brain injury.[1] Among U.S. military personnel, incidence of depression, PTSD, suicide risk, and TBI symptom severity are all significantly increased as the number of TBIs sustained increases.[2] Alterations in brain energy metabolism, local neuroinflammation, and fluid balance are among the mechanisms implicated for contributing to repeated TBI-related adverse outcomes. Therefore, a subsequent injury after an initial TBI is associated with severe cumulative consequences.

Recently, it has been determined that the adaptive immune system can develop immunological memory to CNS injury and activate production of autoantibodies with pathogenic potential. Studies have detected autoantibodies in biofluids from individuals with CNS injury as well as from animal subjects in TBI preclinical models. However, the contribution of immunological memory responses after repeated TBI events has not been defined. **Our goal in this project is to determine whether TBI establishes immunological memory and whether a repeated injury results in a secondary adaptive immune response.**

T and B lymphocytes are primary components of the adaptive immune system, yet the role of lymphocytes in TBI is not well understood. Evidence in other CNS injuries suggests that T and B cells play significant roles in post-traumatic inflammation, degeneration, or repair. Since T and B lymphocytes have both potential neuroprotective and neuropathogenic roles, elucidating the response of the adaptive immune system to TBI is needed to better understand the mechanisms of repeated TBI. Modulating the immune system after TBI may innovate treatment strategies for improved recovery after TBI. **Our hypothesis is that an initial TBI results in immunological memory and that a subsequent TBI potentiates adaptive immune alterations.**

A. **Animal Protocol Number**:

B. **Animal Protocol Title**: (b)(4) (b)(4)

C. **Principal Investigator**: Dr. (b)(6) Assistant Professor, APG

D. **Performing Organization**: Uniformed Services University of the Health Sciences

E. **Funding**:

F. **Objective and Approach**:

To test this hypothesis, we will complete experiments for the following specific aims:

Aim 1: Determine adaptive immune components that are altered in a murine model of repeated TBI. The changes in adaptive immune system components after TBI are not well defined. We will profile lymphocyte populations by flow cytometry to determine whether memory cell populations are altered. Expression analysis of lymphocyte populations will also be accomplished to detect transcripts associated with immunological memory responses.

Aim 2: Determine the status of adaptive immune repertoire alteration as a function of autoantibody presence. Studies have detected autoantibodies in subjects after CNS injury and TBI. The level and nature of autoantibodies after repeated TBI has

not been defined. We will measure serum autoantibody levels from TBI subjects using 1-dimensional gel and ELISA-based methodologies. Clonotypic enrichment in lymphocyte repertoire will be profiled by sequencing receptor immunoglobulins.

Aim 3: Demonstrate CNS-directed lymphocyte proximity to the brain after repeated TBI. The blood-brain barrier establishes immunoprivilege to mitigate self-reactive responses to host tissue. CNS antigen-mediated responses and lymphocyte chemotaxis to the brain after TBI may initiate autoimmune reactions. We will determine the immune and inflammatory status of the choroid plexus to assess its modulating role of lymphocyte populations in the CNS after TBI. Furthermore, we will analyze T cell receptor repertoire status of the choroid plexus to detect if repeated brain injury results in increased infiltration of CNS-specific lymphocyte populations to the brain.

G. **Indexing Terms (Descriptors):** Adaptive immune system; TBI; Brain injury; lymphocyte; memory B cell;

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August 31, 2015

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on August 31, 2015:

Animal Protocol Title: "The Impact of Blast Exposure on Mouse Daily Activities"

USUHS Protocol Number: (b)(6)

Expiration Date: August 30, 2018

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

Revised

PROTOCOL TITLE: The impact of blast exposure on mouse daily activities

GRANT TITLE (if different from above): UCSF/USUHS Partnership to develop tau prion therapeutics for chronic traumatic encephalopathy

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Defense Health Agency, DoD

EARLIEST ANTICIPATED FUNDING START DATE: August 1, 2015

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6) | APG (Department) | (b)(6) (Office/Lab Telephone) | 7/13/15 (Date)

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and i[illegible]tific research practice.

(b)(6) | [illegible] (Title) | (b)(6) (Telephone) | 07.15.2015 (Date)

Research Unit Chief / Dept. Head Signature
Typed Name: (b)(6) M.D., Ph.D.

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) | APG (Department) | (b)(6) (Telephone) | 7/13/2015 (Date)

Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) | LAM (Department) | (b)(6) (Telephone) | 7/15/15 (Date)

Attending/Consulting Veterinarian Signature
Typed Name (b)(6) VMD

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6)

ANIMAL PROTOCOL TITLE: The impact of blast exposure on mouse daily activities

GRANT TITLE (if different from above): UCSF/USUHS Partnership to develop tau prion therapeutics for chronic traumatic encephalopathy

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6) B.S., (b)(6) M.D.

TECHNICIANS(S): (b)(6) M.D., (b)(6) M.S., (b)(6) Ph.D., (b)(6) M.D.

I. NON-TECHNICAL SYNOPSIS:

Blast exposure is a common type of traumatic brain injury (**TBI**) experienced by military service members in theater; particularly those who have served in Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF). Many of these service members report experiencing significant changes in behavior and mood post-injury. Clinical reports have documented that a subset of these TBI patients demonstrate changes in overall daily activity, including changes in arousal and attention, excessive daytime sleepiness, as well an unrestful sleep at night. In post mortem studies, these clinical observations have been linked to injury in a brain area called the hypothalamus, which contains brain cells that control arousal and sleep-wake cycles. We will study in laboratory mice how exposure to blast wave(s) affects daily activity of the mice, and study how the blast may change certain chemicals in the brain. We hope this research will shed light on the effects of overpressure shock waves on behavioral homeostatic mechanisms that could play an important role in recovery from TBI.

II. BACKGROUND:

II.1. Background:

Due to military conflicts overseas, in recent years there has been increasing interest in the effects of *blast overpressure* on the brain. Animal models of blast injury are being employed to investigate the physical and neuropsychological symptoms faced by military personnel exposed to blast waves resulting from the detonation of explosive devices (Goldstein *et al*, 2012). Animal models of blast have used both rats and mice, and these studies have aided in the elucidation of the acute and chronic gross neuropathological and behavioral symptoms that arise after exposure to blast overpressure (Ahlers *et al*, 2012; Cernak *et al*, 2011; De Gasperi *et al*, 2012; Goldstein *et al*, 2012; Koliatsos *et al*, 2011; Rubovitch *et al*, 2011; Tweedie *et al*, 2013; Valiyaveettil *et al*, 2013; Xie *et al*, 2013). Animal blast models show promise for providing critical information in line with the goals of the CNRM in exploring and developing treatments for TBI and post-traumatic stress disorder (**PTSD**)-related symptoms in military personnel and veterans. The CNRM Pre-Clinical Studies Core has a newly-designed, state-of-art Advanced Blast Simulator (**ABS**; ORA Inc, Fredericksburg, VA) for investigators to employ in research examining TBI and PTSD. The area of blast effects on daily activity homeostasis has received no prior attention. Pre-clinical modeling may be an important approach for determining the mechanisms that underlie arousal and activity changes after TBI.

Based on prior evidence from clinical studies and animal research, the hypothalamic hormone, orexin, is known to play a significant role in activating many pathways throughout the central nervous system that maintain arousal during the waking state. Excessive sleepiness in narcoleptic patients, for example, has been linked with loss of orexin neurons. Similar findings have been observed in Parkinson patients who report experiencing sleep attacks and difficulty staying awake. Furthermore, close to half of TBI patients report experiencing sleep disorders, which have adverse effects on recovery and quality of life. Orexin has been shown to decrease following TBI—both in the acute setting and chronically as measured in the CSF of patients (Baumann *et al*, 2005b; Baumann *et al*, 2007). Post mortem studies further delineate loss of orexin neurons in patients with known TBI (Baumann *et al*, 2009). Like orexin, melanin concentrating hormone (**MCH**) plays a role in sleep-wake regulation, where overall it promotes the induction of REM sleep. Postmortem studies of patients with TBI have also shown a decrease in MCH neurons along with orexin neurons (Baumann *et al*, 2009), linking both of these neuropeptides to arousal and general activity levels, as well as sleep-wake cycle regulation. This project will examine the impact of blast exposure on mouse daily activity cycles and whether or not blast alters brain levels of orexin and MCH.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: Biomedical Research Database (BRD)

II.2.2. Date of Search: July 13, 2015

II.2.3. Period of Search: 1998-2009

II.2.4. Key Words and Search Strategy: All fields: Rodent, behavior, blast; orexin, melanin concentrating hormone, mice. Results: 2 hits

II.2.5. Results of Search:

The BRD database includes thousands of records of previous research programs. No hits were found relating to the full set of keywords, "Rodent, behavior, blast; orexin, melanin concentrating hormone, mice." A second search was performed for the words, mouse, blast TBI and orexin. No hits were found. A third search was performed to see if any studies had been performed on orexin and this search derived two hits. One project was entitled, "Molecular connections between arousal and metabolic disease: Orexin and Modafinil" that investigated chronic pharmacological activation of orexin-A and related pathways on metabolic status and obesity. A second project entitled, "Role of CRF and orexin in driving ethanol consumption," investigated the "mechanistic relationship between acute exposure to corticotrophin releasing factor (CRF) and orexin and how forced abstinence and extinction from ethanol self-administration alters the properties of CRF receptors and orexin receptor-dependent potentiation of NMDA receptors in the ventral tegmental area." **Conclusion**: No related hits indicate there is no overlap of this protocol with previous research programs in the DoD.

II.2.6. Literature Source(s) Searched: NIH RePORTER

II.2.7. Date of Search: July 14, 2015

II.2.8. Period of Search: Projects through fiscal year 2015

II.2.9. Key Words and Search Strategy: Orexin, traumatic brain injury, mice

II.2.10. Results of Search:

The NIH website, RePORTER, was used. The keywords orexin, mice and traumatic brain injury produced no hits. The keywords orexin and traumatic brain injury produced one hit. The hit was NIH grant R01NS065069-07, entitled "Mechanisms underlying tauopathy following traumatic brain injury." The project description does not directly mention orexin, but this group recently

published a paper related to this support (Willie *et al*, 2012). **Conclusion**: No related hits indicated there is no overlap with previous research federally sponsored research programs.

II.2.11. Literature Source(s) Searched: PubMed
I.2.7. Date of Search: July 14, 2015
II.2.12. Period of Search: >24 million references: 1949-2015.
II.2.13. Key Words and Search Strategy: Orexin, mice, and traumatic brain injury
II.2.14. Results of Search:

PubMed was used with the keywords, mice, orexin and traumatic brain injury, and six hits were derived. One recent publication described the trouble with wakefulness seen in mice after fluid percussion injury (Lim *et al*, 2013). A second finding was the paper just noted above in II.2.6, where reduced wakefulness was seen after controlled cortical impact TBI (Willie *et al*, 2012). A third report describes changes in orexin receptor immunostaining in the brain region surrounding the injury site from controlled cortical impact (Mihara *et al*, 2011). A 4th report was a metanalysis of the effects of various factors on locomotor activity. It reports that "genetic and pharmacological alterations that enhance the dopamine, orexin, histamine, cannabinoids systems or that antagonize the cholinergic system induce an increase in locomotor activity" (Viggiano, 2008). Finally the 5th and 6th publications were reviews (Baumann and Bassetti, 2005a; Harrington, 2014). With the keywords orexin and traumatic brain injury, 29 hits were obtained. In general, these publications were reports that orexin is related to activity levels, and that TBI has a deleterious impact on arousal and activity and/or sleep-wake cycles. However, none of these publications related to the possible effect of blast overpressure on activity and orexin in a pre-clinical model. Conclusion: There are no reports of the effects of blast on orexin and general activity levels in mice as described in this protocol.

III. OBJECTIVE\HYPOTHESIS:

The objective of this project is to assess the effects of blast TBI (single vs. multiple events) on overall behavioral activity and putative brain systems that regulate activity and sleep-wake cycles. Using a mouse model of blast TBI, we hope this will allow us to elucidate the effects of blast injury on daily behavior cycles, cognition, emotion, and memory, and what impact blast has upon hypothalamic neurons that regulate arousal and sleep. Specifically, we will assess brain tissue post-injury for changes in orexin and melanin concentrating hormone neurons. This study will also allow us to assess any significant differences between single exposure and multiple exposures to blast injury. It is hypothesized that exposure to blast overpressure will reduce mouse overall activity levels and produce fragmented periods of sleep and wakefulness. We predict these changes will correlate with additional signs of TBI, including cognitive deficits and impairment of memory function, and a decrease in brain levels of orexin and melanin concentrating hormone.

IV. MILITARY RELEVANCE:

The *relevance* of this research is to assist in meeting a major objective of the CNRM: "to address the current needs of the medical community to better diagnose and intervene for the prevention of the long term consequences resulting from traumatic brain injury, particularly in the context experienced by service members in Operation Iraqi Freedom (**OIF**) and Operation Enduring Freedom (**OEF**)." According to a respected RAND Corporation report (Tanielian and Jaycox, 2008), as many as 18.5% of the service members returning from these conflicts meet the criteria for a diagnosis of either PTSD or depression, and 19.5% of service members have reported experiencing a probable TBI during deployment. These estimates translate to

approximately 320,000 cases of "probable TBI" and 300,000 cases of reported symptoms for depression or PTSD. Most cases of TBI in OIF and OEF arise from blast injury associated with the detonation of explosive devices in the form of non-contact closed head injury. Scientific research related to developing a better understanding of the biological processes and treatment for blast-induced TBI and/or psychological trauma is necessary for the care of our service members. Specific consequences of TBI include arousal and sleep-wake disorders, which plague up to 43% of people who have experienced TBI (Baumann *et al.* 2006; Castriotta *et al.* 2007). The laboratory mouse, using this injury model, is expected to advance our knowledge of biological mechanisms of brain injury that result in behavioral activity disturbances. Uncovering such mechanisms will allow us to develop strategies for treatment and improve the quality of life for service members with TBI.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

Overall experimental design. The ABS will be used to create a mouse model of blast TBI, from which we will study disruptions in activity and sleep-wake regulation; akin to what has been documented in clinical studies following concussion and impact TBI. The main studies (Experiments 1, 3-5) will comprise four treatment groups of mice: **1.** Mice exposed to a single blast injury. **2.** a second group exposed to multiple blast injuries, and **3.** and **4.** will be groups of mice serving as sham-treated animals for comparison of results obtained for the first and second groups, respectively. As outlined in these experiments, we will assess the effects of blast overpressure on overall behavioral activity, sleep-wake cycles, cognition, emotion, and memory, and neuroanatomical changes.

Table 1. Summary of Experiments 1-5

Experiment	Purpose of Study	Blast Exposure(s)	Home Cage Activity	Behavior Testing	Histology/ Biochemistry	Length of Study
1	Short-term brain changes	Yes	No	No	Yes	7 days
2a	Pilot study of Experiment 3	No	AnyCage	No	No	7 days
2b	Pilot study of Experiment 4	No	Telemetry	No	No	14 days
3	Activity, behavior, and brain changes after blast(s)	Yes	AnyCage	Yes	Yes	30 days
4	Activity, behavior, and brain changes after blast(s)	Yes	Telemetry	Yes	Yes	37 days
5	Long-Term Behavior and brain changes after blast(s)	Yes	No	Yes	Yes	90 days

Table 1 is an overview of the experiments. Experiment 1 (described below) will evaluate short-term changes in brain levels of orexin and melanin concentrating hormone (**MCH**) in the first seven days after exposure to a single blast or repeated blast (1 blast/day for 5 days). Experiment 2 will be a pilot study to verify that the instrumentation (explained below) is

properly working before starting Experiments 3 and 4. Finally, Experiment 5 will evaluate the long-term effects of blast exposure on behavior.

Due to the relative novelty of this approach to blast-related TBI symptoms, two experimental approaches will be used to measure mouse activity. In Experiment 3, mice (across the four treatment groups mentioned above) will be evaluated for daily activity patterns with the Stoelting Company's AnyCage home cage chamber (hereafter described as "chamber"). In Experiment 4, animals will be evaluated for daily activity using the DSI PhysioTel® ETA-F10 transmitter system (hereafter abbreviated as "telemetry system"). The "chamber" approach consists of placing individual mice in an AnyCage home cage, which is an environmentally-regulated, ventilated chamber with a computer that controls the chamber's light-dark cycle. The apparatus also monitors daily food and water intake and running wheel activity, and an infrared camera system measures overall movement. The "telemetry system" employs an implantable device that, under sterile conditions, is positioned subcutaneously in the nape of the mouse. Screws are implanted in the skull to monitor EEG. The mouse is then placed in a standard LAM mouse home cage that is positioned on top of a DSI PhysioTel® Receiver that records radio frequency signals broadcast by the transmitter to monitor EEG, as well as activity as the mouse freely moves about its home cage.

V.1.1. Experiment 1. Short term effects of blast exposure on brain changes in levels of orexin and MCH. The first experiment will evaluate changes in levels of the neuropeptides, orexin and MCH, in the hypothalamus. Separate groups of 30 mice each (**Table 2**) will sustain 1 or 5 blast exposures and on Days 1, 3, or 7 after exposure 10 mice per time point from these samples will be euthanized as described in **V.4.6.Euthanasia**. The brain tissue will be used for immunohistochemistry and western blotting to measure levels of orexin and MCH as a function of treatment and time after injury.

Table 2. Experiment 1. Short Term Effects of Blast

Experimental Treatment	No. of Mice	Pain/Distress Category
1 Blast Exposure	30	D
5 Blast Exposures	30	D
1 Sham Treatment	30	C
5 Sham Treatments	30	C

V.1.2. Experiment 2. Pilot studies to validate use of the AnyCage system and the telemetry system, and refinement of surgical procedures.

The purpose of Experiment 2 will be to verify and refine experimental procedures for measurement of activity. No mice in this study will sustain blast TBI. In Experiment 2A (**Table 3**), a total of 20 mice will be used to ensure the operation of the AnyCage system. Specifically, untreated mice will be placed in individual AnyCage chambers to ensure the software and movable parts in the chamber are working properly. In Experiment 2B, 20 mice will be used to practice the surgical implantation procedure of the telemetry units and to ensure the units are working properly and recorded by the receiver systems. Experiments 3 and 4 (described below) will only take place when we have full procedural success in Experiment 2.

Table 3. Experiment 2. Pilot Studies

Experimental Treatment	No. of Mice	Pain/Distress Category
2A. AnyCage	20	C
2B. Telemetry	20	D

V.1.3. Experiment 3. Effects of blast exposure on daily activity in the AnyCage Chamber. As summarized in **Table 4**, a study will be performed to determine the effects of blast TBI on daily activity, feeding, drinking, and wheel running as evaluated by placement of mice in the AnyCage chambers. Briefly, 20 mice per treatment group will be individually housed in an AnyCage chamber beginning on Day 1 and randomly assigned to sustain one or five blast(s), or one or five sham treatment(s). Following each treatment, they will be placed back in individual AnyCage chambers for a total of 30 days.

Table 4. Experiment 3. Activity Effects of Blast		
Experimental Treatment	No. of Mice	Pain/Distress Category
1 Blast Exposure	20	E
5 Blast Exposures	20	E
1 Sham Treatment	20	E
5 Sham Treatments	20	E

As described later, these animals will receive behavioral testing on Day 8 (Open Field Test), Day 10 (Zero Maze), Days 14-17 (Morris Water Maze) and Day 21 (Porsolt Swim Test). All of the animals in this Experiment are in the Pain/Distress Category E, since that will be tested in the Porsolt Swim Test, where the mouse is placed in an inescapable water trough.

V.1.4. Experiment 4. Effects of blast exposure on daily activity and EEG activity as measured by telemetry.

The experimental design for Experiment 4 (**Table 5**) is identical to what was described for Experiment 3. However, in Experiment 4, the DSI PhysioTel® ETA-F10 transmitter system will be used. One week before blast exposure, animals will be deeply anesthetized and the transmitter will be implanted subcutaneously. Following implantation, the skin incision will be sutured. The two leads from the transmitter will be threaded underneath the animal's skin to attach one wire each to a screw placed over the midline of the skull and over the middle of the parietal bone. The

Table 5. Experiment 4. Short Activity and EEG Effects of Blast		
Experimental Treatment	No. of Mice	Pain/Distress Category
1 Blast Exposure	20	E
5 Blast Exposures	20	E
1 Sham Treatment	20	E
5 Sham Treatments	20	E

scalp wound will then be sutured so the mouse will not have access to the leads. A total of 20 mice per treatment group will then be placed back into their individual home cages that are positioned above the DSI receivers to allow recording of EEG and behavioral activity. On Day 1 (one week after transmitter implantation) animals will be randomly assigned to sustain one or five blast(s), or one or five sham treatment(s). Following each treatment, they will be placed back in their home cage, and they will remain there for 30 days. As described later, these animals will receive behavioral testing on Day 8 (Open Field Test), Day 10 (Zero Maze), Days 14-17 (Morris Water Maze) and Day 21 (Porsolt Swim Test). All of the animals in this Experiment are in the Pain/distress Category E, since that will be tested in the Porsolt Swim Test, where the mouse is placed in an inescapable water trough.

V.1.5. Experiment 5. Long term effects of blast exposure on daily activity.

Experiment 5 will evaluate the long-term effects of blast exposure on behavioral performance and histopathology. Mice in this experiment (**Table 6**) will receive behavioral testing as described in Experiments 3 and 4, but the testing will occur at a later time to determine whether or not there are long-term behavioral impairments from blast exposure. Please note these animals will not be evaluated in the AnyCage or telemetry activity units.

Table 6. Experiment 5. Short Activity and EEG Effects of Blast

Experimental Treatment	No. of Mice	Pain/Distress Category
1 Blast Exposure	20	E
5 Blast Exposures	20	E
1 Sham Treatment	20	E
5 Sham Treatments	20	E

These animals will receive behavioral testing on Day 68 (Open Field Test), Day 70 (Zero Maze), Days 74-77 (Morris Water Maze) and Day 81 (Porsolt Swim Test). All of the animals in this Experiment are in the Pain/distress Category E, since that will be tested in the Porsolt Swim Test, where the mouse is placed in an inescapable water trough.

V.2. Data Analysis:

Based on prior experience, a total of 10 mice for each of the four treatment groups in Experiment 1 should be sufficient. The estimate for the required number of animals needed for Experiment 1 is based upon a recent study we performed in mice that received a closed head injury (b)(6) (b)(6) In this study, there were 8 mice that each received sham treatment or a single closed head concussive injury. An analysis of variance was performed, and indicated there was a significant difference in staining density for orexin in the mouse hypothalamic region as a function of injury (mean difference=23.597, standard deviation of the residuals=13.474, $F_{1,14}$=12.264, p=0.004, power=0.891). I performed simulations using the SigmaPlot program for estimation of power of a similar analysis of variance, but in this case when there are four treatment groups and 10 mice per group. Power estimation indicated that with the aforementioned mean and standard deviation of residuals, power is approximately 0.891. For Experiments 3-5, a sample size of 20 mice/treatment group appears to be ample for behavioral testing and for what is usually reported in publications of mouse behavior testing (number of subjects per group is usually in the mid-teens). This estimate appears to be in line with suggestions by internationally recognized experts. For example, Crawley suggests that "N = 20 mice or more per genotype are often needed to complete the first set of behavioral tests in a new line of mutant mice" (Crawley, 2007).

Statistical Analyses: Food and water intake, and wheel running periods recorded in the AnyCages will be compared across treatment groups. Activity data acquired in the AnyCages will be evaluated to compare any changes during the light and dark phases (Pack *et al*, 2007). Data for activity and EEG wave function will employ the software supplied by the vendor of the transmitters (Data Science International, 2006). The SPSS statistical analysis program (IBM, version 20.0) will be used to compute the arithmetic mean and standard deviation of multiple measures of activity, EEG response and each behavior test employed. In addition, a repeated measures single-factor analysis of variance (ANOVA) will be used to assess whether or not performance on each test is the same when comparisons are made of repeated testing results. Calculations will be performed to determine whether or not the sample sizes/group were adequate to obtain sufficient power (≥0.70). If the data suggests there is variation between the

testing of each cohort of animals, a nested ANOVA can be used to assess whether or not there are differences between testing events on repeated tests. Finally, to assess some degree of the relationship of performance on tests within an experiment, correlation coefficients will be computed for each animal's score on each subtask. Neuropathological data also will be summarized based upon visual inspection and descriptions of level of pathology.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

Animals are required for this research to assess *in vivo* cellular and molecular effects of blast-induced TBI and resulting behavioral performance. These data will provide a means for relating behavioral responses to central nervous system function *in vivo*. Non-animal alternatives do not replicate the three dimensional structure or temporal changes that occur as a function of complex behavioral responses to tasks presented to a live animal, including the evaluation of how TBI affects daily activity cycles. Further, computer modeling and *in vitro* methods will not address experimental questions of the complex, real-time changes and responses that occur during experience.

V.3.2. Animal Model and Species Justification:

By consensus of members of the CNRM, C57Bl/6 mice have been chosen as the suggested species and animal model for mouse TBI research (Anon., 2009). This is based upon the previous use of this strain in innumerable studies and their suitability as biomedical models for understanding central nervous system functions in normal and pathological states in humans and in other animals. The mouse is used for these studies because of their well-understood anatomy and endocrine responses, and the very extensive database of prior physiological research that can be integrated with this new information.

It is necessary to use mice instead of a "lower" species since the research results should be directly associated with intense efforts in other laboratories throughout the world to try and further our understanding and eventual treatment effectiveness for psychological stress and brain injury. Mice are commonly used small animal models for the study of neurobiological and genetics research, including the impact of physical injury and environmental stress upon neuron connectivity, experiential effects on synaptic plasticity, signal transduction, and for assessing treatments to reduce neurological damage and improve quality of life. In the case of murine transgenic and gene knockout models in neuroscience and behavioral research, "wild type" C57Bl/6 mice often serve as the genetic "background" or control for comparison of genetic and genetic/environmental effects (Crabbe, 1999; Crawley *et al*, 1997; Mishina and Sakimura, 2007). Recent work suggests that male and female mice exhibit similar behavioral impairments after TBI (Tucker *et al*, 2015a; Tueker *et al*, 2015b). Half of the mice per treatment group will be of each sex. A sample size of 10/sex/group will permit us to explore possible sex differences resulting from TBI, but this is not expected.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	*Mus musuclus*	*Mus musuclus*
V.3.3.2. Strain/Stock:	C57Bl/6	C57Bl/6
V.3.3.3. Source/Vendor:	(b)(4)	

V.3.3.4. Age:	8-12 weeks		8-12 weeks
V.3.3.5. Weight:	20-25 grams		20-25 grams
V.3.3.6. Sex:	Female		Male
V.3.3.7. Special Considerations:	None		None
V.3.4. Number of Animals Required (by Species):	Mice: 200		Mice: 200

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:

Four refinements will be performed in our protocol. **1.** After delivery to the University, rodents will be acclimated to the animal facility for 5-7 days before use. **2.** Investigator handling before the commencement of TBI procedures and behavioral testing will be used, to relieve distress from the potential combination of handling distress and the testing procedures. **3.** Prior to the use of animals in the ABS, the parameters of the blast are measured to ensure that the shock wave characteristics are consistent across days and approximate blast levels described in the literature for animal research. **4.** Analgesics and anesthetics will be used to decrease possible pain and distress resulting from surgery and blast.

V.3.5.2. Reduction:

We will perform pilot studies using the smallest number of animals possible to ensure proper operation of the AnyCage chambers and the telemetry system before using mice. This will ensure use of a larger cohort of animals will derive useful and reliable data. The number of animals for the larger scale studies (Experiments 3-5) is based on experience from working with rodent TBI models and behavioral assessments. If we find that variability in results is low and replicable results are obtained with a fewer number of animals, fewer animals may be used. Finally, we may obtain data that suggests that behavioral results are quite similar for the two sham groups in Experiments 3-5. Specifically, separate groups of mice are exposed to a one-time sham treatment while other mice will receive sham treatment (brought to the ABS room, anesthetized) five times. We may discover we can halve the number of animals in these groups as a means of reduction.

V.3.5.3. Replacement:

Biological response to central nervous system injury, psychological stress, and experimental treatments is highly complex; no alternative to *in vivo* experimentation is available or practical. We use a mouse model versus a nonhuman primate model or other higher species. Mice are the least sentient and derived animal model we could use for this study and still obtain results that are applicable to the mission of the research.

V.4. Technical Methods:
V.4.1. Pain / Distress Assessment:
V.4.1.1. APHIS Form 7023 Information:
V.4.1.1.1. Number of Animals:

	Species #1 (mouse)*	Species #2
V.4.1.1.1.1. Column C:	80	–
V.4.1.1.1.2. Column D:	80	–
V.4.1.1.1.3. Column E:	240	–

*Explanation of pain categories: 60 sham-treated mice used in Experiment 1 (Pilot Study) will receive no invasive or distressful treatment. They are Category C since they will only receive isoflurane treatments as the sham procedure for blast treatment and be placed in the AnyCage chambers. 60 mice in Experiment 1 are considered Category D. These mice will sustain blast exposure, but given analgesic as a safeguard to any pain. In Experiment 2, 20 mice are considered Category C since they will receive no treatment or surgery and will be housed in the AnyCage chambers. The remaining 20 mice in Experiment 2 are Category D since they will sustain surgical implantation of the transmitter, but they will be provided with analgesia to alleviate any pain from the surgical procedure. The 240 mice in Experiments 3-5 are assigned as Category E. They will be tested in the Porsolt Swim Test apparatus. As described below, this test involves inescapable placement in water, where the mouse must swim to maintain itself above water. Since blast exposure and surgery will include analgesia treatment, animals in this group do not meet the criterion of Category E for unalleviated pain from the sham treatment or blast exposure or transmitter implant, but from participation in the Porsolt Forced-Swim Test.

V.4.1.2. Pain Relief / Prevention:V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

Anesthesia for Blast and Sham Procedures: For the blast and sham procedures, mice will receive the inhalant anesthetic isoflurane + 100% oxygen (0.5-4% isoflurane, induction; 0.5-3% isoflurane, maintenance). Isoflurane will be delivered using a precision vaporizer via a rodent anesthesia machine. Induction will occur in an appropriately-sized clear viewing chamber. Following induction, rodents will be moved to the ABS device and anesthesia will be maintained via a nose cone. Waste anesthetic gases will be passively scavenged using a charcoal filter. Lack of paw-pinch reflex will be used to ensure adequate depth of anesthesia prior to beginning the procedure. Close attention will be given to ensure the rodents remain unconscious and are unresponsive and, if needed, supplemental administration of anesthetic will be given. Should experimental procedures with the ABS require an extended period of unconsciousness for the mice, we request an alternative to isoflurane anesthesia by the administration of an intraperitoneal injection of sterile ketamine (90-100 mg/kg) and xylazine (10 mg/kg) in the ventral caudal abdomen with a 26-28 gauge needle. Following blast or sham procedure, the mice will be monitored to verify anesthesia recovery as evident by ambulation and exploring their cage. During induction with anesthesia and during the recovery period, a warming pad is placed under the cage.

Anesthesia for Surgical Implantation of the ETA-F10 Transmitter: Animals undergoing surgical implantation of a telemetry transmitter will be anesthesized with ketamine (80 90-100 mg/kg b.w.) and xylazine (10 mg/kg b.w.) intraperitoneally in the ventral caudal abdomen with a needle size range of 26-28 gauge. Close attention will be given to ensure the rodents remain unconscious and are unresponsive to a paw-pinch and, if needed, supplemental administration of anesthetic ketamine and xylazine will be given. Following transmitter implantation, the mice will be monitored to verify anesthesia recovery as evident by ambulation and exploring their cage. Following the administration of anesthesia, during the implantation surgery, and during the recovery period, a warming pad is placed under the cage.

Analgesia: Mice exposed to the ABS exhibit a similar awakening time to what is observed in sham-treated animals, and they immediately begin exploring and sniffing behavior. Animals will be observed again one hour after home cage placement to ensure there are no signs of discomfort (difficulty breathing, hunched posture, trembling, lack of reaction to gentle touching (lethargy), or seizure activity). If needed, the LAM veterinarian will be consulted should there be signs of continued discomfort or distress. From previous observation of >100 mice in ABS studies, animals are not expected to experience pain or discomfort from the ABS procedure. However, mice will be provided with acetaminophen (Tylenol) in their drinking water (1 mg/ml; ~200 mg/kg b.w.) for 24 hours as a precaution. A similar procedure will be initiated in mice following implantation of the transmitter and cranial screws. Mice will be provided with acetaminophen (Tylenol) in their drinking water (1 mg/ml; ~200 mg/kg b.w.) for 24 hours.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

Animals will be returned to the animal facility after all procedures. They will be monitored in their cages for complications, such as lethargy, reluctance to ambulate, inability to obtain feed, and seizures. If applicable, mice that will be returned to the animal facility after ABS procedures will be monitored in their cages for evidence of peritonitis or other complications following intraperitoneal injection of ketamine and xylazine (if these medications are employed instead of isoflurane). It is expected that complications will not be encountered. Any animal that experiences problems will be evaluated and treated or euthanized as determined by research staff or PI and Veterinarian.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PubMed
V.4.1.3.2. Date of Search: July 9, 2015
V.4.1.3.3. Period of Search: 1986-2015
V.4.1.3.4. Key Words of Search: Keywords: ("Models, Theoretical"[Mesh] OR "In Vitro"[Publication Type] OR "cell line"[mh] OR "Cells, Cultured"[mh] OR "virtual cell"[All] OR "Computer Simulation"[Mesh] OR "Cadaver"[mh] OR "ex vivo"[All]) AND ("Laboratory Animal Science"[mh] OR "Animal Rights"[All] OR "Animal Care Committees"[All] OR "Animal Use Alternatives"[mh] OR "Animal Testing Alternatives"[mh] OR "Animal Welfare"[mh] OR "Animal Experimentation"[mh]) AND ("reduction"[All] AND "refinement"[All] AND "replacement"[All]) OR "3 Rs"[All] OR "Stress, Psychological"[mh] OR ("Pain"[mh] AND ("Analgesics"[mh] OR "Analgesia"[mh] OR "Anesthesia and Analgesia"[mh])) AND ("Brain Injuries"[mh] OR "Euthanasia, Animal"[mh]) AND ("Mice"[mh] OR "Rodentia"[mh])
V.4.1.3.5. Results of Search: A total of 22 references were identified with this search. None of the results were relevant to alternative methods for biomedical studies in mouse core facilities or alternatives to behavioral testing.

V.4.1.3.6. Sources Searched: Agricola
V.4.1.3.7. Date of Search: July 9, 2015
V.4.1.3.8. Period of Search: No date limits.
V.4.1.3.9. Key Words of Search: Rodent, behavior, injury

V.4.1.3.10. Results of Search: No hits were returned for this search. However, the PI is aware of alternative approaches, including recent article by Combes, which was reviewed (Combes, 2013), and earlier articles by Harting and Zurlo (Hartung and Zurlo, 2012), Flecknell (Flecknell, 2002), and Stewart and Martin (Stewart and Martin, 2003). These publications do not offer an alternative to animal models of traumatic brain injury or *in vivo* behavioral testing.

V.4.1.3.11. Sources Searched: ALTWEB; CAAT (Johns Hopkins Center for Alternative for Animal Testing); (Searched at http://altweb.jhsph.edu/)
V.4.1.3.12. Date of Search: July 9, 2015
V.4.1.3.13. Period of Search: 2000-present.
V.4.1.3.14. Key Words of Search: Rodent, behavior, injury, blast
V.4.1.3.15. Results of Search: This search returned no hits.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: The mice used in Experiments 3-5 will receive behavioral testing (see Tables 1 and 7). This testing involves use of the Porsolt Forced-Swim Test, which may elicit a moderate degree of distress. As described below, this test involves placing the mouse in a water-filled cylinder for a 2- and then a 4-minute trial. Mice must tread water to remain fully afloat (note that some mice quickly realize they essentially can remain still and float). It is necessary to perform this test with no tranquilization, since the animal must experience the challenge while fully awake and aware.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:
V.4.3.1. Pre-surgical Provisions: N/A
V.4.3.2. Procedure: The mice in Experiments 2B and 4 will receive implantation of an ETA-F10 transmitter for telemetric measurement of activity patterns and EEG in their home cage. The surgical procedure has been described previously by Born and colleagues (Born *et al*, 2014). After administration of anesthetic (see V.4.1.2.1. Anesthesia for Surgical Implantation of the ETA-F10 Transmitter) and ensuring the mouse is unresponsive, sterile technique is used to make a midline sagittal incision (~1 cm) from the posterior edge of the skull and down the nape. First, the fur is shaved from the nape and scalp of the mouse, the skin is washed with Betadine, wiped clean, and then dauhed with 70% ethanol. A subcutaneous pocket is made to position the transmitter (~1.6 g, 1.1 cm^3) in the dorsal midline of the animal, and two insulated wires from the transmitter are then connected to two small stainless steel screws (#80×1/8 in., Small Parts, Miami Lakes, FL) that contact the dura mater over the frontal and parietal bones of the skull (stereotaxic coordinates relative to bregma suture: recording electrode, +2.5 mm ANT, 2.5 mm LAT, and ground electrode, -1.0 mm POST, 1.0 mm LAT). Dental cement will be placed on the wire-screw connection to ensure contact. The incision is then closed using surgical glue (3M Vetbond Tissue Adhesive), the wound will be wiped again with ethanol and the incision is covered with a light coat of antibiotic cream.
V.4.3.3. Post-surgical Provisions: Mice will be provided with acetaminophen (Tylenol) in their drinking water (1 mg/ml; ~200 mg/kg b.w.) for 48 hours after surgery. Each day, they will be handled and visually inspected to ensure there is no sign of tenderness or swelling at the incision. If needed, the LAM veterinarian will be consulted should there be signs of continued discomfort or distress.
V.4.3.4. Location: (b)(6)
V.4.3.5. Surgeons: (b)(6)

V.4.3.6. Multiple Major Survival Operative Procedures: N/A
V.4.3.6.1. Procedures:
V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:
Anesthetic injections will be administered as described in V.4.1.2.1. Anesthesia/Analgesia/ Tranquilization. At the completion of all experiments, mice will be euthanized as described in V.4.4.2 Biosamples.

V.4.4.2. Biosamples:
Following completion of behavioral testing and activity analysis, approximately one-half of the mice will receive transcardial perfusion to obtain immunohistochemistry biosamples. In this case, the animals will be anesthetized as described in Section V.4.1.2.1. Once the animals are deeply anesthetized and unresponsive to paw pinch, a midline thoracotomy is made to expose the heart, a 20-22 gauge blunt tip needle connected to a tube (¼ inch ID and $^{1}/_{16}$ inch wall) inserted into the left ventricle through the apex and the right atrium incised. After an initial washout with 20-30 ml of cold 100 mM phosphate buffered saline (PBS), transcardial perfusion with 50-75 ml cold 4% paraformaldehyde in PBS will be delivered through a gravity perfusion system. The brain will then be removed from the calvarium and post-fixed before tissue sectioning for immunohistochemical staining.

Approximately one-half of the mice will be directly euthanized and the brains will be removed from the calvarium to obtain samples of the cerebral cortex (fronto-occipital region and entorhinal region), hippocampus, diencephalon, and brain stem. Samples are quickly frozen and stored in liquid nitrogen before preparation for western blotting. Mice will be anesthetized as had been described in V.4.1.2.1. Anesthesia for Surgical Implantation of the ETA-F10 Transmitter Section.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification:
To ensure long term identification, ear punches will be performed at the time of transmitter implantation, ABS, or sham treatment when the mice are unconscious.

V.4.4.6. Behavioral Studies:
The schedule for behavior testing is outlined in **Table 7**. The mice in Experiments 3-5 will undergo behavioral assessment. The mice in Experiments 3 and 4 will commence testing on Day 8 post-injury or sham treatment, where each animal will be tested in an open field (**OF**) environment for measurement of exploratory and locomotor behaviors. Then, on Day 10 they will be evaluated for performance on the Zero (**Ø**) Maze. On Days 14 through 17, each animal will be tested using the Morris Water Maze for spatial memory. Finally, on Day 28, mice will be tested in the Porsolt Forced-Swim Test. For the three-month study (see Experiment 5 in lower portion of Table 7), the mice will be tested on days as listed.

Table 7. Blast/Sham Treatment and Behavioral Testing Schedule for Mice in Experiments 3-5

Experiments 3 and 4							
Treatment	Day 1	Day 5	Day 8	Day 10	Days 14-17	Day 28	Day 30
	Monday	Friday	Monday	Thursday	Monday-Thursday	Monday	Wednesday
1x Blast	–	Blast	OF	Ø Maze	MWM	Porsolt	Euthanize
5x Blast	Blast Days 1-5		OF	Ø Maze	MWM	Porsolt	Euthanize
1x Sham	–	Sham	OF	Ø Maze	MWM	Porsolt	Euthanize
5x Sham	Sham Days 1-5		OF	Ø Maze	MWM	Porsolt	Euthanize
Experiment 5							
Treatment	Day 1	Day 5	Day 68	Day 70	Days 74-77	Day 88	Day 90
	Monday	Friday	Monday	Thursday	Monday-Thursday	Monday	Wednesday
1x Blast	–	Blast	OF	Ø Maze	MWM	Porsolt	Euthanize
5x Blast	Blast Days 1-5		OF	Ø Maze	MWM	Porsolt	Euthanize
1x Sham	–	Sham	OF	Ø Maze	MWM	Porsolt	Euthanize
5x Sham	Sham Days 1-5		OF	Ø Maze	MWM	Porsolt	Euthanize

Open field testing: Animals will be placed in an open field environment (size: 40 cm x 40 cm) and allowed to move freely for up to one hour. A camera over the apparatus is paired to Any-Maze video tracking software (Stoelting, Co, Wood Dale, IL) and the position of the animal is tracked for the duration of the test session. The software is able to provide a wealth of information regarding the behavior of the animal in the open field environment, including but not limited to the total distance traveled, movement speed, time spent immobile, and time/distance in user-defined physical zones of the open field arena. In addition, photobeam arrays allow measurement of movement in the z-plane (rearing). These measures allow conclusions regarding exploration, locomotion and anxiety-like states of the rodents.

Zero maze: Mice are placed in a darkened (walled) sector of the zero maze for five minutes. The apparatus is in the form of a circle (60 cm in diameter), elevated 50 cm above the floor. Two quadrants of the circular maze are darkened and enclosed, and the other two quadrants are "exposed. When placed in the maze, mice usually spend less time in the "open zones" due to perceived risk of falling and exposure to predators. Thus, reduced time exploring the open quadrants is considered an indicator of anxiety-like behavior. The time the mouse spends in the light versus the dark zones of the apparatus is measured as attest of mild anxiety (Kulkarni *et al*, 2007).

Morris Water Maze: The Morris Water Maze is a standard test of the rodent's spatial memory capabilities and has been used successfully in TBI experiments performed by many investigators in the CNRM. A Stoelting Morris Water Maze (diameter 4.0 m) tank is filled with tap water (25°C) to a depth of 20-30 cm. A clear plastic platform is placed in one of the "quadrants" of the tank floor and visual cues (solid geometric shapes) are placed on the room walls in locations that can be viewed by animals in the pool. On Day 1 (Day 8 post-injury or sham treatment), a single pre-training trial is first used where the mouse is gently placed on the platform in the tank and the mouse remains there for 15 seconds. If the mouse jumps into the water during this time, it is allowed a brief swim (<60s) before being guided back to the platform. On Day 2, training trials begin where the mouse is placed near the side of the tank in a location away from the platform, and is allotted 90 seconds to swim to the platform. Upon finding the platform, the mouse remains on the platform for 15 seconds. If the mouse does not reach the platform in the allotted 90 seconds, they are gently guided to the platform and allowed

to remain there for 15 seconds. A total of four trials, separated by 1-2 min, are performed each day. Swimming movements of the mouse are tracked, and swim speed (distance before reaching the platform) and latency to find the platform are calculated by Any-Maze software. Finally on the fourth day of training (Day 11) approximately one hour after the final training trial, a single trial is performed with the platform removed from the tank. The time spent in the pool quadrant where the platform was located is measured, compared to time spent in the other three quadrants. After all trials, mice are placed in a clean cage underneath a heating lamp until thoroughly dried (approximately five minutes) to maintain thermoregulation.

Additional measurements are possible with the Morris Water Maze. Beginning the next day, *reversal training trials* will take place in which the location of the hidden platform is moved to the opposite side of the tank (Zhao *et al*, 2012). The pre-training phase is omitted, and each mouse receives four 90-sec trials for four days. A second probe trial will take place approximately one hour after the final reversal training trial. Finally, immediately after the reversal probe trial, a single visible platform trial will be conducted. A pipette with a very visible "flag" will be secured to the platform. The latency of the mouse to navigate to the cued platform is recorded (maximum of 90 sec).

Forced-Swim Test: The Porsolt Forced-Swim Test (Porsolt *et al*, 1978) evaluates behavioral despair/learned helplessness. The mouse is placed in a cylindrical container of water (10 cm deep, 25±1°C) and the amount of time the mouse swims is recorded. A pre-exposure session of two minutes immediately precedes the test trial, which is four minutes in duration (the total duration of the exposure is six minutes). Mice tend to float with their heads above water if they stop swimming; however, they will be continuously monitored during the test and any animal that appears to be in danger of sinking will be immediately removed from the water by the experimenter.

V.4.4.7. Other Procedures:

Overpressure (blast)-induced traumatic brain injury

Mice will receive TBI by an overpressure shock wave generated by the ABS. All mice are first anesthetized as described in Section V.4.1.2.1 and weighed. Mice are individually secured in a holder that is inserted inside the distal end of the shock tube. After sustaining a short duration of shock wave stimulation (<10 msec), mice will be returned to their home cages after the procedure and monitored until they regain consciousness. Sham-treated animals will receive anesthesia and be placed in the holder at the distal end of the shock tube, but no shock wave will be produced. All animals will be monitored at least once per day following TBI or sham procedures. Animals are not expected to experience pain or discomfort from TBI, but will be provided with acetaminophen (Tylenol) in their drinking water (1 mg/ml; ~200 mg/kg b.w.) as a precaution. Animals will be observed again one hour after home cage placement to ensure there are no signs of discomfort (difficulty breathing, hunched posture, trembling, lack of reaction to gentle touching (lethargy), or seizure activity). If needed, the LAM veterinarian will be consulted should there be signs of continued discomfort or distress.

For some experiments when mice sustain shock wave overpressure in the ABS, a high speed camera (Motion Pro Y4, IDT) and image acquisition software (Motion Studio, IDT) will be used to capture motion at a rate of 4,500 frames per second. Each video "clip" is approximately 2 seconds in duration. Subsequently, the acquired videos are imported into motion analysis software (Proanalyst Professional Edition, Xcitex) to determine device, and/or head and body movement. This will be performed following the procedures outlined in USUHS IACUC Policy #25 (USUHS, 2013). Video images will be stored on a dedicated workstation that is password protected. In addition, to ensure availability of the data should the hard drive in the workstation

fail, a backup external drive will be employed. The workstation is located in Room (b)(6) (ABS anteroom). The hard drive is in a secured area of the LAM facility in Room (b)(6) which has a locking door, and is stored in a locked cabinet. The videotaping will be performed by Dr. (b)(6) in the mouse surgery suite (Room (b)(6) or ABS testing suite (Room (b)(6) and viewed in Rooms (b)(6) Only Dr. (b)(6) and Dr. (b)(6) will have access to the videotapes as they analyze and summarize the findings. The data analysis phase is labor-intensive and will require retaining the video recordings until all data analysis is completed, but we request retaining the images for three years after the completion of this protocol (e.g., August 1, 2018 if this is the date for initial approval of this protocol + 3 years to September 30, 2021). At that time the images will be erased from the workstation's hard drive and the external hard drive. Please note the measures are converted to numerical values in a spreadsheet and this information will be retained. Actual video images will never be utilized in a presentation at USU or outside the University. Should there be any need for a depiction of a rodent in a publication or a lecture, drawings will be created in place of actual images. It is expected that the data presented in graphical format will be adequate for readers or lecture attendees to visualize motion parameters.

V.4.4.8. Tissue Sharing:

Excess mouse tissue will be made available to other investigators in our or other labs.

V.4.5. Study Endpoint:

The study endpoint is survival for the previously described durations (1 or 3 months), at which time animals will be euthanized.

V.4.6. Euthanasia:

Euthanasia will be performed in accordance with "AVMA Guidelines for the Euthanasia of Animals: 2013 Edition." Methods of euthanasia on this protocol include decapitation and perfusion. Mice are first anesthetized for these procedures. Euthanasia will be performed by the PI or his associates in B2051.

Perfusion: Following anesthesia per Section V.4.1.2.1, we will ensure animals are deeply anesthetized and unresponsive to paw pinch. A midline thoracotomy is made to expose the heart, a 20-22 gauge blunt tip needle connected to a tube (¼ in ID and $^{1}/_{16}$ in wall) inserted into the left ventricle through the apex and the right atrium incised. After an initial washout with 20-30 ml of cold 100 mM phosphate buffered saline (PBS), transcardial perfusion with 50-75 ml cold 4% paraformaldehyde in PBS will be delivered through a gravity perfusion system. The brain will then be removed from the calvarium and post-fixed before tissue sectioning for immunohistochemical staining.

Decapitation: Following anesthesia per Section V.4.1.2.1, we will ensure animals are deeply anesthetized and unresponsive to paw pinch then use a rodent guillotine to decapitate the anesthetized rodents. The decapitation equipment will be cleaned and maintained to ensure the proper function by routinely checking to ensure it is not rusted or blunt. It will be either sharpened or replaced if it does not function properly. A log book will be kept of the maintenance of the guillotine.

In the event that LAM personnel must euthanize an animal for humane reasons, cylinderized carbon dioxide will be used in the LAM necropsy room. Using a clean cage, carbon dioxide will be administered at a rate to replace greater than 20% of the cage volume per minute. Once the

animals have not moved or breathed for at least one minute they will be removed from the chamber. Euthanasia may be ensured by cervical dislocation or opening of the thorax.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s)	(b)(6)	Room Number(s)	(b)(6)
Building(s)		Room Number(s)	
Building(s)		Room Number(s)	

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No __X__

Fluid Restriction: Yes _____ No __X__

V.5.1.3. Exceptions: Experiments 2A and 3 will employ the Stoelting Company's (Wood Dale, IL) ANY-Maze Cage chambers. There are eight of these located in Room (b)(6) that can be used to individually house eight mice for continuous measurement of activity as a function of time. The apparatus is a home cage encased within an isolation chamber that limits external influences from the housing facility and other animal activities and odors. The chamber is equipped with an infrared-sensitive light and LEDs that provide continuous tracking of the animal via a built-in camera linked to Any-Maze tracking software. As noted, the chambers collect continuous data related to behaviors, including but not limited to, movements and spontaneous activity, wheel-running activity, diurnal activities, rearing behaviors, and food and water consumption. A computer controls the light-On and light-OFF period similar to the housing rooms in LAM, and a temperature sensor maintains a constant chamber temperature. Investigators will open the chambers once per day to visually inspect the cage and animal and provide fresh food and water, as needed. The cage is provided with wood shaving bedding, as well as nestlet material, which will be exchanged once per week. At the completion of each run with a particular mouse, the chamber will be thoroughly cleaned, where the home cage, feeding hoppers, running wheels, and water tubes are replaced with sanitized pieces that were cleaned in the LAM cage washer. Finally, after cleaning, the NovaLUM device from Charm Sciences, Inc., will be used to swipe surfaces in the cage to monitor for the presence of ATP as a measure of sanitation hygiene. Records from the readings will be documented, and if any surface is found to have detectable amounts of ATP, it will be cleaned again.

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:

The PI or research staff will observe the mice following all procedural manipulations. LAM personnel will observe the animals in Room (b)(6) at least once a day for general health and husbandry condition. For the AnyCage systems (b)(6) investigators will open the chamber doors once per day to visually inspect the cage and animal and provide fresh food and water, if needed. Any animal observed to be lethargic, losing weight observed by weight recording every other day, exhibiting gross neurological deficits (such as inability to eat or drink, continual circling, seizures, etc.) or any other signs of distress or illness will be evaluated by the PI and/or veterinary staff, then euthanized as previously described.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: The mice in Experiments 1 and 5 will be group-housed. Animals will be provided routine enrichment as described in LAM SOPs with the exception of V.5.3.2. below.

V.5.3.2. Enrichment Restrictions: For Experiments 2, 3-5, request to singly house mice with wood shaving bedding and nestlet material, but not plastic tunnels or other toys. The AnyCages and telemetry units require single housing to enable recording of data. Regarding enrichment materials, the AnyCages have bedding, nesting material, and a running wheel, which will provide enriching activity for the mice. However, inclusion of plastic tunnels will interfere with data collection. The telemetry systems are standard LAM mouse home cages with wood shavings and nesting material. EEG and activity recording requires a flat cage floor to not alter or lose signal, which might occur should a plastic object be placed in the home cage.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
ABS traumatic brain injury	(b)(6)	All individuals have experience with animal models of TBI.	All individuals will receive extensive on-site training from the manufacturer of the ABS
Rodent behavioral testing		(b)(6) has > 15 years of experience in rodent handling and behavioral testing and an M.S. in Behavioral Neuroscience. (b)(6) has > 2 years of experience in rodent handling and behavioral testing from UC Irvine.	Personal laboratory experience. (b)(6) be trained by (b)(6) (b)(6)
IP injections		(b)(6) has many years of experience in rodent handling and drug administration.	All listed investigators have taken the Rodent Handling and Procedural Techniques.*
Euthanasia by decapitation		(b)(6) experience with rodent decapitation >30 years. He will instruct listed colleagues on this procedure.	Performed similar procedures for recent Protocols (b)(6) (b)(6)
Intracardial perfusions		(b)(6) and (b)(6) have many years of experience with perfusions. (b)(6) has experience from when she was an undergraduate, but she will observed and be trained by Drs. (b)(6)	Personal laboratory experience. (b)(6) will receive training from the approved personnel on this protocol.
Inhalant anesthesia administration		(b)(6) and (b)(6) have many years of experience with the adminstraiton of inhalant anesthesia..	Personal laboratory experience. (b)(6) will receive training from the approved personnel on this protocol.

*All listed investigators have attended the USUHS Investigator Training Course: Dr (b)(6) on August 17, 2005, (b)(6) completed several online USUHS Investigator Training Courses on October 28, 2009 and attended the Rodent Handling and Procedural Techniques on November 19th, 2009. She is previously approved to perform the above procedures under (b)(6) has completed the online USUHS Investigator Training Course on September 9, 2008 and the Rodent Handling and Procedural Techniques on September 24, 2009. She is approved to perform the above procedures under protocol (b)(6) Dr (b)(6) completed the Investigator Training course on November 19, 2004 and the training of the Collaborative Institutional Training Initiative (CITI) Program. "Investigators, Staff and Students – Lab Animal Research." on May 14, 2014 (b)(6) completed several USUHS online investigator training courses including "Working with the IACUC" (non-VA) on April 23, 2013 and the USUHS Investigator Training Courses on June 6, 2013 (b)(6) completed USUHS online training on June 2nd, 2015 and attended the USUHS Investigator Training Course on July 2nd, 2015.

VII. BIOHAZARDS/SAFETY:

To minimize exposure to animal allergens when working with lab animals, lab personnel will wear lab coats and/or scrubs, gloves and masks. We will always wear lab coats and gloves when

we contact toxic chemicals and perform some steps in a fume hood to minimize risk of exposure to these teratogens. Potential fumes from fixative are minimized by allowing the fixative to be constantly washed down the sink drain.

The Environmental Safety & Occupational Health (**EHS**) Office performed a recent hazard assessment of the noise levels produced by the ABS. Before their visit the staff was already wearing hearing protection devices. EHS determined that the hearing protection practices are sufficient. As a precaution, they propose to develop a hearing conservation program, where ABS operators will receive annual hearing tests.

VIII. ENCLOSURES:

Literature search results for Sections II.2 (Searches for unnecessary duplication) are available upon request.

Attachment: Policy 025 (related to Section 4.4.7. Other Procedures, ABS Characterization).

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6)

Pr

7-15-15

Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6)

Prin

7-15-15

Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Prin

7-15-15

Date

X. PROTOCOL ABSTRACT:

A. **Animal Protocol Number:** (b)(6)

Animal Protocol Title: The impact of blast exposure on mouse daily activities

C. **Principal Investigator:** (b)(6) Ph.D.

D. **Performing Organization:** Uniformed Services University of the Health Sciences

E. **Funding:** Defense Health Agency, DoD

F. **Objective and Approach:**

The goal of this research is to determine the impact of traumatic brain injury (TBI) on mouse daily activity-rest cycles. Previous research has shown that TBI can cause changes in overall arousal, agitation and irritability, motor activity, and disturbances in sleep-wake cycles. The studies here will determine whether or not exposure to a single or repeated atmospheric overpressure shock wave (similar to the shock wave generated by blasts of explosives in combat settings) disrupts "normal" activity in mice during waking and sleeping hours.

A series of behavior tests will assess mouse performance to evaluate the behavioral changes from an exposure to an overpressure shock wave to gather basic information about the effects of blast on daily activity rhythms, including motor activity in the home cage, wheel running activity, and food and water intake patterns. It is predicted that much like in patients suffering from TBI these activity patterns will be altered by injury. The data collected will then be summarized using conventional statistical approaches (mean, standard deviation, analysis of variance, correlation and regression analyses). We will use as few rodents as possible while still using enough to make valid conclusions. To do this initial research we must use animals since cells or tissues do not reflect the natural activity cycles of a live organism. We hope that this basic research and use of animals will aid in understanding some of the causes for the long-term effects of brain injury upon warfighters returning home to their towns, work, and families, and that this research may allow us to provide better treatments to help suffering soldiers.

G. **Indexing Terms (Descriptors):** Animal, mice, traumatic brain injury, behavioral activity, orexin, melanin concentrating hormone

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November 06, 2015

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS

SUBJECT: IACUC Approval of Protocol - Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on November 06, 2015:

Animal Protocol Title: "Targeting the Ubiquitin Proteasome System to Treat Spinal Muscular Atrophy"

USUHS Protocol Number: (b)(6)

Expiration Date: November 05, 2018

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a post approval meeting with LAM (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Targeting the ubiquitin proteasome system to treat spinal muscular atrophy (*Mus musculus)*

GRANT TITLE (if different from above): Targeting the ubiquitin proteasome system to develop treatment for neurological diseases

Revised

USUHS PROJECT NUMBER: (b)(6)
FUNDING AGENCY: Intramural

EARLIEST ANTICIPATED FUNDING START DATE: Current

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6)	APG	(b)(6)	4/24/2015
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	Ph.D	(b)(6)	10/07/2015
Research Unit Chief / Dept. Head Signature	Title	Telephone	Date

Typed Name: (b)(6) M.D., Ph.D.

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)	PMB	(b)(6)	10/9/2015
Statistician Signature	Department	Telephone	Date

Typed Name (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)	LAM	(b)(6)	7 OCT 2015
Attending/Consulting Veterinarian Signature	Department	Telephone	Date

Typed Name: MAJ (b)(6) DVM, MPH

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

ANIMAL PROTOCOL TITLE: Targeting the ubiquitin proteasome system to treat spinal muscular atrophy (*Mus musculus*)

GRANT TITLE (if different from above): Targeting the ubiquitin proteasome system to develop treatment for neurological diseases

USUHS PROJECT NUMBER:
CO-INVESTIGATOR(S):

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

Goals of Study:
1) To treat mice with drugs that are potential therapeutics for spinal muscle atrophy (SMA). Building on our previous results using trichostatin A, we would like to confirm and expand our experiments using both trichostatin A and bortezomib to study the therapeutic potential of these drugs, alone and in combination.
2) To conduct behavioral testing on these mice in order to determine if there are any improvements in motor function after drug treatment.
3) To interrogate drug mechanisms of action and the role SMN plays in the development and maintenance of motor neurons using primary neuronal cultures from SMA model mice.

II. BACKGROUND:

II.1. Background:

The goal of this study is to determine the mechanism of the disease spinal muscular atrophy (SMA) and to identify novel therapeutics to treat the disease. SMA is a disorder that is characterized by degeneration of motor neurons of the spinal cord leading to muscle paralysis and atrophy. The disease is caused by mutation of the 'survival of motor neuron' (SMN) gene which leads to decreased levels of the SMN protein. The exact function of the SMN gene is yet to be completely determined, but it is believed to be involved in RNA processing. Still, the question remains why reduced levels of the SMN protein affects only motor neurons. In humans SMN is encoded by two genes, SMN1 and SMN2. SMA is caused by deletions and other mutations of the SMN1 gene. All SMA patients lack a functional copy of SMN1 but retain at least one copy of SMN2. Rodents on the other hand, have only one copy of the SMN gene and inactivation of the SMN gene in mice leads to very early cell death in mouse embryos suggesting an important role of SMN in development. (b)(6) lab at Ohio State University has created SMA mice which lack the SMN gene that have been rescued from early death by introducing the human SMN2 transgene [1]. These mice serve as an excellent model system to study SMA and could be used to test possible therapeutics. We will be treating animals with drugs that have been proven to increase both SMN transcript and protein levels in cell culture in order to determine if they will help treat the disease in whole animals and alleviate the severity of the disease.

The purpose of this study is to study the effects of increasing expression of the SMN protein on transgenic SMA model mice. Genetic, histological, and primary tissue culture studies, as well as treating animals with potential therapeutic drugs will be performed.

1. Mouse Model.
(b)(6) lab at Ohio State University created an SMA mouse model which lack the smn gene but carries two copies of the human SMN2 transgene, which rescues lethality [1]. These mice (SMN delta7;SMN2;Smn-/-) aka Delta 7, survive for 14 days on average providing a therapeutic window. At 5 days of age, Delta7 mice have considerable difficulty righting themselves when placed on their backs. The weakness becomes progressively more pronounced over the next week of life. By 10 days of age, it is clear that SMA mice homozygous for the SMNdelta7 and SMN2 transgenes have difficult ambulating and often fall over while walking. When they do walk, they display an abnormal gait and shakiness (fibrillation) of the hind limbs. Our lab will be using SMA mice along with unaffected littermates (wild type and heterozygous mice) to study the role SMN plays in the development and maintenance of motor neurons. These mice serve as an excellent model system to study SMA and could be used to test possible therapeutics.

2. Drug Treatments. The following pharmaceutical grade drugs compounds will be used in this study: Trichostatin A (TSA) from Enzo and Bortezomib from Millenium Pharmaceuticals. Our previous studies demonstrated that repeated daily doses of TSA increased both *SMN2*-derived transcript and SMN protein levels in neural tissues and muscle. When TSA was delivered daily beginning on post--natal day 5, after the onset of weight loss and motor deficit, there was improved survival, attenuated weight loss, and enhanced motor behavior. These results indicated that HDAC inhibitors activate SMN2 gene expression in vivo and have an ameliorating effect on the SMA disease phenotype when started after disease onset[2]. SMN has recently been shown to be turned over by the proteasome and treatment of cells in culture with proteasome inhibitors results in elevated levels of SMN [3, 4]. Both drugs (TSA and bortezomib) will be administered in combination to determine any synergistic effects.

3. Primary neuron cultures. SMA is a motor neuron disease. Primary neurons with low SMN levels are the best tools to study the underlying molecular events that lead to motor neuron death when SMN is deficient. We will isolate and culture motor neurons from affected, heterozygous and wild type embryos and perform RNA sequencing to identify key molecular changes in neurons that could lead to cell death.

II.2. Literature Search for Duplication:
II.2.1. Literature Source(s) Searched: BRD
II.2.2. Date of Search: June 9, 2015
II.2.3. Period of Search: 1998-2015
II.2.4. Key Words and Search Strategy:
Keywords: Mouse, Spinal muscular atrophy, protein degradation, bortezomib, trichostatin A—Results: 0 hits
Keywords: Animal, Mouse, SMA or ubiquitination—Results: 0 hits
II.2.5. Results of Search:
The BRD database includes 12,797 records of previous research programs. No hits were found that identified the terms with protein degradation and spinal muscular atrophy.

Conclusion: No related hits indicated there is no overlap with previous research programs in the DoD.

II.2.6. Literature Source(s) Searched: FEDRIP
II.2.7. Date of Search: June 9, 2015
II.2.8. Period of Search: 1986-2015.
II.2.9. Key Words and Search Strategy:
Animal, Mouse, SMA, protein degradation, bortezomib, trichostatin A, proteasome or ubiquitination
II.2.10. Results of Search:
The keywords, "Animal, Mouse, protein degradation, proteasome or ubiquitination," produced 1 hit. With just the keywords, "SMA and proteasome," there were 0 hits.
Conclusion: Finding no other related hits indicates there is no overlap with previous federally sponsored research programs.

II.2.6. Literature Source(s) Searched: Pubmed
II.2.7. Date of Search: June 1, 2015
II.2.8. Period of Search: 1986-2015.
II.2.9. Key Words and Search Strategy:
Animal, Mouse, SMA, protein degradation, bortezomib, trichostatin A, proteasome or ubiquitination
II.2.10. Results of Search:
The keywords, "Animal, Mouse, protein degradation, proteasome or ubiquitination," produced three hits. With just the keywords, "SMA and proteasome," there were three hits. All three are our previous studies. This proposal builds on our previous work showing that the ubiquitin proteasome system is a potential therapeutic target for treating SMA.
Conclusion: Finding no other related hits indicates there is no overlap with previous research programs.

III. OBJECTIVE\HYPOTHESIS:

The *objective* of this study is to determine the mechanism of the disease, spinal muscular atrophy (SMA), and identify therapeutics that could help treat the disease. SMA is a disorder that is characterized by degeneration of motor neurons of the spinal cord leading to muscle paralysis and atrophy. We will be treating animals with drugs that have been proven to increase both SMN transcript and protein levels in cell culture in order to determine if they will help treat the disease in a whole animal and alleviate the severity of the disease.

IV. MILITARY RELEVANCE:

The military relevance of this protocol relates to the goals of basic, laboratory research to develop therapies that prevent or reduce the long term consequences of spinal muscular atrophy. Better understanding of the biological mechanisms of spinal muscular atrophy may lead to more effective treatments for our service member families and civilians.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

General Approach: The planned experiments have the following goals.

In Vivo:

1) 5 day old (P5) spinal muscular atrophy (SMA) model mice and unaffected littermates and the moms who will be nursing the pups will be transferred from our breeding protocol.

2) To treat mice with drugs that are potential therapeutics to treat SMA. Building on our previous results using trichostatin A, we would like to confirm and expand our experiments using both trichostatin A and bortezomib to study the therapeutic potential of these drugs, alone and in combination.

3) To conduct behavioral testing on these mice in order to determine if there are any improvements in motor function after drug treatment.

In Vitro:

1) To determine any changes in the survival of motor neuron (SMN) gene transcript and protein levels from various tissues of drug treated mice.

2) To look histologically at spinal cord and muscle tissues for any cellular improvements after drug treatment.

3) Pregnant females at E12 will also be transferred from the breeding protocol in order to isolate motor neurons from embryos for in vitro assays. We will study primary cultures of motor neurons from normal and SMA mice in order to learn more about the mechanism of this motor neuron disease.

V.1.1. Experiment 1.1: *Evaluation of compounds that may alter SMN protein levels in SMA mice.*

Experiment 1.1: Drug Treatments: The drug injections will be limited to the histone deactylase (HDAC) inhibitor Trichostatin A (TSA) and the proteasome inhibitor bortezomib (Table 1). Both drugs are solubilized in DMSO. Therefore SMA mice will be treated with DMSO defined as "vehicle" treated mice to serve as our baseline control. Our previous study demonstrated that repeated daily doses of TSA causes increases in both SMN2-derived transcript and SMN protein levels in neural tissues and muscle. When TSA was delivered daily beginning on post-natal day 5, after the onset of weight loss and motor deficit, there was improved survival, attenuated weight loss, and enhanced motor behavior. These results indicated that HDAC inhibitors activate SMN2 gene expression in vivo and have an ameliorating effect on the SMA disease phenotype when started after disease onset. We postulate that we could synergistically improve the disease phenotype by treating these animals with the proteasome inhibitor bortezomib in combination with trichostatin A. SMN has recently been shown to be turned over by the proteasome and treatment of cells in culture with proteasome inhibitors results in elevated levels of SMN. We hypothesize that increasing SMN levels with trichostatin A and inhibiting SMN turnover will further improve the disease phenotype. Both drugs will be administered by themselves or in combination to determine any synergistic effects. Starting at P5 litters and mom will be transferred from breeding protocol and drugs will be administered, daily in the case of trichostatin A and every 48 hours for bortezomib, intraperitoneally using a 33 gauge needle (Hamilton microliter syringe #705 (20 mm (1 in.)); smallest needle available for the volumes of drug to be used). Drugs will be dissolved in 5% DMSO. Vehicle treated animals will be administered 5% DMSO v/v in water. TSA will be dissolved in DMSO to a concentration of 4 µg/µl. SMA mice and their WT and heterozygous littermates will receive 10 mg/kg in a volume of 2.5 µl/g body weight (maximum 35µl). Mice will be genotyped at P10 to confirm which are affected by SMA. Unaffected mice will be used as controls. The intraperitoneal injection would allow rapid diffusion and fast onset of the drug. Pups will be observed and weighed daily from P5 to P20 (20 days). We have performed several published studies using this administration regimen with this mouse model at the designated times [2, 5, 6]. When each mouse has

completed the drug study, (day P20) fluid and food supplements will he supplied as recommended by veterinarians. The major source of nutrients for pups under study will be provided by the female breeder. Based on our experience with these mice we believe that the SMA affected pups are still able to latch onto the mom and feed. Since untreated mice survive 14 days on average, the number of days of survival past day 14 will be observed; representing the experimental endpoint. Study end-point is survival until mice are moribund after which mice will he euthanized and tissues collected. 20% loss in body weight is very early in the progression of the disease in SMA mouse models. We have found that a 30% loss in body weight, calculated based on the maximum weight achieved is a better estimate of moribundity and will allow us to better evaluate the efficacy of drug treatment. This maximum weight is usually achieved at postnatal day 11. If no ability to right after 1 minute OR greater than 30% weight loss (from maximum weight), pup will be euthanized (age P5-P21).

Table 1: Experimental Drugs

Name	Concentration	Dose & Volume	Side-effects
Trichostatin A Vehicle: DMSO	#10 mg/kg/day (Range of possible doses 0.5-10 mg/kg/day) Starting at P5 Ending at P20	IP vol. 10-12 ul/g mice ~ 20 µl total volume	Rough hair coat; diarrhea; lethargy/inactivity
Bortezomib (PS-341) Vehicle: DMSO	*0.15 mg/kg/2 day (Range of possible doses 0.1-1 mg/kg/2 days) Starting at P5 Ending at P20	IP vol. 10-12 ul/g mice ~ 20 µl total volume	Diarrhea; peripheral neurotoxicity.

Experiment 1.2: Starting at postnatal day 5 animals will also be used for behavioral testing of motor function using the time to right test (for motor coordination and balance). The same cohort of animals described in Experiment 1.1 will undergo behavioral testing here.
Procedures: Animals will be transferred from the animal holding room to the experimental room and allowed to habituate to the experimental room for 60 min.

Righting time: Righting time will be measured daily starting at P5 until study endpoint. Right ting time is defined as the better of 2 trials of the time for the pup to turn over to its front after being placed completely on its back (maximum 30 seconds).

Following each procedure mice will be monitored and weighed daily. The PI will consult with LAM veterinarians on a case by case basis to determine pain and distress in all animals. Animals deemed to be in distress will be treated or euthanized by LAM personnel, PI or his staff based on designated endpoint criteria outline **V.4.5**

Table 2: Experiment 1					
Injection	**Total number of experimental animals**	**Injection site (number of experimental animals)**	**Endpoint days (number of experimental animals)**	**Analysis**	**Pain category (number of animals)**
Vehicle (DMSO)	240	Intraperitoneal (60)	Medical endpoint (outline V.4.5)	Survival and behavior	C(45) E(15)
Bortezomib		Intraperitoneal (60)			C(45) E(15)
TSA		Intraperitoneal (60)			C(45) E(15)
TSA + Bortezomib		Intraperitoneal (60)			C(45) E(15)

Approximately 25% of litters will be SMA pups and 75% phenotypically normal mice. We will need approximately 15 TSA, 15 bortezomib, 15 TSA + bortezomib and 15 vehicle (DMSO) treated SMA mice). All SMA mice are category E. All phenotypically normal mice (wild type and hets) are category C.

Therefore, total SMA mice to be treated = 60
Total mice to be treated = 4 x 60 = 240
Total pregnant moms to be used (assuming 6 pups per litter) = 40

Total number of animals = (240 +40) = 280

Flow Outline of Experiments:

The animals used for these experiment are affected SMA mice. SMN delta7; SMN2:Smn -/-.These mice represent only 25 % of each litter; therefore the number of animals required will be 4 times the number of SMA pups. Heterozygous and wild type mice will serve as controls for experiments (Category C). We use the heterozygotes and wild type animals as controls as well as any unforeseen adverse events that may occur due to the drugs and not the disease course.

Flowchart Title: Survival and motor function studies in SMA mice treated with TSA/Bortezomib (**Experiment 1**)

A) **Total number of animals= 280** (40 litters with average litter size of 6 pups + 40 pregnant moms)

(Approximately 25% will be SMA pups and 75% wild type and heterozygous mice. We will require 15 TSA, 15 bortezomib, 15 TSA + bortezomib and 15 vehicle (DMSO) treated SMA mice)

Litter of pups:
25% SMA pups (SMN2+/+/SMNdelta7+/+/mSmn-/-)
75% normal littermates (SMN2+/+/SMNdelta7+/+/mSmn+/- &
SMN2+/+/SMNdelta7+/+/mSmn+/+).

At postnatal day 5 (P5), mice will randomly be assigned to receive daily intraperitoneal injections of vehicle (DMSO), TSA (10 mg/kg) and/or .15 mg/kg bortezomib every other day.

Pups observed, weighed, and treated daily from P5 to P20 (16 days). Genotyped at P10

↓

Pup is euthanized if moribund as defined by inability to right during a 1 minute observation period OR greater than 30% weight loss (from maximum weight achieved).

↓

At P21 pups will be weighed and weaned (if appropriate). Treated pups will then be observed daily until moribund (approximately 21 days) for evidence of any residual drug toxicity such as lethargy, weight loss, exhibiting gross neurological deficits (such as inability to eat or drink, continual circling, seizures, etc.) or any other signs of distress or illness. Pup will be euthanized if moribund as defined by inability to right during a 1 minute observation period OR greater than 30% weight loss (from maximum weight achieved). If pup shows severe respiratory distress, it will be euthanized.

Experiment 2

2.1 Biochemical analysis of SMA tissue

Starting at P5 drugs will be administered, daily in the case of trichostatin A and every 48 hours for bortezomib, intraperitoneally until P12 (doses as described in Experiment 1). Mice will be genotyped at P10 to confirm affected (SMN delta7 +/+;SMN2+/+;Smn-/-). Pups (SMA, het and wild type) will be deeply anesthetized with 2-5% isoflurane at P12. Depth of anesthesia will be verified by pinching all four paws with forceps to make sure that there is no withdrawal reflex. Mice will then be euthanized by cervical dislocation. The brain, spinal cord, liver and hindlimb skeletal muscle will be dissected and quickly frozen. RNA and protein will be extracted from each tissue section by standard procedures. RNA transcript levels will be measured by quantitative RT-PCR and protein levels will be measured by Western blot.

Histology of spinal cords and muscle.

2.2 Hematoxylin-eosin stains will be performed on muscle sections.

1. Starting at P5 drugs will be administered, daily in the case of trichostatin A and every 48 hours for bortezomib, intraperitoneally (doses as described in Experiment 1). Mice will be genotyped at P10 to confirm affected (SMN delta7 +/+;SMN2+/+;Smn-/-). Pups will be deeply anesthetized with 2-5% isoflurane. Depth of anesthesia will be verified by pinching all four paws with forceps to make sure that there is no withdrawal reflex. Deeply anesthetized animals will then be

transcardially perfused briefly with cold 1X PBS. The tibialis anterior muscle and spinal cord tissue will be dissected out, mounted on wooden blocks, and flash frozen in liquid nitrogen-cooled isopentane.
2. 10-12 um thick transverse muscle sections will be cut and mounted on Superfrost slides, and air dried.
3. Sections will be fixed with 100% ethanol, rinsed with water and stained with hematoxylin, rinsed and stained with eosin, rinsed and dehydrated with ethanol and cleared in xylene before mounting in Permount for light microscopy.

Immunocytochemistry will be performed on motor neurons (same mice used in histology of spinal cords section above).
1. After the animals are euthanized by transcardial perfusion, the spinal cord will be dissected. Ventral roots, lumbar spinal cord and the brain stem will be post-fixed 24 h in 100% ethanol as outlined above.
2. The brain stem and spinal cord will then be embedded in paraffin.
3. Serial sections (6 um) will be mounted on glass slides and stained with Nissl's stain.
4. Ventral roots will be post-fixed in 1% osmium tetroxide, rinsed with phsophate buffer and dehydrated.
5. Ventral roots will then be embedded in Spurr resin and 0.5 um sections will be cut and stained with toluidine blue and visualized by light microscopy.

Table 3: Experiment 2

Injection	Total number of experimental animals	Injection site (number of experimental animals)	Endpoint days (number of experimental animals)	Analysis	Pain category
Vehicle (DMSO)	160	Intraperitoneal (40)	12	Histology, Biochemistry	D(30) E(10)
Bortezomib		Intraperitoneal (40)			D(30) E(10)
Trichostatin A		Intraperitoneal (40)			D(30) E(10)
TSA + Bortezomib		Intraperitoneal (40)			D(30) E(10)

Litter of pups:
25% SMA pups (SMN2+/+/SMNdelta7+/+/mSmn-/-)
75% normal littermates (SMN2+/+/SMNdelta7+/+/mSmn+/- & SMN2+/+/SMNdelta7+/+/mSmn+/+).

We will need approximately 10 TSA, 10 bortezomib, 10 TSA + bortezomib and 10 vehicle (DMSO) treated SMA delta 7 mice) - **5 SMA delta 7 pups per treatment group for biochemistry and 5 for histological analysis.** All SMA mice are category E. All normal mice (wild type and het - SMN2+/+/SMNdelta7+/+/mSmn+/- & SMN2+/+/SMNdelta7+/+/mSmn+/+) are category D.

Total SMA mice (SMN delta7 +/+;SMN2+/+;Smn-/-) to be treated = 40
Total animals (given that 25% of each litter will be SMA mice) = 4x40 =160
Total pregnant moms to be used (assuming 6 pups per litter) = 27

Total animals Experiment #2 = 187

Experiment 2
Total number of animals= 187

Flowchart Title: Biochemistry and histology studies in SMA mice treated with TSA/Bortezomib

A) **Biochemistry** - *SMN2* transcript and SMN protein level determination following drug treatment

- 5 TSA, 5 bortezomib, 5 TSA + bortezomib and 5 vehicle (DMSO) treated SMA mice will be used for these experiments.
- Animals will be euthanized.
- The brain, spinal cord, liver and hindlimb skeletal muscle will be dissected and quickly frozen.
- RNA and protein will be extracted from each tissue section by standard procedures.
- RNA transcript levels will be measured by quantitative RT-PCR and protein levels will be measured by Western blot.

B) **Histology**

5 TSA, 5 bortezomib, 5 TSA + bortezomib and 5 vehicle (DMSO) treated SMA mice) will be used to determine histopathological (muscle and nerve) changes following drug treatment.

↓

Animals will be deeply anesthetized then transcardially perfused briefly will cold PBS.

↓

The spinal cord and hind limb skeletal muscle will be quickly removed and frozen and stained.

↓

The muscle slices will be stained with hematoxylin-eosin and immunocytochemistry will be performed on spinal cord slices with Nissl's stain.

Experiment 3

Primary cultures of motor neurons will be generated according to the following protocol from Dr. (b)(6) Pregnant females will be transferred from the breeding protocol at E12, anesthetized no later than E14 using 2-5% isoflurane. Depth of anesthesia will be verified by pinching all four paws with forceps to make sure that there is no withdrawal reflex. Deeply anesthetized animals will then be euthanized by cervical dislocation. The abdomen of the euthanized animal will be sprayed with 70% ethanol prior to performing a caesarian dissection and removal of the uterus. The uterus will be placed in a culture dish and the embryos removed. Embryos collected from the uterus will be placed in a

sterile petri dish in cold basal media and decapitated (Section V4.6). Both neurons and astrocytes will be isolated from sections of the CNS for use in primary cultures. For experiment #3 we use 12 embryos of each genotype, which gives a yield of 80,000-120,000 motor neurons per genotype. Primary neurons do not survive in culture very long so this experiment will be performed once per year.

Table 4: Experiment 3

Genotype	Total number of embryos	Endpoint days	Analysis	Pain category
SMN delta7 $^{+/+}$;SMN2$^{+/+}$;Smn$^{+/+}$	144	E14	Primary culture	C
SMN delta7 $^{+/+}$;SMN2$^{+/+}$;Smn$^{+/-}$			Primary culture	C
SMN delta7 $^{-/-}$;SMN2$^{-/-}$;Smn$^{-/-}$			Primary culture	C

We will need approximately 12 wild type embryos (SMN delta7 $^{-/-}$;SMN2$^{-/-}$;Smn$^{+/+}$), 24 heterozygous embryos (SMN delta7 $^{+/+}$;SMN2$^{+/-}$;Smn$^{+/-}$) and 12 affected SMN delta7 embryos (SMN delta7 +/+;SMN2+/+;Smn-/-) per year (See calculations below). Pregnant females undergoing embryo harvesting would be considered category D. Embryos will be considered category C.

In order to get 12 affect pups, which constitute 25% of each litter, we will need (4 x 12) 48 embryos.
Average litter size – 6 embryos
Therefore, we will need (48 embryos/6) 8 pregnant females to get enough SMA embryos.

Total number of embryos = 8 pregnant females x 6 embryos per litter = 48 embryos.
Total pregnant females required = 8 per year x 3 = <u>24</u>
Total embryos required = 48 per year x 3 = <u>144</u>

<u>Total animals and embryos Experiment #3 = 168</u>

<u>Experiment 3: Flow outline of experiments</u>

Primary cultures of motor neurons

8 embryos per group (per year)

↓

Pregnant moms will be euthanized and E14 embryos will be removed and euthanized.

↓

Spinal cords will be removed and motor neurons will be cultured

V.2. Data Analysis:

For Experiment1, we plan to use sample sizes of 15 animals per group in the comparison of vehicle or proteasome inhibitor treated mice for survival studies. A sample size of 15 per group will have 80% power to detect a difference between groups of 1.1 standard deviations between groups, based on a two-sample t test with a 5%, two-sided significance level. Wahlsten et al (7) indicate that effect sizes of around 1 standard deviation are commonly observed in mouse neurobehavioral studies. Furthermore, in analysis of survival outcomes 15 animals per group will have 80% power to detect a 2/3 reduction in death rate based on a log rank test with 5%, two-sided significance level. For Expt. 2, a sample size of 5 per treatment group will have 80% power to detect a difference between groups of 2.0 standard deviations between groups, based on a two-sample t test with a 5%, two-sided significance level. Experiments 3 is cell culture based approaches that will generate motor neuron for in vitro analysis. Animal use prediction for experiments 3 is based on our data that indicate we can generate 80,000-120,000 motor neurons per genotype from E14 embryos. These numbers of cells are predicted to be sufficient for the desired quantitative qualitative gene expression analysis.

Statistical Analyses: The SigmaStat 2.03 (SPSS) statistical analysis program will be used to compute the arithmetic mean and standard deviation of surgical and treatment groups on each test measurement. For most experiments a one-way analysis of variance (ANOVA) will then be used to assess whether or not performance on each behavioral test differs between experimental groups. For experiments in which individual mice are tested multiple times on the same behavioral test, a one-way ANOVA with repeated measures will be performed. If the ANOVA indicates differences, Tukey's test will be used to compare individual means. In addition, if the data suggest variation between the testing of each cohort of mice, a nested ANOVA can be used to assess whether or not there are differences between testing events on repeated tests. To assess some degree of the relationship of performance on tests within an experiment, correlation coefficients can be computed for each animal's score on each subtask.
Survival and biochemical data will be analyzed using the GraphPad Prism software package (version 6.05; GraphPad Software) and compared statistically with the log-rank test (Kaplan–Meier survival curves), the two-tailed Student's t test or one-way analysis of variance followed by the Newman -Keuls multiple comparison *post hoc* test.

V.3. Laboratory Animals Required and Justification:

1) Animal models provide the only means to study the mechanism of neurological diseases that cannot be accomplished in humans or in cultured cells.
2) In order to have direct relevance for the human disease, the proposed experiments must be carried out in an animal model of SMA. There is no known cellular or computer model that

recapitulates the human disease. Live animals are also required in order to examine the pathology and biochemistry of the normal compared to SMA neuromuscular apparatus.
3) From our cell culture data, we have disease modifying genes that may be promising targets for therapeutics that modulate muscle atrophy and the SMA phenotype and perhaps treat SMA. However, they first need to be validated in an animal model before being presented as an option for drug therapy. Although there are other animal models of SMA (drosophila and zebrafish), the genetically engineered mouse is the animal model of SMA that best mimics the human disease genetically and pathologically. As the purpose of this protocol is to better understand the pathophysiology of the human disease, these studies must be done in the mouse animal model.

V.3.1. Non-animal Alternatives Considered:
1) Animal models provide the only means to study the mechanism of a neurological disease that cannot be accomplished in humans or in tissue culture.
2) Animal models are also the next step for testing drugs that have been shown to work in tissue culture to treat a disease, but need further testing to verify the effectiveness of the drugs prior to trials in humans.
In order to have direct relevance for the human disease, the proposed experiments must be carried out in an animal model of SMA. There is no known cellular or computer model that recapitulates the human disease. Live animals are also required in order to examine the pathology and biochemistry of the normal compared to SMA neuromuscular apparatus.

V.3.2. Animal Model and Species Justification:
Although there are other animal models of SMA (drosophila and zebrafish), the genetically engineered mouse is the animal model of SMA that best mimics the human disease genetically and pathologically. As the purpose of this protocol is to better understand the pathophysiology of the human disease, these studies must be done in the mouse animal model. The SMN delta7+/+;SMN2+/+;Smn-/- (developed by Arthur Burghes at OSU) lack the endogenous mouse Smn gene but express two copies of the human SMN2 transgene. The retention of the SMN2 gene is the genetic circumstance relevant to humans. Phenotypically, they closely mimic the severe human disease phenotype. The SMN delta7$^{+/+}$;SMN2$^{+/+}$;Smn$^{-/-}$ mice, weight loss and weakness begins at approximately P5 and progressive weakness continues until death at approximately P14. . The major source of nutrients for pups under study will be provided by the female breeder. Based on our experience with these mice we believe that the SMA affected pups are still able to latch onto the mom and feed. Death likely occurs due to respiratory failure secondary to severe muscle weakness, just as in the human disease.

Mus musculus is chosen as a suitable species for this research. This is based upon the previous use of this species in innumerable biomedical studies and their suitability for understanding central nervous system functions in normal and pathological states in humans and in other animals. The mouse is used for these studies because of their well-understood anatomy and endocrine responses, and the very extensive database of prior physiological research that can be integrated with this new information. It is necessary to use mice instead of a "lower species" since the research results should be directly associated with intense efforts in other laboratories throughout the world to try and further our understanding and eventual treatment effectiveness for neurological disorders. Mice are a commonly used small animal model for the study of neurobiological genetics research, including the impact of physical injury and environmental stress upon neuron connectivity, experiential effects on synaptic plasticity, signal transduction, and for assessing treatments to reduce neurological damage and improve quality of life.

V.3.3. Laboratory Animals

		Strain #1
V.3.3.1.	**Genus & Species:**	*Mus musculus*
V.3.3.2.	**Strain/Stock:**	FVB/ SMN delta7+/+;SMN2+/+;Smn+/-
V.3.3.3.	**Source/Vendor:**	In house breeding protocol
V.3.3.4.	**Age:**	P5 pups, moms (Expt. 1 and 2) and pregnant females (Expt. 3)
V.3.3.5.	**Weight:**	2-14 grams pups; 25-35 grams post-parturient and pregnant females
V.3.3.6.	**Sex:**	Equal numbers of males and females pups + 91 pregnant/nursing females
V.3.3.7.	**Special Considerations:**	Nursing mothers transferred with litters for experiments 1 and 2. At study endpoint, mothers will be transferred back to breeding protocol. Exp. 3 moms euthanized and pups used at E12

V.3.4. Number of Animals Required 633 (542+ 91 pregnant/nursing females transferred from breeding protocol)

ANIMALS	Total Number
	TOTAL
Experiment 1	280
Experiment 2	187
Experiment 3	168
Total number	**635**

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:
Mice will be acclimated to investigator handling before any procedures to relieve distress from the potential combination of handling distress, surgical procedures and behavioral testing. Also, mice will be anesthetized prior to euthanasia by cervical dislocation.

V.3.5.2. Reduction:
We are attempting to perform a series of measurements of biological functions and mouse behavior that requires making assurances that there is sufficient number of animals to derive valid measures

of statistical data. Power calculations have been performed to determine the minimum number of animals needed to reach statistical significance. Data derived from this protocol will be available to other investigators via peer-reviewed scientific publications.

V.3.5.3. Replacement:
We are using a rodent model (mouse) versus a nonhuman primate model. The mouse model is the least sentient and derived animal model we could use for this study and still obtain acceptable results. Biological response to central nervous system injury and experimental treatments is highly complex: no alternative to *in vivo* experimentation is available or practical.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals: 635

V.4.1.1.1.1.	**Column B:**	67 (pregnant and nursing females Expts. 1 and 2)
V.4.1.1.1.2.	**Column C:**	324 (Expts. 1 and 3)
V.4.1.1.1.3.	**Column D:**	120 control mice (Expt.2) and 24 (pregnant females from Expt. 3)
V.4.1.1.1.4.	**Column E:**	100 (Expts. 1 and 2)

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: Inhalation anesthetics will be used immediately prior to cardiac perfusion-fixation and cervical dislocation (experiment #2). For inhalation anesthetic, oxygen will be mixed with isoflurane delivered via precision vaporizer using a rodent anesthesia machine. Mice will be placed in an induction chamber and exposed to a mixture of O_2 and isoflurane (2-5%) to induce general anesthesia. Following induction, mice will be removed from the chamber and maintained on 0.25-3% isoflurane administered by nosecone during procedures. We will monitor the depth of anesthesia by noting response to toe pinch (pedal withdrawal reflex) and rate and quality of respirations. Waste anesthetic gases will be passively scavenged using a charcoal canister. Anesthetics will be administered either by the PI or his staff.

Experiment #3: For embryo collection: Pregnant females will be anesthetized no later than E14 using 2-5% isoflurane. Depth of anesthesia will be verified by pinching all four paws with forceps to make sure that there is no withdrawal reflex. Deeply anesthetized animals will then be euthanized by cervical dislocation. The abdomen of the euthanized animal will be sprayed with 70% ethanol prior to performing a caesarian dissection and removal of the uterus and collection of embryos.

Experiment #2: For biochemical analysis: On the day of euthanasia, mice will be exposed to a

mixture of O_2 and isoflurane (2-5%) to induce general anesthesia prior to cardiac perfusion or cervical dislocation.

Experiment #2: Histology of spinal cords and muscle. Hematoxylin-eosin stains will be performed on muscle sections. Mice will be deeply anesthetized with 2-5% isoflurane. Depth of anesthesia will be verified by pinching all four paws with forceps to make sure that there is no withdrawal reflex. Deeply anesthetized animals will then be transcardially perfused briefly with cold 1X PBS. The muscle and spinal cord tissue will be dissected out, mounted on wooden blocks, and flash frozen in liquid nitrogen-cooled isopentane.

The PI will consult with DLAM veterinarians on a case by case basis to determine pain and distress in all animals. Animals deemed to be in distress will be treated or euthanized by LAM personnel, PI or his staff.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: N/A

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PubMed
V.4.1.3.2. Date of Search: June 9, 2015
V.4.1.3.3. Period of Search: 1986-2015.
V.4.1.3.4. Key Words of Search: SMA, Spinal Muscular Atrophy, pain, distress, mouse
V.4.1.3.5. Results of Search: A total of 13 references were identified with this search. None of the searches were relevant to alternative methods for investigations of SMA in mice.

V.4.1.3.6. Sources Searched: Agricola
V.4.1.3.7. Date of Search: June 9, 2015
V.4.1.3.8. Period of Search: No date limits.
V.4.1.3.9. Key Words of Search: SMA, Spinal Muscular Atrophy, pain, distress, mouse
V.4.1.3.10. Results of Search: Twenty-five references were returned by this search, but none of the hits were relevant.
V.4.1.3.11. Sources Searched: ALTBIB
V.4.1.3.12. Date of Search: June 9, 2015
V.4.1.3.13. Period of Search: Any year up to 2000
V.4.1.3.14. Key Words of Search: SMA, Spinal Muscular Atrophy, pain, distress, mouse
V.4.1.3.15. Results of Search: No hits

V.4.1.3.16 Literature Source(s) Searched: PubMed
V.4.1.3.17 Date of Search: June 1, 2015
V.4.1.3.18 Period of Search: Presently, >21.3 million references; 1949-2015.
V.4.1.3.19. Key Words and Search Strategy: Cardiac perfusion and mice and pain alternative
V.4.1.3.20 Results of Search:
No published articles provide a painless procedure for cardiac perfusion.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: SMA is a severe motor neuron disease affecting young humans and mice that causes premature death because of muscle weakness and respiratory failure. There is currently no treatment for this disease in humans or mice and consequently no means by which to relief the distress experienced by SMA mice as part of the natural history of their disease. The aim of our study is to improve survival (hopefully thereby minimizing distress in these mice) using treatment with TSA and bortezomib.

Survival is the outcome measure used in human clinical trials of therapeutic agents. In this mouse study in order to minimize distress we will define survival as an endpoint before death (but representing inevitable death) and pups will be euthanized when they reach this endpoint. In our experience the combined features of severe motor dysfunction and weight loss indicate an irreversible condition that inevitably leads to death. We will make all efforts to minimize distress by euthanizing animals once they reach these endpoints

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: Primary cultures of motor neurons (Expt. 3) will be generated according to the following protocol from Dr (b)(6) Pregnant females will be transferred from the breeding protocol at E12, anesthetized no later than E14 using 2-5% isoflurane. Depth of anesthesia will be verified by pinching all four paws with forceps to make sure that there is no withdrawal reflex. Deeply anesthetized animals will then be euthanized by cervical dislocation. The abdomen of the euthanized animal will be sprayed with 70% ethanol prior to performing a caesarian dissection and removal of the uterus. The uterus will be placed in a culture dish and the embryos removed. Embryos collected from the uterus will be placed in a sterile petri dish in cold basal media and decapitated.

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: Housing in LAM, behavioral testing in (b)(6) located in the Pre-Clinical Studies Core.

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:
Mice will receive intraperitoneal injections of **A)** either 0.15 mg/kg of Bortezomib or **B)**10 mg/kg TSA. **C)** vehicle (5% DMSO solution) or **D)** both TSA (10 mg/kg) + Bortezomib (0.15

mg/kg). TSA will be dissolved in 5% DMSO to a concentration of 4 µg/µl. SMA mice and their wild type and heterozygous littermates will receive 10 mg/kg in a volume of 2.5 µl/g body weight (maximum volume: 35 µl) using a 33-gauge needle (Hamilton microliter syringe #705 (20 mm (1 in.): smallest needle available for the volumes of drug to be used). Control animals received equal volumes of vehicle alone. Bortezomib is FDA approved for treating multiple myeloma and is available in pharmaceutical grade. Pharmaceutical grade TSA is obtained from Enzo Pharmaceuticals.

V.4.4.2. Biosamples:

Tail snips (~5 mm) in length will be removed from the distal tip of the tail using sharpened scissors cleaned with ethanol immediately prior to use for each animal. The tail sample will be transferred into a microcentrifuge tube or 96 well plate for subsequent analysis by PCR. Hemostasis will be ensured following the procedure by applying gauze with gentle finger pressure to the distal tail. Samples will be taken at postnatal day 10 (Expt. 1, 2 and 3) and animals of the appropriate genotype that will be used for breeding (heterozygotes) will be marked with a unique identifying ear tag.

Following treatment (Expt.2), the mice will be euthanized and brain tissue will used for assessment of levels of protein ubiquitination, for protein expression of ubiquitination-related enzymes, as well as for a series of biomarkers conventionally used in the lab to evaluate motor neuron disease. For these needs, animals are euthanized by individuals on this protocol, and the brain is removed from the calvarium to obtain samples of the cerebral cortex (fronto-occipital region and entorhinal region), hippocampus, diencephalon, and brain stem. Samples are stored in liquid nitrogen before preparation for western blotting or for measuring mRNAs.

Transcardial perfusion for immunohistochemistry biosamples (Expt. 2). In addition, some of the mice will be used for immunohistochemistry. Following anesthesia per Section V.4.1.2.1, we will ensure mice are deeply anesthetized and unresponsive to paw pinch. A midline thoracotomy is made to expose the heart, and a 20-22 gauge blunt tip needle connected to a tube (¼ in ID and 1/16 in wall) is inserted into the left ventricle through the apex, and the right atrium is incised. After an initial washout with 30-500 ml of cold 100 mM phosphate buffered saline (PBS), transcardial perfusion with 100-250 ml cold 4% paraformaldehyde in PBS will be delivered through a manual gravity perfusion system until adequate perfusion reached. The brain will then be removed from the calvarium and post-fixed before tissue sectioning for immunohistochemical staining (cf., V.4.4.2. Biosamples).

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification:
All animals will be identified with cage cards.

V.4.4.6. Behavioral Studies:
Mice (Experiment 1) will be evaluated on the following tests daily starting at P5:

1. **Righting time:** Defined as the best of 2 trials of the time for the pup to turn over to its front after being placed completely on its back (maximum 30 seconds).

V.4.4.7. Other Procedures: Deeply anesthetized mice will be fixed by transcardial perfusion (Expt. 2) as outlined in **V.4.4.2.**

V.4.4.8. Tissue Sharing:
Excess mouse tissue will be made available to other investigators in our or other labs.

V.4.5. Study Endpoint:
Study end-point is survival until the age required for research sampling (approximately 14 days for untreated mice) after which mice will be euthanized and tissues collected. 20% loss in body weight is very early in the progression of the disease in SMA mouse models. We have found that a 30% loss in body weight is a better estimate of moribundity and will allow us to better evaluate the efficacy of drug treatment. Study end-point for treated mice: if no ability to right after 1 minute OR greater than 30% weight loss (from maximum weight), pup will be euthanized (age P5-P21).

Any mouse observed to be lethargic, losing weight, exhibiting gross neurological deficits (such as inability to eat or drink, continual circling, seizures, etc.) or any other signs of distress or illness will be evaluated by the PI and/or veterinary staff then euthanized as previously described.

V.4.6. Euthanasia:
Euthanasia will be performed in accordance with the guidelines of the "2013 Report of the AVMA Panel on Euthanasia." Methods of euthanasia on this protocol include cervical dislocation and perfusion. Mice are anesthetized before these procedures. Euthanasia will be performed by the PI or the staff in the CNRM Pre-Clinical Studies Core.

Perfusion (Expt. 2): Following anesthesia per Section V.4.1.2.1, we will ensure mice are deeply anesthetized and unresponsive to paw pinch. A midline thoracotomy is made to expose the heart, and a 20-22 gauge blunt tip needle connected to a tube (¼ in ID and 1/16 in wall) is inserted into the left ventricle through the apex, and the right atrium is incised. After an initial washout with 30-500 ml of cold 100 mM phosphate buffered saline (PBS), transcardial perfusion with 100-250 ml cold 4% paraformaldehyde in PBS will be delivered through a manual gravity perfusion system until adequate perfusion reached. The brain will then be removed from the calvarium and post-fixed before tissue sectioning for immunohistochemical staining (cf., V.4.4.2. Biosamples).

Cervical dislocation (Expt.2): Following anesthesia per Section V.4.1.2.1, we will ensure mice are deeply anesthetized and unresponsive to paw pinch. Cervical dislocation will be performed by placing a large hemostat behind the base of the anesthetized mouse's skull and pulling back sharply on the tail. Following dislocation mice will be examined to confirm respiratory arrest and the lack of a heartbeat. This method of euthanizing treated mice mice is preferred over CO_2 given the variability in time to die of neonatal mice following CO_2 exposure and the requirement to harvest tissue for biochemical analysis immediately after euthanizing mice [8].

Embryo harvesting (Expt. 3): Primary cultures of motor neurons will be generated according to the protocol from (b)(6)
Pregnant females will be transferred from the breeding protocol at E12, anesthetized no later

than E14 using 2-5% isoflurane. Depth of anesthesia will be verified by pinching all four paws with forceps to make sure that there is no withdrawal reflex. Deeply anesthetized animals will then be euthanized by cervical dislocation. The abdomen of the euthanized animal will be sprayed with 70% ethanol prior to performing a caesarian dissection and removal of the uterus. The uterus will be placed in a culture dish and the embryos removed. Embryos collected from the uterus will be placed in a sterile petri dish in cold basal media. Each embryo will be decapitated at the infracranial notch just below the base of skull using fine-tip forceps or small curved scissors with the aid of a dissection microscope.

In the event that LAM personnel must euthanize an animal for humane reasons, cylinderized carbon dioxide will be used in the LAM necropsy room. Using a clean cage, carbon dioxide will be administered at a rate to replace greater than 20% of the cage volume per minute. Once the mice have not moved or breathed for at least one minute they will be removed from the chamber. Euthanasia may be ensured by cervical dislocation or opening of the thorax.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol (5 mice per cage).

V.5.1.1. Study Room:

Building(s)	(b)(6)	Room Number(s)	(b)(6)
Building(s)		Room Number(s)	
Building(s)		Room Number(s)	

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No __X__

Fluid Restriction: Yes _____ No __X__

V.5.1.3. Exceptions: None.

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
The PI or research staff will observe the mice twice daily following all experimental procedures. LAM personnel will observe the mice at least once a day for general health and husbandry condition. Any mouse observed to be lethargic, losing weight, exhibiting gross neurological deficits (such as inability to eat or drink, continual circling, seizures, etc.) or any other signs of distress or illness will be evaluated by the PI and/or veterinary staff then euthanized as previously described.

V.5.2.2. Emergency Veterinary Medical Care:
All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays. In the case of an emergency health problem, if the responsible person (e.g. PI) is not available or if the

investigator and veterinary staff cannot reach consensus on treatment, the veterinarian has the authority to treat the animal, remove it from the experiment, institute appropriate measures to relieve severe pain or distress, or perform euthanasia if necessary.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species. Animals are grouped-housed. Standard nestlets, tunnels, or toys provided by LAM will be provided in each home cage.

V.5.3.2. Enrichment Restrictions: None.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Perfusion-fixation, intraperitoneal injection, c-section, anesthesia, euthanasia.	(b)(6)	Over 10 years experience with mouse behavior studies, IP injections, perfusion-fixation, euthanasia , mouse surgeries including hysterectomy.	USU LAM Investigator/animal handler training class February 4, 2014. Animal handling and surgery training at the NIH (2006).
Perfusion-fixation, intraperitoneal injection, euthanasia.		Lab technician with over 3 years experience with IP injections, perfusion-fixation, euthanasia	USUHS LAM Investigator/animal handler training class August 24, 2013.

VII. BIOHAZARDS/SAFETY:

To minimize exposure to animal allergens when working with lab animals, lab personnel will wear lab coats and/or scrubs, gloves and masks. Potential fumes from fixative are minimized by allowing the fixative to be constantly washed down the sink drain. This procedure will be carried out in a chemical fume hood.

VIII. ENCLOSURES:

Literature search results for Sections II.2 (Searches for unnecessary duplication) are available upon request.

Cited References

1. Le, T.T., et al., *SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN.* Hum Mol Genet, 2005. **14**(6): p. 845-57.
2. Avila, A.M., et al., *Trichostatin A increases SMN expression and survival in a mouse model of spinal muscular atrophy.* J Clin Invest, 2007. **117**(3): p. 659-71.
3. Burnett, B.G., et al., *Regulation of SMN Protein Stability.* Mol Cell Biol, 2009. **29**(5): p. 1107-1115.
4. Kwon, D.Y., et al., *The E3 ubiquitin ligase mind bomb 1 ubiquitinates and promotes the degradation of survival of motor neuron protein.* Mol Biol Cell, 2013. **24**(12): p. 1863-71.

5. Kwon, D.Y., et al., *Increasing expression and decreasing degradation of SMN ameliorate the spinal muscular atrophy phenotype in mice.* Hum Mol Genet, 2011. **20**(18): p. 3667-77.
6. Narver, H.L., et al., *Sustained improvement of spinal muscular atrophy mice treated with trichostatin A plus nutrition.* Ann Neurol, 2008. **64**(4): p. 465-70.
7. Wahlsten, D., *Planning genetic experiments: Power and sample size*, in *Neurobehavioral Genetics: Methods and Applications*, B.C. Jones and P. Mormede, Editors. 1999, CRC Press: Boca Raton.
8. Pritchett, K., et al., *Euthanasia of neonatal mice with carbon dioxide.* Comp Med, 2005. **55**(3): p. 275-81.

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 10-13-15

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 10-13-15

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL** or **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature

10.13.15

Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number: (b)(6)

B. Animal Protocol Title: Targeting the ubiquitin proteasome system to treat spinal muscular atrophy (*Mus musculus*)

C. Principal Investigator (b)(6) Ph.D.

D. Performing Organization: Uniformed Services University of the Health Sciences

E. Funding: USUHS Intramural funds

F. Objective and Approach:

The goal of the work is to improve treatments for neurodegenerative diseases by studying the role of the ubiquitin-proteasome pathway in the brain. To approach this problem we will use laboratory mice. We will test if inhibiting the proteasome drugs can increase the stability of proteins critical to neuronal survival and protect against the effects of neurodegeneration. In addition, we will determine if the ubiquitin proteasome system is affected by toxic insults to the brain. This is an important step for establishing whether or not ubiquitin-dependent degradation has a critical role in neurodegeneration, and whether changes in ubiquitination activities in the brain can reduce central nervous system injury and eventually be used to help patients with neurodegeneration.

The data collected will be summarized using conventional statistical approaches (mean, standard deviation, analysis of variance, correlation and regression analyses). We will use as few mice as possible while still using enough to make valid conclusions. To do this initial research we must use animals since cells or tissues will not reflect the actual biology and behavioral responses of a live organism. We hope that this basic research and use of animals will aid in understanding some of the causes for the long-term effects brain injury has upon the war fighter returning home to their town, work, and families, and that this research may allow us to provide better treatments to help suffering soldiers.

G. Indexing Terms (Descriptors): Animal, mice, spinal muscular atrophy, ubiquitination, protein ubiquitination, behavioral testing, anatomy

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
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June 14, 2016

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) by Designated Member Review on June 14, 2016:

Animal Protocol Title: "Acetate Supplementation Using Glyceryl Triacetate as an Adjunct Therapy for Hemorrhagic Shock and Injury in Mice (Mus musculus)"

USUHS Protocol Number: (b)(6)

Expiration Date: June 13, 2019

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206 ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

Revised

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Acetate Supplementation using Glyceryl Triacetate as an Adjunct Therapy for Hemorrhagic Shock and Injury in Mice (*Mus musculus*)

GRANT TITLE (if different from above): Same

USUHS PROJECT NUMBER/ DAI GRANT NUMBER: (b)(6)

FUNDING AGENCY: NIH-USU Collaborative Program called CHIRP

EARLIEST ANTICIPATED FUNDING START DATE: Active from February 2016

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6)	APG	(b)(6)	4/14/2016
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review [illegible] research practice.

(b)(6)	Vice Chair	(b)(6)	14 Apr 2016
Research Unit Chief / Dept. Head Signature Typed Name:	Title	Telephone	Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)	APG	(b)(6)	4/14/2016
Statistician Signature Typed Name:	Department	Telephone	Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics. All signatures are required prior to submission to the IACUC Office.

(b)(6)	LAM	(b)(6)	4/14/16
Attending/Consulting Veterinarian Signature Printed Name: (b)(6) VMD	Department	Telephone	Date

The aims of the supporting grant must be submitted along with the 3206. If funding is from NIH please also submit the Vertebrate Animal Section (VAS).

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) **Ph.D.**

PRINCIPAL INVESTIGATOR EMAIL: (b)(6)

ANIMAL PROTOCOL TITLE: Acetate Supplementation using Glyceryl Triacetate as an Adjunct Therapy for Hemorrhagic Shock and Injury in Mice (*Mus musculus*)

GRANT TITLE (if different from above): Same

USUHS PROJECT NUMBER: (b)(6)

DAI GRANT NUMBER: Processing in progress

CO-INVESTIGATOR(S): (b)(6) Ph.D., (b)(6) M.Sc, (b)(6) Ph.D. (b)(6) Ph.D. and (b)(6) M.D. (awaiting investigator and rodent handling training).

TECHNICIANS(S): None

I. **NON-TECHNICAL SYNOPSIS:** Hemorrhage is responsible for about 30-40% of all traumatic deaths and approximately 50% of battlefield deaths worldwide. Studies have suggested that the ability of tissues to deal with hemorrhage related problems such as hypoxia and hypothermia, is critical and that robustness of this ability differs among individuals, suggesting involvement of genetic or epigenetic mechanisms. Recently it has been shown that acetylation of the transcription factor HIF-2α controls erythropoietin production during pathophysiological stress induced by oxygen deprivation. In earlier studies we have developed acetate supplementation using glyceryl triacetate (GTA), which generates acetate and glycerol on hydrolysis in vivo, to improve outcomes after traumatic brain injury. Based on these and other studies we have found that oral GTA administration is an effective way to increase acetate levels in all tissues and that GTA has no detectable toxic effects on chronic usage even at relatively high doses in animals and children. In subsequent studies, acetate supplementation using GTA has been shown to induce anti-inflammatory effects in multiple rat model systems. Histone deacetylase inhibitors including valproic acid and trichostatin, which increase protein acetylation status in general and histone acetylation status in particular, have anti-inflammatory and protective effects in hemorrhagic shock. This is due in part to the fact that patients who survive acute episodes of blood loss experience systemic inflammatory response syndrome triggered by ischemia-reperfusion injury and organ damage. Based on these earlier findings, we propose to develop oral acetate supplementation using GTA as a way to augment acetylation-based regulatory mechanisms involved in the response to and recovery from bodily injury, blood loss and reperfusion injury. Our immediate goal is to develop GTA treatment as an adjunct therapy for hemorrhagic shock and injury. We and others have shown that acetyl-coenzyme A synthetase-1 (enzyme name: AceCS1, gene: Acss2) is primarily involved in generating acetyl-coenzyme A (acetyl-CoA) for acetylation reactions, especially in cell nuclei, and we currently have an ongoing breeding colony of mice in which the Acss2 gene is knocked out for studying the related genetic

mechanisms. Using this gene knockout model we plan to determine the genetic and epigenetic regulatory mechanisms involved in the acetylation-based regulation of stress responses to blood loss and injury, and the beneficial effects of GTA treatment on survival from hemorrhagic shock and injury. Our central hypothesis is that enhancing acetylation-based endogenous genetic/epigenetic mechanisms is an effective way to minimize chronic disability and death due to hemorrhagic shock. We propose to test this hypothesis using GTA-mediated acetate supplementation in a mouse model of hemorrhagic shock with concomitant injury and determine the associated genetic/epigenetic mechanisms using our Acss2 gene knockout mouse model. The long-term goal of the proposed studies is to improve survival for patients with traumatic hemorrhage and associated shock via regulation of genetic and epigenetic response mechanisms and to provide knowledge that will lead to genetically-informed personalized treatments.

II. BACKGROUND:

II.1. Background: The proposed research is focused on determining the effectiveness of acetate supplementation with GTA in improving recovery from hemorrhagic shock (HS) with concomitant control cortical impact (CCI) -injury to the brain and subsequent ischemia-reperfusion injury (IRI) in wild type mice and Acss2 gene knockout mice. Acss2 encodes the nuclear-cytoplasmic form of the enzyme that converts acetate to acetyl-CoA, and it is the only known enzyme capable of generating acetyl-CoA from acetate in the cytoplasm and nucleus of cells. This enzyme has been found to be critical for acetylation of the transcription factor HIF-2α, and the promotion of erythropoietin production in response to severe anemia (Xu et al., 2014; Chen et al., 2015), making this enzyme an important target for the treatment of HS and IRI. In subsequent studies, we plan to study the genetic/epigenetic mechanisms involved in the protective effects of increased glyceryl triacetate (GTA)-mediated acetylation using the HS and concomitant injury model system in these mice. Hemorrhage is responsible for about 30-40% of all traumatic deaths and approximately 50% of battlefield deaths worldwide (Klemcke et al., 2011). Therefore, the long term goal of the proposed studies is to improve survival for patients with traumatic hemorrhage and associated IRI via acetylation based regulation of genetic and epigenetic response mechanisms and to provide knowledge that will lead to genetically-informed personalized treatments. In pursuit of this long term goal, genetic and epigenetic studies will be carried out using human tissue samples from the CHIRP repository based on the results from the proposed studies in mice.

Individual variations in response to HS: During HS multiple physiological mechanisms come into play to maintain blood pressure and tissue perfusion for as long as possible. When these mechanisms fail or are not effective, tissue perfusion is diminished to critical levels which can subsequently produce end organ injury resulting from hypoxia, hypercapnia or hypothermia. Reperfusion results in additional injury due to excessive inflammatory responses and oxidative damage, among other mechanisms. It has been known for some time that there is considerable variability in the ability of patients to survive these conditions and genetic and epigenetic factors are sure to play critical role in this inter-person variability. As early as 1970, Kim and Shoemaker investigated possible mechanisms underlying physiological differences between survivors and fatalities among HS patients (Kim and Shoemaker, 1970). These and

subsequent studies have indicated that cardiac index, oxygen delivery and tissue oxygen utilization were highly divergent between the groups (Shoemaker, 1976; Bishop et al., 1995; Bishop et al., 1993). Other studies have shown association between patient survival and inflammatory cytokines and heat shock proteins (Roumen et al., 1993; Pittet et al., 2002). Increased survival has been associated with higher respiration rates, arterial PO2, saturation of hemoglobin, blood O2 content, blood pH and lower lactate levels in rat models (Torres Filho et al., 2010; Reynolds et al., 2008). Later studies by other investigators working on cardiovascular function, blood pressure regulation and hypertension have indicated that the variability in the survival time could be accounted for by genetic variability (polymorphism) (Rapp, 2000; Cowley, Jr., 2003). Based on more recent evidence, it seems likely that multiple epigenetic mechanisms affecting transcription factor activation and gene expression are also important components of this inter-individual variability.

Studies have indicated that females are less susceptible to cerebral injury, independent of the effects of sex hormones (Semenas et al., 2010b). In a recent study using a clinically relevant model of severe hemorrhage and circulatory arrest in sexually immature piglets, it was demonstrated that females displayed an enhanced hemodynamic response, cardio-protection, and increased survival (Semenas et al., 2010a). Interestingly, this cardio-protective effect was observed in spite of comparable estradiol and testosterone levels in the male and female animals, indicating an innate gender-related cardio-protection, independent of sex hormone effects. Recent studies also suggest sex-differences in the expression of several enzymes and proteins, such as nitric oxide synthase and superoxide dismutase. Therefore, it is likely that the observed sex benefit in females is multifactorial and relates to the expression of a diverse group of vascular factors, inflammatory mediators and metabolic enzymes. However, possible epigenetic mechanisms involved in these sex-related but sex hormone independent effects remain uncertain.

Regulation of stress erythropoiesis by an acetylation based epigenetic mechanism: Erythropoietin (EPO) is synthesized extensively in the kidney and liver of adult mammals and is involved in controlling erythrocyte production. Synthesis of EPO is regulated by the stress-responsive transcription factor hypoxia-inducible factor-2 (HIF-2). New studies have shown that effective HIF-2 dependent induction of EPO during hypoxia requires acetylation of the HIF-2α subunit and that the lysine acetyltransferase CREB-binding protein (CBP) is required for this HIF-2α acetylation (Xu et al., 2014; Chen et al., 2015). These studies further showed that in acutely anemic mice the nuclear-cytoplasmic form of acetyl-CoA synthetase (enzyme; AceCS1, gene; Acss2) is required in conjunction with CBP for HIF-2 acetylation, CBP-HIF-2α complex formation, CBP-HIF-2α recruitment to the EPO enhancer and efficient induction of EPO gene expression. Furthermore, acetate supplementation with GTA augmented stress erythropoiesis in an Acss2-dependent manner in acutely anemic mice. Additionally, such acetate supplementation increased EPO expression and resting hematocrit in acquired and inherited chronic anemia models in mice. These studies emphasize the regulatory role of transcription factor acetylation in the control of EPO gene expression during pathophysiological states marked by tissue hypoxia, and the key role played by Acss2 in mediating this response.

Acetate supplementation using GTA has anti-inflammatory effects: GTA does not induce acetylation at all protein acetylation sites, but rather has been shown to induce acetylation at specific lysine sites on some histone and non-histone proteins. The reason for this specificity is still uncertain. We compared the effects of GTA and equimolar levels of acetate and glycerol and have shown they have different inhibitory effects on the growth of certain cancer cell lines in culture (Long et al., 2013). Multiple studies have shown that acetate supplementation using GTA has unique anti-inflammatory effects. Most of these studies have focused on the brain using several model systems. In one study, acetate supplementation with GTA was found to attenuate lipopolysaccharide (LPS)-induced neuroinflammation (Reisenauer et al., 2011). In rats subjected to LPS-induced neuroinflammation, a daily treatment with GTA (6g/kg) by oral gavage reduced the percentage of CD-11b-positive microglia by three-fold compared to rats subjected to neuroinflammation and given water. In another study, acetate supplementation using GTA reduced microglial activation and brain IL-1β levels in a rat model of Lyme neuroborreliosis (Brissette et al., 2012). These studies showed that acetate supplementation utilizing GTA is an effective treatment to reduce injury phenotype and progression in Lyme neuroborreliosis. In a subsequent study, acetate supplementation with GTA was found to modulate histone acetylation and decrease IL-1β expression in a rat model of neuroinflammation (Soliman et al., 2012a). In this study, a 28-day dosing regimen of GTA was used for acetate supplementation and the effect on brain histone acetylation and IL-1β expression was determined. It was found that long term acetate supplementation increased the proportion of brain histone H3 acetylated at lysine 9 (H3K9), histone H4 acetylated lysine 8 and histone acetylated lysine 16. Consistent with these findings, acetate supplementation effectively reversed the decrease in H3K9 acetylation that was caused by the LPS-induced neuroinflammation. Further, LPS-induced neuroinflammation increased IL-1β message (10-fold) and protein (1.3 fold), and the levels of this pro-inflammatory cytokine were reduced to control levels by acetate supplementation with GTA. Based on these studies, it can be concluded that dietary acetate supplementation attenuates microglial activation by effectively reducing pro-inflammatory cytokine expression by a mechanism that might involve a promoter site-specific pattern of histone acetylation and possibly acetylation of other transcription factor proteins as well. The proposed studies will determine if this anti-inflammatory effect of GTA is also operative in other tissues including liver, lung, intestine, heart and kidney.

Histone deacetylase inhibition creates a pro-survival phenotype during HS: A number of studies from different laboratories have shown that histone deacetylase (HDAC) inhibitors improve survival in HS victims (reviewed in Halaweish et al., 2015). These drugs act by improving the acetylation status of proteins including histones and transcription factors. In one study, pretreatment with valproic acid (300mg/kg, twice), a HDAC inhibitor, prolonged the survival of severely hypotensive rats by 5 times in a lethal hemorrhage model (Gonzales et al., 2006). Also, acetylation status of histone and non-histone proteins was increased in the heart. In models of HS and polytrauma, treatment with HDAC inhibitors has been shown to provide a significant survival advantage, even in the absence of fluid resuscitation. Multiple polytrauma models studies included two-hit models involving severe hemorrhage and sepsis, and HS combined with traumatic brain injury. However, chronic treatment of HDAC inhibitors is not encouraged due to the effects on global histone acetylation status especially with

pan-HDAC inhibitors. Therefore, efforts are under way to develop isoform selective inhibitors as therapeutic agents (Cheng et al., 2015).

The research plan involves setting up a mouse model of HS based on reduction in mean arterial pressure (MAP) rather than volume since it is more reliable than blood volume reduction (Pfeifer et al., 2013). The HS model will be set up using wild type and Acss2 gene knockout mice following a recently published procedure which combines experimental injury with HS (Sodhi et al., 2015). However, rather than bilateral femur fracture, we will use CCI injury to the brain as the concomitant injury as a model of polytrauma (Dennis et al., 2009). The bilateral femur fracture model is not suitable for chronic studies, whereas CCI injury represents a good model for polytrauma in conjunction with HS. Therefore we plan to use the CCI injury as the additional injury. The levels of cytokines and erythropoietin will be measured in blood, brain, lung, gut, liver, kidney and heart using antibody based protein array methods. Subsequently, the studies will be repeated under optimized conditions based on results from the first set of experiments to investigate the genetic and epigenetic mechanisms involved. Toward this goal, we will use a combination of open chromatin mapping with formaldehyde-assisted isolation of regulatory elements (FAIRE-seq) and enhancer and transcription factor mapping using chromatin immune-precipitation (ChIP-seq). Also, RNA-seq will be used to obtain the gene expression profiles under this condition. All these analyses will be done using multiple tissue samples.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: DTIC, FEDRIP, PUBMED

II.2.2. Date of Search: March 12-14, 2016

II.2.3. Period of Search: All published work (no limit)

II.2.4. Key Words and Search Strategy: Key words were used in sets of three. They are: 1) mice, acetate supplementation and -hemorrhagic shock/injury, 2) mice, Glyceryl triacetate -and hemorrhagic shock/injury, 3) mice, hemorrhagic shock/injury and genetic mechanisms and 4) mice, hemorrhagic shock/injury and epigenetic mechanisms.

II.2.5. Results of Search: 1) PUBMED: No articles were found for key word combinations #1 and #2 and #4. One article was found for combination #3, but it was not relevant for the proposed studies. 2) DTIC: One article was found for key word combinations #1. This work is relevant to the proposed research, but not directly related or repeat of the proposed research. No article was found for combination #2, #3 and #4. FEDRIP: Three articles were found for key word combinations #2 and 444 articles for combination #1, #3 and #4. However no article was found when the search was restricted to scholarly publications.

III. OBJECTIVE\HYPOTHESIS: Our central hypothesis is that enhancing acetylation-based endogenous genetic/epigenetic mechanisms is an effective way to minimize chronic disability and death due to hemorrhagic shock. We propose to test this hypothesis using GTA-mediated acetate supplementation in a mouse model of

hemorrhagic shock with concomitant injury and determine the associated genetic/epigenetic mechanisms using our Acss2 gene knockout mouse model.

IV. MILITARY RELEVANCE: Hemorrhage is responsible for about 30-40% of all traumatic deaths and approximately 50% of battlefield deaths worldwide. Studies have suggested that the ability of tissues to deal with hemorrhage related problems such as hypoxia and hypothermia, is critical and that robustness of this ability differs among individuals, suggesting involvement of genetic or epigenetic mechanisms. The long-term goal of the proposed studies is to improve survival for patients with traumatic hemorrhage and associated shock via regulation of genetic and epigenetic response mechanisms and to provide knowledge that will lead to genetically-informed personalized treatments. As such this project is highly relevant to the health and survival of our military personnel in battlefield situations.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures: As mentioned above, the research plan in this protocol involves setting up a mouse model of HS based on reduction in mean arterial pressure (MAP) rather than volume since it is more reliable than blood volume reduction (Pfeifer et al., 2013). The HS model will be set up using wild type and Acss2 gene knockout mice following a recently published procedure which combines experimental injury with HS. We will use CCI injury to the brain as the concomitant injury as described earlier (Dennis et al., 2009). The levels of cytokines and erythropoietin will be measured in blood, brain, lung, gut, liver, kidney and heart using antibody based protein array methods to determine the acetylation associated protective mechanisms in this model system. Subsequently, studies will be repeated under optimized conditions based on results from the first set of experiments to investigate the genetic and epigenetic mechanisms involved. Toward this goal, we will use a combination of open chromatin mapping with formaldehyde-assisted isolation of regulatory elements (FAIRE-seq) and enhancer and transcription factor mapping using chromatin immune-precipitation (ChIP-seq). Also, RNA-seq will be used to obtain the gene expression profiles under this condition. All these analyses will be done using multiple tissue samples. We are confident that these preliminary studies will provide reliable leads to pursue our long term goal of providing knowledge that will lead to genetically-informed personalized treatments.

V.1.1. Experiment 1: To determine the protective effects of acetate supplementation with GTA in the mouse model of HS and experimental trauma by analyzing survival, inflammatory responses, end-organ pathology and related chronic responses in wild type and Acss2 gene knocked out mice.

The experimental design and methods are described below briefly:

1. Mouse HS/resuscitation and polytrauma model: Both wild type and Acss2 -/- mice will be used for these studies. All experiments will be done using males approximately 10 weeks of age (~25 g). Mice will be anesthetized and subjected to polytrauma consisting of CCI injury to the brain followed by 60 min HS (Dennis et al., 2009). For comparison purposes, animals with HS alone also will be used. The mice will be treated with GTA (5 gm/kg) intragastrically for three different durations. GTA will be administered twice per day, spaced 4 to 6 hours apart with treatment lasting between 1

and 12 days and the response to injury will be evaluated by multiple methods on the days designated (see Table below). On the designated days animals will be divided into two groups randomly for biochemical and histochemical analyses (4 wild type and 4 Acss2 -/- mice for each procedure). The levels of pro-inflammatory cytokines including IL-1β and TNF-α, as well as erythropoietin, will be measured in blood on the designated days. For ELISA studies the mice will be euthanized and blood will be drawn. The mice will be perfused with PBS to remove the remaining blood, and the brain, small intestine, lungs, liver, kidney and heart will be extracted and analyzed by several methods including antibody based protein array methods and ELISA assays. For histochemical and immunohistochemical analyses mice will be anesthetized and perfused transcardially with 10% neutral buffered formalin and the same organs will be processed for standard histopathology as well as immunohistochemistry to detect end organ pathology and determine the effectiveness of GTA and acetylation-associated protective mechanisms in this model system.

For the surgical procedures mice will be anesthetized with 3-5% isoflurane and maintained with 2% isoflurane in 100% O2 throughout the experiments. Under sterile conditions, a right groin exploration will be performed and the right femoral artery and vein will be exposed and catheterized using sterile polyethylene (PE-50) tubing (Harvard Apparatus). The catheters will be connected to a blood pressure transducer for continuous arterial blood pressure monitoring during the procedure. The mice will be placed in a stereotaxic frame and a 5 mm bone flap will be removed over the parietal cortex. Injury will be produced with a 3 mm flat steel tip with a velocity of 5 m/sec and a penetration depth of 1 mm (Dennis et al., 2009). The bone flap will be replaced and HS will be induced over the course of 15 min by withdrawing blood (approximately 2.0 ml/100gm) until we achieve a MAP between 30 and 35 mm Hg. The MAP will be maintained in this range for 60 min. At the end of the 60 min HS period, mice will be resuscitated to a MAP of at least 60 mm Hg. This will be done over 15 min with their remaining shed blood plus two times the maximum shed blood in lactated Ringer's solution. The catheters will be removed and mice will be taken off isoflurane and returned to their cages. Sham-operated mice will undergo anesthesia and femoral cannulation only.

The mice will be sacrificed for ELISA or histological analyses on the days indicated in Table below. The mice will be deeply anesthetized with a pentobarbital based preparation (Fatal-Plus solution, 75-100 mg/kg, i.p.) and transcardially perfused with PBS (50 ml) for biochemical analyses, or with 10% neutral buffered formalin (60 ml) for histological and immunohistochemical analyses. Blood will be collected by cardiac puncture.

For biochemical analyses tissues (brain, liver, lungs, kidney and small intestine) will be dissected rapidly, frozen on dry ice and stored at -80°C until used. For histology and immunohistochemistry the same tissues will be removed and postfixed in 4% paraformaldehyde overnight at 4°C for histopathological and immunohistochemical analyses. We plan to have 8 mice in each group so that four mice can be used for ELISA/biochemical analysis and four can be used for histopathological and immunohistochemical analyses.

The CNRM SOP #1 Brain Injury Models will be followed for the CCI work in mice. The pain will not be alleviated in the CCI groups (pain category E) since the drugs used can interfere with the inflammatory responses/mechanisms, the primary focus of the proposed studies.

2. GTA Treatment: GTA treatment will be done as described in our earlier studies (Arun, P et al, 2010a). Experimental groups and conditions are given in Table below. Animals will receive the first dose of GTA intragastrically (5g/kg) 30 minutes after resuscitation from HS using a blunt tipped intragastric feeding needle passed down the esophagus. On subsequent days the treated animals will be given the same dose twice a day (morning and evening) until sacrifice. Untreated injured animals will be given an equal volume of water. Groups of 16 mice for each treatment condition will include 8 wild type male mice and 8 Acss2 -/- male mice randomly selected from our breeding colony. Females will be used exclusively for breeding purposes. The three time points will allow us to differentiate between short term and long term effects of GTA treatment on the responses to HS, CCI and IRI.

Injury	Treatment length	Treatment	Analyses
Sham = 16 (8 wild type and 8 Acss2) x 3 (time points) x 2 (water or GTA) x 2 (repeating) = 192	1, 4 or 12 days	water or GTA	ELISA & histology
HS only 16 (8 wild type and 8 Acss2) x 3 (time points) x 2 (water or GTA) x 2 (repeating) = 192	1, 4 or 12 days	water or GTA	ELISA & histology
HS + CCI =16(8 wild type and 8 Acss2) x 3 (time points) x 2 (water or GTA) x 2 (repeating) = 192	1, 4 or 12 days	water or GTA	ELISA & histology

Table 1: Treatment groups and procedures. Animals in the experimental groups will receive either GTA or an equivalent volume of water. The experiments will be conducted on equal numbers of male wild type and Acss2 -/- mice (n = 8 per group for a total of 16 animals per treatment condition). At the end of the designated treatment periods, groups of 8 mice at each time point under each condition will be divided randomly into a group for ELISA measurements and a group for histology and immunohistochemistry (4 wild type and 4 Acss2 -/- mice for each). All animals (total = 576) will be listed under category E since analgesics cannot be used to alleviate the pain in any of the groups including the sham.

3. Cytokine and hormone quantitation by enzyme-linked immunosorbent assay (ELISA): Concentrations of cytokines such as IL-6, TNF-α will be measured using ELISA assays. Other HS-related proteins such as erythropoietin and HMGB1 in the plasma and in tissues will also be measured using ELISA. The CHIRP Laboratory Core has facilities for multiplex ELISAs and this resource will be utilized for our ELISA based studies. At the time points noted (Table 1) mice will anesthetized with Fatal Plus and blood will be drawn by cardiac puncture. The animals will be perfused with approximately 50 ml of PBS to remove remaining blood and tissues will be removed including brain, lungs, liver, kidney, small intestine and heart. Tissues will be frozen rapidly in liquid nitrogen and stored at -80 °C until analyzed.

4. Histochemical and Immunohistochemical analyses to detect multiple organ pathology: Fixation and tissue processing will be done during different time points in the study (Table 1). Mice will be deeply anesthetized with a pentobarbital based preparation

(Fatal-Plus Solution, 75-100 mg/kg, i.p.) and transcardially perfused with PBS (50 ml) followed by 4% paraformaldehyde (100 ml). Tissues including brain, kidney, heart, liver, lungs, and small intestine will be removed and post-fixed in 4% paraformaldehyde overnight at 4 °C, then transferred to a solution of 20% sucrose in PBS. After 18 hours the sucrose solution will be replaced with a fresh 20% sucrose solution and kept for 72 h, and then tissues will be frozen with dry ice and stored at -80-°C until sectioning. Frozen organs will be cryostat-cut at 5-20 µm, mounted onto glass slides. Some of the slides will be used for H & E staining which will be done at Biomedical Instrumentation Center at USUHS. For the immunostaining, endogenous peroxidase will be blocked by Peroxidase Block solution (DAKO). For specific protein detection, sections will be incubated at 0-4-°C overnight in presence of the respective primary antibodies and the immunoreactivity will be detected by using the appropriate Vectastain peroxidase kits according to the manufacturer's instructions.

V.1.2. Experiment 2: To repeat the studies using an optimal time point and perform genetic and epigenetic analyses in blood and different tissues such as liver, heart and kidney to understand the genetic and epigenetic mechanisms involved.

In order to understand the genetic and epigenetic mechanisms involved in the demonstrated protective effects of GTA, we plan to use a combination of open chromatin mapping with formaldehyde-assisted isolation of regulatory elements (FAIRE-seq), and enhancer and transcription factor mapping using chromatin immune-precipitation using anti-pan-acetyl lysine antibodies (ChIP-seq). Also, RNA-seq will be used to obtain the gene expression profiles in response to GTA in wild type and Acss2 -/- mice. We will use an optimal duration for GTA treatment based on the results of Experiment 1 and studies will be repeated under conditions where a maximal response was found in wild type mice using the optimal model system conditions.

Injury	Treatment length	Treatment	Analyses
Sham = 8 (4 wild type and 4 Acss2) x 2 (water or GTA) x 2 (repeating) = 32	Optimum time point	water or GTA	Genetic and epigenetic analyses
HS only = 8 (4 wild type and 4 Acss2) x 2 (water or GTA) x 2 (repeating) = 32	Optimum time point	water or GTA	Genetic and epigenetic analyses
HS + CCI = 8 (4 wild type and 4 Acss2) x 2 (water or GTA) x 2 (repeating) = 32	Optimum time point	water or GTA	Genetic and epigenetic analyses

Table 2: Genetic and epigenetic analyses. Details of the experiment are as given in table 1. Studies will be repeated using the optimal time point from experiment 1. All animals (total = 96) will be listed under category E since analgesics cannot be used to alleviate the pain in any of the groups including the sham.

V.1.3. Experiment 3. To repeat the experiment 2 using female mice of the same age.

The experiment will be done exactly as described in experiment 2, but with female mice.

V.2. Data Analysis: Data will be presented as mean ± SEM. Parametric data (cytokine levels etc.) will be analyzed using a paired 2-tailed t test for the paired data or a 1-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons post-test. Nonparametric data (histology data, immunohistochemical data) will be analyzed using a Wilcoxon signed rank test for paired data or a Kruskal-Wallis test (nonparametric ANOVA). An associated probability value of $P < 0.05$ will be considered significant.

Power analysis using the quantitative data obtained from our earlier studies have indicated that for behavioral studies a sample size of 10 animals per group will have 80% power to detect a difference of 1.5 standard deviations between groups assuming a 5% significance level, and should be sufficient for the outcomes we intend to measure; power calculations were performed using nQuery Advisor® 4.0 software. Similar calculations have indicated that 5-6 animals/group is sufficient for biochemical and immunohistochemical studies. The total number of animals for each experiment has been calculated based on the above considerations.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: The specific nature of the project does not permit use of non-animal alternatives. The global physiological changes occurring in different cell types in the in-vivo injury models cannot be reproduced in single cell culture system, or any alternatives.

V.3.2. Animal Model and Species Justification: Mouse model of hemorrhagic shock is one of the most commonly used animal models for these preclinical studies. Also, controlled cortical impact (CCI) in the rodents is one of the most extensively used models of TBI in the literature and this allows us to compare our results with those from other established laboratories.

V.3.3. Laboratory Animals

ALTERNATIVES CONSIDERATIONS: Does the protocol have any provisions that would qualify it to be identified as one that Refines, Reduces, or Replaces (3R's) the use of animals in relation to other protocols or procedures performed in the past?

Y/N (circle) **SECTION V.3.5.**

Exceptions to the *Guide for the Care and Use of Laboratory Animals* (Please check all applicable):

[ ] Use of Paralytics (V.4.1.2.3.)
[ ] Prolonged Restraint (V.4.2.)
[ ] Multiple Major Survival Surgery (V.4.3.6.)
[X] Use of Non-pharmaceutical grade chemicals (V.4.4.1.)
[ ] Use of Complete Freund's Adjuvant (V.4.4.3.)
[ ] Death as an endpoint (V.4.5.)
[ ] Food/Water Restriction (V.5.1.2.

[ ] Single Housing of Social Species (V.5.1.3)
[ ] Restriction of Environmental Enrichment (V.5.3.2.)
[ ] Drug Use/Controlled Substances (Appendix A)

IDENTIFICATION OF SPECIES AND STRAIN: In accounting for animal numbers, please ensure that the strain of animal as well as the species is identified. If more than one strain of any species will be used, please list each proposed strain in a separate column. If more than two species/strains are to be used, duplicate Sections V.3.3.1 – V.3.4 , and Section V.4.1.1.1, on subsequent pages to cover all requested strains.

		Species/Strain #1	Species/Strain #2
V.3.3.1.	**Genus & Species:**	*Mus musculus*	*Mus musculus*
V.3.3.2.	**Strain/Stock:**	C57/BL6	Acss2 -/-,
V.3.3.3.	**Source/Vendor:**	LAM approved vendors and Breeding in house	Breeding in house (Breeding protocol APG-15-561).
V.3.3.4.	**Age:**	Adult	Adult
V.3.3.5.	**Weight:**	20-25g	20-25g
V.3.3.6.	**Sex:**	**Male and female**	**Male and female**
V.3.3.7.	**Special Considerations:**	None	Homozygous mice only will be used for the proposed studies
		425 (includes 10% extra for unexpected losses)	**425(includes 10% extra for unexpected losses)**

V.3.4. Number of Animals Required (by Species/Strain):

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: In the initial studies conducted to test the effect of GTA on traumatic brain injury using the CCI model, we have focused on the obvious behavioral, biochemical and histological parameters which are known to be well associated with TBI. We have found that most of the animals recover fast and start running around in apparent normal way in a matter of hours. Occasionally, some (less than 10%) are found to show a greater impairment. As a refinement, we plan to look for such animals and euthanize them since they are likely to be outliers in the study as well. Also, animals will be acclimated before the behavioral studies. All the animals will receive local anesthesia during -catherization of the artery and vein. We do not plan to provide

any post-operative analgesia for these animals because use of analgesics can interfere with the goal of the proposed studies.

V.3.5.2. Reduction: We have considered reducing the total number of control animals by avoiding separate control groups. However, in view of the variations between animals, we do not feel comfortable in comparing with a control group done on another day. Therefore, we are unable to reduce the number of animals in the control group. Also, we have tried to use the minimum number of animals for each experiment based on our prior experience with GTA (earlier protocols) and statistical analysis.

V.3.5.3. Replacement: *In silico* methods and tissue culture approaches cannot be used for evaluation of the questions studied here, since these actions can only be observed in an intact living animal. At the present time, animals lower than rodents in the phylogenetic scale cannot be used to model hemorrhagic shock and/or brain injury in human, because of wide anatomic and physiological differences.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment: NA

V.4.1.1. APHIS Form 7023 Information: NA

V.4.1.1.1. Number of Animals:

	Species/Strain #1	Species/Strain #2
V.4.1.1.1.1. Column C:		
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	**425**	**425**

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: For CCI model of TBI, mice will be initially anesthetized with 3-5 % isoflurane in 100% O2 in a vented anesthesia chamber. Mice under anesthesia will be placed on a stereotaxic instrument -and continuous isofluorane anesthesia (2%) will be given using a nose cone. That mice are under anesthesia will be determined by their lack of response to pinching the toes.

We do not plan to use analgesia for these studies. One of the main purposes of these studies is to evaluate the anti-inflammatory and healing effects/mechanisms of GTA mediated acetylations on CCI and HS induced injuries. Since most of the pain relief agents have some type of anti-inflammatory mechanisms, pain relief efforts in experiment will interfere with the goal of this study.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Animals will be adapted to their environment for at least 5 days prior to the experiments.

V.4.1.2.3. Paralytics: NA

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PubMed, Agricola

V.4.1.3.2. Date of Search: 3/20/2016

V.4.1.3.3. Period of Search: 1966-2016

V.4.1.3.4. Key Words of Search: animal, mouse, pain, analgesia, transcardial perfusion, controlled cortical impact model, hemorrhagic shock

V.4.1.3.5. Results of Search: A total of 452 publications were obtained when -key words animal, mouse, pain and analgesia -were used. However, none appeared when transcardial perfusion or controlled cortical impact or hemorrhagic shock was included in the search. All of the 452 publications involved studies on different aspects of pain, including neural mechanisms and newer pain relieving agents. When key words mouse and transcardial perfusion were combined, 30 publications describing studies involving the use of transcardial perfusion were obtained. None of them provided any alternatives to the propcsed -painfu' procedures. Agricola: the key words combination animal, mouse, pain analgesia provided no publications unlike in the case of PUBMED and therefore adding the other key words was not attempted.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: One of the main purpose of these studies is to evaluate the anti-inflammatory and healing effects/mechanisms on GTA mediated acetylations on CCI and HS induced injuries. Since -most of the pain relief agents have some type of anti-inflammatory mechanisms, pain relief efforts in experiment will interfere with the goal of this study.

V.4.2. Prolonged Restraint: NA

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: The surgical procedures will be performed aseptically following the LAM guidelines. Anesthesia will be induced by isoflurane vapor in 100% oxygen in a commercially available induction chamber. Once anesthesia is induced, animals will be transferred to the designated surgery area and anesthesia will be maintained via nose cone. See Section V 4.1.2.1 Anesthesia/Analgesia/Tranquilization for details.

When the animal is anesthetized, its head will be shaved with a hair clipper. The head will be cleaned with betadine and alcohol, each repeated 3 times alternately. Then the animal wil. be placed into a stereotaxic frame (appropriate frame size will be used for mice) after treating its pressure points such as ears and nose with lidocaine jelly. A heated pad with thermister controlled feedback will be put between the animal's body and the base of the stereotaxic frame. Ophthalmic ointment will be applied to the eyes

to prevent dryness. The body will be covered with a sterile drape with an opening to expose the head.

V.4.3.2. Procedure: Animal surgery, anesthesia and CCI-TBI and HS: For the surgical procedures mice will be anesthetized with 3-5% isoflurane and maintained with 2% isoflurane in100% O2 throughout the experiments. Under sterile conditions, a right groin exploration will be performed and the right femoral artery and vein will be exposed and catheterized using sterile polyethylene (PE-50) tubing (Harvard Apparatus). The catheters will be connected to a blood pressure transducer for continuous arterial blood pressure monitoring during the procedure. The mice will be placed in a stereotaxic frame and a 5 mm bone flap will be removed over the parietal cortex and kept in a sterile location for reuse. Injury will be produced with a 3 mm flat steel tip with a velocity of 5 m/sec and a penetration depth of 1 mm (Dennis et al., 2009). The bone flap will be replaced and the scalp will be closed using sutures. Subsequently, HS will be induced over the course of 15 min by withdrawing blood (approximately 2.0 ml/100gm) using a heparinized syringe (to avoid clotting) until we achieve a MAP between 30 and 35 mm Hg. The MAP will be maintained in this range for 60 min. At the end of the 60 min HS period, mice will be resuscitated to a MAP of at least 60 mm Hg. This will be done over 15 min with their remaining shed blood plus two times the maximum shed blood in lactated Ringer's solution via the same catheters. The catheters will be removed and the incision closed by sutures: sutures will be removed in 4-5 days or as soon as the wounds are healed. Mice will be taken off isoflurane and returned to their cages. Sham-operated mice will undergo anesthesia and femoral cannulation only.

We plan to use a heparinized syringe for withdrawal of blood. Majority of studies in the literature have used the heparinized syringe approach rather than infusion of a heparin solution before the procedure. In view of the fact that heparin infusion could interfere with clotting mechanism with a chance for interference with experimental outcome we opted to use the heparinized syringe approach. **We are planning to catheterize both femoral arteries and a femoral vein.** One femoral artery will be used to draw the blood and other will be used for continuous measurement of MAP. Femoral vein is reserved for reinjection and resuscitation. This method is expected to represent the clinical scenario better.

A search of the literature (Jespersen, B. et al, 2012) showed that the loss of mobility and potential pain in the affected limb because of loss of blood flow after closure of both the major arterial supply and venous return are not real concerns in the proposed model. Distress to the animal is minimal in this procedure (Jespersen, B. et al, 2012).

V.4.3.3. Post-surgical Provisions: After CCI-TBI surgery/HS, animals will be kept in warm condition by a heated blanket or light until they recover from anesthesia. The animals will be observed twice daily after surgery and recovery by our research staff for 4-5 days.

V.4.3.4. Location: (b)(6) (b)(6) facilities at USUHS

V.4.3.5. Surgeon: Dr (b)(6)

V.4.3.6. Multiple Major Survival Operative Procedures: NA

V.4.3.6.1. Procedures: NA

V.4.3.6.2. Scientific Justification: NA

V.4.4. Animal Manipulations:

V.4.4.1. Injections:
1. Fatal Plus (50-85 mg/kg), Intraperitoneal, volume = 100-200 µl for mice, needle size = 25-27 gauge needle for mice.
2. Isoflurane for anesthesia – volatile anesthetic-2-5% in 100% oxygen-FDA approved agent.
3) GTA administration by oral gavage (5 g/kg)– not FDA approved., GRAS status given by FDA, GTA is used extensively in preclinical studies. The PI's group has used GTA for acetate supplementation studies during the last 10 years.
4) Heparin contained in heparinized syringe. FDA approved. Extensively used in similar studies.

V.4.4.2. Biosamples: Tissues such as brain, lung, liver, kidney, heart and blood will be collected post mortem.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: The animals will be identified by cage card. However, individual animals after procedure will be given a particular identifying tattoo **during surgery** on their pinna of the ear, tail, arms or legs.

V.4.4.6. Behavioral Studies: None planned.

V.4.4.7. Other Procedures: Oral gavage: Treatment of mice with GTA to test effectiveness against HS and injury will be done via oral gavage. The details are given in the experimental method section V.1.1 Experiment 1. Animals will be held in a supine position and the blunt needle will be inserted into the mouth, directed at a dorsal caudal direction and gently lowered into the stomach. Projecting the needle to the top part of the mouth will avoid any chance of entering the respiratory tract and the lung. We have extensive experience in doing oral gavage in mice based on our ongoing and prior work.

V.4.4.8. Tissue Sharing: Tissues may be shared with other investigators upon request.

V.4.5. Study Endpoint: Study Endpoint: The animals will be euthanized for tissue collections and also for histological analysis at the designated time points previously described.

Early Endpoint: Any of the following will represent a reason for early euthanasia: inability to ambulate or reach food or water, self-mutilation, continuous circling, or persistent labored respiration or gasping, loss of 20% of weight below baseline (weight taken on alternate days), prostration/inactivity for up to 24 hours. These signs, if observed by animal facility personnel and no study personnel can be reached, may be taken by the veterinarian as cause for euthanasia.

V.4.6. Euthanasia: Some animals in each group will be euthanized by decapitation following injection of a ~75-100 mg/kg dose of pentobarbital based euthanasia solution. Others will be euthanized by transcardial perfusion following euthanasia solution administration (Fatal Plus, 75-100mg/kg or other pentobarbital based euthanasia solution, Intraperitoneal, 200-300µl volume, 25 gauge needle).

Transcardial perfusion will be done as described in our publications (Ariayannur et al, 2010; Arun et al, 2010 b; Mathew et al, 2005; Madhavarao et al, 2009). Briefly, animals will be deeply anesthetized with Fatal Plus (75-100 mg/kg), and the heart will be exposed by opening up the thoracic cavity after confirming that the animal is fully under anesthesia. To monitor the depth of anesthesia we will test whether the animal has lost its toe pinch (pedal withdrawal) reflex. The animals will be perfused with either 10% neutral buffered formalin or 4% freshly depolymerized paraformaldehyde through the left ventricle using a peristaltic pump. The right atrium will be cut and approximately 50-75 ml of fixative solution will be passed through the circulatory system. Following the perfusion, tissues will be removed to a container of fresh fixative solution prior to histopathological analysis.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs.

V.5.1.1. Study Room: CCI-TBI will be conducted in LAM's surgery room or CNRM facilities.

Building(s) (b)(6) Room Number(s) (b)(6) (b)(6)

V.5.1.2.

Special Husbandry Provisions: No separate housing required after surgery

Food Restriction: Yes _____ No X

Fluid Restriction: Yes _____ No X

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: The animals will be observed daily according to LAM SOPs.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Intragastric administration	(b)(6)	Ph.D. 8 years exp. in mice Ph.D., 2 years exp. in mice	Investigator training, 2003, Rodent handling class, 2003 Investigator training, 2014, Rodent handling class,2014
IP injection		Ph.D., 2 years exp. in mice M.Sc., 3 years exp. in mice	Investigator training, 2014, Rodent handling class,2014 Investigator training, 2013, Rodent handling class, 2013
decapitation		Ph.D., 2 years exp. in mice M.Sc., 3 years exp. in mice	Investigator training, 2014, Rodent handling class,2014 Investigator training, 2013, Rodent handling class, 2013
transcardial perfusion		Ph.D., 2 years exp. in mice	Investigator training, 2014, Rodent handling class,2014

	(b)(6)	M.Sc., 3 years exp. in mice	Investigator training, 2013, Rodent handling class, 2013
CCI		Ph.D. 8 years exp. in mice	Investigator training, 2003, Rodent handling class, 2003
HS		M.D., to be trained by our collaborator at WRAIR.	awaiting investigator and rodent handling trainings

VII. BIOHAZARDS/SAFETY: Gowns, gloves, and masks will be used for personal protection and sharp items will be disposed of in separate containers as per safety rules. The volatile anesthetic agents will be properly closed and removed from the procedure area. Chemicals and hazardous waste used in this protocol will be handled in accordance with all applicable state and federal guidelines, regulations, and standing operating procedures. Waste anesthetic gas (isoflurane) will be scavenged passively and exposure to investigators will be minimized using the set up used in the LAM operating room since the surgeries will be done in one of those operating rooms.

A. Zoonotic Disease:None
B. Safety Hazards:None
C. Isoflurane:Yes
D. Isoflurane Exposure: Minimal exposure
E. Sharp Instruments: Yes
F. Infectious Agents that do not cause Zoonoses: None

VIII. ENCLOSURES: References

IX. EXTRAMUAL COLLABORATION:

If there are any collaborations with outside entities, please explain here.

X. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) 4/14/2016

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely,

"Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) [redacted] 4/14/2016

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress ~~**WILL**~~ **or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) [redacted] 4/14/2016

Principal Investigator Signature Date

XI. PROTOCOL ABSTRACT:

Hemorrhage is responsible for about 30-40% of all traumatic deaths and approximately 50% of battlefield deaths worldwide. Studies have suggested that the ability of tissues to deal with hemorrhage related problems such as hypoxia, hypercapnia and hypothermia, is critical and that robustness of this ability differs among individuals, suggesting involvement of genetic or epigenetic mechanisms. Recently it has been shown that acetylation of the transcription factor HIF-2α controls erythropoietin production during pathophysiological stress induced by oxygen deprivation. In earlier studies we have developed acetate supplementation using glyceryl triacetate (GTA), which generates acetate and glycerol on hydrolysis in vivo, to improve outcomes after traumatic brain injury. Based on these and other studies we have found that oral GTA administration is an effective way to increase acetate levels in all tissues and that GTA has no detectable toxic effects on chronic usage even at relatively high doses in animals and children. In subsequent studies, acetate supplementation using GTA has been shown to induce anti-inflammatory effects in multiple rat model systems. Histone deacetylase inhibitors including valproic acid and trichostatin, which increase protein acetylation status in general and histone acetylation status in particular, have anti-inflammatory and protective effects in hemorrhagic shock. This is due in part to the fact that patients who survive acute episodes of blood loss experience systemic inflammatory response syndrome (SIRS) triggered by ischemia-reperfusion injury and organ damage. Based on these earlier findings, we propose to develop oral acetate supplementation using GTA as a way to augment acetylation-based regulatory mechanisms involved in the response to and recovery from bodily injury, blood loss and reperfusion injury. Our immediate goal is to develop GTA treatment as an adjunct therapy for hemorrhagic shock and injury. We and others have shown that acetyl-coenzyme A synthetase-1 (enzyme name: AceCS1, gene: Acss2) is primarily involved in generating acetyl-coenzyme A (acetyl-CoA) for acetylation reactions, especially in cell nuclei, and we currently have an ongoing breeding colony of mice in which the Acss2 gene is knocked out for studying the related genetic mechanisms. Using this gene knockout model we plan to determine the genetic and epigenetic regulatory mechanisms involved in the acetylation-based regulation of stress responses to blood loss and injury, and the beneficial effects of GTA treatment on survival from hemorrhagic shock and injury. Subsequently, we plan to focus efforts on enhancing our understanding of such regulatory mechanisms in humans for improving survival from traumatic hemorrhage and associated shock, providing knowledge that will lead to genetically-informed personalized treatments. Our central hypothesis is that enhancing acetylation-based endogenous genetic/epigenetic mechanisms is an effective way to minimize chronic disability and death due to hemorrhagic shock. We propose to test this hypothesis using GTA-mediated acetate supplementation in a mouse model of hemorrhagic shock with concomitant injury and determine the associated genetic/epigenetic mechanisms using our Acss2 gene knockout mouse model. The long-term goal of the proposed studies is to improve survival for patients with traumatic hemorrhage and associated shock via regulation of genetic and epigenetic response mechanisms and to provide knowledge that will lead to genetically-informed personalized treatments.

A. Animal Protocol Number: (b)(6)

B. Animal Protocol Title: Acetate Supplementation using Glyceryl Triacetate as an Adjunct Therapy for Hemorrhagic Shock and Injury

C. **Principal Investigator**: (b)(6) Ph.D.

D. **Performing Organization**: USUHS

E. **Funding**: CHIRP

F. **Objective and Approach**: Our central hypothesis is that enhancing acetylation-based endogenous genetic/epigenetic mechanisms is an effective way to minimize chronic disability and death due to hemorrhagic shock. We propose to test this hypothesis using GTA-mediated acetate supplementation in a mouse model of hemorrhagic shock with concomitant injury and determine the associated genetic/epigenetic mechanisms using our Acss2 gene knockout mouse model. The long-term goal of the proposed studies is to improve survival for patients with traumatic hemorrhage and associated shock via regulation of genetic and epigenetic response mechanisms and to provide knowledge that will lead to genetically-informed personalized treatments.

G. **Indexing Terms (Descriptors)**: Hemorrhagic shock and injury, Glyceryltriacetate, traumatic brain injury, mouse, animal model, genetic and epigenetic mechanisms, acetyl CoA synthetase gene knockout.

APPENDIX A.

PI NAME: (b)(6) Ph.D.
PROTCOL NUMBER: (b)(6)
DRUGS AND CONTROLLED SUBSTANCES

Please list ALL drugs and controlled substances that will be used under this protocol, indicating the DEA Schedule if known. Provide both the Approved Name and the Proprietary Name of each drug if known.

This list, once approved, will be used by the IACUC and the USU Pharmacy to determine which drugs and controlled substances can be supplied to the Principal Investigator. The Pharmacy will not dispense to the PI any drug that is not included in this list.

Drug Name (Please give both approved and Proprietary Names if possible)	DEA Schedule (I, II, III, or IV) (if known)
1. **Sodium pentobarbital (Fatal Plus)**	II
2.	

Date of Protocol Expiration Date: New Protocol

References

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September 5, 2013

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF MEDICINE

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on September 5, 2013:

Animal Protocol Title: "Gene Therapeutic Approach for Tolerance Induction to Cognate Antigens and EAE in Mice"

USUHS Protocol Number: (b)(6)

Expiration Date: September 4, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc: Office of Research

Revised

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Gene Therapeutic Approach for Tolerance Induction to Cognate Antigens and EAE in Mice

GRANT TITLE (if different from above): Gene Therapeutic Approach for Tolerance Induction

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: NIH/NIAID

EARLIEST ANTICIPATED FUNDING START DATE: 10/1/13

PRINCIPAL INVESTIGATOR: (b)(6) PhD

(b)(6)	MED	(b)(6)	07-24-13
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is cor (b)(6) ractice.

	Chair	(b)(6)	08-02-13
Research Unit Chief / Dept. Head Signature	Title	Telephone	Date

Typed Name: (b)(6) M.D.

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)	MED	(b)(6)	08-02-13
Statistician Signature	Department	Telephone	Date

Typed Name: (b)(6) PhD

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)	LAM	(b)(6)	08-23-13
Attending/Consulting Veterinarian Signature	Department	Telephone	Date

Typed Name (b)(6) VMO

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) Medicine, (b)(6)

ANIMAL PROTOCOL TITLE: Gene Therapeutic Approach for Tolerance Induction to Cognate Antigens and EAE

GRANT TITLE (if different from above): Gene Therapeutic Approach for Tolerance Induction

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6) PhD
(b)(6) PhD
(b)(6) PhD
(b)(6) PhD
(b)(6)

TECHNICIANS(S): (b)(6)

I. **NON-TECHNICAL SYNOPSIS:** Multiple sclerosis (MS) is an immune-mediated disease in which certain cells from the immune system attack and destroy parts of the central nervous system (CNS), in particular the myelin sheath that surrounds nerve fibers and aids in signal transduction and the nerve fibers themselves. This disruption of nerve impulses can be debilitating, with symptoms ranging from mild (limb numbness) to severe (paralysis/loss of vision). While the exact cause of MS is not known, it is believed that a number of factors play a role in disease development, among them an abnormal immune response. It is also believed that infections, genetics and environmental factors may be involved in triggering disease. Our lab has been interested in investigating the role that the immune system plays in MS. We have shown that when antigens are linked to protein, called an immunoglobulin "carrier", they can specifically inhibit the reactivity of the immune system (tolerance) to the linked protein antigens. In this protocol, we will use Experimental Allergic Encephalomyelitis (EAE), a mouse model which resembles MS. This is a well-established experimental model for multiple sclerosis that induces an impaired ability to walk and possibly can cause some muscle paralysis in the mice over a period of days to weeks. Mice will be injected with a protein from mouse brain together with an agent facilitating development of white blood cells (called T cells) that attack brain proteins, leading to tissue destruction. Treatments with modified immune cells from other mice will be tested to determine if such treatments might prevent or alleviate the impaired movement induced by the injection of brain proteins. We also are developing soluble fusion proteins built on the IgG scaffold to facilitate tolerance without cell transfer.

II. **BACKGROUND:**

II.1. Background: Our lab is interested in developing techniques to regulate (eliminate) the immune response to a variety of proteins that are being attacked by the body in autoimmunity. Our goal is to use a type of white blood cell, called B lymphocytes, which have been engineered to display parts of foreign or self materials to the immune system in such a way as to "trick" it into not responding, in order to control autoimmune (anti-self) diseases. We expect, and preliminary results have shown, that transferring these antigens to the blood B lymphocytes will lead to diminished responses to these proteins and curing of autoimmunity in animal models for multiple sclerosis.

Further studies show that we can also trick the immune system by transferring the antigens to the precursor form of B cells found in the bone, the bone marrow cell. The long-term objective is to understand and harness this process so that we can better prevent and reverse the autoimmune attack that occurs in diseases like diabetes or multiple sclerosis (MS). In this protocol, we will use Experimental Allergic Encephalomyelitis (EAE) as an autoimmune disorder resembling MS. It is characterized by a chronic relapsing, and remitting paralysis secondary to central nervous system (CNS) demyelination caused by T cells. Our hypothesis is that tolerance can be induced by our B-cell presentation of foreign or self proteins) delivered via our gene therapy procedure. This somehow tricks the immune system into a state of non-responsiveness (tolerance) and a reversal of autoimmunity. The objective is to optimize this process for future application.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: Biomedical Research Database
RePORTER
Pubmed

II.2.2. Date of Search: July 23, 2013

II.2.3. Period of Search: 1990-2013 (BRD: 1998-2013 only)

II.2.4. Key Words and Search Strategy: Multiple sclerosis/EAE and alternatives to animal use; multiple sclerosis and alternatives to painful procedures; adjuvant alternatives;

II.2.5. Results of Search: No relevant hits; alternatives to footpad injection found and are incorporated. No other relevant hits nor do they represent duplication of work proposed. EAE is an autoimmune neurological disease that cannot be replicated in culture. Moreover, you cannot determine whether a therapy or immunization protocol will work in culture. Mice have been used for studies in immunology for several decades, and are the animal of choice due to extensive knowledge of their genetics, and of murine T and B cells and their interactions. In addition, mice provide useful models for human disease.

III. **OBJECTIVE\HYPOTHESIS:** Our goal is to use an animal model of Multiple Sclerosis (MS) to devise ways to prevent immune responses to nervous system proteins (like myelin). Our hypothesis is that tolerance can be induced by our B-cell presentation of foreign or self proteins) delivered via our gene therapy procedure. This somehow tricks the immune system into a state of non-responsiveness (tolerance) and a reversal of auto-immunity. The objective is to optimize this process for future application.

IV. **MILITARY RELEVANCE:** Autoimmune disorders such as multiple sclerosis (MS) can be severely debilitating. Moreover, there has been an increased incidence of certain autoimmune diseases in veterans of the ongoing wars. Understanding how to reverse these and other undesirable immune responses is important for the general health of the military population and their families. Our research is aimed at reversing the effects of autoimmune diseases and hence is relevant.

V. **MATERIALS AND METHODS:**

- C57Bl/6J (b)(4) male and female, 6-8 weeks of age. These mice are susceptible to MOG-induced EAE

- T-cell receptor transgenic 2D2 mice, are available from (b)(4) Male and female donors, 6-8 weeks of age. These mice possess transgenic T cells that are specific for MOG35-55, a target in EAE.
- Balb/c and DO11.10 are used in an experiment to follow the fate of specific T cells in a non-EAE model, and FoxP3-GFP mice to follow the role of T regulatory cells.

V.1. Experimental Design and General Procedures:

V.1.1. **Experiment 1**: To test whether memory B cells from immune mice are pathogenic or tolerogenic in our gene therapy protocol.

Goal and protocol: To determine whether tolerance to MOG-induced EAE can be induced with B cells from immunized mice, that is, can antigen-specific B cells be tolerogenic or immunogenic for CNS antigen. This is important because autoimmune patients will possess B cells specific for the CNS antigen. This will test the hypothesis that antigen-specific B cell may be tolerogenic or may contribute to pathogenesis whereas non-specific B cells are tolerogenic.

• Mouse strains needed/source: C57BL/6J, (b)(4) male and female, 6-8 weeks of age. These mice are susceptible to MOG-induced EAE (40 donors and 80 recipients per experiment; 360 mice total; JAX)

Experiment I: To determine whether tolerance to MOG-induced EAE can be induced with B cells from MOG-immunized donors; Age = 6-8 weeks

This experiment needs to be performed three times (original plus two repeats) for statistical significance based on the PI's experience with this model. We require:

Group 1: B cells from MOG immunized donors transduced with MOG-IgG (10 donors)
Group 2: B cells from MOG immunized donors transduced with OVA-IgG (10 donors) (specificity)
Group 3: B cells from naive donors transduced with MOG-IgG (10 donors) (tolerant)
Group 4: B cells from naïve donors transduced with OVA-IgG (10 donors) (specificity)

Day -30

Immunize 20 donors (groups 1 and 2) with MOG protein in alum intraperitoneally (i.p.) (10 micrograms in 0.2 ml, 27G needle)

~

Day -9

Prepare B cells from 20 normal C57BL/6J mice (normal polyclonal donors) or from 20 immunized donors, activate with LPS *in vitro*

~

Day -8

Culture activated B cells *in vitro* with MOG35-55 retroviral construct or control, e.g. OVA construct)

~

Day-7

Wash, mix and inject B cells (i.v.; tail; 27G, <0.2 ml) into 4 groups of 20 recipients (80 C57BL/6J)

~

Day 0

Immunization with antigen (MOG35-55) + Adjuvant (complete Freund's adjuvant (CFA)), flanks (s.q.) and base of tail (s.q.) (27G, 0.1 ml total)

~

Daily
Observe clinical symptoms

Day 28 All mice in groups 1-4 will have blood samples taken and will be euthanized to obtain tissues for T-cell proliferation*, record clinical symptoms. Mice are euthanized when they reach stage IV in this protocol if remission does not occur!
This experiment is repeated twice more.

**All euthanized mice are tested for T-cell proliferation in spleen and draining popliteal and inguinal lymph nodes.*

V.1.2. **Experiment 2:** To determine the fate of specific T cells in modulation of EAE (This experiment could not be done in our initial period due to commensals in facility affecting the tolerance protocol).

Goal and protocol: Same general protocol as Experiment 1. We will repeat this basic protocol with an injection of 2D2 T-cell receptor (TCR) transgenic T cells (labeled with carboxy-fluorescein succinimidyl ester, CFSE) into recipients in order to localize their migration under optimum tolerance conditions. In this variation of the basic experiment, MOG-specific TCR transgenic T cells (2D2) will be isolated, labeled with CFSE to trace them, and transferred into normal C57BL/6 recipient mice, which then receive tolerogenic B cells. At various time points during the experiment, mice from each group will be euthanized to obtain lymph node and spleen cells to assay for T cell responses to the test antigen in culture or followed for disease symptoms. The readout is EAE and flow cytometry.

Experiment 2

To determine whether the fate of T cells in tolerance to MOG-induced EAE

Mice;
Each experiment requires 48 C57BL/6 recipient mice, 24 C57BL/6 donor mice and 12 TCR transgenic 2D2 mice. Thus, 24 C57BL/6 donors and 48 recipients (x3) = 216; 12 2D2 donors per expt. (x3=36). Male and female, age 6-8 weeks.

This experiment needs to be performed three times (original plus two repeats) for statistical significance based on the PI's experience with this model.

Day -9
Prepare B cells from 24 normal C57BL/6 mice (normal polyclonal donors), activate with LPS *in vitro*
~

Day -8
Culture activated B cells *in vitro* with MOG35-55 retroviral construct or control, e.g. OVA construct
Day-8
Inject TCR transgenic 2D2 T cells (12 donors) i.v. (27G, <0.2 ml) labeled with CFSE into two groups of 12 recipients; 24 recipients do not receive 2D2 T cells
~

Day-7
Wash and inject B cells into 4 groups of 12 recipients

Group 1: B cells from naive donors transduced with MOG-IgG (plus 2D2 T cells)
Group 2: B cells from naïve donors transduced with OVA-IgG (plus 2D2 T cells)
Group 3: B cells from naive donors transduced with MOG-IgG (tolerant)
Group 4: B cells from naïve donors transduced with OVA-IgG (specificity)

~

Day 0

Immunization with Antigen (MOG35-55) + Adjuvant (CFA), flank (s.q.) and base of tail (s.q.) (27G, 0.1 ml total)

~

Day 3, 7, 14, 21

On days 3,7,14, and 21, three mice per group will be euthanized and spleens removed. Spleen cells will be examined for presence of CFSE-labeled T cells (to follow fate of T cells), both in numbers and for amount of proliferation.

Mice are observed for clinical symptoms on days 7-21 and are euthanized when they reach stage IV in this protocol if remission does not occur!

V.I.3. **Experiment 3:** To determine the fate of specific T cells in B-cell delivered gene therapy for a non-CNS-derived antigen

- Mice needed and source: Balb/c (216) and D011.10 (36), from (b)(4) are used for experiment 3 to follow the fate of specific T cells in a non-EAE model.

Goal and protocol: Same general protocol as Experiment 2. We will repeat this basic protocol with an injection of D011.10 TCR transgenic T cells (CFSE labeled) into recipients in order to localize their migration under optimum tolerance conditions with a non-CNS antigen. To follow the fate of the T cells, Ovalbumin-specific DO11.10 TCR transgenic T cells will be isolated, labeled with CFSE to trace them, and transferred into normal Balb/c recipient mice, which then receive tolerogenic B cells. At various time points during the experiment, mice from each group will be euthanized to obtain lymph node and spleen cells to assay for T cell responses to the test antigen in culture and antibody to OVA.

Experiment 3
To determine whether the fate of specific T cells in tolerance induced by OVA expressing B cells
Mice: Number per expt. Total Balb/c, (b)(4) male and female 72 donors and 144 recipients = 216.
Age =6-8 weeks
TCR transgenic DO11.10 mice 12x3=36 donors; Male and female, age 6-8 weeks.
This experiment needs to be performed three times (original plus two repeats) for statistical significance based on the PI's experience with this model.

Day -2

Prepare B cells from 24 normal Balb/c mice, activate with LPS *in vitro*

~

Day -1

Culture activated B cells *in vitro* with OVA-IgG retroviral construct or control, e.g. OVA construct) or control (MOG or other irrelevant antigen)

~

Day 0

Inject transduced B cells i.v. (27G, <0.2 ml) into Balb/c recipients

~

Day 1

Inject (i.v.) TCR transgenic DO11.10 T cells (12 donors) labeled with CFSE into 2 groups of 12 recipients; 24 mice do not receive DO11.10 T cells as controls

~

Day 3, 7, 14 and 28

Isolate spleen cells from 3 mice per group and examine for presence of CFSE-labeled T cells (to follow fate of T cells), both in numbers and for amount of proliferation. We already know this protocol leads to tolerance to OVA peptides, which is validated by T-cell proliferation to OVA peptide *in vitro*.

V.1.4. **Experiment 4:** To determine the role of FoxP3 T regulatory cells in B-cell delivered gene therapy. Same general protocol as Experiment 3. We will repeat the basic protocol except that DO11.10-FoxP3-GFP donors are used to follow the role of T regulatory cells in tolerance. The rationale is that these donor DO11.10-FoxP3-GFP, a marker of Tregs, will appear green when activated. These mice then receive tolerogenic B cells. At various time points during the experiment, mice from each group will be euthanized to obtain lymph node and spleen cells to assay for the increase in T regulatory (GFP+) cells.

• Mice needed and source: Balb/c [(D)(4)] and DO11.10-FoxP3-GFP (USU), are used as in experiment 3 to follow the role of specific T cells in a non-EAE model.

Experiment 4

To determine the role of regulatory T cells in tolerance

This experiment is repeated once

Day -2

Prepare B cells from 24 normal *Balb/c* mice, activate with LPS *in vitro*

~

Day-l

Culture activated B cells *in vitro* with OVA-Ig retroviral construct or control (e.g. MOG or other irrelevant antigen)

~

Day 0

Inject transduced B cells i.v. (27G, <0.2 ml) into 48 recipients + DO11.10-FoxP3-GFP TCR transgenic to trace Tregs into 24 recipients

~

Day 3, 7, 14 and 28

Isolate spleens from 3 mice per group and check for CFSE staining by flow cytometry (we already know this protocol leads to tolerance to OVA peptides, which is validated by T-cell proliferation to OVA peptide *in vitro*.

Experiment 5

GOAL: To determine whether fusion IgG's we have developed can induce tolerance to MOG-induced EAE

Mice;
Each experiment requires 40 C57BL/6 recipient mice (120 total)
This experiment needs to be performed three times (original plus two repeats) for statistical significance based on the PI's experience with this model.

Day-7
Wash and inject fusion IgG cells into 4 groups of 10 recipients (all injections i.v. 27G, <0.2 ml)

Group 1: Inject with scCD20 IgG MOG (10 micrograms)
Group 2: Inject with scCD20 IgG OVA (10 micrograms)
Group 3: Inject with scCD20 IgG MOG (50 micrograms)
Group 4: Inject with scCD20 IgG OVA (50 micrograms)

~

Day 0
Immunization with antigen (MOG35-55) + Adjuvant, flanks (s.q.) and base of tail (s.q.) (27G, 0.1 ml total)

~

Daily
Observe clinical symptoms

~

Day 28
Sample bleed and/or euthanize to obtain tissues for T-cell proliferation*, record clinical symptoms. Mice are euthanized when they reach stage IV in this protocol if remission does not occur!
This experiment is repeated twice more.

**All euthanized mice are tested to T-cell proliferation in spleen and draining popliteal and inguinal lymph nodes.*

V.2. Data Analysis: The significance of the responses will be analyzed by students' t test when appropriate sized groups or distributions are observed. In other cases, a statistician will be invited to examine the data.

V.3. Laboratory Animals Required and Justification: All of our studies require animals with an intact immune system and therefore cannot be replicated *in vitro*. Mice have been used for studies in immunology for several decades, and are the animal of choice due to extensive knowledge of their genetics, and of murine T and B cells and their interactions. In addition, mice provide useful models for human disease. Indeed, immunization of mice with myelin proteins mimics the symptoms of multiple sclerosis in humans. Thus, these mice are an excellent model for MS. We use mice that express T -cell receptor transgenes in order to examine the role of specific T cells in tolerance, and the fate of the latter in 2 different models, CNS-specific or non-CNS (OVA) reactive.

We also use mice that express a Green Fluorescent Protein (GFP) when a gene for FoxP3 (involved in regulation of immune responses) is expressed. These mice will allow us to follow the consequences of our treatment protocols on the development of regulatory T cells, and importantly will enable us *to* isolate and studies these T cells.

V.3.1. Non-animal Alternatives Considered: The ability to form antibodies or develop autoimmune responses requires *in vivo* immunization. *In vitro* experiments cannot address the questions. *There are NO non-animal alternatives to address these questions.*

V.3.2. Animal Model and Species Justification: Mice have been used for studies in immunology for several decades, and are the animal of choice due to extensive knowledge of their genetics, and of murine T and B cells and their interactions. In addition, mice provide useful models for human disease. Indeed, immunization of mice with myelin proteins mimics the symptoms of multiple sclerosis in humans. Thus, these mice are an excellent model for MS

V.3.3. Laboratory Animals

		Species #1
V.3.3.1.	Genus & Species:	Mouse (*Mus musculus)*
V.3.3.2.	Strain/Stock:	See Table below
V.3.3.3.	Source/Vendor:	See below
V.3.3.4.	Age:	6-12 weeks
V.3.3.5.	Weight:	20-25 g
V.3.3.6.	Sex:	M and F
V.3.3.7.	Special Considerations:	See below

V.3.4. Number of Animals Required (by Species):

Species	Strain	Weight/Age	Sex	Total # *(for 3 yrs)*
Mice	C57BL/6	25g/>6 wks	M/F	**696**
Mice	Balb/c	25g/>6 wks	M/F	**264**
Mice	DO11.10 (Balb/c) TCR transgenics	25g/>6 wks	M/F	**36**
Mice	*DO11.10/FoxP3-GFP knockin	25g/>6 wks	M/F	**96**
Mice	2D2 TCR transgenic	25g/>6 wks	M/F	**36**

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:
All of our studies require animals with an intact immune system and therefore cannot be replicated *in vitro*. Mice have been used for studies in immunology for several decades, and are the animal of choice due to extensive knowledge of their genetics, and of murine T and B cells and their interactions. In addition, mice provide useful models for human disease. Indeed, immunization of mice with myelin proteins mimics the symptoms of multiple sclerosis in humans. Thus, these mice are an excellent model for MS. Appropriate anesthesia will be used when performing retro-orbital blood collection in an effort to reduce pain and distress. Soft bedding, enrichment pads, and gel nourishment will be used in all EAE related experiments. Analgesia is not recommended in our

experiments, as it may strongly influence the fate of T-cell subsets studied in our experiments. Analgesics may interfere with these studies since they are known to modulate the immune response. For example, see Carrigan KA, Saurer TB, Ijames SG and Lysle DT, 2004. Buprenorphine produces naltrexone reversible alterations of immune status *Internat. Immunopharmacol.* 4:419-28 for details and for additional citations. One refinement we intend to incorporate into this protocol is to inject antigen/adjuvant into the flanks and base of tail (SQ) as an alternative to footpad injection.

V.3.5.2. **Reduction:** Our experiments require tissue donors of bone marrow-derived B cells (LPS activated B cells), which will be harvested and retrovirally transfected for transfer. Based on experience, each experimental group needs to have 10-12 mice for statistical and biologic relevance (see references below). To estimate sample size of recipients needed to reach a statistical significance with 95% confidence using a two-tailed method where beta is 0.20, a computer program was used. Mean and standard deviation values from previous experiments performed under this protocol were used in the program. As part of these studies, we need to establish the role of regulatory T cells (and specific T cells) in tolerance.

We use one donor for every 2 recipients, and transfer B cells transduced with viral vectors for Ig fusions proteins with MOG or OVA (control) that are target antigens in MS (as well as control constructs). Therefore, groups of at least 20-24 mice are needed as recipients, and 10-12 mice as donors, and each experiment is repeated for statistical significance. The experiment is repeated for statistical significances based on the PI's experience with this model and precedence in the field.

The estimated total number of mice is based on the principal investigator's extensive experience in this experimental system, the requirement to repeat experiments for validation and to obtain significance. We have requested this annual total of mice as determined by many years of prior experience in this and similar experimental systems (historical justification; see references below). The mice will be used in a series of linked studies that involve the induction of tolerance via gene therapy of transplanted B cells, the fate of target T cells and the role of regulatory T cells, and the assessment of clinical symptoms and immune responses. While it is impossible to detail the outcome of all experiments and to predict the specific fate of any single experimental group ahead of time as experiments are planned based on ongoing results, a typical set of experiments is delineated below. Every effort is made to ensure the scientific utility of data derived from each animal used in this study by monitoring, evaluating and modifying group size as needed to obtain clear and consistent data. Every effort will be made to minimize the numbers of mice used without compromising research results.

V.3.5.3. **Replacement:** No computer or *in vitro* simulation is available as replacement.

V.4. **Technical Methods:** Tissue Harvest: Mice whose tissues will be used as donor will be euthanized via CO_2 asphyxiation as described in Section V.4.6. Spleens and/or lymph nodes are removed aseptically, and B cells are purified using magnetic beads (positive selection) or antibody + complement (negative selection), then stimulated *in vitro* with Lipopolysaccharide (LPS) for retroviral transduction. Retroviral vectors containing the CNS proteins (or OVA) fused to IgG heavy chain are used to transduce the cells via standard procedures. These B cells are then adoptively transferred into naïve or immunized mice of each strain to test our tolerogenic protocol.

Monitoring of EAE: Experimental Allergic Encephalomyelitis (EAE) is an autoimmune disorder characterized by a chronic relapsing, and remitting paralysis secondary to central nervous system (CNS) demyelination caused by T cells, and mice can go into remission even after development of stage III disease. Animals are monitored daily and investigators will inform the animal care staff of

the condition of the animals so that they can be carefully monitored. Mice will be monitored by the above investigators for disease daily and EAE will be scored as follows: (I) flaccid tail, (II) partial paralysis of 1 or 2 limbs or flaccid tail with ataxia, (III) total paralysis of 2 limbs, (IV) paralysis of more than 2 limbs, (V) moribund. All mice will be weighed at stage III of EAE, when paralysis

has developed in both hind limbs, we will start putting paper bedding in the cages (a much softer bedding the conventional one). At this stage, we will also provide saturated gel so that the mice don't become dehydrated. Hydration will be monitored by physical examination. Moreover, at this stage III, the animal will be relocated to a separate cage if there are signs of injury by other animals. Food pellets will also be put on the cage floor for easier access. In animals at grade III or IV, the urinary bladders will be expressed daily by the investigators. Heat supplementation will be provided if necessary.

Note that due to the apparent severity of symptoms relative to access to food, water, urine and possibly fecal retention, we will continue to fully document each animals grade classification with daily observations and performance of functions, i.e., body weight, hydration, evidence that food has been accessed, urine expression, etc. These records will be monitored periodically by a consulting veterinarian, and the IACUC.

Alternative Endpoints: (b)(4)

(b)(4)

(b)(4) Additionally, if animals show continued lack of mobility and access to food or become moribund (i.e. display ruffled fur, immobility using front paws, dehydration, 20% weight loss) they will be euthanized. In order to determine weight loss, mice will be weighed at the time of immunization and then monitored periodically after that.

*This period is based on the fact that a number of mice can recover within this period of time, and that in many cases, additional analysis of the phenotype of cells in the peripheral blood may need to be followed to understand mechanisms (rise in suppressor T cells, cytokine profiles, etc.). Note that the paralysis per se is painless, so that the main issue is access to food and water, which we provide daily. Under other circumstances, however, mice are euthanized at 60 days after immunization.

See below for further details.

Euthanasia & Tissue Harvest: Mice will be euthanized via C02 asphyxiation followed by cervical dislocation. The following tissues will be harvested: spleen and draining lymph nodes (popliteal and inguinal chain):

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals: 1164

		Species: Mus musculus #1 (C57BL/6)	Species: Mus musculus #2 (Balb/c)
V.4.1.1.1.1.	Column C:	228	264
V.4.1.1.1.2.	Column D:		
V.4.1.1.1.3.	Column E:	504	

		Species: Mus musculus #3 (DO11.10)	Species: Mus musculus #4 (2D2)
V.4.1.1.1.1.	Column C:	132	36
V.4.1.1.1.2.	Column D:		
V.4.1.1.1.3.	Column E:		

V.4.1.2. Pain Relief / Prevention:

Experimental Allergic Encephalomyelitis (EAE) is an autoimmune disorder characterized by a chronic relapsing, and remitting paralysis secondary to central nervous system (CNS) demyelination caused by T cells, and mice can go into remission even after development of stage III disease. Animals are monitored daily and investigators will inform the animal care staff of the condition of the animals so that they can be carefully monitored. **At stage III of EAE, when paralysis has developed in both hind limbs, we will start putting paper bedding in the cages (a much softer bedding the conventional one). At this stage, we will also provide saturated gel so that the mice don't become dehydrated. Hydration will be monitored by physical examination. Moreover, at stage III, the animal will be relocated to a separate cage if there are signs of injury by other animals. Food pellets will also be put in the bedding to make easy access to food. In animals at grade III or IV, the urinary bladders will be expressed daily by the investigators. Heat supplementation will be provided if necessary.**

Note: due to the apparent severity of symptoms relative to access to food, water, urine and possibly fecal retention, we will continue to fully document each animals grade classification with daily observations and performance of functions, i.e., body weight, hydration, evidence that food has been accessed, urine expression, etc. These records will be available to be monitored periodically by a LAM veterinarian, and the IACUC.

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: General anesthesia (isoflurane) and topical anesthesia (tetracaine) will be used for retro-orbital bleeding. The procedure for isoflurane anesthesia is as follows: Mice will be anesthetized with isoflurane + 100% oxygen, 500-1000ml/min (0.5-4% induction, 0.5-3% maintenance). Isoflurane will be delivered using a precision vaporizer via a rodent anesthesia machine. Induction will occur in an appropriately-sized clear viewing chamber. Following induction, mice will be moved to the stereotaxic device and maintained via nose cone. Lack of paw-pinch reflex will be used to ensure adequate depth of anesthesia prior to beginning procedure. Waste anesthetic gases will be passively scavenged using a charcoal filter. Topical anesthetic eyedrops will be applied directly to the eye. Analgesia is not recommended in our experiments, as it may strongly influence the fate of T-cell subsets studied in our experiments. Analgesics may interfere with these studies since they are known to modulate the immune response. For example, see Carrigan KA, Saurer TB, Ijames SG and Lysle DT, 2004. Buprenorphine produces naltrexone reversible alterations of immune status *Internat. Immunopharmacol.* 4:419-28 for details and for additional citations. Euthanasia of mice does not require monitoring for pain as the CO2 euthanasia is a non-painful procedure. Collection of blood from the vein tail is a minimal distressful procedure that will take less than 1 min per mouse.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: After tail snips (for genotyping), ear punching (for identification), blood withdrawal, and injections mice will be observed for a period of time to be certain they are not experiencing excessive pain and/or distress or bleeding. For procedures that require it, silver nitrate and/or clean gauze/paper towel will be used to stop any bleeding that occurs. During the course of experiments involving EAE, animals will be observed daily to monitor any paralysis and to ensure that the animals have access to food and water.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: Animal Welfare Information Center (AWIC), PubMed, Agricola

V.4.1.3.2. Date of Search: July 29, 2013.

V.4.1.3.3. Period of Search: 1990-2013

V.4.1.3.4. Key Words of Search: Animal testing, Multiple Sclerosis/EAE animal alternatives, mice, multiple sclerosis, pain, alternatives to complete Freund's adjuvant, alternatives tc footpad immunization, alternatives to painful or distressful procedures in mice

V.4.1.3.5. Results of Search: 3 references, one relevant. For example: Rodrigues DH, Sachs D, Teixeira AL. Mechanical hypernociception in experimental autoimmune encephalomyelitis. Arq Neuropsiquiatr. 2009 Mar;67(1):78-81. Footpad injections replaced by flank and base of tail (SubQ).

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: Injections of adjuvants (complete Freund's adjuvant, CFA) are necessary to obtain significant immune responses to some of our experimental proteins. See V.4.1.2.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: N/A

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: Intravenous (i.v.) Tail Vein Injections: Under constant surveillance, mice will be placed in a clean cage under a heat lamp for 1-3 minutes, so that tail veins will dilate. One at a time, the mice will be removed from the cage, placed in the Mouse Tail Vein Illuminator device (Braintree Scientific) for restraint, tail wiped with a 70% ethanol soaked gauze pad, and using a 27G

needle attached to a 0.5cc syringe injected with 100-200µl cells or fusion constructs diluted in sterile saline depending on the experiment.

Intravenous (i.v.) Retro-Orbital Injection (alternative to tail vein): The animal is anesthetized with isoflurane/tetracaine and placed on a clean paper towel on the bench top with eye to be injected facing up. Using the thumb and forefinger, gently retract the skin around the eye so that the eyeball protrudes slightly. Using a 27G needle attached to either a 0.5cc or 1cc syringe, insert the needle bevel up into the medial canthus until it hits the orbital bone and pull back slightly. The needle should be at a 45 degree angle to the nose. Inject 100-200µl. Slowly remove the needle and apply light pressure with sterile gauze to control any bleeding that may occur. Care should be taken to avoid applying too much pressure around the trachea, which may result in cutting off the animal's airway. Also, avoid inserting the needle too far into the retro-orbital space as this may puncture the orbital bone behind the eye, causing the agent to be injected into the skull. Finally, care must be taken to prevent movement of the needle once it is inserted to avoid rupturing the blood vessels behind the eye. A minimum of one day between injections is required and no more than two injections per mouse (one per eye) will be given. This procedure is well tolerated by the animals and is routinely used in many laboratories.

Intraperitoneal (i.p.) Injections: All i.p. injections will use a 27G needle attached to either a 0.5cc or 1cc syringe in a volume of 200µl. Prior to injection, site will be swabbed with 70% ethanol. Sterile saline will be used as a diluent. (experiment 1, MOG + Alum is ip)

Subcutaneous (s.c.) Injections: All s.c. injections will use a 27G needle attached to a 0.5cc or 1cc syringe. Total volume injected will not exceed 100µl. Prior to injection, site will be swabbed with 70% ethanol.

Blood Collection: Blood collection will occur through the retro-orbital sinus. The procedure is as follows:

Mice are placed in the chamber of the isoflurane vaporizing machine until they are sufficiently anesthetized. Mice are then removed from the chamber by the base of tail and placed on their side on a clean surface. The mice are then grasped by the scruff of the neck using the thumb and index finger. A clean capillary tube is then placed near the posterior portion of the eye and then slowly inserted into the retro-orbital sinus and gently rotated between the fingers until blood flow commences. No more than 200µl shall be collected. Once the capillary tube is removed, gentle pressure will be applied to the eye with a clean gauze pad/towel. Mice will be observed for a sufficient period of time post bleed to confirm bleeding has stopped.

V.4.4.2. Biosamples: Blood collection: 0.2mL blood collected with mouse under anesthesia (isoflurane). Frequency of bleeds is listed within each experiment.

Tissue collection: Draining lymph nodes and spleen will be removed after euthanasia.

V.4.4.3. Adjuvants: Complete Freund's Adjuvant (CFA), Alum

Our lab is interested in developing techniques to regulate (eliminate) the immune response to a variety of proteins that are being attacked by the body in autoimmunity. We are researching ways to tolerize the immune system to prevent destruction of the body's own cells. Specifically, we are studying a mouse model of multiple sclerosis known as EAE. This is a well-established model that requires the use of complete Freund's adjuvant (CFA) to elicit a robust immune response. We have attempted to use alternative adjuvants in the past, however we have found that they do not sufficiently stimulate the immune system.

In our study, the use of CFA will be limited to the initial immunization with antigen. The route of administration will be subcutaneous into the flank and base of tail (0.05mL/site). Prior to immunization, the injection site will be clipped of fur and swabbed with betadine and alcohol. Post injection, animals will be monitored daily for any adverse reactions. If necessary, the injection site will be disinfected and bandaged. If the animal is observed to be in pain/distress and that pain/distress cannot be alleviated, euthanasia will be employed.

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Ear Clip: mice will be restrained by hand and ear(s) will be punched using a commercially available ear punch for identification purposes. Alternatively, mice may have numbered tags attached to their ears.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: N/A

V.4.4.8. Tissue Sharing: N/A

V.4.5. Study Endpoint: Euthanasia. Euthanasia is allowed for veterinary staff if animals appear moribund and PI staff informed.

V.4.6. Euthanasia: Using a compressed gas cylinder containing CO_2 and a flow rate of > 2 liters per minute, introduce 100% CO_2 into the cage containing the mice. Within two minutes the mice should be unconscious. Observe mice for a period to confirm that they have stopped breathing. Remove mice from the cage and ensure death by cervical dislocation.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:
Building(s) (b)(6) Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes		No	X
Fluid Restriction:	Yes		No	X

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Monitor animals on a daily basis for unexpected illness/injury.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Colony management Animal observation Injections Retro-orbital blood collection CO_2 Euthanasia/cervical dislocation Genotyping Anesthesia	(b)(6)	He has fifteen years of experience in mouse related work and will be further trained in all procedures relevant to this protocol by Dr. (b)(6)	USUHS Investigator training, Fall 2010
Animal observation Injections Retro-orbital blood collection CO_2 Euthanasia/cervical dislocation Genotyping Anesthesia		He has twelve years of experience in mouse related work and will be further trained in all procedures relevant to this protocol by Dr. (b)(6)	USUHS Investigator training, Fall 2010
Animal observation Injections Retro-orbital blood collection CO_2 Euthanasia/cervical dislocation Genotyping Anesthesia		He has eight years of experience in mouse related work and will be further trained in all procedures relevant to this protocol by Dr. (b)(6)	USUHS Investigator training, Rodent Handling, Spring 2011
Animal observation Injections Retro-orbital blood collection CO_2 Euthanasia/cervical dislocation Genotyping Anesthesia		She has three years of experience in mouse related work and will be further trained in all procedures relevant to this protocol by Dr. (b)(6)	USUHS Investigator training, Rodent Handling Spring 2011
Animal observation Injections Retro-orbital blood collection		He has five years of experience in mouse related work and will	USUHS Investigator training, Spring 2012

CO_2 Euthanasia/cervical dislocation Genotyping Anesthesia		be further trained in all procedures relevant to this protocol by Dr. (b)(6)	
Animal observation Injections Retro-orbital blood collection CO_2 Euthanasia/cervical dislocation Genotyping Anesthesia	(b)(6)	He has five years of experience in mouse related work and will be further trained in all procedures relevant to this protocol by Dr (b)(6)	USUHS Investigator training, Rodent Handling, Spring 2011
Principal Investigator Animal observation		He has over 40 years of experience in mouse related work.	

VII. BIOHAZARDS/SAFETY:

VIII. ENCLOSURES:

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. **Animal Use:** The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. **Duplication of Effort:** I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. **Statistical Assurance:** I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. **Biohazard\Safety:** I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. **Training:** I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. **Training:** I verify that I have attended the USUHS Investigator/Animal User Training Course.

G. (b)(6) _____ 08/01/13

Principal Investigator Signature — Date

G. **Training:** The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. **Responsibility:** I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

I. (b)(6) _____ 08/01/13

Principal Investigator Signature — Date

Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature

08/01/13
Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: Gene Therapeutic Approach for Tolerance Induction to Cognate Antigens and EAE in Mice

C. Principal Investigator: (b)(6) PhD
(b)(6)

D. Performing Organization: USUHS

E. Funding: NIH/NIAID

I. **F. Objective and Approach:** Multiple sclerosis (MS) is an immune-mediated disease in which certain cells from the immune system attack and destroy parts of the central nervous system (CNS), in particular the myelin sheath that surrounds nerve fibers and aids in signal transduction and the nerve fibers themselves. This disruption of nerve impulses can be debilitating, with symptoms ranging from mild (limb numbness) to severe (paralysis/loss of vision). While the exact cause of MS is not known, it is believed that a number of factors play a role in disease development, among them an abnormal immune response. Our lab has been interested in investigating the role that the immune system plays in MS. We have shown that when antigens are linked to protein, called an immunoglobulin "carrier", they can specifically inhibit the reactivity of the immune system (tolerance) to the linked protein antigens. In this protocol, we will use Experimental Allergic Encephalomyelitis (EAE), a mouse model which resembles MS. This is a well-established experimental model for multiple sclerosis that induces an impaired ability to walk and possibly can cause some muscle paralysis in the mice over a period of days to weeks. Mice will be injected with a protein from mouse brain together with an agent facilitating development of white blood cells (called T cells) that attack brain proteins, leading to tissue destruction. Treatments with modified immune cells from other mice will be tested to determine if such treatments might prevent or alleviate the impaired movement induced by the injection of brain proteins. We also are developing soluble fusion proteins built on the IgG scaffold to facilitate tolerance without cell transfer.

G. Indexing Terms (Descriptors): animals, EAE, MS, tolerance

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
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BETHESDA, MARYLAND 20814-4712
http://www.usuhs.mil

June 19, 2013

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF MEDICINE

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on June 19, 2013:

Animal Protocol Title: "T Cell Trafficking and Retention in an Autoimmune Mouse Model"

USUHS Protocol Number: (b)(6)

Expiration Date: June 18, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Vice-Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

Revised

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: T cell trafficking and retention in an autoimmune mouse model

GRANT TITLE (if different from above): Molecular mechanisms of secondary T cell recruitment to established CNS lesions

USUHS PROJECT NUMBER: (b)(6)
FUNDING AGENCY: (b)(4) USUHS

EARLIEST ANTICIPATED FUNDING START DATE: 10/01/2010

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6) MED (b)(6) 4/4/13
Principal Investigator Signature Department Office/Lab Telephone Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific th good scientific research practice.

(b)(6) Vice Chair Res. (b)(6) 4/17/13
Research Unit Chief / Dept. Head Signature Title Telephone Date
Typed Name:

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the st

(b)(6) MED (b)(6) 4/17/13
Statistician Signature Department Telephone Date
Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) LAM (b)(6) 17 Apr 2013
Attending/Consulting Veterinarian Signature Department Telephone Date
Typed Name (b)(6) DVM

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) MED, (b)(6)

ANIMAL PROTOCOL TITLE: T cell trafficking and retention in an autoimmune mouse model

GRANT TITLE (if different from above): Molecular mechanisms of secondary T cell recruitment to established CNS lesions

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): N/A

TECHNICIANS(S): N/A

I. NON-TECHNICAL SYNOPSIS: Autoimmune diseases are extremely complex disorders that result from the immune system attacking its own tissues. Autoimmune diseases, including multiple sclerosis (MS), affect approximately 10 million individuals in the United States alone. Here we will study one of the more prevalent autoimmune diseases, MS, a disease that can result in severe and chronic disability. MS is an autoimmune disease in which the immune system attacks the myelin sheath that is essential for transduction of nerve impulses in neurons. MS is a progressive disease which can initially cause multiple neurological symptoms and ultimately leads to paralysis, disability and death. The immune processes leading to autoimmunity in MS is initiated and coordinated by T lymphocytes, a type of white blood cell that also directs healthy immune responses. T lymphocytes that are specific for endogenous products (autoantigens) become activated and traffic to the central nervous system (CNS) or brain in the case of MS. The goal of this study is to understand the mechanisms T cells use to traffic to and be retained within the CNS using a mouse model of MS. We will analyze the extrinsic and intrinsic signals that control T cell migration and retention within the CNS during neuroinflammatory disease.

II. BACKGROUND:

II.1. Background:. In these studies, multiple techniques will be utilized to determine how T cell migrate through tissue in the steady state and during inflammation, and their fate following induction of inflammatory lesions. Analysis of the role of T cells in autoimmune disease requires the use of animal models and there are well-characterized models of autoimmune diabetes and MS in mice. For induction of autoimmunity in mice, we will use established models for experimental autoimmune encephalomyelitis (EAE) (the mouse model for MS). To generate a population of autoreactive T cells that can be analyzed and tracked, we will utilize adoptive transfer techniques for induction of EAE. This system will utilize an *in vivo* priming/adoptive transfer method to allow introduction of pathogenic T-cells without the potential experimental complications associated with direct induction of disease. This adoptive transfer model will be utilized to assess the phenotype and functional capabilities associated with T-cell trafficking to the central nervous system, the site of pathogenesis for MS. Further, we will examine the importance of recognized adhesion molecules,

including VLA-4 and LFA-1 and effector functions including interleukin 17 (IL-17) and interferon gamma (IFN-γ) to the pathogenesis of EAE. (b)(4)

(b)(4)

We hypothesize that the experiments outlined in this proposal will allow us to identify the signals and properties of T cells that invade tissues in autoimmune disease and determine their fate once tissue destruction in achieved. These findings will also allow us to identify specific T-cell components necessary for disease induction. Ultimately, the information found through the proposed study would demonstrate novel targets for therapeutic treatment of autoimmune diseases such as MS. All of the animal studies and experiments described in this protocol are supported by either a grant from the (b)(4) or Institutional Start-up Funds.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: DOD Biomedical Research Database, NiH Reporter, PubMed

II.2.2. Date of Search: 03/29/2013 (DOD BRD, NiH Reporter), 5/22/13 (PubMed)

II.2.3. Period of Search: 1990-2013

II.2.4. Key Words and Search Strategy: "Multiple Sclerosis T cells trafficking, Multiple Sclerosis T cells retention, EAE T cells retention" for NIH reporter and DOD BRD, Rodentia"[Mesh] AND (migration OR trafficking OR retention) AND ("Multiple Sclerosis"[Mesh] OR "Encephalomyelitis, Autoimmune, Experimental"[Mesh]) AND "T-Lymphocytes"[Mesh] for PubMed

II.2.5. Results of Search: 242 hits, none duplicative. No relevant results were found. our studies will be describing currently unknown pathogenesis pathways relating to T cell retention within the brain and spinal cord. At this time we are the leading edge of this topic of inquiry. .

III. OBJECTIVE\HYPOTHESIS: Our objective is to use an animal model of Multiple Sclerosis (MS) to identify vital components of nervous system inflammation in order to target these components for therapeutic intervention in humans. Our hypothesis is that T cell retention is a requisite component for induction of autoimmune lesions in the brain and spinal cord. We expect that disruption of the processes utilized by T cells to remain within the nervous system would prevent or even reverse the associated inflammation and clinical symptoms.

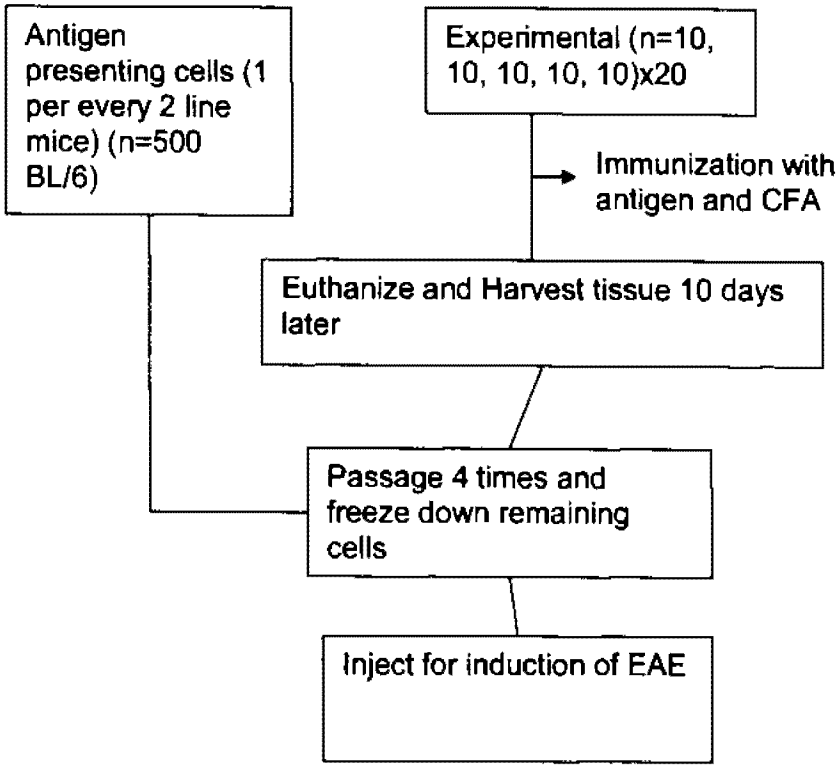
IV. MILITARY RELEVANCE: Multiple sclerosis (MS) can be debilitating and is one of the most common causes of disability in non-elderly adults. Our research is aimed at modulating the effects of neuroinflammation. Understanding how to reverse

neuroinflammatory states such as those found in MS is relevant to the general health of the military population and their families.

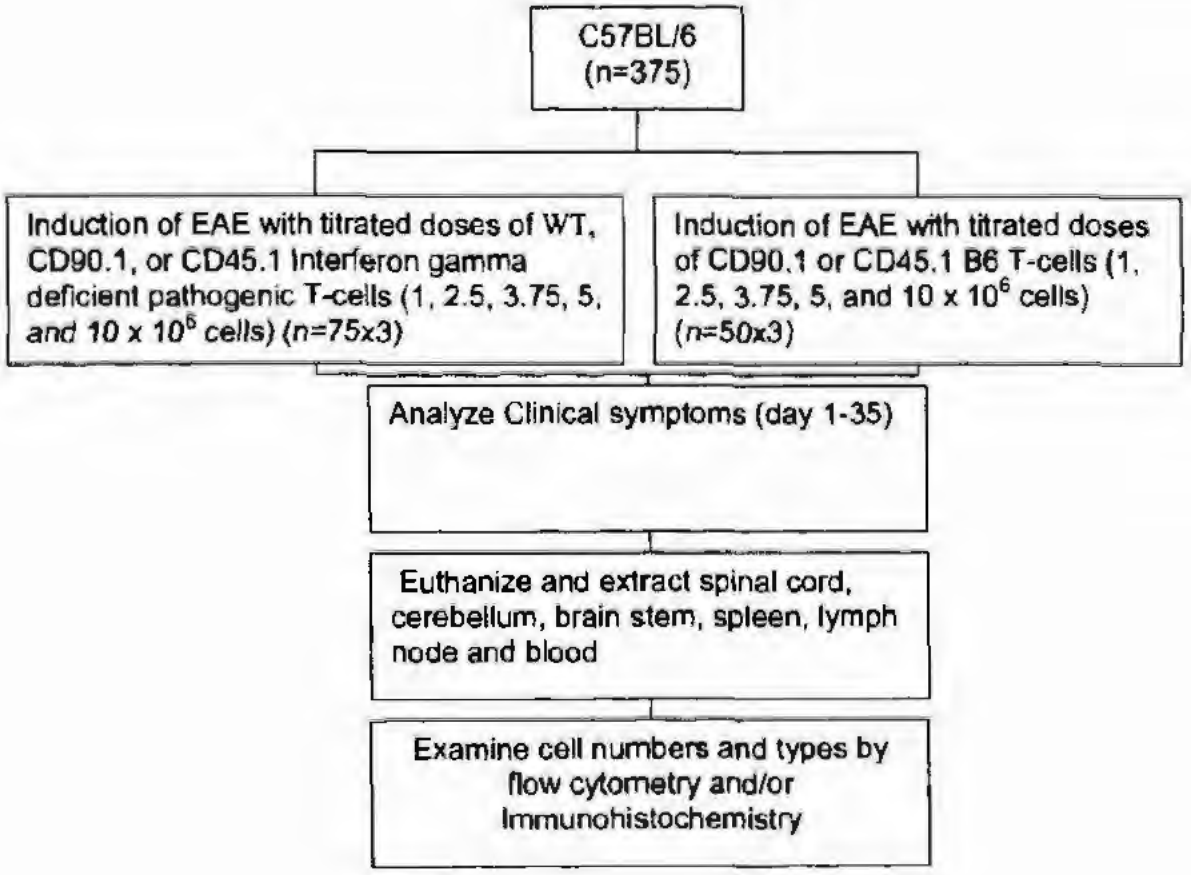
V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1: The objective of this experiment is to generate T cells specific for myelin proteins and control T cells specific for a non-self-antigen (ovalbumin). These T cells will be further propagated by *in vitro* culture to produce pathogenic T cells lines that will subsequently be used for induction of EAE by adoptive transfer *in vivo*. We will generate these T cells in 5 different mouse strains: two different B6 congenic mouse strains that express a T cell-specific marker allele (CD45.1 and CD90.1), in a mouse strain that is deficient in IFN-γ production (IFN-γ KO mice), and in CD45.1 and CD90.1 congenic IFN-γ deficient mice to define the mechanisms of IFN-γ action in EAE induction, maintenance, and resolution. For each line 10 mice (CD90.1, CD45.1, IFN-γ KO, CD45.1 IFN-γ KO, or CD90.1 IFN-γ KO) will be immunized with antigen and adjuvant as described below. Following immunization mice will be rested for 10 days. then euthanized by CO2 asphyxiation/cervical dislocation as described below. Axillary lymph nodes and the spleen will be recovered and treated with peptide, cytokines, and irradiated spleen cells recovered from naive C57BL/6 mice in standardized sterile practices necessary to produce T-cell lines specific for the immunizing antigen. We estimate 20 lines from each group will be required to fulfill the experiments described below. In total we will need 500 C57BL/6 (treated as Category C), and 200 CD90.1, 200 CD45.1, 200 IFN-γ KO, 200 CD90.1 IFN-γ KO, and 200 CD45.1 IFN-γ KO all treated as Category E mice.



V.1.2. Experiment 2: The purpose of these experiments is to determine the relative capacity of the cell lines developed in Experiment 1 to induce EAE *in vivo*. T cell lines specific for myelin proteins will be generated from WT or IFN-γKO congenic mice as described in Experiment 1, and transferred into healthy C57BL/6 host mice, and the induction of EAE will be monitored by analyzing the behavior and clinical signs of EAE as described below to determine whether Classical or Atypical EAE is induced. 5 mice per group will be injected with 1 of 5 titrated doses of pathogenic EAE inducing T-cells (1, 2.5, 3.75, 5, and 10 x 10^6 cells) derived from cell lines established in Experiment 1; Mice will be examined for clinical symptoms as described below. After 35 days (corresponding to classical EAE disease induction, peak, and resolution) mice will be perfused and euthanized as described to allow histochemical analysis of the brain and spinal cord. Experiment will be repeated 3 times to insure adequate representation of the data. This group of experiments will require 375 C57BL/6 mice all treated as Category E mice.

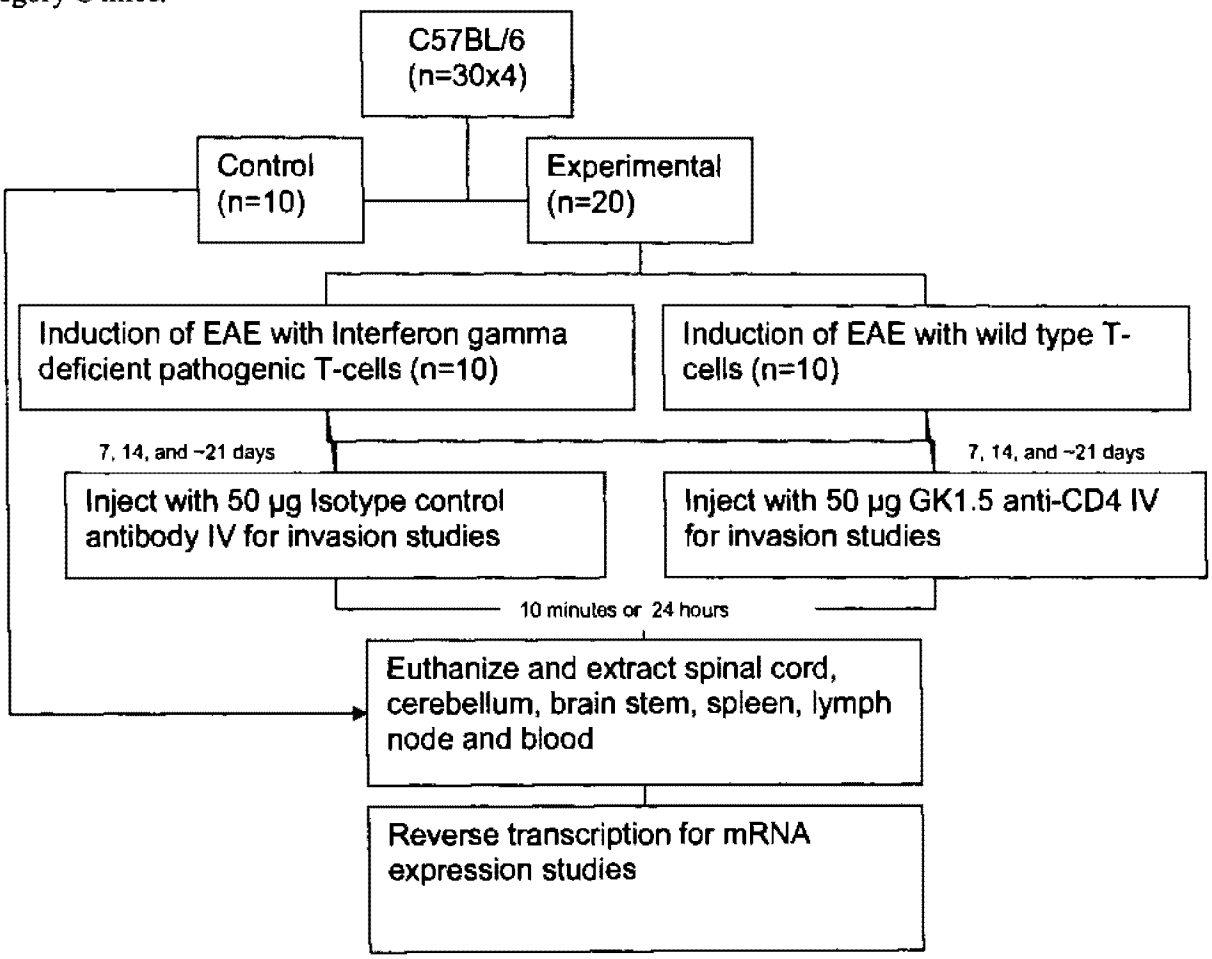


V.1.3. Experiment 3: The purpose of this experiment is to analyze the gene expression pattern in the brain and CNS following induction of the two forms of EAE (classical and atypical forms). Mice will be injected with optimal doses (as determined by experiment 2) of pathogenic T cells derived from either WT or Interferon gamma deficient mice as shown in Experiment 1.

(b)(4)

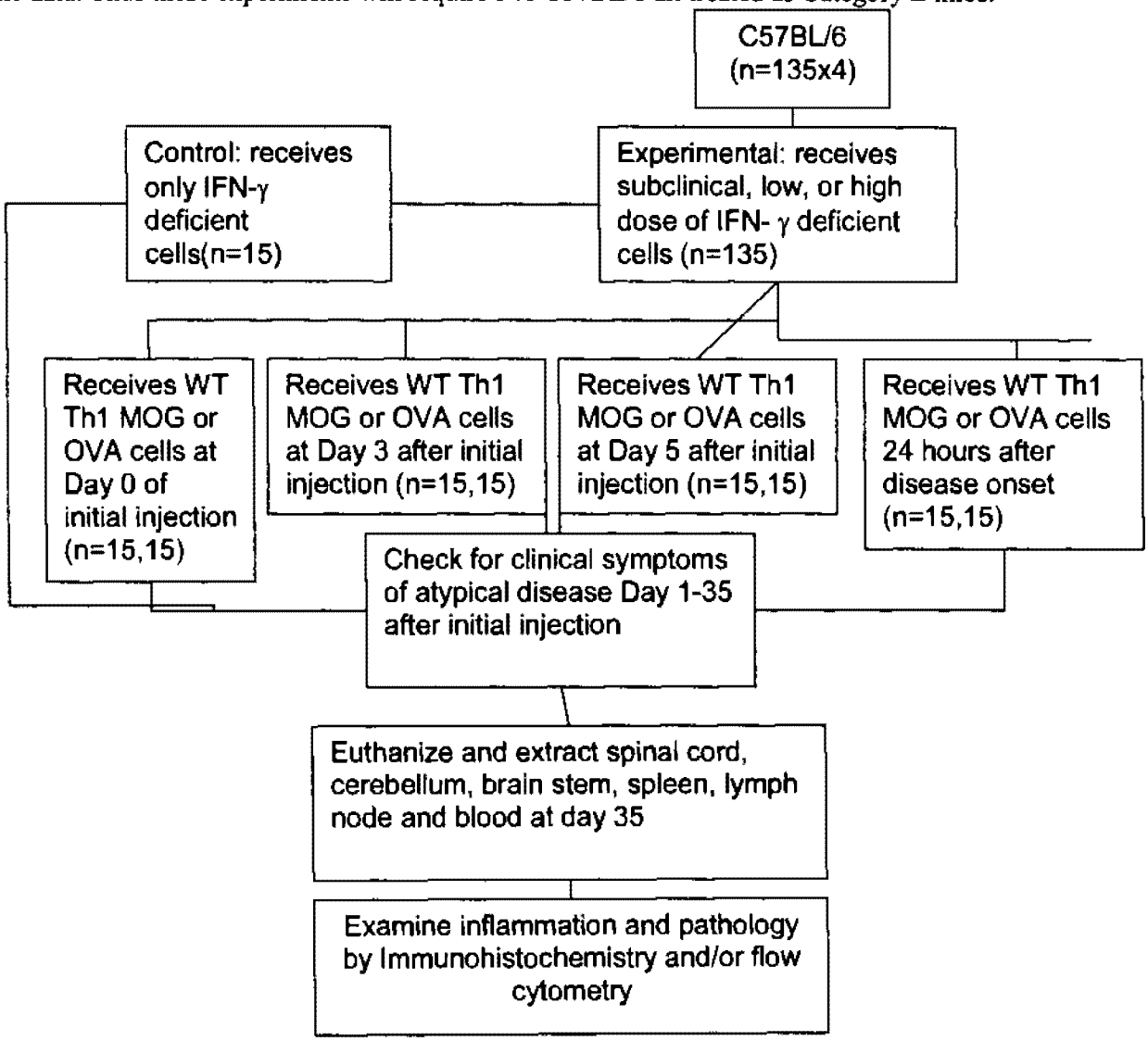
(b)(4) then euthanized and perfused as described in section V.4.6. Harvested tissue will then be utilized for mRNA examination. Experiment will be repeated 4 times to insure adequate representation of the data. This group of experiments will require 120 C57BL/6 mice with 80 treated as Category E mice and 40 as

Category C mice.



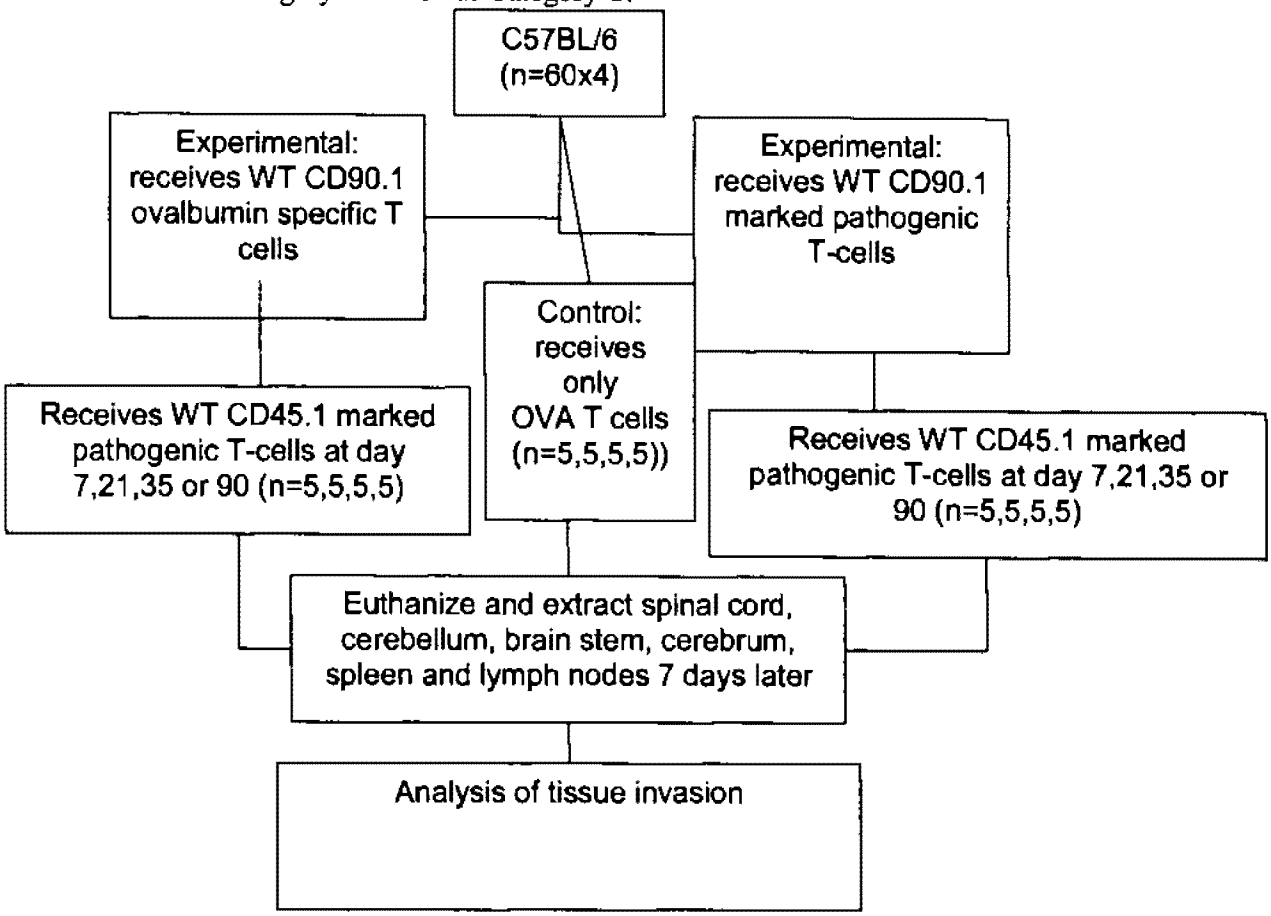
V.1.4. <u>Experiment 4:</u> The objective of the experiment will be to determine the effect lesions induced by IFN-γ deficient effector cells will have on CNS cell expression patterns at various times following induction of the atypical EAE. 5 mice per group will be injected with various doses (subclinical, low disease (peak clinical scores of ~2), high disease (peak clinical scores of 3-4) inducing doses) of pathogenic EAE inducing T-cells derived from Interferon gamma deficient mice in Experiment 1. Mice will then be injected with WT EAE inducing T cells or control T cells at various time points after the initial injection (Day 0, 1, 3, or 5 days after disease onset in control mice). Mice will then be examined for disease symptoms as described below before being euthanized 35 days later. Harvested tissue will be utilized for flow cytometric and histological examination. Experiment will be repeated 4 times to insure adequate representation

of the data. Thus these experiments will require 540 C57BL/6 all treated as Category E mice.



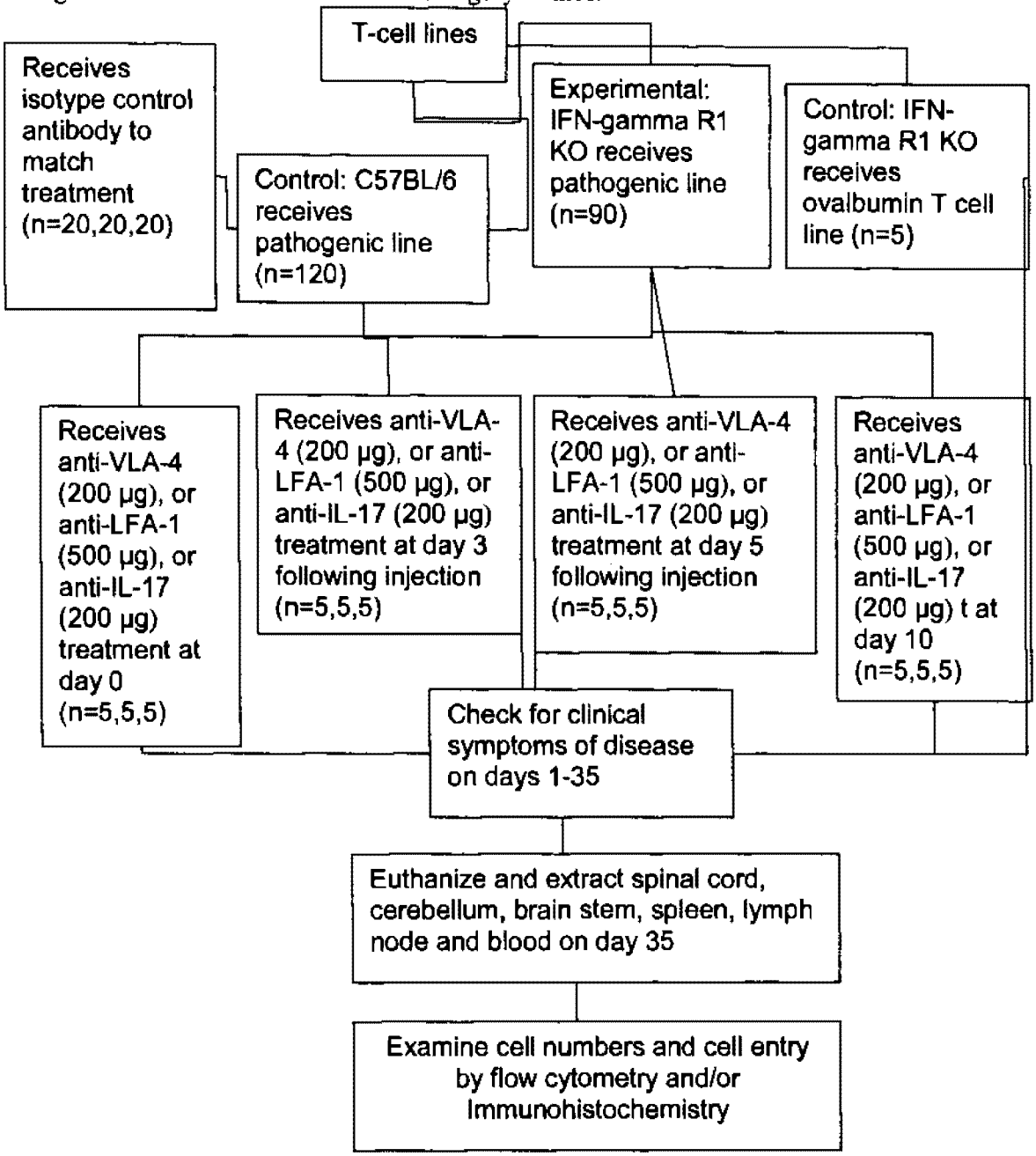
V.1.5. <u>Experiment 5:</u> The objective of this experiment is to determine how prior classical EAE CNS lesion development influence secondary EAE lesion localization within the brain and spinal cord. 5 mice per group will be injected with pathogenic EAE inducing T-cells or ovalbumin specific cells derived from CD90.1 WT mice. Mice will then be injected with WT CD45.1 EAE inducing T-cells 7 days, 21 days, 35 days, and 90 days after the initial injection. Mice will be perfused and euthanized as described in section.4.6 7 days later. Harvested tissue will be utilized for histology and flow cytomtery. Experiment will be repeated 4 times to insure adequate representation of the data. Thus, these experiments will require 240 C57BL/6 mice

with 160 treated as Category E and 80 as Category C.



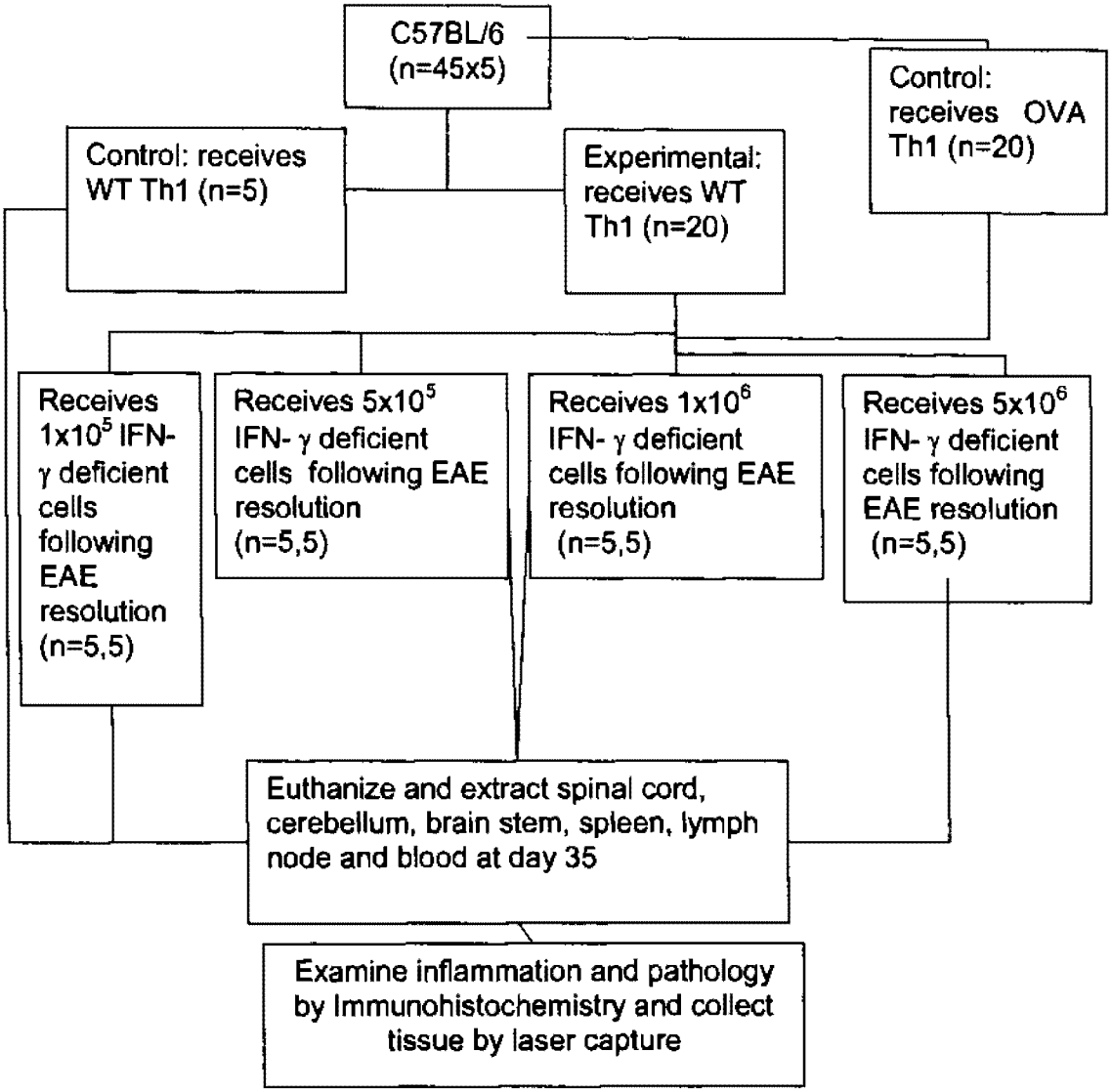
V.1.6. <u>Experiment 6:</u> the objective of this experiment is to determine if induction of atypical EAE disease requires similar molecular markers to those previously reported to be required for induction of typical EAE. Both WT and IFN-γ R1 KO mice will be injected with an optimal dose of pathogenic EAE inducing T-cells derived from WT mice or a control line specific for ovalbumin generated in Experiment 1. 2 days before or after 0, 3, 5, or 10 days mice will be injected with purified antibody specific for a T-cell marker or a control antibody with the same immunoglobulin isotype then perfused and euthanized as described below. Harvested tissue will then be utilized for flow cytometry and/or histological examination. These experiments will be

repeated 4 times for each antibody. These experiments will require 460 C57BL/6 mice and 380 IFN-gamma R1 KO mice all treated as Category E mice.



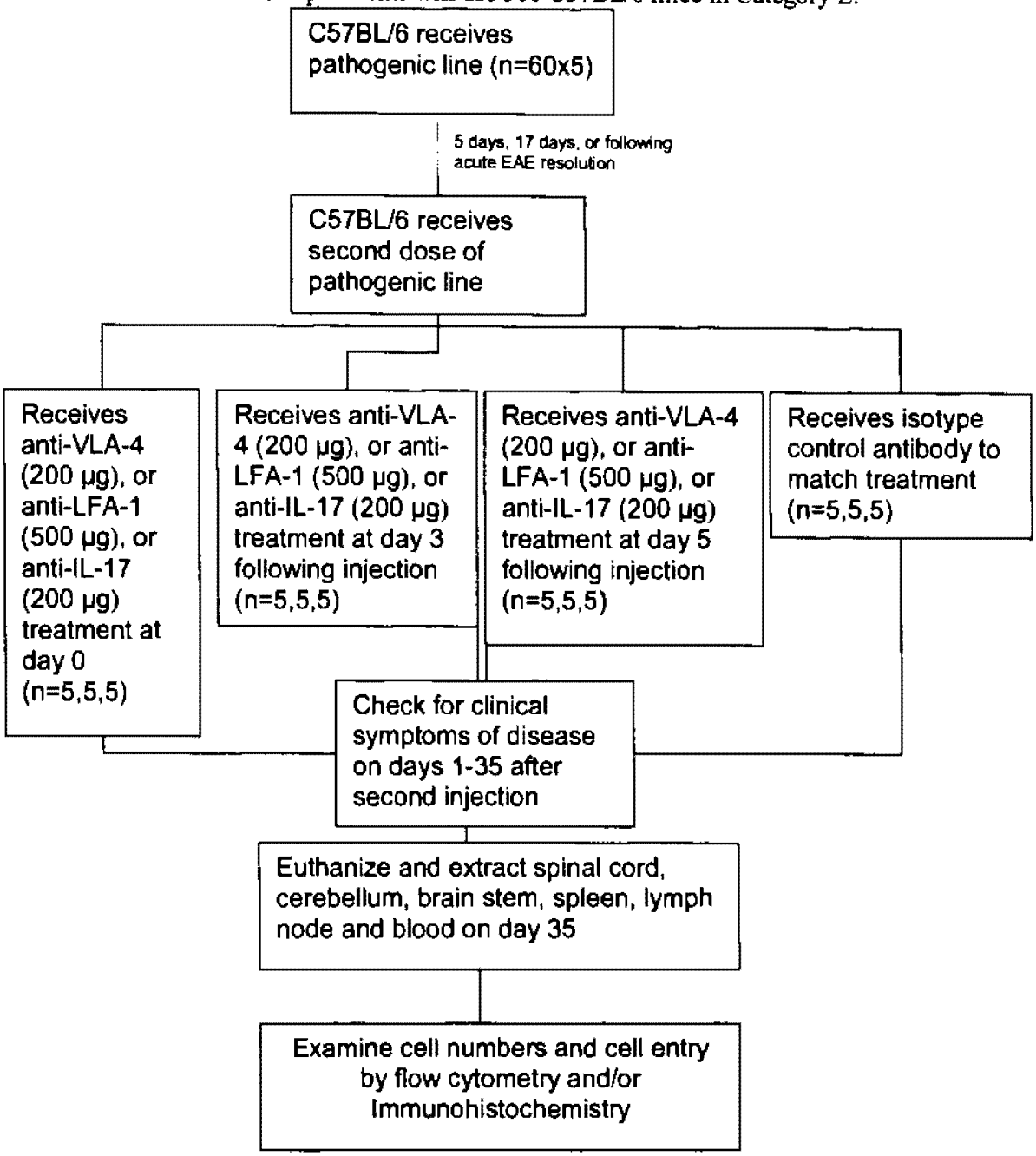
V.1.7. Experiment 7: The objective of this experiment is to determine if IFN-γ deficient effector cells are capable of exacerbating disease directly in lesions already formed by IFN-γ sufficient effector cells or form new lesions. Mice will be injected with pathogenic EAE inducing T-cells or ovalbumin specific T cells derived from CD90.1 or CD45.1 WT mice generated in Experiment 1. Mice will be examined for the development and resolution of EAE. After EAE resolution mice will then be injected with additional doses of pathogenic EAE inducing T-cells derived from Interferon gamma deficient mice. Mice will be examined for

relapse of disease. At the peak of relapse mice will be perfused and euthanized. Harvested tissue will be utilized for histology. Experiment will be repeated 5 times to insure adequate representation of the data. These experiments will require 225 C57BL/6 mice all treated as Category E mice.



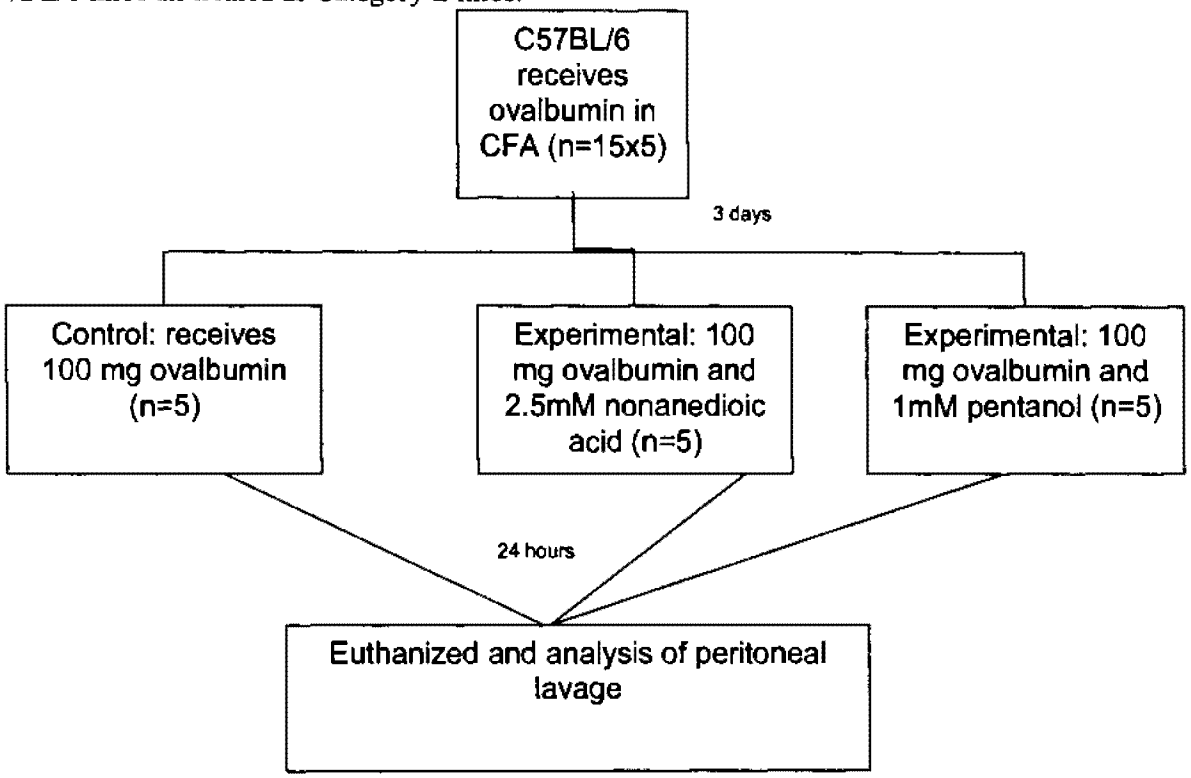
V.1.8. Experiment 8: The objective of this experiment is to determine if induction of secondary EAE disease requires similar molecular markers to those previously reported to be required for induction of typical EAE. C57BL/6 mice will be injected with an optimal dose of pathogenic EAE inducing T cells derived from WT mice. After 5 days (preclinical), 17 days (peak), or following resolution of acute EAE disease mice will receive a second dose of MOG specific EAE inducing cells. Concurrently or on days 3 and 5 after the second encephalitogenic cell dose mice with be injected with purified antibody specific for a T-cell marker or a control antibody with the same immunoglobulin isotype, then perfused and euthanized. Harvested tissue will then be utilized for flow cytometry and/or histological examination. These experiments will

be repeated 5 times. As such these experiments will use 300 C57BL/6 mice in Category E.



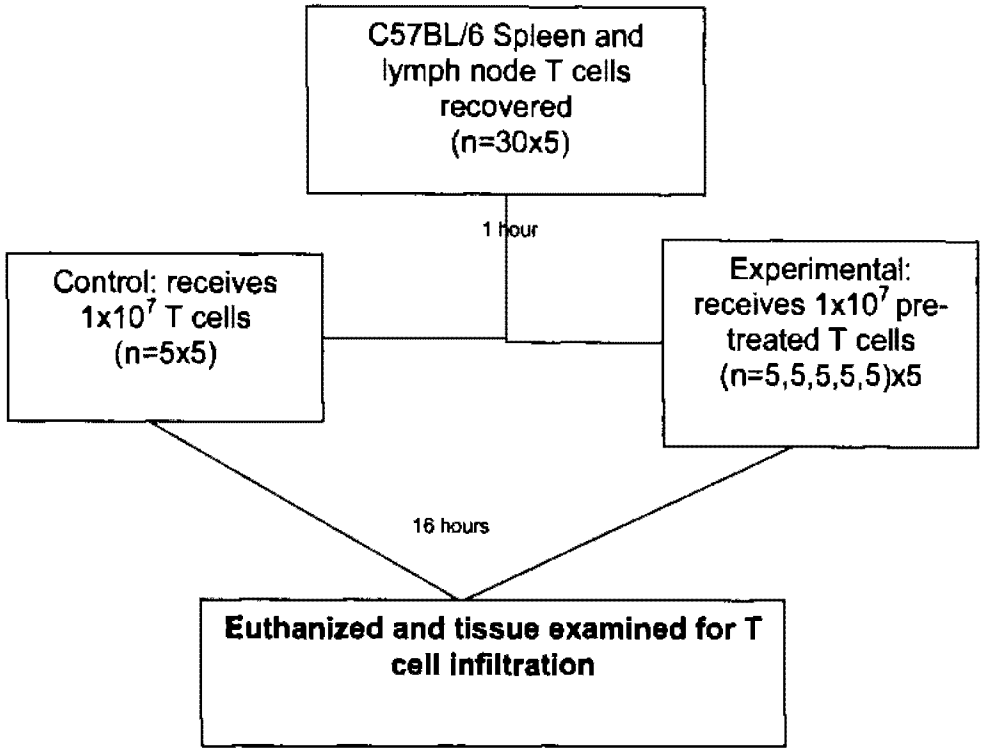
V.1.9. Experiment 9: The objective of this experiment is to determine if administration of nonanedioic acid is sufficient to block antigen specific T cell entry into an inflammatory site. C57BL/6J mice will be immunized subcutaneously (SQ) with 100 mg chicken ovalbumin (Sigma) in complete Freund's adjuvant in two 50 ul volumes as described below in section V.4.4.1 in compliance with USUHS IACUC policy #2 regarding the use of complete Freund's adjuvant. Then, 3 d later, ovalbumin-primed mice will be challenged by intraperitoneal injection of 100 mg ovalbumin dissolved in 250 ul double-distilled water with or without 2.5 mM nonanedioic acid or 1 mM pentanol. After 24 hours these mice will be euthanized by CO2 asphyxiation/cervical dislocation and peritoneal lavage with 5 ml PBS will be done with direct

visualization of the peritoneal sac and its contents to avoid peripheral blood contamination. Harvested lavage will be counted and examined by flow cytometry. Experiment will be repeated 5 times to insure adequate representation of the data. Thus, these experiments will require 75 C57BL/6 mice all treated as Category E mice.



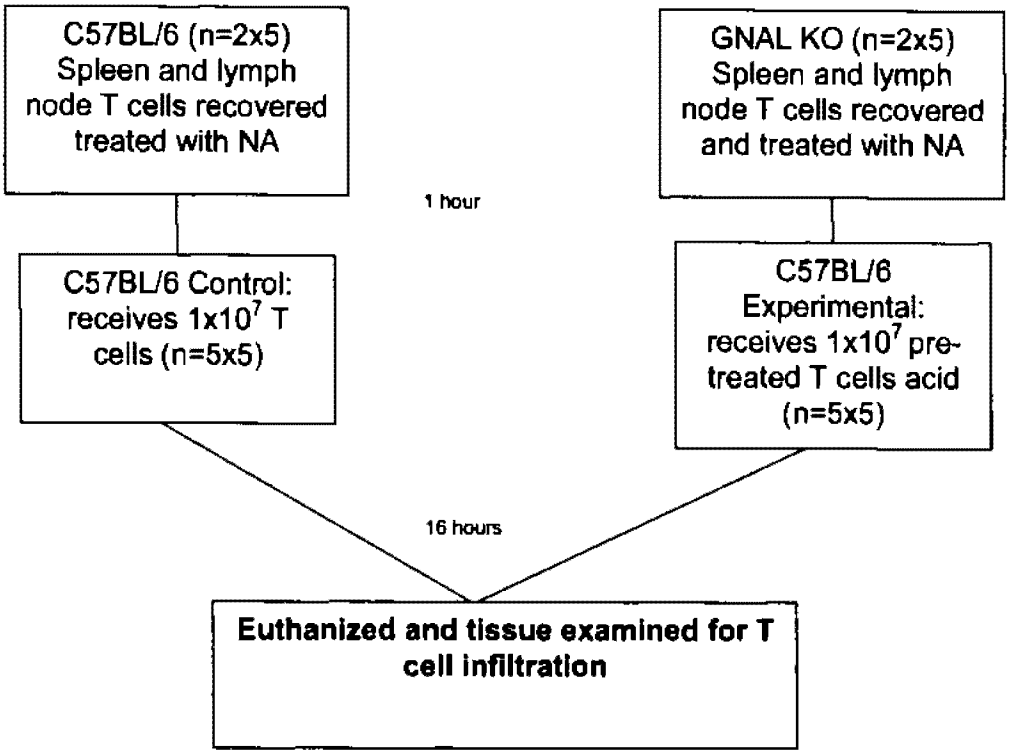
V.1.10. <u>Experiment 10</u>: Objective: To determine if various in vitro treatments are sufficient to block T cell recirculation from peripheral tissue to the lymphoid compartment.. C57BL/6J mice will be euthanized and naïve T cells separated from spleen and lymph nodes. Separated T cells will be incubated in sterile conditions for 1 hour with or without 2.5 mM nonanedioic acid, 1mM pentanol, 10 um sp-5,6-cBIMPS, 10 uM RP-CMAPs, or 1uM FTY720. Cells will then be transferred to mice via subcutaneous injection into the footpad. After 16 hours these mice will be euthanized by CO2 asphyxiation/cervical dislocation and spleen, contra and ipsilateral popliteal, inguinal, and axillary lymph nodes recovered and examined for T cell accumulation. Recovered T cells will be counted and examined by flow cytometry. Experiment

will be repeated 5 times to insure adequate representation of the data. Thus, these experiments will require 300 C57BL/6 mice, with 150 considered Category E and 150 Category C.



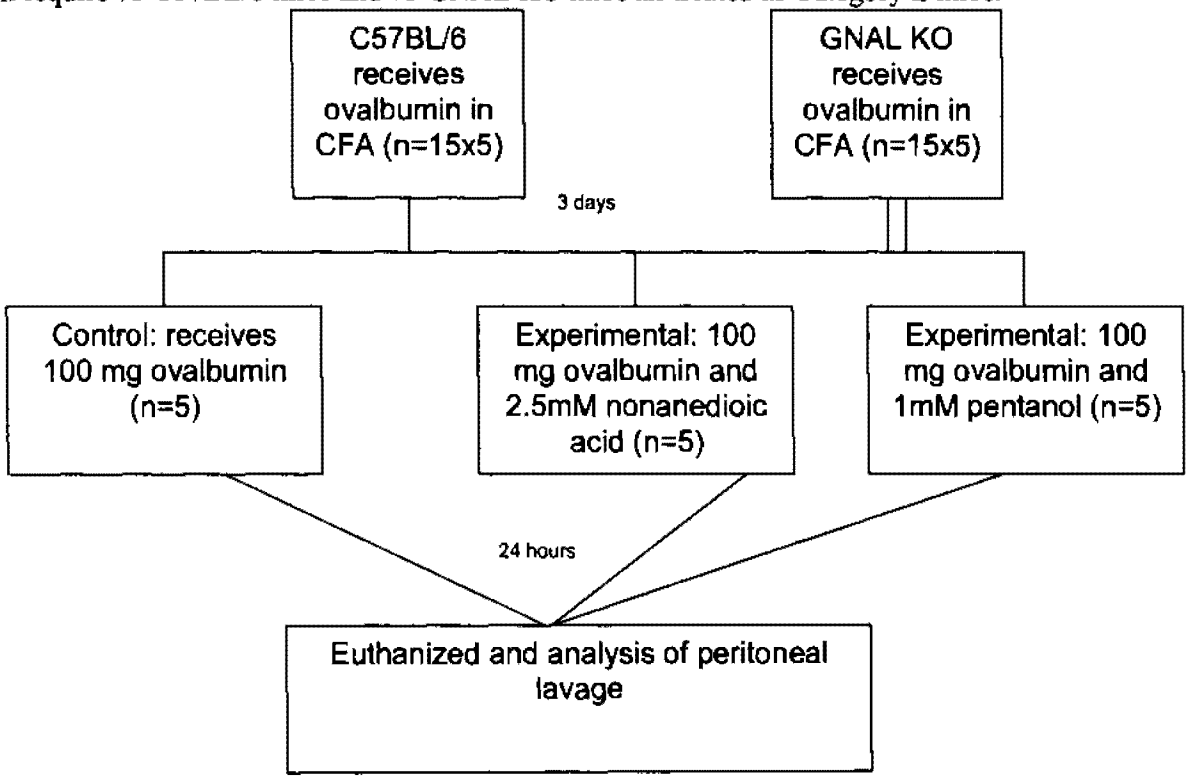
V.1.11. **Experiment 11** the objective of this experiment is to determine if various in vitro treatments are sufficient to block T cell recirculation from peripheral tissue to the lymphoid compartment. C57BL/6J of GNAL KO mice will be euthanized and naïve T cells separated from spleen and lymph nodes. Separated T cells will be incubated in sterile conditions for 1 hour with 2.5 mM nonanedioic acid (NA). Cells will then be transferred to mice via subcutaneous injection into the footpad. After 16 hours these mice will be euthanized by CO2 asphyxiation/cervical dislocation and spleen, contra and ipsilateral popliteal, inguinal, and axillary lymph nodes recovered and examined for T cell accumulation. Recovered T cells will be counted and examined by flow cytometry. Experiment will be repeated 5 times to insure

adequate representation of the data, thus,these experiments will require 60 C57BL/6 mice (10 Category C and 50 Category E) and 10 GNAL KO mice all Category C.



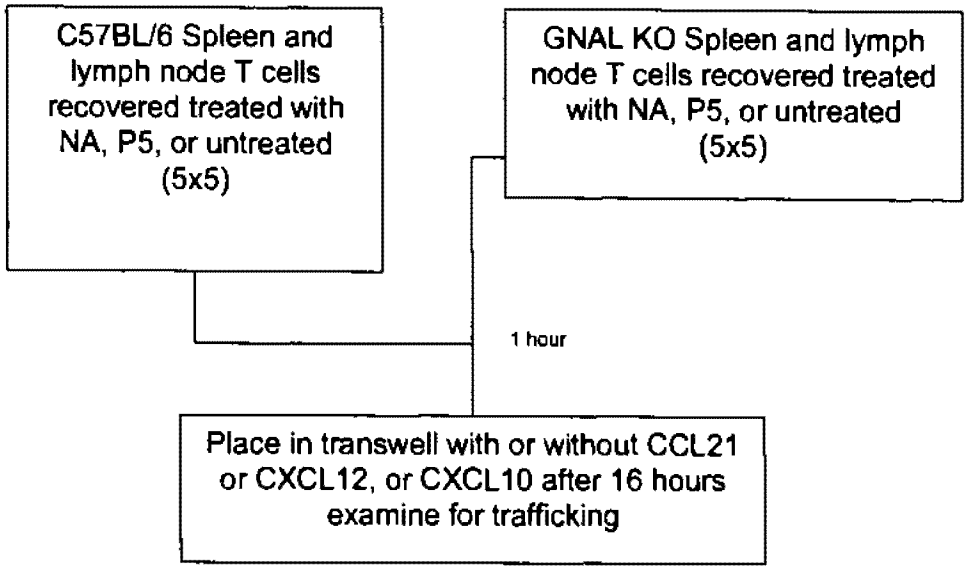
V.1.12. **Experiment 12:** The objective of this experiment is to determine if administration of nonanedioic acid is sufficient to block antigen specific T cell entry into an inflammatory site in the absence of GNAL. C57BL/6J or GNAL KO mice will be immunized subcutaneously (SQ) with 100 mg chicken ovalbumin (Sigma) in complete freunds adjuvant in two 50 ul volumes as described in section V.4.4.1. Then, 3 d later, ovalbumin-primed mice will be challenged by intraperitoneal injection of 100 mg ovalbumin dissolved in 250 ul double-distilled water with or without 2.5 mM nonanedioic acid or 1 mM pentanol. After 24 hours these mice will be euthanized by CO2 asphyxiation/cervical dislocation and peritoneal lavage with 5 ml PBS will be done with direct visualization of the peritoneal sac and its contents to avoid peripheral blood contamination. Harvested lavage will be counted and examined by flow cytometry. Experiment will be repeated 5 times to insure adequate representation of the data. Thus, these experiments

will require 75 C57BL/6 mice and 75 GNAL KO mice all treated as Category E mice.



V.1.13. **Experiment 13**: The objective of this experiment is to determine if administration of nonanedioic acid is sufficient to block T cell entry responses to chemokines in the absence of GNAL. C57BL/6J or GNAL KO mice will be euthanized by CO2 asphyxiation/cervical dislocation. Harvested spleen cells will be examined for trafficking capacity to CCL21, CXCL12, or CXCL10 following treatment with or without 2.5 mM nonanedioic acid (NA) or 1 mM pentanol (P5). Experiment will be repeated 5 times to insure adequate representation of the data. Thus, these experiments will require 25 C57BL/6 mice and 25 GNAL KO mice all treated

as Category C mice.



V.2. **Data Analysis**: All statistical tests will be performed using Prism GraphPad. The significance of the responses will be analyzed by students' t test or analysis of variance (including tukey's subtests) tests as appropriate given the numbers of groups utilized (t test for experiment 11, ANOVA for all others). To determine the appropriate number of mice tested for each experiment we utilized a web based power calculator (http://homepage.stat.uiowa.edu/~rlenth/Power/index.html) and our prior knowledge of the variability within the system and to determine appropriate sample sizes. We set our alpha error rate to correspond with the literature standard of 0.05. We set our beta error rate to the conservative literature standard rate of 0.10. We set the influence of random effects to be within 1.33 standard deviations and the minimal detectable difference to be a 0.8 unit contrast in the observed clinical scores. We made our assumptions based on previously observed variability of EAE clinical scores.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: Alternative methods for studying autoimmune disease outside of *in vivo* approaches do not currently exist. The studies supported by this protocol specifically examine in vivo autoimmune pathogenesis, a phenomena without an in vitro equivalent. Further, our studies will be describing currently unknown pathogenesis pathways. The complex and unknown *in vivo* factors regulating pathogenesis preclude use of current in vitro or computer based models.

V.3.2. Animal Model and Species Justification:

Mice were chosen based on the similarities between the models utilized and human autoimmune disease. In addition, mice are a very well characterized model system, with a multitude of immunologically relevant mouse specific tools available. No smaller, less sentient mammalian species with appropriate genetic components and inflammatory responses are readily available for studying these aspects of autoimmunity. Examination in non-mammalian species is problematic as the autoimmune disease modeled is only known to occur in higher vertebrate species. Current *in vitro* systems do not recapitulate the complex interactions between the cells and extracellular components necessary to model the interactions of T-cells with the target organs of interest. *In silico* models are insufficient for dissection of pathogenic T-cell interactions with target tissues due to the vast number of still unknown players that play a role in the examined processes.

The C57BL/6 (B6) background was chosen for the EAE studies due to the similarities between the disease model and autoimmune mediated CNS disease in humans. The B6 model provides a well described system that has been used in many published studies for examination of mechanisms involved in autoimmune mediated CNS diseases such as MS. Information gained from this system has already proven translatable to improved treatment of human disease. In addition, the B6 background is widely used for production of genetically altered mouse strains providing vital reagents for examination of molecular mechanisms of pathogenesis. As such the reagents necessary for these studies are readily available.

V.3.3. Laboratory Animals

		Species #1
V.3.3.1.	**Genus & Species:**	Mus Musculus
V.3.3.2.	**Strain/Stock:**	See table
V.3.3.3.	**Source/Vendor:**	See table
V.3.3.4.	**Age:**	6-24 weeks
V.3.3.5.	**Weight:**	20-27 g
V.3.3.6.	**Sex:**	Male and Female

V.3.3.7. Special Considerations: N/A

V.3.4. Number of Animals Required (by Species): Mice-4785

Strain	Source	Sex	Total # *(for 3 yrs)*
C57BL/6/CR	(b)(6)	M	3295
IFN-gamma KO		M	200
IFN-gamma KO-CD45.1	*	M/F	100/100
IFN-gamma KO-CD90.1	*	M/F	100/100
IFN-gamma R1 KO	(b)(6)	M	380
C57BL/6-Ly5.2/CR (Called CD45.1 here)		M	200
C57BL/6J-CD90.1		M	200
GNAL KO	*	M/F	55/55

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: All animals with EAE are observed daily for advanced disease. If advanced disease is detected food and water sources on the floor of the cage will be provided. In addition animals will be euthanized if they meet any of the alternate endpoint criteria outlined in section V4.5. Analgesics were considered but cannot be used to relieve pain due to their effects on the immune and neuronal cells being tested.

V.3.5.2. Reduction: The estimated total number of mice is based on a power analysis utilizing the principal investigator's extensive experience in this experimental system, as well as the requirement to repeat experiments for validation of results. Every effort is made to ensure the scientific utility of data derived from each animal used in this study by monitoring, evaluating and modifying group size as needed to obtain clear and consistent data. Every effort will be made to minimize the numbers of mice used without compromising research results.

V.3.5.3. Replacement: All of our studies require animals with an intact immune system, and therefore can not be replicated *in vitro*. For details please see the literature seach for alternatives, the description of data analysis, etc...

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	C57BL/6/CR	γ KO	γ KO CD45.1	γ KO CD90.1	γR1 KO	CD45.1	CD90.1	GNAL KO
V.4.1.1.1.1. Column C:	640	805	0	0	0	0	0	0	35
V.4.1.1.1.2. Column D:									
V.4.1.1.1.3. Column E:	3945	2490	200	200	200	380	200	200	75

V.4.1.2. Pain Relief / Prevention:

- **V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:** Anesthesia will be used for terminal procedures (500 mg/kg 2% avertin). Avertin is used in terminal procedures to prevent disturbance of the T cell and nervous tissue cell subsets studied in our experiments that can occur with other anesthetic methods. Compounding of avertin will occur as follows (2% PBS mixture will be Sterile filtered with a 0.2 micron filter then stored and used under sterile conditions. Compounded Avertin will be kept at 4^0C in dark containers to prevent photodegredation. Soultion color (clear) and pH (>5) will be checked before each use. All solutions will be labeled with date of manufacture and discarded after 4 months.)

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

Monitoring of EAE: Experimental Allergic Encephalomyelitis (EAE) is an autoimmune disorder characterized by a chronic relapsing, and remitting paralysis secondary to central nervous system (CNS) demyelination caused by T cells, and mice can go into remission even after development of stage III disease. Ordinarily, animals will be monitored for clinical symptoms daily and weighed every other day to determine weight loss associated with autoimmune disease. Any mouse that has a clinical score of 4 or an inability to express urine will monitored twice daily until the symptoms have resolved below a clinical score of 4, to functional capacity to void the bladder, or the animal is euthanized. We and other labs have devised a system for classification of disease severity in both atypical and classical EAE. (b)(4) (b)(6),(b)(4) Clinical symptoms will be graded and recorded daily using the following scales:

Classical EAE symptoms

1- Flaccid Tail
2- Delay in righting reflex, hind limb weakness
3- Flaccid paralysis in 1 hind limb
4- Flaccid paralysis in both hind limbs
5- Moribund

Atypical EAE symptoms
1- Tail Rigor
2- Wide stance, leaning, or directed locomotion to a single side, mild ataxia
3- Continual turning to a single side, rigor in a single limb, moderate ataxia
4- Rolling, rigor in multiple limbs, severe ataxia
5- Moribund

Ataxia symptoms
1- Mild ataxia (stumbling)
2- Moderate ataxia (weaving)
3- Severe ataxia (moribund)

Mice will be considered moribund if they are found to be incapable of directed mobility at will. Specifically, mice with paralysis, rigor, or weakness in three or more limbs will be considered moribund. Additionally any mouse that is found to be incapable of directed continuous (lasting longer than 1s) movement due to vertigo or ataxia will be defined as moribund. Any mice with scores higher than a 2 on any EAE disease score scale will be provided with moistened food and transport gel on the cage floor to insure access to food and water. Hydration will be monitored by physical examination. If animals show signs of injury or difficulty ambulating they will be relocated to a separate cage to protect them from potential negative interactions with cagemates. Mice with distended bladders that do not urinate upon handling will be considered incapable of self-elimination and the bladder will be expressed twice daily until symptoms subside or the mouse is euthanized. Skin lesions and ulcers are expected in many of the mice after complete CFA administration. Lesions will be monitored for pus-like discharge. If such a discharge occurs mice will be euthanized.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: AGRICOLA, PubMed

V.4.1.3.2. Date of Search: 04/04/13

V.4.1.3.3. Period of Search: All available.

V.4.1.3.4. Key Words of Search: Alternatives to EAE, Alternatives to encephalomyelitis, Pain EAE
V.4.1.3.5. Results of Search: Agri (0), Pubmed (74) None relevant.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: Some mice used under this proposal will develop EAE following treatment. The neurological symptoms associated with EAE will be experienced without relief for the remainder of the time described in individual experiment descriptions. The induction and development of EAE is vital to all major scientific questions addressed in this proposal. As analgesics work by modulation of neuronal and inflammatory processes treatment with analgesics could compromise our

neuroimmunological findings. It is my belief that the utilization of the EAE system will allow us to address unique questions relating to neuroinflammation that may in turn provide significant human benefit, thus justifying the moderate and temporary pain and distress the animals may experience.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: IP injection - A 27 gauge needle will be used to inject sterile 2% Avertin (Tribromoethanol)/PBS (for anesthesia prior to exsanguination via cardiac puncture), or MOG peptide or Ovalbumin/PBS (for recovery of peritoneal leukocytes), or400 ng of pertussis toxin in PBS (for active induction of EAE) into the recipient mice. All IP injections will be done with 200 ul of volume. IV injection- into lateral tail vein will be done using a 27 gauge needle to adoptive transfer encephalitogenic or control T cells, or to administer purified monoclonal antibodies in PBS. For all IV injections no more than 0.2mls per injection will be administered. SQ injection- For induction of EAE and development of antigen specific cell lines , subcutaneous injections of CFA/PBS emulsion containing MOG peptide or ovalbumin will be done using a 25G needle into the upper trunk ~2cm caudal and ventral to each forelimb in a total volume of 50 µl per site.

Treatment (concentration)	Dose	Frequency	Route	Site	Volume	Needle size
Avertin (2 % in PBS)	500 mg/kg	Once	IP	abdomen	0.2 mL	27 ga ½in
Pertussis Toxin (2 ug/mL)	20 mg/kg	Once	IP	abdomen	0.2 mL	27 ga ½ in
MOG peptide (0.5 mg/ml in	100ug/mouse	Once	IP	abdomen	0.2 mL	27 ga ½ in

PBS)						
Ovalbumin (0.5 mg/ml in PBS)	100ug/mouse	Once	IP	abdomen	0.2 mL	27 ga ½in
T cells (in PBS)	.5-10 x10^6 cells/mouse	up to 2	IV	Lateral tail vein	0.2 mL	26 ga ¾ in
Purified monoclonal antibody (1-100 ug/ml)	1-100 mg/kg	once	IV	Lateral tail vein	0.2 mL	26 ga ¾ in
MOG peptide (0.5 mg/ml in CFA/PBS emulsion)	50ug/mouse	Once	SQ	Upper trunk	0.1mL	25 ga 1in
Ovalbumin (8 mg/ml in CFA/PBS emulsion)	800ug/mouse	Once	SQ	Upper trunk	0.1mL	25 ga 1in

V.4.4.2. Biosamples:

Once mice are euthanized, we will perform aseptic removal of liver, lung, lymph node (LN), pancreas, spleen, and CNS tissue for *in vitro* analysis and T-cell separations using standard techniques. For removal of the spleen an incision will be made near the left flank of the mouse, the spleen lifted up with a forceps and cut away from the tissue fascia with surgical scissors. Lymph nodes (LN) will be removed from the intestines (mesenteric lymph node (MLN)), from peripheral sites such as the popliteal LN (PLN) in the legs, from the upper torso (axillary lymph node (ALN)) and from the pancreatic area (PLN). The central nervous system will be removed in multiple sections, separating the cerebellum and brainstem, the remaining brain, and the spinal cord. Each portion of the CNS will be removed whole following perfusion. The brain will be exposed by removal of skin and the cranium with scissors. The spinal cord will be removed as a piece following removal of the dorsal side of each vertebra using scissors. The lungs, liver, and pancreas will be also be removed following perfusion.

We will obtain mouse peripheral blood for screening of peripheral T cells in mice. For obtaining peripheral blood, mice will be immobilized using a table top cone mouse immobilizerand a sterile 27 gauge needle will be used to prick the tale over a vein. ~20 ul of blood will be removed via tail bleeding into a heparinized capillary tube. Blood will then be stained and examined by flow cytometry. If mice do not immediately stop bleeding following collection pressure will be applied to the site of puncture with a sterile gauze strip until hemostasis is achieved. Each mouse will be screened via blood sampling no more than twice, once for screening purposes and once to examine T cell populations.

I am very experienced with mouse bleeding (13 years' experience), and these procedures are performed efficiently, minimizing discomfort to the mouse. For screening mice, bleeding will be done between 4 and 8 weeks of age. For quality control, transgenic mice will routinely be screened once every month, (or up to once weekly as necessary).

V.4.4.3. Adjuvants: Freund's adjuvant has been commonly used for decades in immunologic studies in mice and while multiple adjuvants are sufficient to induce EAE in rats, CFA is thought to be uniquely capable of reliably generating autoimmunity in mice (Stromnes and Goverman 2006). As such for these studies, no adequate substitute is available. Mice will be treated with CFA containing either the MOG immunodominant peptide or the protein control ovalbumin. We will utilize subcutaneous injections of CFA/PBS emulsion containing MOG peptide or ovalbumin will be done using a 25G needle into the upper trunk ~2cm caudal and ventral to each forelimb in a total volume of 50 µl per site. Mice will be watched for development of adverse reactions and subsequent euthanasia as described in section V.4.5.

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Within each experimental iteration mice will be identified through use of ear punch as outlined below (V.4.4.7). Using a location code for punch placement we can identify up to 99 mice/experiment.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: Mice will be restrained by hand and an ear punch will be utilized to produce specific marks. Prior to use of the ear punch the area to be marked and the punch will be treated with 70% isopropanol. Mice will be marked immediately prior to use and as such mouse age will depend on the age (4-8 weeks)at time of usage.

V.4.4.8. Tissue Sharing: Unused tissues will be available after termination of experimental procedures with the understanding that mice have been manipulated and may have disease-associated tissue inflammation.

V.4.5. Study Endpoint: Euthanasia at pre-determined time. Alternatively mice will be euthanized if they are judged to be moribund or display any of the following criteria associated with EAE development:

i. total weight loss exceeding 20% of body weight at start of experiment.
ii. Inability to rest.
iii. Hunched posture, head tucked into abdomen, lethargy lasting for >24 hours.
iv. Any condition that interferes with fecal or urinary elimination.

or development of other issues sometimes found in the mouse population:

v. Formation of multiple sores on tail.
vi. Diarrhea, if debilitating or prolonged over 3 days.
vii. Spreading alopecia that covers ~25% or more body surface area.
viii. Excessive scratching with associated scabbing
ix. Persistent coughing, labored breathing, or nasal discharge.

x. Jaundice.
xi. Bleeding from any orifice and/or prolapse.
xii. Pus-like discharge from sores.

V.4.6. Euthanasia: In general mice will be euthanized with CO2 from a regulated tank with input at .5-1 liters/minute (10-30% volume displacement per minute) until respiratory arrest is clear followed by cervical dislocation as recommended by the 2013 AVMA Guidelines for the Euthanasia of Animals. For experiments that require harvesting tissue used in determination of mRNA expression the long timing and induction of anoxia associated with CO2 asphyxiation is problematic and so mice will be euthanized by cervical dislocation following high dose (500mg/kg) avertin anesthesia. Mice will be tested for deep anesthesia as evidenced by a lack of response to foot pressure prior to dislocation. Finally, because cervical dislocation disrupts the architecture of the spinal cord, it cannot be utilized as a primary method of euthanasia when intact spinal cords are required. As such, for all studies that utilize CNS tissue, mice will be euthanized by exsanguination via cardiac perfusion under high dose (500mg/kg) avertin anesthesia injected intraperitoneally followed by perfusion with ice cold PBS.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) _____ Room Number(s)LAM Procedure room

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes	_____	No	X
Fluid Restriction:	Yes		No	X

V.5.1.3. Exceptions: None _____ _____

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be cared for by LAM personnel according to LAM SOPs. We will also monitor daily and weigh every other day following development of EAE clinical symptoms. Food and water sources will be placed on the floor of the cage as needed for animals that may have difficulty accessing the normal feed and water sources.

V.5.2.2. Emergency Veterinary Medical Care All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary

technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions: None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Colony management, genotyping, animal observation, injections, tissue sampling, euthanasia	(b)(6) PhD	13 years experience with mouse maintenance and handling including previous experience in all procedures listed in the protocol	(b)(6) IACUC Training 1999 (b)(6) (b)(6) IACUC Training 2004, (b)(6) IACUC rodent handling and investigator training, 2008, USUHS IACUC training 4/4/13

VII. BIOHAZARDS/SAFETY: We will follow all regulations on handling mice as per instructions received during the USUHS IACUC courses. Routine PPE (lab coat, mask, gloves) will be worn when working with animals to reduce the potential exposure to animal allergens. Blood withdrawal and spleen collection (after euthanasia) will be handled in a sterile hood in LAM facility. Sharps will be disposed of as instructed by EHS policy.

VIII. ENCLOSURES: None

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

_____ _____

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) [redacted]

Pr[redacted] 4/17/13
Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) [redacted]

[redacted]ure 4/17/13
Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: T cell trafficking and retention in an autoimmune mouse model

C. Principal Investigator: (b)(6) PhD, Department of Medicine

D. Performing Organization: Uniformed Services University of the Health Sciences

E. Funding: ~$50,000

F. Objective and Approach: The long-term objective is to understand and identify the necessary components involved in T cell mediated autoimmune attack that occurs in neuroinflammatory diseases like multiple sclerosis (MS). In this protocol, we will use Experimental Allergic Encephalomyelitis (EAE) as a demyelinating autoimmune disorder resembling MS. Our hypothesis is that discrete molecular components will be required to retain T cells long term within the CNS. This long term population appears to be involved in the continuation and/or reactivation of lesions within the brain and spinal cord. The objective is to identify vital components of this T cell retention future for future therapeutic targeting. Previous work within the model used here have been shown that information gained in the mouse system is uniquely applicable to multiple sclerosis treatment. However deleterious effects associated with established treatment limit their utility. AS such there is a great need for new and better therapeutics.

G. Indexing Terms (Descriptors): Experimental allergic encephalomyelitis (EAE), multiple sclerosis, mice, autoimmunity

References:

(b)(6)

Stromnes, I. M. and J. M. Goverman (2006). "Active induction of experimental allergic encephalomyelitis." Nat Protoc 1(4): 1810-1819.

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BETHESDA, MARYLAND 20814-4799

May 21, 2014

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF MEDICINE

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Full Committee Review on May 21, 2014:

Animal Protocol Title: "Generation of Therapeutic Human anti-Influenza Virus Antibodies (HAIVA) using DRAG mice"

USUHS Protocol Number: (b)(6)

Expiration Date: May 20, 2017

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: "Generation of Therapeutic Human anti-Influenza Virus Antibodies (HAIVA) Using DRAG mice"

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: USUHS

EARLIEST ANTICIPATED FUNDING START DATE: JAN 01.2014

PRINCIPAL INVESTIGATOR: (b)(6) MD.

(b)(6)	MED	(b)(6)	4/02/2014
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	Professor	(b)(6)	4/2/14
Research Unit Chief / Dept. Head Signature	Title	Telephone	Date

Typed Name (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)	MED	(b)(6)	4/02/2014
Statistician Signature	Department	Telephone	Date

Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)	LAM	(b)(6)	3 April 2014
Attending/Consulting Veterinarian Signature	Department	Telephone	Date

Typed Name (b)(6) DVM

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: Dr. (b)(6)

ANIMAL PROTOCOL TITLE: "Generation of Therapeutic Human anti-Influenza Virus Antibodies (HAIVA) Using DRAG mice"
GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): Drs. (b)(6)

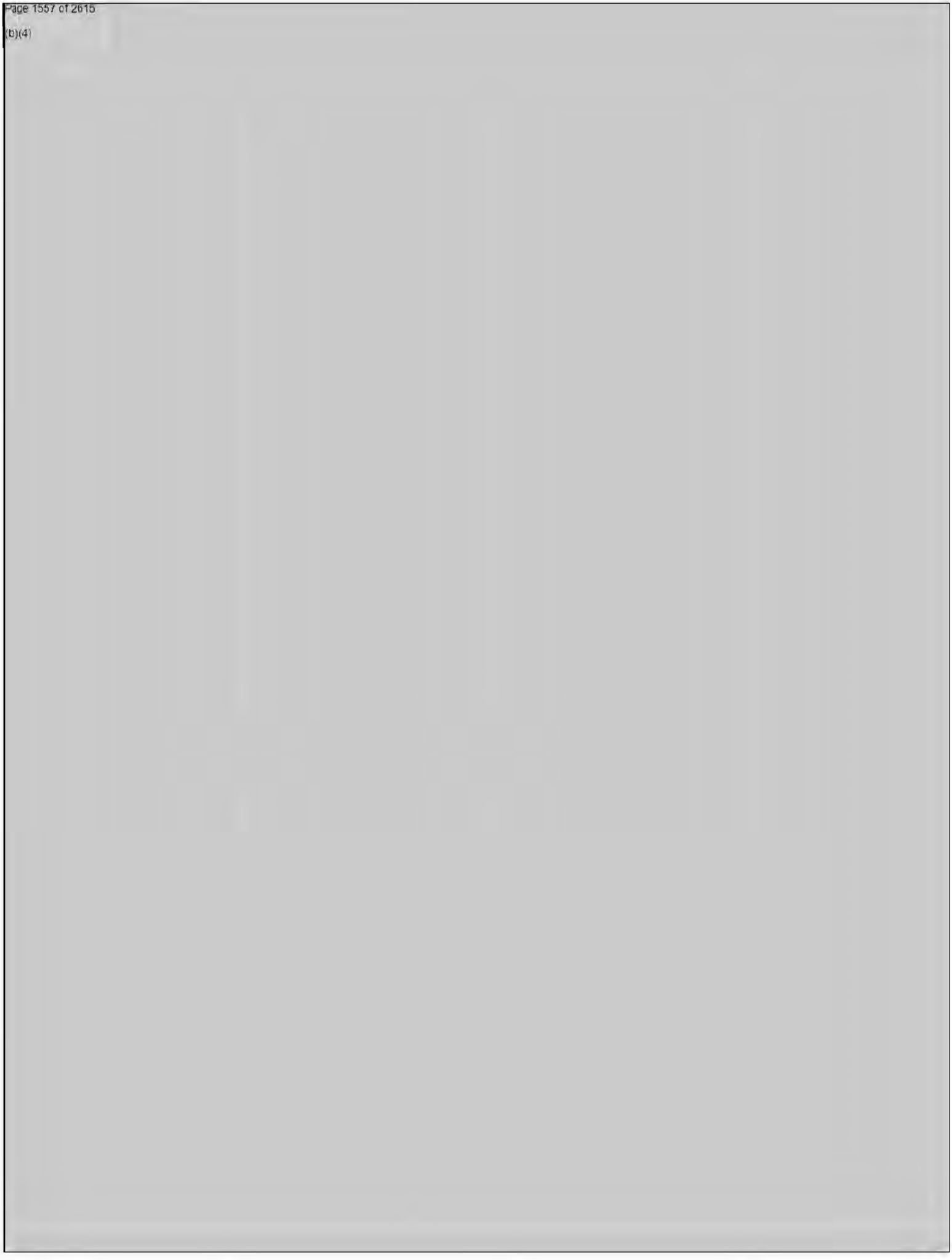
TECHNICIANS(S):

I. **NON-TECHNICAL SYNOPSIS:** Influenza viral vaccination using mixtures of egg-cultured virus strains isolated from the lungs of recently infected people can provide protection to 60-70% of individuals. Thus, conventional flu vaccines require yearly preparation due to seasonal changes in the structure of viral proteins (antigen drift or shift) that lead to immune evasion that result in a less efficiency of a previous year vaccine preparation. The preparation process and testing of a new flu vaccine requires 6-8 months. The vaccine is less efficient in elderly, immunocompromised individuals, and children due to a poor and respectively, immature immune system. Some 3,000 deaths occurred between the years 1986-87 and around 50,000 in the year between 2003-04 by non-endemic, seasonal influenza infection in USA according to the CDC reports(Centers for Disease & Prevention, 2010). Furthermore, current therapeutic procedures for patients with acute flu pulmonary infection rely mostly on palliative treatment that does not address viral clearance from the system, and their efficacy depends greatly on the antiviral resistance to the drug and the immune status of each individual to clear the infection.

Our proposal aims at designing of a new class of anti-viral therapeutics, Human Anti-influenza Virus Antibodies (HAIVA prep) for critical conditions like acute pulmonary infection (with different types of flu viruses), and for vaccination purposes in imminent flu endemics. HAIVA prep consists of a cocktail of "fully human" antibodies able to neutralize a large number of influenza virus strains regardless the seasonal antigen drift and the immune status of an individual. Generation of HAIVA preps relies on our newly developed humanized DRAG mouse (Danner et al., 2011) (patent recently licensed by Jacksons Labs) and our preliminary data showing the possibility of generating fully human antibodies against flu and malaria. HAIVA preparation is designed for human use and to lack the side effects of conventional flu vaccines, ex. Allergy to egg, cardiovascular and kidney complications, joints inflammatory syndrome, etc. HAIVA shelf-life is expected to be several years and highly efficient regardless the seasonal mutations of the virus and the patient immune status. HAIVA-like preparations may greatly impact the way acute flu infections are treated in both the military and civilian populations. They may also represent a novel platform to generate 'universal' vaccines for other types of infections and pathogens of bioterrorism threat.

II. **BACKGROUND:**

(b)(4)



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(b)(4)

(b)(4),(b)(6)

(b)(4),(b)(6)

(b)(4) (b)(6)

(b)(4),(b)(6)

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) [redacted] 04/03/2014

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful re

(b)(6) [redacted] 24/03/2014

Principal Investiga Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

04/03/2014
Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: "Generation of Therapeutic Human anti-Influenza Virus Antibodies (HAIVA) Using DRAG mice"

C. Principal Investigator: Dr. (b)(6)

D. Performing Organization: Uniformed Services University of the Health Sciences

E. Funding: (b)(6)

F. Objective and Approach: The overall objective is to provide efficient protection and virus clearance through heterotypic cross-targeting the conserved regions of viral proteins by a generation of novel human anti-influenza virus antibodies utilizing humanized DRAG mice.

G. Indexing Terms (Descriptors):

(b)(4)

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

October 8, 2014

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF MEDICINE

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on October 8, 2014:

Animal Protocol Title: "Protective Efficacy of Human Malaria Vaccines in Mice (Mus Muscaulus)"

USUHS Protocol Number: (b)(6)

Expiration Date: October 7, 2017

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr (b)(6)

The USUHS bas an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the ahove referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to scbedule a pre-protocol planning meeting (b)(6) Tbis meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: "Protective efficacy of human malaria vaccines in mice (*Mus musculus*)."

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY:

EARLIEST ANTICIPATED FUNDING START DATE: JAN 01 2014

PRINCIPAL INVESTIGATOR: (b)(6) MD.

(b)(6) — MED — (b)(6) — 06/24/14

Principal Investigator Signature | Department | Office/Lab Telephone | Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) — Professor — (b)(6) — 6/24/14

Research Unit Chief / Dept. Head Signature | Title | Telephone | Date

Typed Name (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) — MED — (b)(6) — 06/24/14

Statistician Signature | Department | Telephone | Date

Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) — LAM — (b)(6) — 24 June 2014

Attending/Consulting Veterinarian Signature | Department | Telephone | Date

Typed Name (b)(6) DVM

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: Dr. (b)(6)

ANIMAL PROTOCOL TITLE: "Protective efficacy of human malaria vaccines in mice (*Mus musculus*)"

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): Dr (b)(6)

TECHNICIANS(S):

NON-TECHNICAL SYNOPSIS:

Currently, the pre-clinical animal models that are used to test approaches that target the immune system (i.e., vaccines) are imperfect. Development of humanized mouse models able to generate a surrogate human immune system is a highly pursued goal for investigating human immunology and testing human vaccines prior to clinical trial. Under this protocol we will investigate the immunogenicity and protective efficacy of human malaria vaccines generated in our lab using NMRC genetically engineered, humanized mice. We have demonstrated that genetically modified mice expressing human protein (HLA, and CD80) and that are knockout for mouse immune system (RAG KO, Il2R KO), namely DRAG mice, developed a functional human immune system upon infusion of human hematopoietic stem cells from umbilical cord blood. Our results also indicated that the humanized mice sustain infection with *Plasmodium falciparum* as they also develop human hepatocytes and erythrocytes –the restriction elements for human malaria infection.

To demonstrate the protective efficacy of two different human monoclonal antibodies generated previously in the lab that are specific for Pf CSP (Ab1 and Ab2), we will vaccinate wild type mice that self-cure malaria infection (BALB/c and C57BL/6 mice) as well as our four strains of humanized mouse strains (HLA-DR4, RAG2KO, IL2RgcKO, CD80, NOD mice, HLA-DR4, A2, RAG2KO, IL2RgcKO, NOD mice, HLA-DR4, A2, RAG2KO, AbbKO, C57 mice, and HLA-DR4, AbbKO, NOD) with human monoclonal antibodies Ab1 and Ab2, and further infect these mice with a mouse specific malaria strain, *Plasmodium Yoelii* (a strain that infects the mice but not humans). The P. Y strain has the *Plasmodium Falciparum* CSP transgene (Py-Pf CSP transgene) from *Plasmodium Falciparum* CSP known to infect humans.The Py-Pf CSP transgenic strain does not infect humans, but it does infect mice. The rationale of using human monoclonal antibodies as protective agents against infections has been overtly demonstrated. The use of Ab1 and Ab2 preparations in these mice is to test their protective efficacy against Py malaria carrying Pf CSP transgene. We expect that binding Ab1 and/or Ab2 to the sporozoites will impair their ability to reach the liver. BALB/c and C57BL/6 mouse strains are used as control infectivity. The use of humanized mice is to find out the role of various human immune molecules (see human transgenes expressed in these mice) on the Py infectivity as well as how these

human molecules interfere with Py infection upon Ab treatment.

A number of 70 mice from each strain (BALB/c and C57BL/6 mice as well as HLA-DR4, RAG2KO, IL2RgcKO, CD80, NOD mice, and HLA-DR4, A2, RAG2KO, IL2RgcKO, NOD mice, and HLA-DR4, A2, RAG2KO, AbbKO, C57 mice, and HLA-DR4, AbbKO, NOD) will be used in the experiments as described in Table 1.

In a parallel experiment we will vaccinate the same strains of mice as mentioned above with four different protein fragments from Pf CSP, namely the NH2 terminus of Pf CSP (Ag1), or COOH terminus of Pf CSP (Ag2), or COOH-repeat of Pf CSP (Ag3), or the intact Pf CSP (Ag4). The rationale of using these CSP fragments is to delineate the most immunogenic part of CSP that can induce protective antibodies upon immunization. CSP is known as an immunogenic protein, but mapping the immunogenic sites is still a major challenge. The use of humanized mice will provide insights into the role of each of these human immune molecules (see human transgenes expressed in these mice) on the immunogenicity and protection against malaria infection.

The proposed study is part of a peer-reviewed award "(Development of malaria vaccines for military, of $ 680,000) by MIDRP to the Malaria group from NMRC. Dr (b)(6) project has been assigned as a collaborator sub-award to this main project.

II. BACKGROUND:

II.1. Background:

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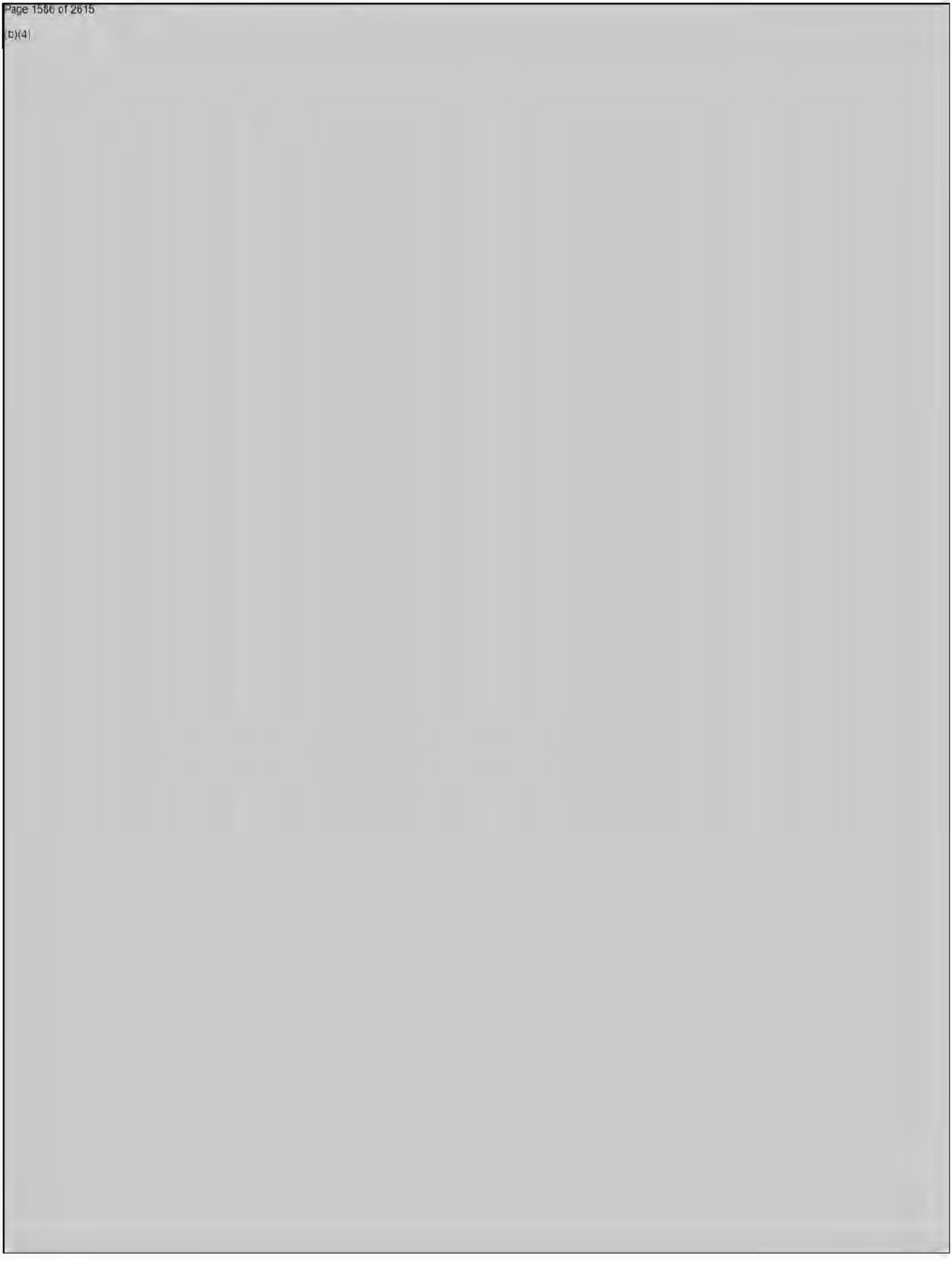

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(b)(4) (b)(6)

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) 06/24/14

Principal Inve Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) 06/24/14

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** are relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not ava[redacted] the objectives of this proposed experiment.

(b)(6)

[redacted]

Principal Investiga[redacted]

06/29/10

Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: "Protective efficacy of human malaria vaccines in mice (*Mus musculus*)."

C. Principal Investigator: Dr. (b)(6)

D. Performing Organization: Uniformed Services University of the Health Sciences

E. Funding: (b)(6)

F. Objective and Approach: The overall objective is to test the immunogenicity and protective efficacy of human vaccines in humanized mice.

G. Indexing Terms (Descriptors):

(b)(4)

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

January 14, 2014

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF MILITARY EMERGENCY MEDICINE

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on January 14, 2014:

Animal Protocol Title: ""Biomarkers for Detection of Heat Intolerance in Mice

USUHS Protocol Number: (b)(6)

Expiration Date: January 13, 2017

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc: Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Biomarkers for Detection of Heat Intolerance in Mice

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Office of Naval Research

EARLIEST ANTICIPATED FUNDING START DATE: ongoing

PRINCIPAL INVESTIGATOR:

(b)(6) — MEM — (b)(6) — 10/31/2013

Principal Investigator Signature | Department | Office/Lab Telephone | Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) — Chair — (b)(6) — 1 Nov 13

Research Unit Chief / Dept. Head Signature | Title | Telephone | Date

Typed Name:

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) — MEM — (b)(6) — 10/31/2013

Statistician Signature | Department | Telephone | Date

Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) — LAM — (b)(6) — 6 Nov 13

Attending/Consulting Veterinarian Signature | Department | Telephone | Date

Typed Name (b)(6)

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) Military & Emergency Medicine (b)(6)

ANIMAL PROTOCOL TITLE: Biomarkers for Detection of Heat Intolerance in Mice

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6)

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

Heat injury continues to be a significant problem confronting the military, particularly during recruit training, and remains a common cause of preventable non-traumatic related death. Identifying these heat-vulnerable individuals is important for reducing heat-related injuries in outdoor constructions and military operations. The overall goal of this project is to determine the biomarkers that can be used to detect heat intolerance. In this protocol, we will continue our previous work to test the effects of two antioxidants, quercetin and curcumin, on the heat stress responses of key organs, which play an important role in maintaining thermal homeostasis, of heat-tolerant versus heat-intolerant mice.

II. BACKGROUND:

II.1. Background:

Prolonged exposure to high temperatures may cause heat-related illnesses, such as cramps, syncope, exhaustion or even stroke in some individuals. Heat-related injuries remain a threat to the health and operational effectiveness of military personnel [1]. Heat injury victims experience long-term complications that may include multi-system organ (liver, kidney, muscle) and neurologic damage, as well as reduced exercise capacity and heat intolerance. Animal and human research suggest late or untreated heat injury may result in organ damage that continues for weeks to months and possibly even after clinical symptoms or biomarkers have returned to normal. Current military return-to-duty guidelines are based on "best guess" estimates and clinical anecdotes, rather than valid biomarkers of recovery and scientific evidence. Thus, the predictive and prognostic biomarkers, which can be used to identify heat-intolerant individuals and to determine recovery after heat injury, will improve military force readiness. Our previous work showed that heat-intolerant (INT) mice were associated with altered regulation of tissue heat shock proteins (HSPs) at translational and transcriptional levels compared to heat-tolerant (TOL) mice [2, 3]. In this protocol, we will further assess the role of HSPs in key organs, including heart, liver and skeletal muscles, in regulating heat tolerance in mice fed a diet containing quercetin (Que) and curcumin (Cur). Both Que and Cur are plant-derived natural products and have antioxidant/anti-inflammatory properties. Oxidative stress and inflammation have been implicated in heat stress.

Importantly, it has been shown that Que [4] inhibits but Cur [5] enhances induction of HSPs by heat acclimation (HA).

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:
Biomedical Research Database; RePORT; PubMed
II.2.2. Date of Search: 10|22|2013
II.2.3. Period of Search:
all available years
II.2.4. Key Words and Search Strategy:
"heat" AND (tolerance" OR "intolerance" OR "stress") AND ("antioxidant" OR "flavonoid" OR "quercetin" OR curcumin")
Results include work done in rodents relevant to regulation of heat shock proteins (HSP).
BRD 0 hit; RePORT 0 hit; PubMed 407 hits

II.2.5. Results of Search:
No previous studies were found that duplicate the proposed studies. Heat intolerance has been shown to be associated with hypothalamo-pituitary-adrenocortical axis impairment in rats [6]. The effects of quercetin on heat acclimation and HSP content in blood cell has been examined in humans [4]. Peripheral organs such as liver, heart and skeletal muscles are known to play an important role in maintaining thermal homeostasis during heat stress. Little information is available on how an antioxidant intervention influences the responses of these organs to heat stress.

III. OBJECTIVE\HYPOTHESIS:
The objective of the proposed studies is to establish key biomarkers that can be used to identify mice that are heat intolerant. We hypothesize that heat intolerance is associated with inflammation and oxidative stress that induce hyperthermia and tissue injury during heat stress. Heat exposure should cause greater metabolic activities and stress-related changes in INT mice, compared to TOL mice. Anti-inflammatory or antioxidant therapy should reduce hyperthermia and tissue stress in response to heat shock. Thus, we propose to establish the biomarker profile of heat-intolerance and test the hypothesis by comparing heat-induced functional and/or biochemical changes in INT and TOL mice in the absence and presence of antioxidants. The proposed studies in this protocol will continue our work about the effects of selected dietary antioxidants, Que and Cur, on heat tolerance in mice.

IV. MILITARY RELEVANCE:
Heat related injury remains a threat to the health and operational effectiveness of military personnel who are heat-intolerant. Heat injury victims experience long-term complications. In this project, we will examine various biomarkers that can be potentially used to identify heat-intolerance. The information from this project will help develop strategies to detect heat-susceptible individuals and to improve military force readiness.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:
The experiments will be conducted using adult male C57BL/6J mice (b)(4) (b)(4) All animals will be housed in the USUHS LAM facility and allowed free access to water and food. We previously reported that INT mice were characterized by increased cardiovascular activities; and activation of heat shock proteins (HSPs) and glucocorticoid receptor in multiple organs in response to heat exposure. The proposed studies will test the effects of Que and Cur on heat stress in control (**Experiment 1**) and heat-acclimated mice (**Experiment 2**). Both Que and Cur are natural plant extracts and commonly present in fruits and vegetables. The proposed doses of Que and Cur (oral administration) in mice are comparable to the relatively low amount used in human studies, in which, for example, supplemental quercetin intake ranges from 1,000 mg (~14 mg/kg/day for a 70 kg person) [7] to 2,000 mg (~28 mg/kg) [4]. Neither Que nor Cur is listed as an ingredient in lab animal diets (Harlan Laboratories).

Animals. Mice will be surgically implanted with a temperature transponder (G2 E-Mitter, Mini Mitter Corp, Bend, OR) in abdominal cavity and a pressure transmitter (PA-C10, Data Science International, St. Paul, MN) (catheter placed into the left carotid artery and the transmitter body tunneled subcutaneously to a small pouch along the right ventral flank) under anesthesia (1.5-2% isoflurane). At least 2 weeks will be allowed for recovery. The body weight of the animals will be recorded 2-3 times each week. To be included in experimentation, mice will have to regain pre-surgical body weight. Their heat tolerance will be determined through a heat tolerance test described below. Mice with a peak core temperature of >= 42.0 °C during the test will be considered "INT" while those with a peak core temperature of <= 41.0 °C, "TOL" [2, 3].

V.1.1. Experiment 1: Effects of Que and Cur on heat tolerance
As illustrated in Figure 1, each mouse will undergo two heat tolerance tests separated by at least 10 days. The study includes three groups of mice that will receive regular food (control) or foods containing Que or Cur between the two heat tests. Spirometry measurements will be performed on control mice within 24 hrs before and 2 hrs after heat test. Each group requires 15 INT mice. Our previous studies showed ~31% of C57BL/6J mice are INT. **Total for Exp 1: mice = 45 x 3 groups = 135**

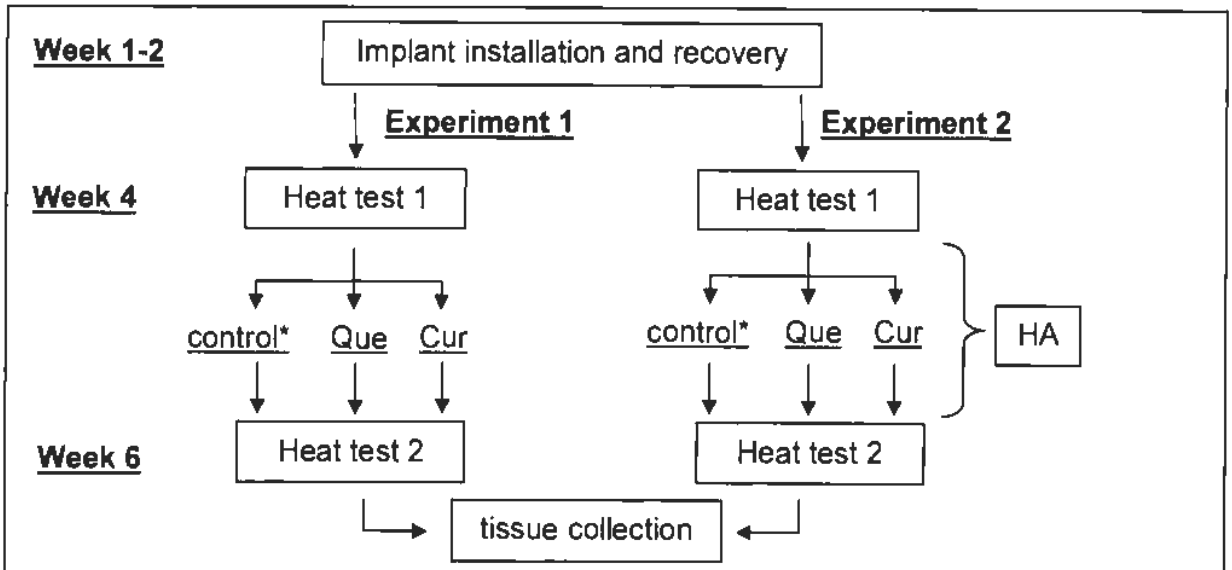


Figure 1. Experimental groups and procedures. Animals in each experiment will undergo two heat tests (an interval of at least 10 days). All mice will be placed on normal diet (control) or diet containing Que or Cur and mice in Experiment 2 will receive heat acclimation training (HA) between the two tests. *spirometry before and after heat test

Heat tolerance test. The test will be performed in an environmental chamber (Thermo Forma 3950, Marietta, OH). All mice will be placed in the chamber (in their regular cages with both food and water) overnight before each heat test. All heat tests will be conducted in the morning (0830-1200). As soon as a stable baseline measurement is obtained, heat will be turned on. The chamber temperature will gradually rise until it reaches the preset temperature, 39.5 °C (this heating process takes ~1 hr). Heat exposure will continue until whichever comes first, core temperature ≥42.4 °C or 3 hours (including chamber heating).

Dietary treatments. Mice will be placed on regular chow or chow containing Que or Cur. We estimate that, on average, mice eat approximately 2.5 grams of mouse chow per day. We prepare dried mouse chow pellets containing 0.6% Que or Cur (in weight), resulting in a daily dose of ~1.5 mg/mouse/day (60 mg/kg body wt/day). The pellets will be prepared by mixing Que or Cur with powdered mouse chow in a blender. Water will be added to make a thick paste sheet that is cut into squares and dried in an oven at 85° F until it returns to original weight.

Spirometry. Each mouse will be placed in an airtight polypropylene chamber (iWorx). The mouse chamber air is circulated (open loop) via a pump at a rate of ~250 ml/min during acclimation, which allows rapid temperature equilibration between the mouse and environmental chambers and sufficient fresh air supply (mouse resting minute ventilation: ~23 ml). The chamber (1.4L) provides a comfortable room that permits animals to calm down quickly and changes in gas concentration can be effectively detected. We will allow animals sufficient time (usually <20 min) to acclimate in the chamber. Closed-flow respirometry (~15 min) will be used to measure VO_2 and carbon dioxide release (VCO_2) at a flow rate of ~150 ml/min. Excurrent O_2 and CO_2 will be recorded continuously.

V.1.2. Experiment 2: Effects of Que and Cur on heat acclimation
The experimental groups and measurements are similar to Experiment 1 (Figure 1). In addition, mice will receive HA training between the two heat tests. Similarly, each group requires 15 INT mice. About ~31% of mice are expected to be INT. **Total for Exp 2: mice = 45 x 3 groups = 135**

HA training. Mice will be subjected to an ambient temperature of 33 °C in the environmental chamber (in their regular cages with foods and water) for 5 hrs daily for 10 consecutive days. This HA is considered mild. Higher temperatures and longer exposure (24hrs daily for 30 days) have been used in rodent studies.

Upon completion of each experiment, animals will be euthanized for collection of arterial blood (via the right carotid artery), urine, heart, liver kidneys and soleus and gastrocnemius muscles under isoflurane anesthesia.

V.2. Data Analysis:
Based on our experience, the number of mice requested is necessary to accomplish the research goals. Two-way ANOVA with repeated measures will be used to evaluate the differences among groups in each Experiment. Post hoc subgroup comparisons will be performed after significant effects. Using reasonable estimations of the variance of each of the measurements based on previous studies, we determined the proposed sample sizes to have an 80% power to detect a difference in mean variables of 1.2 standard deviations with a significant level of 0.05 (two-tailed).

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:
Heat tolerance in a living animal is a result of the complex interaction involving multiple organs/systems. This cannot be sufficiently modeled in a non-living system (i.e. computer modeling) or simple living unit (i.e. cultured cells) and thus, requires the use of in vivo model system.

V.3.2. Animal Model and Species Justification:
Rodents have been commonly used in heat stress studies. It is desirable to characterize heat intolerance in a mouse model of heat stress, which offers an additional advantage due to the ability to use genetic engineering, an important tool to study the mechanisms for heat tolerance in the future.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	Mus musculus	
V.3.3.2. Strain/Stock:	C57 BL/6J	
V.3.3.3. Source/Vendor:	(b)(4)	
V.3.3.4. Age:	2-3month	
V.3.3.5. Weight:	20-30g	

V.3.3.6. Sex: M

V.3.3.7. Special Considerations: none

V.3.4. Number of Animals Required (by Species):

Mice 270

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:

Possible pain and distress will be reduced by the use of an implanted device to record temperature and blood pressure. Alternatives (i.e. rectal probe for temperature) would be more stressful. All the procedures (i.e. installation of telemetry probes, terminal blood or tissue collection) that cause pain will be completed under anesthesia. Pain reliever will be administered to animals following surgeries.

V.3.5.2. Reduction:

We design experiments and prepare tissue samples in a way that allows us to conduct several physiological measurements in a given animal and multiple assays in a given tissue rather than adding animals.

V.3.5.3. Replacement:

Ex vivo studies, tissue culture methods, and computer simulations were considered but rejected since they are not suitable techniques to address the goals of the project. These techniques cannot replicate the complexity of in vivo temperature regulation mechanisms.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:		
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	270	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

Isoflurane (4% induction, 2% maintenance) will be used during surgeries and terminal tissue collections. Analgesic will be given for the first 24 hrs post surgery and longer if needed. Buprenorphine will be administered subcutaneously between the shoulders blades or along the back at a dose of 0.05-0.1mg/kg (0.01 ml/mouse) q12hr using a 27-29 gauge needle in a 1 cc syringe.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

Animals will be continuously monitored for their activity after heat tests. They will be transferred back to their housing unit once they return to normal activity.

V.4.1.2.3. Paralytics:

none

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched:

PubMed, Altweb

V.4.1.3.2. Date of Search:

10/22/2013

V.4.1.3.3. Period of Search:

All available dates

V.4.1.3.4. Key Words of Search:

Pubmed: heat stress OR exposure AND mice

Altweb: body temperature measurement

V.4.1.3.5. Results of Search:

No alternative procedure or method, which results in less pain or stress, was available.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

The proposed study will expose conscious mice to high ambient temperature (39.5 ± C) for up to 180 min including ~1hr chamber heating. This process is needed to investigate heat tolerance that cannot be effectively assessed under anesthesia or analgesia. Our preliminary results show that majority of the animals remained fully active during entire heat exposure and core temperatures rapidly returned to baseline levels following heat exposure. The discomfort caused by this process should be very limited.

V.4.2. Prolonged Restraint:

none

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions:

The skin of the incision sites will be prepared (shaving, hair removal) and treated with betadine and alcohol. The surgeon will follow all aseptic procedures (wear a lab coat and gloves and use sterilized instruments). The animal's body temperature will be maintained at ~37 °C with a heating pad.

V.4.3.2. Procedure:

Animals will be periodically tested for absence of a response to toe pinch to ensure the appropriate depth of anesthesia during the surgical operation. The following procedures will be performed during a single surgery.

Blood pressure telemetry probe installation: A midline incision will be made on the anterior neck area. The left carotid artery will be carefully isolated and freed from the muscle and tissue around the trachea. The implantable catheter of the telemetry probe (Data Science International, St. Paul, MN) will be securely installed into the carotid artery. The telemetry probe body (weight: 1.4 gm) will be implanted into a subcutaneous pocket in the right flank. The incision will be closed with sutures wound clips.

Body temperature telemetry probe installation: A midline incision will be made to access to abdominal cavity under anesthesia after the area is shaved/cleaned/sterilized. A telemetry probe for temperature and heart rate (weight: 1.1 g. Respironics, Inc) will be

placed in the cavity before closing the incision with absorbable suture (muscle) and wound clips (skin).

V.4.3.3. Post-surgical Provisions:
Animals will be carefully monitored following the implant surgery. They will return to their housing unit after they become awake and fully mobile. Animals will be surveyed for signs of infection or pain twice a day for two days after the surgery.

V.4.3.4. Location:
G-175

V.4.3.5. Surgeon:
(b)(6)

V.4.3.6. Multiple Major Survival Operative Procedures:
none

V.4.3.6.1. Procedures:
N/A

V.4.3.6.2. Scientific Justification:
N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:
none

V.4.4.2. Biosamples:
Upon completion of the studies, we will collect blood via a carotid catheter, urine via a bladder puncture and tissues/organs (hindlimb muscle, liver, kidneys and heart, etc) under terminal anesthesia.

V.4.4.3. Adjuvants:
none

V.4.4.4. Monoclonal Antibody (MAbs) Production:
none

V.4.4.5. Animal Identification:
Tail tattoo (will be performed under anesthesia at the time of surgical implantation). Cage cards

V.4.4.6. Behavioral Studies:
none

V.4.4.7. Other Procedures:
none.

V.4.4.8. Tissue Sharing:
none

V.4.5. Study Endpoint:
The endpoint for mice in Experiments 1 and 2 will be up to 5 weeks following surgical implantation. Although not expected, any animals that are in poor health (i.e. inactive, sign of persistent pain or stress, body weight loss >15%) will be euthanized upon the suggestion of or in consultation with the LAM veterinary staff.

V.4.6. Euthanasia:
Upon completion of experiments, animals will be anesthetized with isoflurane. Euthanasia will be accomplished by exsanguination under deep anesthesia for terminal blood and tissue collection. The euthanasia procedure is in compliance with the 2013 AVMA Guidelines on Euthanasia.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations:
Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:
Building(s) (b)(6) Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No √

Fluid Restriction: Yes _____ No √

V.5.1.3. Exceptions:
none

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
Animals will be observed and cared for by the Center for LAM personnel according to USUHS Center for LAM SOPs.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions:
none

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
all protocol procedures	(b)(6)	15 yrs research experience using rodents	attended USUHS Investigator Training Course in March 2008
recovery assessment		>10 yrs experience in rodent research	attended USUHS Investigator Training Course in 1982?
all protocol		3 yrs experience in	attended USUHS

procedures	(b)(6)	rodent research	Investigator Training Course in July 2010.

VII. BIOHAZARDS/SAFETY:

All personnel handing animals will wear protective gloves, lab coat and mask at all times to minimize exposure to allergens. During surgical procedures, isoflurane vapor will be scavenged by a canister that connects to mouse anesthesia mask.

VIII. ENCLOSURES:

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 10/31/2013

Principal Investigator Signature — Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 10/31/2013

Principal Investigator Signature — Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) _____ 10/31/2013

Principal Investigator Signature **Date**

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title:

Biomarkers for Detection of Heat Intolerance in Mice

C. Principal Investigator:

(b)(6)

D. Performing Organization:

Uniformed Services University of the Health Sciences

E. Funding:

Office of Naval Research

F. Objective and Approach:

Heat-related injuries remain a threat to the health and operational effectiveness of military personnel. The objective of the proposed research is to establish key biomarkers that can be potentially used to identify individuals susceptible (heat intolerance) to heat stress. The proposed studies will determine the biomarkers of heat intolerance by comparing the physiological and biochemical changes of heat-tolerant and -intolerant mice in response to heat exposure. Our previous studies have shown that heat exposure causes stress-related changes in multiple organs of heat-intolerant mice. In this protocol, we will determine if these changes are associated with oxidative stress by testing the effects of selected dietary antioxidants on heat tolerance and acclimation in mice. Specifically, we will examine the effects of heat exposure and acclimation on mice fed a normal food or food containing quercetin or curcumin. The proposed research will provide information useful for developing strategies to detect and protect individuals susceptible to heat injury

G. Indexing Terms (Descriptors):

heat stress, tolerance, acclimation, antioxidants, mice

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

October 2, 2015

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF MILITARY EMERGENCY MEDICINE

SUBJECT: IACUC Approval of Protocol - Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on October 2, 2015:

Animal Protocol Title: "Application of Low Dose Inhaled Carbon Monoxide to Attenuate Diet-Induced Obesity and Insulin Resistance in Rats (Rattus Norvegicus)"

USUHS Protocol Number: (b)(6)

Expiration Date: October 1, 2018

Supporting Grant(s) Number: TBD

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc: Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Application of low dose inhaled carbon monoxide to attenuated diet-induced obesity and insulin resistance in rats (*Rattus norvegicus*)

Revised

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: TBD

FUNDING AGENCY: USUHS intramural start-up and JPC-5 (CANDS)

EARLIEST ANTICIPATED FUNDING START DATE: ongoing

PRINCIPAL INVESTIGATOR: LCDR (b)(6)

(b)(6) _____ MEM (b)(6) 14AUG2015

Principal Investigator Signature | Department | Office/Lab Telephone | Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) _____ Acting Chair (b)(6) 8.17.15

Research Unit Chief / Dept. Head Signature | Title | Telephone | Date

Typed Name: Dr. (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) _____ MEM (b)(6) 14AUG2015

Statistician Signature | Department | Telephone | Date

Typed Name:

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) _____ LAM (b)(6) 8/21/15

Attending/Consulting Veterinarian Signature | Department | Telephone | Date

Typed Name: LT (b)(6) VMD

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) Military & Emergency Medicine (b)(6)
(b)(6)

ANIMAL PROTOCOL TITLE: Application of low dose inhaled carbon monoxide to attenuate diet-induced obesity and insulin resistance in rats (*Rattus norvegicus*)

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER:

CO-INVESTIGATOR(S): (b)(6)

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

The prevalence of obesity in the U.S. general population and military continues to increase. Obesity increases the risk of developing insulin resistance, diabetes, and cardiovascular disease. Dysregulated mitochondrial function and altered structure have been reported to be associated with obesity related conditions. Diet and exercise are recommended in the prevention and treatment of obesity and resulting diseases, but are not reversing the trend. The use of alternative prevention and treatment strategies, in conjunction with exercise, may be required to slow the rise in obesity and diseases. Low dose inhaled carbon monoxide (CO) has successfully been used to increase the production of new mitochondria, thus may protect against the onset of obesity associated diseases. The overall goal of this project is to determine whether low dose inhaled CO is efficacious in preventing diet-induced obesity and insulin resistance in rats by preserving mitochondrial structure and function.

II. BACKGROUND:

II.1. Background:

In the U.S., the prevalence of overweight and obesity combined is approximately 70%, and the prevalence of obesity is approximately 35%, rates that have more than doubled from 1976-1980 (1). In the U.S. Military, the combined prevalence of overweight and obesity is 60.5% and the prevalence of obesity is 12.9%, a 2.5 fold increase in obesity from 1990 (18). While there is a genetic component to the pathology of obesity and associated diseases, much of the cause can be attributed to excess energy intake and insufficient physical activity. Diet-induced obesity is associated with an increase in fat accumulation within visceral adipocytes, chronic low-grade inflammation, an increase in plasma free fatty acids that leads to ectopic fat accumulation and dysregulated lipid and glucose oxidation in the liver and skeletal muscle, and insulin resistance (17). The underling etiology of insulin resistance or prediabetes has been reported to be attributed to an increase in mitochondrial reactive oxygen species (ROS) and a reduction in the cellular antioxidant defenses (3, 5). If unabated, the increase in ROS may lead to impaired regulation of mitochondrial morphology, a reduction in mitochondrial biogenesis and a reduction in respiratory capacity, all of which have been linked to the

development of type 2 diabetes (6, 10, 12, 13). Although lethal at high concentrations, low-dose carbon monoxide (CO) may provide a safe, relatively non-invasive treatment option for obesity and its associated metabolic diseases. By stimulating the production of antioxidant enzymes (heme oxygenase-1 (HO-1) and mitochondrial superoxide dismutase (SOD2)), and hydrogen peroxide, CO serves to protect against oxidative damage and inflammation, and stimulates mitochondrial biogenesis (14, 21). The proposed work is expected to identify how CO alters mitochondrial dynamics in skeletal muscle of rats fed a high-fat diet for 10 weeks. The results from this work will advance our understanding of diet-induced obesity and mitochondria dysfunction. Perhaps most intriguing will be the therapeutic potential that may be afforded by CO.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:
Biomedical Research Database; RePORT; PubMed

II.2.2. Date of Search: 08/13/2015
II.2.3. Period of Search:
All available years

II.2.4. Key Words and Search Strategy:
(("Mitochondria"[Mesh] OR mitochondria) AND ("Carbon Monoxide"[Mesh] OR "inhaled carbon Monoxide" OR "inhaled CO")) OR (("Insulin Resistance"[Mesh] OR "Diabetes Mellitus"[Mesh] OR "Overweight"[Mesh] OR "insulin resistance" OR overweight OR obesity OR obese OR diabetes) AND ("Carbon Monoxide"[Mesh] OR "inhaled carbon Monoxide" OR "inhaled CO")) AND ("Rodentia"[Mesh] OR rats OR rat OR rattus)

Biomedical Research Database 0 hit; RePORT 0 hit; MEDLINE/PubMed 151 hit

II.2.5. Results of Search:
No previous studies were found that duplicate the proposed research. Only one study has examined the efficacy of inhaled CO on diet-induced obesity in mice (7). By exposing mice to 28 parts per million (ppm) or 200 ppm daily for 30 weeks, CO was determined to be effective in attenuating diet-induced obesity and improving glycemia in the first 10 weeks, but not with continued high-fat feeding. In this study, an exercise component was not included, and mitochondria morphology and respiratory capacity measures were not performed. Furthermore, CO was administered every day vs. intermittently, thus this amount over this duration could have in fact lead to ROS production, inflammation and mitophagy, masking any protection that may exist in the long-term.

III. OBJECTIVE\HYPOTHESIS:

The overall objective of this research is to explore the potential use of low dose inhaled CO on attenuating the development of diet-induced obesity and insulin resistance in rats, with and without exercise since moderate-intensity aerobic and high-intensity interval training increases mitochondria number and quality, respiratory capacity and whole body oxygen consumption (VO_2) (8, 15, 20, 23). The central hypothesis is that

CO will protect against diet-induced obesity and insulin resistance by preserving mitochondrial morphology, mitochondrial biogenesis, and respiratory capacity.

IV. MILITARY RELEVANCE:

Obesity related illnesses are likely to increase in the military with rising obesity rates. This will impact operational readiness, both fitness to perform and equipment requirements, e.g., the racks on ships will be too small for the girth of a sailor. In addition, the number of available healthy recruits that meet body fat and fitness standards will become fewer, impacting force readiness. Finally, Tricare and the VA system will be affected by the financial burden of treating obesity associated diseases. Research aimed at understanding the molecular mechanisms of obesity, and potential adjunct prevention and treatment options are, therefore, warranted.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

Animals: The experiments will be conducted using male obese prone Sprague Dawley (OP-CD) rats aged 8-9 weeks old (b)(4) All animals will be doubly housed in the USUHS LAM facility on a reverse 12 h light and 12 h dark schedule, and allowed free access to water and food. Animals will be randomized to 1 of 5 groups after pre-study testing **(Figure 1)**: sedentary (CON), high-fat sedentary (HF), high-fat exercise training (HF-EX), high-fat sedentary carbon monoxide (HF-CO), or high-fat exercise training + carbon monoxide high-fat (HF-EXCO).

Exercise: Seven days upon arrival, all rats will be habituated to endurance exercise on a 4 lane rodent motorized treadmill (Columbus Instruments). The lanes are 43.8 cm in length and 12 cm wide with the dividers between lanes 12.7 cm in height. The rats will perform 3 practice sessions to condition them to run on the treadmill. The sessions will consist of the rats being placed on the lanes with the speed increased to 9 m/min, a very slow pace, for 10 min at an incline of 0° (19). On the back of each treadmill lane, there is an electrical shock grid (11.4 x 12 cm) that can produce a stimulus current of 0 – 1.5 mA (adjustable), a voltage of 163V, at a repetition rate (adjustable) of 1, 2 or 3 Hz. Since electrical stimulation can yield significant stress on the animal, the current will not exceed 0.5 mA and the rate will not exceed 1 Hz over a period of 5 sec. If the animals fail to run for a period of 5 sec, then the exercise session will be terminated. To circumvent reliance on negative reinforcement, chocolate will be provided following all exercise sessions which will positively condition the animals to perform the treadmill exercise. Following the 3 familiarization sessions, rats will undergo a test to determine the animals maximal aerobic exercise capacity (VO_2 max) using a modular enclosed metabolic treadmill (Columbus Instruments). The only difference between this unit and the regular treadmills is that the treadmill is enclosed in Plexiglas for determination of oxygen uptake (VO_2) and carbon dioxide production (VCO_2) using open circuit spirometry. The animals will be placed into this unit for 5-10 min to determine basal steady state VCO_2 and VO_2, followed by a 15 min warm-up at 40-50% of the estimated VO_2 max, which has been reported to correspond to a treadmill speed of 12-14 m/min at an incline of 25° (24). The belt speed will be increased by 1.8 m/min every 2 min until VO_2 levels-off (generally 8-15 min), or the animal refuses to run for 5 sec. The total testing time will be ≤ 45 min, including the time needed to establish steady state VCO_2

and VO_2. Since 10-15% of rats refuse to run on a treadmill (11), this pre-study familiarization and VO_2 max testing will be used to randomize the animals into the exercise training (HF-EX and HF-EXCO) and non-exercise (CON, HF and HF-CO) groups. During the 10 week protocol, the HF-EX and HF-EXCO groups will undergo aerobic exercise training three times/week, consisting of an 18 min warm-up at 40-50% of the animals measured VO_2 max, followed by 6 alternating intervals of 4 min at 85-90% and 2 min at 50% of the animals VO_2 max, serving as an active recovery period (9). Should the animals fail to complete this protocol, adjustments will be made to the intensity so that the volume of work performed is similar amongst the animals, i.e., lower speed, longer duration. In addition, a VO_2 max test will be performed in the HF-EX and HF-EXCO groups every 2 weeks with the training workload adjusted for subsequent training sessions. After each training session and VO_2 max test, the animals will receive a small piece of chocolate as positive reinforcement (24). The rats in the CON, HF and HF-CO groups will perform a weekly 10 min treadmill training session at 9 m/min and 0° incline to maintain treadmill familiarity.

Diet: All animals will consume a low-fat control diet upon arrival until the start of the 10 week protocol. After all pre-study testing is complete and the animals have been randomized to CON, HF, HF-EX, HF-CO and HF-EXCO groups, the CON group will continue to receive the low-fat control diet, whereas the HF, HF-EX, HF-CO and HF-EXCO groups will be fed a high-fat diet comprised of 43% carbohydrates, 42% fat, and 15% protein ad libitum until the completion of the study.

Body mass: Weights will be recorded for all animals weekly using a calibrated scale.

Resting whole body oxygen consumption (VO_2) and carbon dioxide production (VCO_2): Prior to, at week 5, and following the 10 week protocol (after an overnight fast), conscious rats will be placed in the modular enclosed metabolic treadmill for 30 min for continuous recording of VO_2 and VCO_2. These values will be used to calculate the respiratory exchange ratio (VCO_2/VO_2) and energy expenditure (kcal/h) (4).

Intraperitoneal glucose tolerance test (IPGTT): Prior to and following the 10 week protocol, and after an overnight fast, approximately 5-10 µL of blood will be collected from the tail vein (time 0) from conscious, restrained rats. The rats will be placed in a rat restrainer and the tail will be swabbed with alcohol to expose the blood vessel. Finger pressure will be applied approximately 5 cm from the tip of the tail, and a 21-23 gauge butterfly needle will be inserted into the tail vein with blood collected into microvette tube. The total time of restraint will be approximately 1-3 min. Next, 2 g of glucose/kg body weight (99.5% glucose from Sigma-Aldrich dissolved in distilled water) will be administered intraperitoneally via a 1-2 mL syringe with a 21 gauge needle. The same blood collection procedure will be repeated 15, 30, 60, 90 and 120 min post-glucose injection for determination of blood glucose using reagent strips and an automated handheld glucometer. The rats will be placed back in their cages, without access to food, in-between blood collection over the 2 h IPGTT.

Baseline and post-study blood collection for serum lipids and insulin: Prior to and following the 10 week protocol, and after an overnight fast, approximately 400 µL of blood will be collected from the lateral saphenous vein into a 500 µL microvette for later

analysis of serum lipids (total cholesterol, triglycerides, high density and low density lipoproteins) and insulin. This sample will be collected at the same time as the first tail vein blood draw (time 0) for the IPGTT to avoid an additional day of fasting and stress. The rats will be placed in a restrainer; the lower limb will be shaved with an electric razor and swabbed with alcohol to expose the vein prior to puncturing with a 21-23 gauge needle.

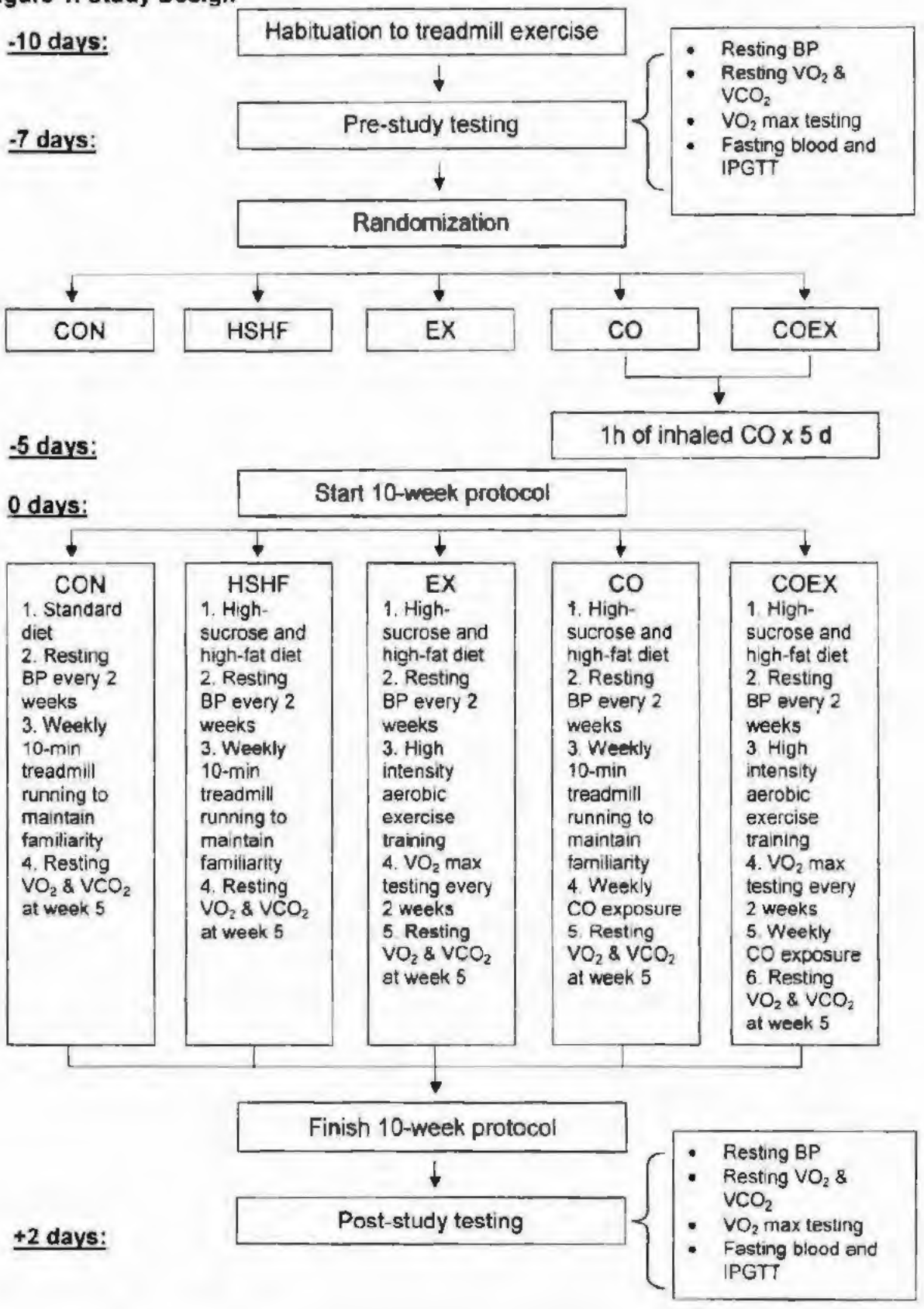
Carbon monoxide (CO) exposure: Two rat cages containing 2 rats in each will be placed in an enclosed plastic box that will be placed underneath a ventilated hood. Carbon monoxide (250 ppm balance air) will be supplied to the enclosed box via an inlet port for 1 h x 5 consecutive days before the 10 week protocol, and once per week for 1 h during the 10 week protocol. An outlet port with tubing will be ventilated into the hood. A portable CO analyzer will be placed inside the enclosed box to verify that the CO is maintained at 250 ppm for 1 h. A carbon monoxide concentration of 250 ppm has previously been reported to raise carboxyhemoglobin (HbCO) levels to ~13% in rats (22), with levels <20% considered to be safe (2). Only animals in the HF-CO and HF-EXCO groups will receive the CO treatment.

Carboxyhemoglobin levels: Of the animals that receive CO (HF-CO and HF-EXCO), before and after a CO treatment, approximately 100 μL of blood will be collected from the tail vein in a subset of animals (n = 6-8), as described above, in order to determine the concentration of carboxyhemoglobin in whole blood.

Resting blood pressure: Before the 10 week protocol, during weeks 2, 4, 6, 8 and following the 10 week protocol, resting blood pressure will be measured in all of the rats using a rat tail-cuff system (Kent Scientific). Rat tail-cuff blood pressure monitoring is considered non-invasive and has been extensively used by researchers, i.e., > 1000 PubMed hits. Rats will be placed in a restrainer that are appropriate for body mass with the tails exposed and placed on a heating pad to maintain tail temperature between 32-35° C for 15 min, which will be monitored with an infrared thermometer. Next, an occlusion cuff will be placed near the base of the tail and a volume pressure-recording sensor cuff will be placed distal to the occlusion cuff. Once the cuffs are secure, inflation-deflation cycles will commence with the first 5 serving as acclimation cycles, followed by 5 measurements used for data analysis. The total time of restraint is approximately 20-25 min. Once the measurements are complete, the animals will be immediately placed back into their housing cages with the appropriate food and water.

Tissue and blood collection: Upon collection of all post-study data, animals will be euthanized in an induction box using isoflurane. The animals will not be in contact with gauze dampened with isoflurane. Blood from the left ventricle and tissues (skeletal muscle, bone, heart, lung, liver and brain, and epidydmal fat pads) will be harvested for later analyses.

Figure 1. Study Design



V.2. Data Analysis:
A two factor repeated measures ANOVA will be used to determine whether inhalation of low dose CO is as effective as aerobic exercise training in attenuating diet induced obesity and insulin resistance. Since this experiment is novel, sample size estimates are based off of studies that explored the effects of a high-fat diet on obesity and metabolic dysregulation in rats (16, 25). From these studies, we anticipate that 10 weeks on a high-fat diet will result in a mean difference in weight gain of ~100 ± 55 g (SD) compared to rats fed standard chow, requiring a minimum of 9 rats per group to achieve 80% power with a 0.05 significance level (two-tailed). Since not all rats are trainable (~10-15%), and to ensure that there is sufficient tissue available for mitochondrial morphology, respiratory capacity and biogenesis measures, and for preliminary CO exposure and exercise testing, we estimate that approximately 15 animals in each group will be needed to complete this study.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:
Obesity and the metabolic sequela is complex, involving multiple organs/systems. This cannot be sufficiently modeled in a non-living system (computer models) or cultured cells and thus, requires the use of in vivo model system.

V.3.2. Animal Model and Species Justification:
Rats are a good species to explore the effects of diet-induced obesity, specifically because this outcome and metabolic complications are observed within a short period of time, i.e., 5-12 weeks. While these changes also occur in mice, the skeletal muscles that are preferentially recruited during aerobic exercise training are too small in mice for all of the proposed, necessary, measurements. Only males will be used for this research to avoid the potential confounding effects of estrogen and progesterone on energy homeostasis.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	*Rattus norvegicus*	
V.3.3.2. Strain/Stock:	Sprague Dawley (OP-CD); strain code: 463	
V.3.3.3. Source/Vendor:	(b)(4)	
V.3.3.4. Age:	8-9 weeks	
V.3.3.5. Weight:	~250-300 g	
V.3.3.6. Sex: Females will not be used in this pilot study due to the role that estrogen and progesterone play in regulating	M	

glucose and lipid metabolism, and insulin sensitivity, serving to protect from diet-induced obesity. However, once we characterize the effects of CO on obesity and insulin resistance in male rats, we will then consider studying females, but this will be several years down the road. Furthermore, the entire study design would have to be changed, lengthened, to ensure that a high proportion of the female rats become obese and insulin resistant.

V.3.3.7. Special Considerations: None

V.3.4. Number of Animals Required (by Species): Rats 75

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:

Possible pain and distress will be reduced by habituation to the rodent treadmill and the blood pressure tail-cuff system. There is no pain or discomfort associated with inhaling 250 ppm of CO balanced with room air. The animals will be restrained during blood collection to minimize distress.

V.3.5.2. Reduction:

The proposed experiments are designed to maximize the number of measurements that can be made to support the objectives. In addition, we will collect additional organs not intended for study in this research (brain, heart and lung), but quite possibly for future experiments which will ultimately reduce the number of animals required for those protocols. Since this research has not previously been carried out, reducing the number of animals, or use of animals, is not possible.

V.3.5.3. Replacement:

In vitro models were considered, but deemed inappropriate to test the proposed hypothesis since the objectives are to examine in vivo diet-induced obesity.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2

V.4.1.1.1.1. Column C:

V.4.1.1.1.2. Column D:

V.4.1.1.1.3. Column E: 75

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:
None

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:
Animals will be monitored for 30 min following tail-vein blood collection. Following blood pressure tail-cuff monitoring, resting VO_2 and VCO_2 monitoring, CO exposure and treadmill exercise, animals will be immediately returned to their housing units. Since the electrical shock grid can induce redness and bleeding to the tails, all of the rat tails will be monitored. Although unlikely, in the event that a tail becomes necrotic, the rat will be removed from the study and euthanized using isoflurane.

V.4.1.2.3. Paralytics:
None

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched:
PubMed, Altweb

V.4.1.3.2. Date of Search:
8/12/2015

V.4.1.3.3. Period of Search:
All available dates

V.4.1.3.4. Key Words of Search:
(treadmill* OR forced running) AND ("Rodentia"[Mesh] OR rats OR rat OR rattus) AND (alternative OR substitute OR replace* OR allev*) AND (pain OR distress OR stress OR shock OR motivation)
PubMed 43 hit; Altweb 38 hit

V.4.1.3.5. Results of Search:
No alternative procedure or method, which results in less pain or stress, was available. Habituation and positive-reinforcement are the best approaches for alleviating stress.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
High-intensity aerobic exercise training and VO_2 max testing. During the 10 week protocol, the HF-EX and HF-EXCO groups will undergo aerobic exercise training three times/week, consisting of an 18 min warm-up at 40-50% of the animals measured VO_2 max, followed by 6 alternating intervals of 4 min at 85-90% and 2 min at 50% of the animals VO_2 max, serving as an active recovery period (9). This intensity is high, but

serves to reduce the frequency by which the animals need to perform exercise training (i.e., 3 days per week vs. 5 days per week) and still obtain the benefits. Should the animals fail to complete this protocol, the intensity will be reduced (lower speed) and the duration will be increased so that the volume of work performed is similar between all animals. Although not anticipated to occur, if the intensity does need to be reduced, the exercise session will never last longer than 90 min including the warm-up period. Moreover, if the animals fail to run for 5 consecutive sec with electrical stimulation provided (0.5 mA at a frequency of 1 Hz) the exercise session will be terminated. When performing VO_2 max testing (all animals), as described in section V.1., if the animals VO_2 plateaus, or they fail to run for 5 consecutive sec with electrical stimulation, same as above, the test will be terminated. Therefore, whenever an animal cannot perform, the exercise session or test will be terminated.

V.4.2. Prolonged Restraint:
None

V.4.3. Surgery:
V.4.3.1. Pre-surgical Provisions:
No surgeries will be performed.

V.4.3.2. Procedure:
N/A

V.4.3.3. Post-surgical Provisions:
N/A

V.4.3.4. Location:
N/A

V.4.3.5. Surgeon:
N/A

V.4.3.6. Multiple Major Survival Operative Procedures:
None

V.4.3.6.1. Procedures:
N/A

V.4.3.6.2. Scientific Justification:
N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:
Prior to and following the 10 week protocol, and after an overnight fast, 2 g of 99.5% glucose/kg body weight (dissolved in distilled water) will be administered intraperitoneally via a 1-2 mL syringe with a 21 gauge needle.

V.4.4.2. Biosamples:
For the IPGTT conducted before and after the 10 week protocol, 5-10 µL of blood will be collected from the tail vein (time 0) from conscious, restrained rats. The rats will be placed in a restrainer and the tail will be swabbed with alcohol to expose the blood vessel. Finger pressure will be applied approximately 5 cm from the tip of the tail, and a 21-23 gauge butterfly needle will be inserted into the tail vein with blood collected into microvette tube. The total time of restraint will be approximately 1-3 min. For serum lipids, 400 µL of blood will be collected from the lateral saphenous vein into a 500 µL microvette. This sample will be collected at the same time as the first tail vein blood draw (time 0) for the IPGTT to avoid an additional day of fasting and stress. The rats will be placed in a restrainer for no ≥ 5 min; the lower limb will be shaved with an electric razor and swabbed with alcohol to expose the vein prior to puncturing with a 21-23 gauge needle. Upon completion of the protocol, we will collect blood from the left ventricle and tissues (skeletal muscle, brain, heart, lung, liver, brain and epidydmal fat pads) under terminal anesthesia.

V.4.4.3. Adjuvants:
None

V.4.4.4. Monoclonal Antibody (MAbs) Production:
None

V.4.4.5. Animal Identification:
Ear tags and cage cards

V.4.4.6. Behavioral Studies:
None

V.4.4.7. Other Procedures:
None

V.4.4.8. Tissue Sharing:
None

V.4.5. Study Endpoint:
The endpoint for all animals will be up to 10 days following the 10 week protocol to ensure that all post-study data is collected. Although not expected, any animals that are in poor health (i.e., inactive, sign of persistent pain or stress, body weight loss >15%) will be euthanized upon the suggestion of or in consultation with the LAM veterinary staff.

V.4.6. Euthanasia:
Upon completion of experiments, animals will be euthanized with isoflurane for terminal tissue collection.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. The animals will be housed 2 per cage.

V.5.1.1. Study Room:
Building(s) (b)(6) Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes ___X___ No _____

Fluid Restriction: Yes _____ No ___X___

In order to assess the animal's ability to transport and oxidize glucose, measure fasting serum lipids, and assess resting energy expenditure, the animals must be fasted during their light phase. This will occur on two occasions before, and after the 10 week protocol. On one occasion, blood collection and the IPGTT will be performed, and on the other occasion, resting VO_2 and VCO_2 measurements will be collected. The test diets produced by Harlan (TD.08485 and TD.88137) will be procured by LAM, and provided by the investigators throughout the study. Animals will be housed on a reverse light cycle in order for the investigators to conduct exercise testing and training.

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
Animals will be observed and cared for by the Center for LAM personnel according to USUHS Center for LAM SOPs.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples may include tunnels and nylabones for rats.

V.5.3.2. Enrichment Restrictions:
None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or	Name of person	Qualifications of person	Specific training in this

procedure (e.g., tail vein injections, euthanasia)	performing activity	performing activity (e.g., research technician, 2 yrs experience)	activity or procedure (e.g., rodent handling class, 1999)
All protocol procedures	(b)(6)	6 yrs experience in rodent research	Attended USUHS investigator training course in 2015
All protocol procedures		>20 yrs experience in rodent research	Attended USUHS investigator training course in 2008
All protocol procedures		>20 yrs experience in rodent research	attended USUHS investigator training course in 2014
IPGTT, blood collection, tail-cuff blood pressure monitoring, exercise training, tissue collection		4.5 yrs experience in rodent research.	Attended USUHS animal handling class and investigator training course in 2011
IPGTT, blood collection, tail-cuff blood pressure monitoring, exercise training, tissue collection		None. Will be trained by the PI and (b)(6)	Attended USUHS animal handling class and investigator training course in 2015
IPGTT, blood collection, tail-cuff blood pressure monitoring, exercise training, tissue collection		None. Will be trained by the PI and (b)(6)	Attended USUHS animal handling class and investigator training course in 2015

VII. BIOHAZARDS/SAFETY:

All personnel handing animals will wear protective gloves, lab coat and mask at all times to minimize exposure to allergens. Personnel will follow universal sharps procedures to reduce risk of injury.

VIII. ENCLOSURES:

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 14 AUG 2015

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 14 AUG 2015

Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature

14AUG2015

Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title:
Application of low dose inhaled carbon monoxide to attenuate diet-induced obesity and insulin resistance

C. Principal Investigator:
(b)(6)

D. Performing Organization:
Uniformed Services University of the Health Sciences

E. Funding:
USUHS Office of Research (Start-up) and JPC-5 (CANDS)

F. Objective and Approach:
The prevalence of obesity in the U.S. general population and military continues to increase. Obesity increases the risk of developing insulin resistance, diabetes, and cardiovascular disease. Dysregulated mitochondrial function and altered structure have been reported to be associated with obesity related conditions. Low dose inhaled carbon monoxide (CO) has successfully been used to increase the production of new mitochondria, thus may protect against the onset of obesity associated diseases. Obese-prone Sprague Dawley rats will be fed a high-fat diet for 10 weeks and subjected to low dose inhaled CO (250 ppm), high-intensity aerobic exercise training, or both. Measures will include fasting lipids, insulin, and glucose tolerance, blood pressure, resting VO_2 and VCO_2, and VO_2 max testing. Moreover, indicators of skeletal muscle mitochondrial biogenesis and morphology, and respiratory function will be determined. Data will be analyzed using a two-way repeated measures ANOVA. The novelty of this experiment precludes the alternative use of cell culture or simulated models. This research will determine whether low dose inhaled CO is efficacious in preventing diet-induced obesity and insulin resistance in rats by preserving mitochondrial structure and function.

G. Indexing Terms (Descriptors):
Inhaled carbon monoxide, animals, rats, exercise, diet-induced obesity, insulin resistance, skeletal muscle, mitochondrial

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July 12, 2012

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF MICROBIOLOGY

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on July 12, 2012:

Animal Protocol Title: "Pathogenicity of Shiga Toxin-Producing E.coli and Ricin Intoxication (Mice)"

USUHS Protocol Number: (b)(6)

Expiration Date: July 11, 2015

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

Learning to Care for Those in Harm's Way

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Pathogenicity of Shiga toxin-producing *E. coli* and ricin intoxication (mice)

GRANT TITLE (if different from above): "Pathogenicity of Shiga Toxin-producing *E. coli*", "Shiga Toxin and Ricin Interaction with Enterocytes and Rescue of Target Cells", "Antibacterial and Anti-Shiga Toxin Expression Activities of Morpholino Compounds against EHEC O104 H4 and Related Prokaryotes", "Susceptibility of Recombinant Inbred Mice to Shiga Toxins and *E. coli* O157:H7"

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: NIAID, DTRA, USUHS

EARLIEST ANTICIPATED FUNDING START DATE: Current

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6)		MIC	(b)(6)	
Principal Investigator Signature		Department	Office Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)		Chair	(b)(6)	
Dept. Head Signature Typed Name: (b)(6) Ph.D.		Title	Telephone	Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)		MIC	(b)(6)	5/14/12
Statistician Signature Typed Name: (b)(6) Ph.D.		Department	Telephone	Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)	LAM	(b)(6)	6-12-12
Attending/Consulting Veterinarian Signature Typed Name	Department	Telephone	Date

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

ANIMAL PROTOCOL TITLE: Pathogenicity of Shiga toxin-producing *E. coli* and ricin intoxication (mice)

GRANT TITLE (if different from above): "Pathogenicity of Shiga Toxin-producing *E. coli*", "Shiga Toxin and Ricin Interaction with Enterocytes and Rescue of Target Cells", "Antibacterial and Anti-Shiga Toxin Expression Activities of Morpholino Compounds against EHEC O104:H4 and Related Prokaryotes", "Susceptibility of Recombinant Inbred Mice to Shiga Toxins and *E. coli* O157:H7"

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6) Ph.D.; (b)(6) Ph.D.; (b)(6) (b)(6) Ph.D.; (b)(6) Ph.D.; (b)(6) Ph.D.; (b)(6) Ph.D.; LTC (b)(6) VMD; Ms. (b)(6) Ms. (b)(6) Ms. (b)(6)

TECHNICIANS(S): Mr. (b)(6) Mr. (b)(6) Mr. (b)(6) Ms. (b)(6)

I. **NON-TECHNICAL SYNOPSIS:** Shiga toxin (Stx)-producing Escherichia coli (STEC) are the causative agent of "hamburger disease" because they are often transmitted through undercooked hamburgers. STEC O157:H7 are food- and water-borne bacteria that are the most common infectious cause of bloody diarrhea in the United States. The hemolytic uremic syndrome (HUS), which can occur as a result of STEC infection, is the most frequent cause of acute kidney failure in U.S. children. The STEC make one or more Stxs (Stx1, Stx2 or variant of those) that are key factors in the development of the HUS. The genes that encode the Stxs are present on bacteriophages, which are viruses that infect *E. coli*; these viruses can infect other bacteria and transfer the toxin genes. Evidence that such transmission may occur became evident in 2011 when a large food-borne outbreak of bloody diarrhea and HUS occurred in Germany. The outbreak isolate was an enteroaggregative *E. coli* strain that had most likely acquired the stx_2 phage from a more typical STEC. Stxs act to halt protein synthesis in the target cell, which causes the cell to die. The goals of the studies described here are as follows: 1) evaluate the potency of different Stxs; 2) determine if the *stx* phage can be transferred from one bacterial strain to another in the intestines; 3) study the role of Stx2 in the unusual disease caused by the German outbreak strain; 4) investigate the systemic effects of ricin; 5) identify host factors that play a role in Stx or *E. coli* disease; and 6) determine if antibodies against Stxs or ricin can protect against exposure to the respective toxin. All of the mouse studies described herein will be performed in full accordance with the Institutional Animal Care and Use Committee at USUHS.

II. BACKGROUND:

II.1. Background: Our experience with Shiga toxins (Stxs), STEC-infection animal models, and ricin that led to this proposal will be briefly reviewed.

Escherichia coli O157:H7 and other Stx-producing E. coli (STEC) cause food- and water-borne outbreaks and sporadic cases of intestinal disease manifest as diarrhea and/or hemorrhagic colitis (bloody diarrhea). About 5-10% of children infected with these agents subsequently develop a sequela called the hemolytic uremic syndrome (HUS) that is characterized by hemolytic anemia, thrombocytopenia, and kidney dysfunction and/or failure. The capacity of STEC to cause bloody diarrhea and HUS derives from the activity of the Stxs. There are two major types of Stxs expressed by *E. coli* associated with human disease: Stx1 and Stx2 (or variants thereof). These cytotoxins block eukaryotic translation and are structurally similar; however, Stx1 and Stx2 differ in relative toxicity both for Vero cells and for mice. Stx2 and its variants are more toxic than Stx1 when injected in pure form into mice or when STEC is fed orally to mice pre-treated with streptomycin (str) to reduce facultative bowel flora or gavaged into mice with an intact commensal flora. We have found that STEC strains that produce a particular variant of Stx2, called Stx2dact, are exquisitely virulent in the str-treated mouse oral challenge model. Furthermore, Stx2dact exhibits increased cytotoxicity or "activation" after incubation with crude mucus isolated from mice or humans or with purified elastase.

We are interested in the contribution of Stx type to disease and will test strains that produce more than one toxin type for virulence in mice and then evaluate the pathogenesis of derivatives of those strains that are mutated in one toxin type. Additionally, the potency of Stx subtypes can be measured by oral gavage or by intraperitoneal or intravenous injection into mice. Studies to determine the relative potency of toxin subtypes are important to estimate the potential virulence of STEC strains in humans. We will examine the virulence of human outbreak strains such as the German O104:H4 isolate in our mouse models to directly ask about the pathogenicity of such isolates. Furthermore, because the O104:H4 strain likely acquired the capacity to produce Stx2 through lysogeny with an stx_2-encoding phage, we will examine if the O104:H4 phage can be transmitted to other bacterial strains within the intestines of mice or if a phage cured derivative of O104:H4 can become lysogenized again with the toxin-encoding phage *in vivo*.

We found that Stx2 has the capacity to enhance STEC colonization in tissue culture and in mice. Stxs bind to globotriaosylceramide (Gb3) on the surface of host cells. In addition, we demonstrated that eukaryotic cells treated with Stx2 exhibit an increase in cell-surface localized nucleolin (a host factor that interacts with intimin, a common STEC adhesin). In the proposed studies, we will characterize the capacity of Stx1 to augment STEC colonization in a manner similar to that of Stx2. In addition, we will address our hypothesis that nucleolin plays a role in the Stx-mediated enhancement of colonization by STEC strains in mice.

Previous studies in our laboratory and others showed that Gb3 expression on host cells is elevated in the presence of butyrate, a metabolite produced by some commensal gut flora. We found that a high fiber diet (10% guar gum) causes an increase in intestinal butyrate levels, which may lead to increased cell surface Gb3 and enhanced Stx binding and transit through the gut. Therefore we will examine the effect

of alterations in the amount of fiber in the mouse diet on susceptibility to toxin alone or to infection with STEC strains.

Our studies in mice have largely been restricted to the outbred CD-1 and inbred BALB/c and C57BL/6 strains. Recently a large number of BXD mouse strains (parental mice = C57BL/6J and DBA/2J) were established that can be used to determine genetic markers that correlate with susceptibility to infection or intoxication. Although many investigators believe that there must be host genetic factors involved in the development of the HUS, we have not previously had a model to ask questions about what those host factors might be. In these studies we plan to assess the relative susceptibility of the BXD mice to intoxication by Stx1 or Stx2, or to colonization by O157 strains that produce either Stx1 or Stx2.

Ricin is a plant toxin that has the same mode of action as the Stxs. No vaccine is currently licensed to protect individuals from infection or disease caused by STEC or from intoxication with ricin. We have begun to develop and test anti-ricin and anti-Stx therapeutics that rely on a) the conjugation of antibody fragments to the cell-binding domain of ricin or Stx; or, b) small molecule inhibitors of ricin (obtained from a collaborator); or, c) morpholino compounds that are either anti-bacterial or will suppress toxin expression from STEC strains. We will examine the protective capacity of the inhibitors in tissue culture and mice.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: Pubmed, Biomedical Research Database, NIH RePORTER

II.2.2. Date of Search: 6-7 May 2012

II.2.3. Period of Search: Pubmed: 1948-present, English filter
Biomedical Research Database: 1998-2009
NIH RePORTER: 1998-2012 active projects

II.2.4. Key Words and Search Strategy:
1. "Shiga toxin OR Verotoxin" AND "mouse OR mice" AND "infection OR intoxication"
2. "Ricin" AND "mouse OR mice" AND "intoxication"

II.2.5. Results of Searches:

Pubmed: "Shiga toxin OR Verotoxin" AND "mouse OR mice" AND "infection OR intoxication"
Results: A total of 150 publications were found. Ten publications are from our laboratory. The remaining studies focused on diagnostics and detection (5 reports), pathogenesis of STEC and Stx (33), host response and interactions with host cells (24), therapeutics (22), antibiotic treatment of STEC infection (12), vaccine development (19), the use of probiotics as alternative therapies (6), epidemiology (1), and topics not relevant to the search (13). Of particular interest are 20 publications that discuss various mouse models to assess STEC infection or Stx intoxication. Two papers investigated the use of *Citrobacter rodentium* strains that cxpress Stx as a means to study the role of Stx in infection. Five of the twenty papers are from our laboratory.

They describe the streptomycin-treated CD-1 mouse model that we use to investigate morbidity and mortality caused by STEC, the intact commensal flora BALB/c mouse model that we use to study the capacity of EHEC strains to colonize the gut, and the CD-1 mouse model of Stx intoxication. An additional six publications used various mouse models to emulate the hemolytic uremic syndrome that is a sequel in humans. Several publications, at least three of which are from our laboratory, demonstrated the capacity of antibodies that neutralize Stx activity to protect against STEC infection. These studies provide a strong foundation to support our studies in Experiment V.1.6., which utilize neutralizing antibodies as components of potential therapies to counter STEC disease.

Pubmed: "Ricin" AND "mouse OR mice" AND "intoxication"
Results: A total of 34 publications were identified, none of which are from our laboratory. The studies focused on vaccines (4), anti-ricin therapeutics (9), detection of ricin (3), ricin toxicity (4), the host response to ricin (3), the mechanism of action of ricin (5), and topics not relevant to the search (5). None of the reports investigated the mechanisms by which ricin transits throughout the body or the novel liposome-receptor-antibody approach that we will test as a therapeutic.

Biomedical Research Database: "Shiga toxin OR Verotoxin" AND "mice OR mouse"
Results: A total of 29 projects were identified and all of them are from our laboratory.

Biomedical Research Database: "Ricin" AND "mouse or mice"
Results: A total of 72 projects were identified in this search, of which four are from our laboratory. The studies focused on bioassays and detection (12), toxicology of ricin (4), a skin patch vaccine (8), recombinant ricin subunit vaccines (18), vaccines that protect against multiple bioweapons (2), and therapeutics and countermeasures to treat ricin exposure (18). Six were deemed irrelevant to the topic of the search. The projects identified in this search do not overlap with our proposed experiments.

NIH RePORTER: "Shiga toxin OR Verotoxin" AND "mice OR mouse"
Results: A total of 22 projects were identified in this search, of which 5 are not relevant. The remaining projects addressed therapeutics (5), innate immunity (2), diagnostics (1), Stx-encoding phage (1), EHEC pathogenesis (5), and animal models of STEC disease (3). None of these projects overlap with our proposed experiments.

NIH RePORTER: "Ricin" AND "mouse or mice"
Results: Ten studies were returned in this search and six were deemed irrelevant to the studies proposed here. Two addressed therapeutics, one related to innate immunity, and one was focused on diagnostics. Our proposed studies do not overlap with the studies identified here.

III. OBJECTIVE\HYPOTHESIS: The goals of the studies outlined in this protocol are 1) to characterize the roles of Shiga toxins in the pathogenesis of EHEC disease; 2) to determine the level of nucleolin expression in the intestine in response to EHEC infection; 3) to assess the contribution of specific diets to the pathogenesis of STEC, 4)

to identify host biomarkers of EHEC disease using advanced recombinant inbred (ARI) mice with defined genotypes; and 5) to measure the safety and efficacy of therapeutic compounds designed to ablate the lethal effects of Stx or ricin.

IV. MILITARY RELEVANCE: *E. coli* O157:H7 and *E. coli* O104:H4 have the potential to simultaneously infect large numbers of people who ingest as few as 100 organisms in common source food- or water-borne outbreaks (examples: July 1996 outbreak in Japan that affected ~10,000 people, 2006 outbreak in the U.S. linked to spinach, 2011 outbreak in Germany linked to fenugreek seeds). In addition, the rate of secondary transmission of *E. coli* O157:H7 is high. Therefore, large-scale infection of soldiers with *E. coli* O157:H7 or another Shiga-toxin producing *E. coli* (STEC) isolate would likely result in an incapacitating illness among troops. Furthermore, Stx and other Stx family members are considered potential biological warfare/terrorist threats as highlighted by the CDC- and DoD-mandated select agent restrictions on use, storage and shipment of Stxs and Stx-expressing clones.

In addition to the Stxs, our laboratory studies ricin, a toxin derived from the castor bean. Ricin is also select agent and is considered to be a potential bioterrorism weapon. Our laboratory has designed potential therapeutics against ricin and will need to test the safety and efficacy of these treatments.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures: For the majority of the studies proposed here, we will use one of the following mouse models of EHEC infection or Stx or ricin intoxication.

V.1.A. Infection with Enterohemorrhagic *E. coli* (EHEC) to assess virulence: Six- to eight-week-old mice will be given drinking water that contains streptomycin (str, 5 g/liter) or ampicillin (amp, 5-10 g/L) to reduce normal facultative flora. [**Note**: from published studies, we know that after 1 day of str or amp treatment, the number of facultative bacteria drops from about 10^8 to less than 10^2 CFU/g of feces, and the number of anaerobic bacteria remains constant at about 10^9 CFU/g of feces (3, 6).] Mice will then be fasted overnight. An overnight fast is needed to reduce the volume of the stomach contents so that the entire inoculum can be administered. In addition, we have found that mice fed by Pipetman or weigh boat are more likely to ingest the inoculum if they have been fasted. The next morning, groups of five mice will be fed 10^2 to 10^{10} CFU of the *E. coli* strain (str or amp resistant) to be tested in 25-100 µl of 20% sucrose. The oral infection will be accomplished either by holding the mice and allowing them to eat the material as delivered from a Pipetman tip, by placing weigh boats with the inocula in the cages of the animals, or by oral gavage with a 20 gauge, 1 ½" disposable gavage needle. The mice readily ingest the bacterial suspension which is prepared by harvesting the bacteria by centrifugation, washing the bacterial pellet twice in phosphate buffered saline (PBS, pH 7.4), and suspending the washed bacteria in 1 ml of sterile 20% (w/v) sucrose. After inoculation the animals will then be returned to their cages and permitted food and water containing 5 g/liter str or amp *ad libitum*. Mice will be weighed daily and any mice that lose more than 30% of their initial body weight will be euthanized. The endpoint for this study is extreme morbidity or mortality, as defined in Section V.4.5, and the experiment will be continued for 21-28 days. The timing of the

inoculations will be planned such that the majority of the illnesses will occur during normal business hours (0600-1900) to facilitate more frequent monitoring of the animals so as to permit euthanasia as soon as mice are extremely moribund. Death due to infection typically occurs 3-6 days post-inoculation, depending on the EHEC strain used. Inoculations will be done on the morning of the first day of the experiment so as to permit maximum monitoring during the times of day when we typically observe the most deaths. If we observe that more deaths occur between 1900 and 0600 in the experiments described here, we will change the inoculation time such that deaths will occur during the day and/or we will monitor the mice every 4-6 hrs between 1900 and 0600. Mice will be monitored for signs of extreme morbidity (see Section V.4.5 below for definition of extreme morbidity) as described in Section V.3.5.1. Any mice that exhibit extreme morbidity will be euthanized immediately by inhalational isoflurane overdose followed by cervical dislocation.

V.1.B. Infection with EHEC to monitor colonization. Six- to eight-week-old mice will be fasted overnight. An overnight fast is needed to reduce the volume of the stomach contents so that the entire inoculum can be administered. In addition, we have found that mice fed by Pipetman or weigh boat are more likely to ingest the inoculum if they have been fasted. The next morning, groups of five mice will be fed 10^8 to 10^{10} CFU of the *E. coli* strain to be tested in 25-100 µl of 20% sucrose. The oral infection will be accomplished either by holding the mice and allowing them to eat the material as delivered from a Pipetman tip, by placing weigh boats with the inocula in the cages of the animals, or by oral gavage with a disposable gavage needle. Once the mice are fed, they will be returned to their cages and allowed food and water *ad libitum*. Mice orally inoculated in this way may be further monitored in the following ways: 1) the morbidity due to O157 infection will be assessed through observation of physical symptoms (Section V.3.5.1), 2) the degree to which a given strain colonizes the intestine will be determined by the number of CFU that persist in the feces for up to 14 days after infection (mice will be placed individually into empty cages and approximately 1 g of feces will be collected, homogenized in 10 ml of 1% PBS, and 10-fold serial dilutions of the homogenate prepared and plated on agar media that contains the appropriate antibiotics); 3) bacteremia will be monitored by enumeration of bacteria in the blood (blood will be collected either once by tail vein bleed, or, if the mice are to be euthanized for tissue collection, blood will be collected immediately after the mice are euthanized); and/or 4) specific sites of colonization and/or toxin localization will be determined by examining tissues collected from infected animals. Mice will be weighed daily and any mice that lose more than 30% of their initial body weight will be euthanized. The endpoint for this study is euthanasia 7-21 days post-inoculation. While we do not anticipate significant morbidity or mortality in these mice, the mice will be monitored for signs of extreme morbidity (see Section V.4.5 below for definition of extreme morbidity) as described in Section V.3.5.1. Any mice that exhibit extreme morbidity will be euthanized immediately by inhalational isoflurane overdose followed by cervical dislocation. Death due to infection in this model is uncommon, but occurs 3-6 days post-inoculation.

V.1.C. Intoxication with Stx or ricin. Six to eight week old male or female mice will be intoxicated by i.p. injection of Stx or ricin in sterile phosphate-buffered saline using a 26

gauge needle attached to a tuberculin syringe or by oral gavage with a 20 gauge 1.5" gavage needle. Mice will be weighed daily and any mice that lose more than 30% of their initial body weight will be euthanized. The endpoint for this study is extreme morbidity or mortality, and the experiment will be continued for 14-21 days. The timing of the inoculations will be planned such that the majority of the illnesses will occur during normal business hours (0600-1900) to facilitate more frequent monitoring of the animals so as to permit euthanasia as soon as mice are extremely moribund. Death due to intoxication typically occurs 2-3 days post-inotoxication. Inoculations will be done on the morning of the first day of the experiment so as to permit maximum monitoring during the times of day when we typically observe the most deaths. If we observe that more deaths occur between 1900 and 0600 in the experiments described here, we will change the inoculation time such that deaths will occur during the day and/or we will monitor the mice every 4-6 hrs between 1900 and 0600. Mice will be monitored for signs of extreme morbidity (see Section V.4.5 below for definition of extreme morbidity) as described in Section V.3.5.1. Any mice that exhibit extreme morbidity will be euthanized immediately by inhalational isoflurane overdose followed by cervical dislocation.

V.1.1. Experiment 1. Characterization of the roles of Shiga toxin variants in the pathogenesis of STEC disease. Our EHEC virulence (V.1.A) model and our EHEC colonization model (V.1.B) will be used to investigate the involvement of Stx variants in the pathogenesis of STEC-mediated disease.

V.1.1.1. Assessment of the impact of Stx2dact versus Stx2c on STEC virulence when the toxins are expressed in otherwise isogenic backgrounds. We previously demonstrated that STEC B2F1, a strain that produces Stx2dact, exhibits enhanced virulence in our mouse model of STEC infection. Stx2dact is the most potent form of Stx2, and we theorize that the increased activity of this toxin variant is due to the fact that it is activated by intestinal mucus. One hypothesis that we will test in aim #2 of our NIH grant is whether a derivative of strain B2F1 that produces Stx2c (Stx2c is less potent than Stx2dact on tissue culture cells), rather than Stx2dact, is attenuated in str-treated CD-1 mice. Our second mouse virulence study will compare EHEC strain 86-24 (Stx2+) to 86-24 making Stx2dact. *These latter studies, in which 86-24 producing Stx2dact will be tested for virulence in mice, will be done in the ABSL-3 Suite once the Suite is available, as requested by the Division of Select Agents and Toxins at the CDC* (see attached letter, enclosure 2).

These studies will be performed exactly as described in V.1.A above.

V.1.1.1.1: Investigation of contribution of Stx2c to B2F1 virulence.
Group 1: 5 mice at 10^1 CFU B2F1
Group 2: 5 mice at 10^2 CFU B2F1
Group 3: 5 mice at 10^4 CFU B2F1
Group 4: 5 mice at 10^6 CFU B2F1
Group 5: 5 mice at 10^1 CFU B2F1 making Stx2c only
Group 6: 5 mice at 10^2 CFU B2F1 making Stx2c only
Group 7: 5 mice at 10^4 CFU B2F1 making Stx2c only

Group 8: 5 mice at 10^6 CFU B2F1 making Stx2c only
Group 9: 5 mice at 10^8 CFU B2F1 making Stx2c only
Group 10: 5 mice at 10^{10} CFU B2F1 making Stx2c only

5 mice/group x 10 groups x 4 iterations = 200 CD-1 mice

V.1.1.1 2: Investigation of contribution of Stx2dact to 86-24 virulence.
Group 1: 5 mice at 10^8 CFU 86-24
Group 2: 5 mice at 10^{10} CFU 86 24
Group 3: 5 mice at 10^2 CFU 86-24 making Stx2dact only
Group 4: 5 mice at 10^4 CFU B2F1 making Stx2dact only
Group 5. 5 mice at 10^6 CFU B2F1 making Stx2dact only
Group 6: 5 mice at 10^8 CFU B2F1 making Stx2dact only
Group 7: 5 mice at 10^{10} CFU B2F1 making Stx2dact only

5 mice/group x 7 groups x 4 iterations – 140 CD-1 mice

Total number of mice (V.1.1): 200 + 140 = 340 CD-1 mice

V.1.1.2. Feeding of novel Stx2dact-producing strains to mice. Our laboratory is involved in an ongoing characterization of STEC isolates identified in California or locally. When we identify an isolate that makes Stx2dact, the IACUC will be notified. We will assess the virulence of that isolate in male CD-1 mice at doses of 10^4 to 10^{10} CFU/mouse. We may receive up to 5 such strains/year. CD-1 mice will be inoculated orally with each strain as described in V.1.A.

Group 1: 5 mice at 10^4 CFU
Group 2: 5 mice at 10^6 CFU
Group 3: 5 mice at 10^8 CFU
Group 4: 5 mice at 10^{10} CFU

5 mice/group x 4 groups x 5 strains/year x 3 years = ***300 CD-1 mice***

V.1.1.3. Assessment of virulence of STEC outbreak isolates. Our laboratory is uniquely poised to rapidly characterize the virulence of STEC isolates recovered during outbreaks with our currently approved procedures or modifications thereof. In studies approved as a major modification to (b)(6) we recently developed a new mouse model of infection for the unique *E. coli* O104:H4 strain (C227 11) that caused a serious STEC outbreak linked to contaminated fenugreek seeds that occurred in Germany in May 2011. When we receive a new outbreak isolate, we will assess the virulence of that isolate in our str treated male CD-1 mouse model (V.1.A) and we will investigate the capacity of the strain to colonize the gut in our ICF BALB/c mouse model (V.1.B). In our extensive experience with these models, we have found that a minimum of four experiments with each mouse model is necessary to accurately compare the virulence of each outbreak strain to that of our prototype STEC strains 86-24 or B2F1. Additional

studies will be necessary to characterize the role of Stx(s) in the capacity of each strain to colonize the gut and to cause disease. We may receive up to 3 such strains/year.

V.1.1.3.1: Infection of str-treated CD-1 mice with outbreak strain to assess virulence.
Group 1: 10 mice infected with 10^{10} CFU 86-24 or 10^{6} CFU B2F1
Group 2: 10 mice infected with 10^{6} CFU outbreak strain
Group 3: 10 mice infected with 10^{8} CFU outbreak strain
Group 4: 10 mice infected with 10^{10} CFU outbreak strain

10 mice/group x 4 groups x 4 iterations x 3 strains/year x 3 years = 1440 male CD-1 mice

V.1.1.3.2: Infection of str-treated CD-1 mice with Stx- derivative(s) of outbreak strain to assess contribution of Stx to virulence.
Group 1: 10 mice infected with lethal dose of outbreak strain identified above
Group 2: 10 mice infected with same dose of Stx-negative derivative of outbreak strain

10 mice/group x 2 groups x 4 iterations x 3 strains/year x 3 years = 720 male CD-1 mice

V.1.1.3.3: Infection of ICF BALB/c mice with outbreak strain to assess colonization of the gut.
Group 1: 10 mice infected with 10^{10} CFU 86-24 or 10^{6} CFU B2F1
Group 2: 10 mice infected with 10^{10} CFU outbreak strain

10 mico/group x 2 groups x 4 iterations x 3 strains/year x 3 years = 720 BALB/c ***mice***

V.1.1.3.4: Infection of ICF BALB/c mice with Stx- derivative(s) of outbreak strain to assess the contribution of Stx to colonization of the gut.
Group 1: 10 mice infected with 10^{10} CFU outbreak strain
Group 2: 10 mice infected with 10^{10} CFU of Stx-negative derivative of outbreak strain

10 mice/group x 2 groups x 4 iterations x 3 strains/year x 3 years = 720 BALB/c ***mice***

Total number of mice (V.1.3)
1440 + 720 = 2160 male CD-1 mice
720 + 720 = 1440 female BALB/c mice

V.1.1.4. Characterization of roles of each Stx variant in STEC disease caused by strains that encode more than one Stx. Some STEC strains encode for and produce more than one Stx. To investigate the contribution of each Stx variant to colonization and pathogenesis, we will challenge mice with the parent strain or isogenic strains that do not produce one or both Stx variants. The strains that we will test in our str-treated CD-1 virulence model (V.1.A) and in our ICF BALB/c model (V.1.B) are as follows:

- 933 (Stx1, Stx2) and isogenic derivatives that are Stx1-Stx2+, Stx1+Stx2-, Stx1-Stx2-;
- B2F1 (Stx2dact1, Stx2dact2) and its isogenic derivatives that are Stx2dact1-Stx2dact2+, Stx2dact1+Stx2dact2-, Stx2dact1-Stx2dact2-;
- K3995 (Stx2, Stx2c) and its isogenic derivatives that are Stx2-Stx2c+, Stx2+Stx2c-, Stx2-Stx2c-

V.1.1.4.1. Infection of str-treated CD-1 mice to assess virulence of Stx-derivatives.
Group 1: 10 mice infected with lethal dose of parent strain
Group 2: 10 mice infected with same dose of parent strain missing one Stx
Group 3: 10 mice infected with same dose of parent strain missing the other Stx
Group 4: 10 mice infected with same dose of parent strain missing both Stxs

10 mice/group x 4 groups x 4 iterations x 3 parent strains = ***480 male CD-1 mice***

V.1.1.4.2: Infection of ICF BALB/c mice to assess capacity of Stx- derivative(s) to colonize the gut.
Group 1: 10 mice infected with parent strain
Group 2: 10 mice infected with same dose of parent strain missing one Stx
Group 3: 10 mice infected with same dose of parent strain missing the other Stx
Group 4: 10 mice infected with same dose of parent strain missing both Stxs

10 mice/group x 4 groups x 4 iterations x 3 parent strains = ***480 BALB/c mice***

V.1.1.4.3: Infection of ICF BALB/c mice to determine the site of colonization of parent strain and Stx- derivatives.
Group 1: 10 mice infected with parent strain
Group 2: 10 mice infected with same dose of parent strain missing one Stx
Group 3: 10 mice infected with same dose of parent strain missing the other Stx
Group 4: 10 mice infected with same dose of parent strain missing both Stxs

10 mice/group x 4 groups x 2 iterations x 3 parent strains = ***240 BALB/c mice***

Total number of mice (V.1.1.4)
480 male CD-1 mice
480 + 240 = 720 female BALB/c mice

Total number of mice for V.1.1:
340 + 300 + 2160 + 480 = 3280 male CD-1 mice
1440 + 720 = 2160 female BALB/c mice

V.1.2. Experiment 2: Investigation of the role of a high fiber diet in STEC pathogenesis. Previous studies in our laboratory and others showed that the receptor for Stxs *in vitro* and *in vivo* is Gb3, which is on the surface of host cells in the intestines and kidney. Gb3 expression on host cells is elevated in the presence of butyrate, a metabolite produced by some commensal gut flora. We recently found that a high fiber diet (10% guar gum) causes an increase in intestinal butyrate levels, which may lead to increased cell surface Gb3 and enhanced Stx binding and transit through the gut. To test this hypothesis, we will employ a modified version of our BALB/c ICF model (V.1.B). Fourteen days prior to challenge with 86-24 (produces Stx2) or its isogenic Stx2-derivative TUV86-2, mouse chow will be changed from the standard diet provided by LAM (3.5% plant fiber) to custom diets from (b)(4) that contain either 2% guar gum (low fiber) or 10% guar gum (high fiber) as the sole fiber source. In some experiments, 5 µM butyrate will be co-injected with the inoculum (EHEC or Stx). In other experiments, mice will be challenged with derivatives of 86-24 and TUV86-2 that express green fluorescent protein (GFP) or red fluorescent protein (RFP) to permit *in vivo* imaging with our Carestream Multispectral FX Pro *in vivo* imaging system. The Carestream Multispectral FX Pro has a large dynamic range and a highe signal:noise ratio for fluorescence; in addition, it has x-ray capability that permits more accurate signal localization. At each time point, up to five mice will be anesthetized with isoflurane and placed on a heated platform within the imaging box of the Carestream Multispectral FX Pro for imaging as described in Section V.4.1.2.1.

V.1.2.1: Infection of mice fed custom diets to assess colonization, adherence, Gb3 expression, and/or Stx binding, and to characterize the mouse intestinal microbiome. Female BALB/c mice will be fed a low fiber or a high fiber diet for 14 days prior to challenge by oral gavage with 86-24 or TUV86-2 as described in V.1.B above. In some studies, colonization of the gut will be assessed daily for up to 14 days by bacterial enumeration of homogenized fecal samples. In other studies, mice will be euthanized at specific times post-challenge and intestines and kidneys will be harvested for immunohistochemistry and/or fluorescent microscopy.

V.1.2.1.1: Intestinal colonization of mice fed custom diets and challenged with EHEC.
Group 1: 10 mice, low fiber diet, 86-24 challenge
Group 2: 10 mice, high fiber diet, 86-24 challenge
Group 3. 10 mice, low fiber diet, TUV86-2 challenge
Group 4: 10 mice, high fiber diet, TUV86-2 challenge

10 mice/group x 4 groups x 3 iterations = 120 female BALB/c mice

V.1.2.1.2: Harvest of tissues from mice fed custom diets and challenged with EHEC. Mice will be fed one of the two diets for 14 days, challenged with 86-24 or TUV86-2 (or their GFP-expressing derivatives), and euthanized 6 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 168 h post-inoculation. Intestines and kidneys will be harvested and prepared for immunohistochemistry.

Group 1: 5 mice, low fiber diet, 86-24 challenge
Group 2: 5 mice, high fiber diet, 86-24 challenge
Group 3: 5 mice, low fiber diet, TUV86-2 challenge
Group 4: 5 mice, high fiber diet, TUV86-2 challenge

10 mice/group x 4 groups x 7 time points x 2 iterations = 560 female BALB/c mice

V.1.2.1.3: Characterization of the mouse intestinal microbiome in mice fed custom diets and challenged with STEC. We will use Illumina sequencing of fecal 16S rDNA from mice fed high or low fibers diets to assess the effects of diet and STEC on the microbiome. Fecal samples will be collected from mice daily for 14 days prior to and 14 days after challenge with 86-24 or sterile saline
Group 1: 8 mice, low fiber diet, sterile saline challenge
Group 2: 8 mice, high fiber diet, sterile saline challenge
Group 3: 8 mice, low fiber diet, 86-24 challenge
Group 4: 8 mice, high fiber diet, 86-24 challenge

8 mice/group x 4 groups x 2 iterations = 64 female BALB/c mice

Total number of mice (V.1.2.1) = 120 + 560 = 64 = 744 female BALB/c mice

V.1.2.2. Use of an intestinal loop model to assess EHEC adherence to intestinal epithelium, Stx binding to Gb3, Stx transit through the intestinal epithelium, nucleolin redistribution to the epithelial cell surface, and Stx-mediated induction of apoptosis. An intestinal loop model will be used to investigate direct interactions between EHEC, Stx, and the intestinal epithelium. The procedure that we will use is a modification of a protocol that was described by (b)(6) *et al.* for analysis of the effects of *Clostridium perfringens* epsilon toxin on different segments of the intestines (2). The procedure is described in detail in Section V.4.3.2. In brief, female BALB/c mice will be anesthetized and their peritoneal cavities will be exposed. The intestines will be removed and loops will be formed. EHEC, Stx, and/or butyrate will be injected into the loops, the intestines will be placed back into the peritoneum, and the incision will be closed with sutures. Topical and general analgesia will be provided during the 6 hour time course of the study. All procedures will be conducted in a certified Biosafety Cabinet in our satellite animal room. (b)(6) At the study endpoint, mice will be euthanized by an overdose of inhaled isoflurane followed by cervical dislocation and intestines will be harvested for further analysis.

1.2.2.1. Inoculation of intestinal loops with EHEC strains 86-24 or TUV86-2. Each group will include 5 mice inoculated with 86-24, 5 mice inoculated with TUV86-2, and 2 mice inoculated with sterile saline in a particular intestinal site, for a total of 12 mice per ligature site.

Group 1: 12 mice with loop in duodenum/jejunum
Group 2: 12 mice with loop in ileum
Group 3: 12 mice with loop in cecum

Group 4: 12 mice with loop in ascending colon
Group 5: 12 mice with loop in distal colon

*12 mice/group x 5 groups x 3 iterations = **180 female BALB/c mice***

1.2.2.2: Inoculation of intestinal loops with Stx1 or Stx2. Each group will include 5 mice inoculated with 500 ng Stx1, 5 mice inoculated with 500 ng Stx2, and 2 mice inoculated with sterile saline in a particular intestinal site, for a total of 12 mice per ligature site.

Group 1: 12 mice with loop in duodenum/jejunum
Group 2: 12 mice with loop in ileum
Group 3: 12 mice with loop in cecum
Group 4: 12 mice with loop in ascending colon
Group 5: 12 mice with loop in distal colon

*12 mice/group x 5 groups x 3 iterations = **180 female BALB/c mice***

1.2.2.3: Inoculation of intestinal loops with butyrate and EHEC strain 86-24. Each group will include 5 mice inoculated with 86-24, 5 mice inoculated with 5 µM butyrate diluted in sterile saline + 86-24, and 2 mice inoculated with 5 µM butyrate diluted in sterile saline in a particular intestinal site, for a total of 12 mice per ligature site.

Group 1: 12 mice with loop in duodenum/jejunum
Group 2: 12 mice with loop in ileum
Group 3: 12 mice with loop in cecum
Group 4: 12 mice with loop in ascending colon
Group 5: 12 mice with loop in distal colon

*12 mice/group x 5 groups x 3 iterations = **180 female BALB/c mice***

1.2.2.4: Inoculation of intestinal loops with butyrate and Stx1 or Stx2. Each group will include 5 mice inoculated with 500 ng Stx1 or Stx2, 5 mice inoculated with 5 µM butyrate diluted in sterile saline + 500 ng Stx1 or Stx2, and 2 mice inoculated with 5 µM butyrate diluted in sterile saline in a particular intestinal site, for a total of 12 mice per ligature site.

Group 1: 12 mice with loop in duodenum/jejunum
Group 2: 12 mice with loop in ileum
Group 3: 12 mice with loop in cecum
Group 4: 12 mice with loop in ascending colon
Group 5: 12 mice with loop in distal colon

*12 mice/group x 5 groups x 2 toxins x 3 iterations = **360 female BALB/c mice***

Total number of mice (V.1.2.2) = 180 + 180 + 180 + 360 = 900 female BALB/c mice

Total number of mice (V.1.2) = 744 + 900 = 1644 female BALB/c mice

V.1.3. Experiment 3: Acute intoxication with Stxs. We recently optimized our mouse models of acute Stx intoxication after i.p. or oral administration of purified toxin. We will intoxicate mice via the i.p. or oral routes to confirm our preliminary LD_{50} observations. In addition, we will intoxicate mice with fluorescently-labeled Stx and use *in vivo* imaging to monitor Stx transit through the mouse after oral intoxication.

V.1.3.1. Intraperitoneal administration of Stx variants to calculate LD_{50} values. Stx variants will be administered to BALB/c mice via i.p. injection with a 26 gauge needle attached to a tuberculin syringe. Mice will be closely monitored for morbidity and mortality, and any mice that exhibit two or more signs of morbidity will be euthanized by isoflurane overdose followed by cervical dislocation. We will determine the i.p. LD_{50} for Stx2c, buffer-treated (BT)-Stx2dact, and elastase-treated (ET)-Stx2dact. One group of mice intoxicated with 5 LD_{50} of Stx2 (5 ng) will serve as the positive control group in each experiment. The LD_{50} will be calculated with Probit analysis.

V.1.3.1.1: Intraperitoneal administration of Stx2 and Stx2c

Group 1: 5 mice at 10 ng Stx2
Group 2: 5 mice at 0.5 ng Stx2c
Group 3: 5 mice at 1 ng Stx2c
Group 4: 5 mice at 2 ng Stx2c
Group 5: 5 mice at 5 ng Stx2c
Group 6: 5 mice at 10 ng Stx2c
Group 7: 5 mice at 20 ng Stx2c
Group 8: 5 mice at 50 ng Stx2c

5 mice/group x 8 groups x 2 iterations = 80 BALB/c mice

V.1.3.1.2: Intraperitoneal administration of Stx2, BT-Stx2dact, and ET-Stx2dact.

Group 1: 5 mice at 10 ng Stx2
Group 2: 5 mice at 0.5 ng BT-Stx2dact
Group 3: 5 mice at 1 ng BT-Stx2dact
Group 4: 5 mice at 2 ng BT-Stx2dact
Group 5: 5 mice at 5 ng BT-Stx2dact
Group 6: 5 mice at 10 ng BT-Stx2dact
Group 7: 5 mice at 0.5 ng ET-Stx2dact
Group 8: 5 mice at 1 ng ET-Stx2dact
Group 9: 5 mice at 2 ng ET-Stx2dact
Group 10: 5 mice at 5 ng ET-Stx2dact
Group 11: 5 mice at 10 ng ET-Stx2dact

5 mice/group x 11 groups x 2 iterations = 110 BALB/c mice

Total number of mice (V.1.3.1) = 80 + 110 = 190 female BALB/c mice

V.1.3.2. Oral gavage of Stx variants to calculate LD_{50} values. Stx variants will be administered via oral gavage as in V.1.A above and mice will be closely monitored for morbidity and mortality. Any mice that exhibit two or more signs of morbidity will be euthanized by isoflurane overdose followed by cervical dislocation. We will determine the oral LD_{50} for Stx2c, buffer-treated (BT)-Stx2dact, and elastase-treated (ET)-Stx2dact. One group of mice intoxicated with 5 LD_{50} of Stx2 (17 µg) will serve as the positive control group in each experiment.

V.1.3.2.1: Oral gavage of Stx2 and Stx2c
Group 1: 5 mice at 17 µg Stx2
Group 2: 5 mice at 5 µg Stx2c
Group 3: 5 mice at 10 µg Stx2c
Group 4: 5 mice at 20 µg Stx2c
Group 5: 5 mice at 50 µg Stx2c
Group 6: 5 mice at 100 µg Stx2c

5 mice/group x 6 groups x 2 iterations = 60 BALB/c mice

V.1.3.2.2: Oral gavage of Stx2, BT-Stx2dact, and ET-Stx2dact.
Group 1: 5 mice at 17 µg Stx2
Group 2: 5 mice at 2 µg BT-Stx2dact
Group 3: 5 mice at 5 µg BT-Stx2dact
Group 4: 5 mice at 10 µg BT-Stx2dact
Group 5: 5 mice at 20 µg BT-Stx2dact
Group 6: 5 mice at 50 µg BT-Stx2dact
Group 7: 5 mice at 2 µg ET-Stx2dact
Group 8: 5 mice at 5 µg ET-Stx2dact
Group 9: 5 mice at 10 µg ET-Stx2dact
Group 10: 5 mice at 20 µg ET-Stx2dact
Group 11: 5 mice at 50 ng ET-Stx2dact

5 mice/group x 11 groups x 2 iterations = 110 BALB/c mice

Total number of mice (V.1.3.2): 60 + 110 = 170 BALB/c mice

V.1.3.3: *in vivo imaging of BALB/c mice orally intoxicated with Stx2.* We hypothesize that Stx2 travels from the stomach to the small intestine, where it can transit across the epithelium and enter the bloodstream. Once in the circulation, Stx2 travels to the kidney and binds to its receptor Gb3 on the surface of kidney cells. Intoxication studies with fluorescently-labeled Stx2 are needed to investigate the sites in the body to which the toxin travels and the mechanism by which Stx2 binds to and transits through the gastrointestinal epithelium. Female BALB/c mice will be orally intoxicated with 1 LD_{50} Stx and imaged throughout the course of the study in our Carestream Multispectral FX Pro *in vivo* imaging system. At specific time points, mice will be euthanized and organs will be imaged *ex vivo* prior to harvest and fixation for immunohistochemistry and immunofluorescence.

Group 1: 5 mice, 1 LD_{50} Stx2, euthanize 4 h post-intoxication
Group 2: 5 mice, 1 LD_{50} Stx2, euthanize 8 h post-intoxication
Group 3: 5 mice, 1 LD_{50} Stx2, euthanize 12 h post-intoxication
Group 4: 5 mice, 1 LD_{50} Stx2, euthanize 24 h post-intoxication
Group 5: 5 mice, 1 LD_{50} Stx2, euthanize 48 h post-intoxication
Group 6: 5 mice, 1 LD_{50} Stx2, euthanize 72 h post-intoxication
Group 7: 5 mice, 1 LD_{50} Stx2, euthanize 96 h post-intoxication

5 mice/group x 7 groups x 3 iterations = 105 female BALB/c mice

Total number of mice (V.1.3) = 190 + 170 + 105 = 465 female BALB/c mice

V.1.4. Experiment 4: Characterization of the virulence of C227-11, an O104:H4 STEC outbreak strain. C227-11 is an enteroaggregative *E. coli* (EAEC) strain that acquired the *stx2* phage. EAEC strains typically form biofilms in the host intestines and cause mild to moderate colitis. Acquisition of the *stx2* phage and subsequent expression of Stx2 conferred upon C227-11 unique properties of both EAEC and STEC strains. In studies approved as a major modification to (b)(6) we developed a mouse model of C227-11 virulence in which C57BL/6 mice are inoculated by oral gavage and treated with ampicillin (amp) in their water beginning 4 h after challenge (C227-11 is naturally amp-resistant). Infected mice lose up to 25% of their body weight throughout the 28 day infection, though only ~50% succumb to the infection. In the studies proposed here, we will identify the site of C227-11 colonization in the mouse gut, assess the virulence of phage-cured and *stx2*- derivatives of C227-11, and determine whether transmission of *stx*-encoding phages occurs *in vivo*. All of the studies in Experiment 4 will be conducted as described in V.1.A above with two modifications. Six to eight week-old female C57BL/6 mice will be used in place of male CD-1 mice. In addition, mice will be treated with 10 g/L amp rather than str, and the amp-water treatment will commence 4 hrs after challenge rather than the day before.

V.1.4.1. Oral infection of C57BL/6 mice with C227-11 to determine the site of colonization in the gut. Five mice per group will be challenged by oral gavage with C227-11. One group of mice each will be euthanized at 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h, and 120 h. Intestines will be harvested and bacteria in each compartment will be enumerated.
Group 1: 5 mice, euthanized at 3 h
Group 2. 5 mice, euthanized at 6 h
Group 3: 5 mice, euthanized at 12 h
Group 4. 5 mice, euthanized at 24 h
Group 5: 5 mice, euthanized at 48 h
Group 6. 5 mice, euthanized at 72 h
Group 7: 5 mice, euthanized at 96 h
Group 8: 5 mice, euthanized at 120 h

*5 mice/group x 8 groups x 2 iterations = **80 female C57BL/6 mice***

V.1.4.2. Assessment of the contribution of Stx2 to the pathogenesis of C227-11. The intensity of infection and the frequency of HUS in the 2011 C227-11 outbreak suggest that Stx2 contributed significantly to the severity of disease. To test this hypothesis, we will infect 6 to 8 week-old C57BL/6 mice orally by gavage or pipet feeding with C227-11, a phage-cured derivative of C227-11, or a *stx2* deletion mutant of C227-11. Colonization will be monitored by bacterial enumeration of homogenized fecal samples collected daily. Morbidity and mortality will be monitored regularly and mice that exhibit two or more signs of morbidity will be euthanized by isoflurane overdose and cervical dislocation.
Group 1: 10 mice inoculated with C227-11
Group 2: 10 mice inoculated with phage-cured C227-11
Group 3: 10 mice inoculated with *stx2* deletion mutant of C227-11

10 mice/group x 3 groups x 4 iterations = ***120 female C57BL/6 mice***

V.1.4.3. Transmission of *stx2* phages *in vivo*. Evolutionary studies of EAEC strains suggest that the *stx2* phage in C227-11 (ΦO104) was acquired from an O157 EHEC strain that contains a *stx2* phage (ΦO157). To test the transmissibility of the phages *in vivo*, we will infect C57BL/6 mice with one of the following three pairs of strains in which the phage contains a gene the encodes kanamycin (kan) resistance (donor first, recipient second): amp-resistant C227-11 and str-resistant *E. coli* K-12, amp-resistant C227-11 and phage-cured str-resistant 86-24, and str-resistant 86-24 and phage-cured amp-resistant C227-11. The experiments will be conducted with and without a dose of ciprofloxacin (cipro) that is sublethal to the bacteria. A sublethal dose of cipro, which is a dose of cipro that will kill some but not all of the bacteria, has been shown to induce excision of the phage from the bacterial chromosome and formation of new virions that could be transferred to other bacteria. A dose of 40 µg cipro or saline will be administered i.p. in a volume of 0.1 ml with a 26 gauge needle attached to a tuberculin syringe on days 2, 3, 4, and 5 post-inoculation (7). Fecal samples will be collected daily and plated on media selective for the donor, the recipient, and any kan-resistant recipients (indicates transmission of the phage from donor to recipients).

V.1.4.3.1: Transmission of ΦO104 from C227-11 to E. coli K-12.
Group 1: 5 mice inoculated with C227-11, treated with saline
Group 2: 5 mice inoculated with *E. coli* K-12, treated with saline
Group 3: 10 mice inoculated with equal doses of C227-11 and *E. coli* K-12, treated with saline
Group 4: 5 mice inoculated with C227-11, treated with cipro
Group 5: 5 mice inoculated with *E. coli* K-12, treated with cipro
Group 6: 10 mice inoculated with equal doses of C227-11 and *E. coli* K-12, treated with cipro

[(5 mice/group x 4 groups) + (10 mice/group x 2 groups)] x 3 iterations = 120 female C57BL/6 mice

V.1.4.3.2. Transmission of ΦO104 from C227-11 to phage-cured 86-24
Group 1: 5 mice inoculated with C227-11, treated with saline
Group 2: 5 mice inoculated with phage-cured 86-24, treated with saline
Group 3: 10 mice inoculated with equal doses of C227-11 and phage-cured 86-24, treated with saline
Group 4: 5 mice inoculated with C227-11, treated with cipro
Group 5: 5 mice inoculated with phage-cured 86-24, treated with cipro
Group 6: 10 mice inoculated with equal doses of C227-11 and phage-cured 86-24, treated with cipro

[(5 mice/group x 4 groups) + (10 mice/group x 2 groups)] x 3 iterations = 120 female C57BL/6 mice

V.1.4.3.3. *Transmission of ΦO157 from 86-24 to phage-cured C227-11.*
Group 1: 5 mice inoculated with 86-24, treated with saline
Group 2: 5 mice inoculated with phage-cured C227-11, treated with saline
Group 3: 10 mice inoculated with equal doses of 86-24 and phage-cured C227-11, treated with saline
Group 4: 5 mice inoculated with 86-24, treated with cipro
Group 5: 5 mice inoculated with phage-cured C227-11, treated with cipro
Group 6: 10 mice inoculated with equal doses of 86-24 and phage-cured C227-11, treated with cipro

[(5 mice/group x 4 groups) + (10 mice/group x 2 groups)] x 3 iterations = 120 female C57BL/6 mice

Total number of mice (V.1.4.3) = 120 + 120 + 120 = 360 female C57BL/6 mice

Total number of mice (V.1.4): 80 + 120 + 360 = 560 C57BL/6 mice

V.1.5. Experiment 5: Identification of host biomarkers that play a role in STEC and/or Stx-mediated disease. The purpose of these studies is to identify potential host factors that are involved in the pathogenesis of STEC infections. We will use 49 strains of advanced recombinant inbred (ARI) mice for detailed and high throughput systems genetics and systems biology studies of host interactions with STEC. These mice offer the most robust and practical means to identify networks of genes and pathways involved in regulating the host response. The mice, called BXD mice, result from crosses of C57BL/6 and DBA2/J mice for several generations, then backcrosses of the progeny to generate genetic mosaic mice. The genome sequences of the parent strains are known, as are the locations of the crossovers for each strain of BXD mice. Thus, this panel of mice can be used to identify specific traits that are linked to host susceptibility to Stx intoxication and/or EHEC infection. The BXD mice to be used in these studies will be obtained from Dr. (b)(6) at the (b)(6) or from (b)(4) In addition, we will continue our studies to investigate the role of Carcinoembryonic Antigen (CEA) in the pathogenesis of STEC infection. CEA is a protein expressed by human mucosal epithelial cells; the gene that encodes CEA is not present in mice. CEA Tg mice are more susceptible to colonization of mucosal

surfaces by bacteria. The increased susceptibility occurs because CEA suppresses exfoliation of mucosal epithelial cells, which are the site of attachment for mucosal pathogens, including *E. coli* O157:H7. We hypothesize that CEA Tg mice will be colonized more efficiently by *E. coli* O157:H7 because the mucosal cells to which the bacteria have attached will not be exfoliated as much as mucosal cells from wild-type mice.

V.1.5.1. Initial screen to identify host biomarkers involved in colonization of the gut with EHEC. We tested colonization in 25 strains of BXD mice as part of MIC-09-137. A minimum of 30 strains of mice need to be infected before quantitative trait loci (QTL) linked to colonization can be identified. Groups of 3 C57BL/6J, DBA/2J, or different strains of BXD mice will be infected orally with 86-24 ($Stx2^+$) or TUV86-2. Colonization of the intestines will be monitored daily by collection of feces and enumeration of the CFU present in the feces. In addition, mice will be monitored for bacteremia 7 days post-inoculation by blood collection from the tail vein followed by enumeration of bacteria in the blood. We do not expect to find bacteremia; however, one group reported that EHEC-infected mice may become septicemic, a finding that we have not duplicated.

V.1.5.1.1. Final preliminary experiments to generate sufficient data for QTL mapping. A minimum of 10 mice from each BXD strain need to be infected prior to QTL mapping, and we have not reached that threshold with some strains of BXD mice. We anticipate the need to conduct 5 additional experiments, described below, to complete our initial screen.

Group 1: 3 C57BL/6J mice
Group 2: 3 DBA/2J mice
Groups 3-7: 3 mice from each of 5 different BXD strains

3 mice/group x 7 groups/experiment x 5 experiments = 105 mice

V.1.5.1.2. Second round of screening to identify host biomarkers involved in colonization of the gut with EHEC. Once BXD strains that exhibit a different colonization phenotype than the parent strains have been identified, a second round of colonization experiments will be conducted to confirm the results of the initial screen. Groups of 5 BXD mice each will be infected with 86-24 or TUV86-2 and monitored for colonization and bacteremia as above. Groups of 3 C57BL/6J and DBA/2J mice will serve as controls. Every attempt will be made to combine experiments such that control mice can be used for multiple experimental groups of BXD mice. Up to 12 BXD strains will be tested in the second round of screening.

Group 1: 3 C57BL/6J mice
Group 2: 3 DBA/2J mice
Groups 3-5: 5 mice from each of 3 different BXD strains

21 mice/experiment x 4 experiments x 2 iterations = 168 mice

V.1.5.2. Second round of screening to identify host biomarkers involved in Stx2 intoxication. In studies conducted under MIC-09-137, we identified a region of chromosome 12 that contains a QTL that is involved in the number of days of survival after systemic Stx2 intoxication. The studies here will take advantage of BXD strains that are genetic mosaics for the parental strains in the region of chromosome 12 that contains the QTL. Groups of 5 BXD mice each will be intoxicated with Stx2 and monitored for symptoms of intoxication as in Section V.4.5. Groups of 3 C57BL/6J and 3 DBA/2J mice will serve as controls. Every attempt will be made to combine experiments such that control mice can be used for multiple experimental groups of BXD mice. A total of 6 BXD strains will be tested in the second round of screening. We anticipate that 2-4 strains of BXD can be tested in a particular experiment; however, many BXD strains do not breed well, so simultaneous availability of multiple strains is not always achievable.

Group 1: 3 C57BL/6J mice
Group 2: 3 DBA/2J mice
Groups 3-4: 5 mice from each of 2 different BXD strains

16 mice/experiment x 3 experiments x 2 iterations = 96 mice

V.1.5.3: Characterization of the involvement of CEA in the pathogenesis of STEC infection. CEA Tg mice were generated on the C57BL/6J background, so C57BL/6J mice will serve as the control in this experiment. Mice will be treated with str-water prior to and during the experiment, which will be carried out as described in V.1.A. Fecal samples will be collected daily to assess intestinal colonization and to quantify epithelial cell shedding.

Group 1: 10 CEA Tg mice inoculated with 86-24
Group 2: 10 C57BL/6J mice inoculated with 86-24

Total number of mice = (10 CEA Tg mice + 10 C57BL/6J mice) x 5 iterations = ***50 CEA Tg mice and 50 C57BL6/J mice***

Total number of mice (V.1.5):
15 + 24 + 18 + 50 = 107 C57BL/6J mice
15 + 24 + 18 = 57 DBA/2J mice
75 + 120 + 60 = 255 BXD mice
50 CEA Tg mice

V.1.6: Experiment 6: Measurement of the efficacy of therapeutic compounds designed to ablate the lethal effects of Stx. The objective of these studies is to test the efficacy of therapeutics designed to inactivate Stxs after entry into the gut or more distal target cells. We will engineer toxin-neutralizing antibodies or toxin inhibitors that can be selectively delivered into toxin-sensitive cells. The first approach involves administration of toxin-neutralizing monoclonal antibodies after infection or intoxication. Secondly, we theorize that conjugation of StxB1 or StxB2 to the exterior of liposomes

will target and deliver liposome-encapsulated inhibitory RNA molecules (iRNAs) to the cytoplasm of toxin-sensitive cells. The iRNAs will be designed to block the enzymatic activity of the toxins inside the cell. Finally, we hypothesize that peptide-linked phosporodiamidate morpholino oligos (morpholinos), which bind specific RNA sequences in the region of the translational start site and block translation, can be used to inhibit growth of and/or Stx production by STEC strains *in vivo*. The efficacy of these inhibitors will be tested in mice once their efficacy has been demonstrated in tissue culture cells. We anticipate testing up to 9 compounds over the next 3 years.

V.1.6.1: Determination of capacity of monoclonal antibodies against Stx2 to rescue mice from systemic intoxication with Stx2 variants or from infection with STEC strains that produce one or more Stx2 variants. We have previously shown that mouse monoclonal antibody 11E10 anti-Stx2 has the capacity to rescue Stx2-intoxicated and 86-24- or B2F1-infected CD-1 mice from morbidity and mortality. In the current studies, we will test the capacity of 11E10 to rescue male CD-1 mice from intoxication with Stx2c and Stx2dact and from infection with K3995 (Stx2, Stx2c) and C227-11 (Stx2).

V.1.6.1.1: Monoclonal antibody-mediated rescue from mortality of male CD-1 mice intoxicated with Stx2c or Stx2dact. Groups of five male CD-1 mice will be intoxicated with 5 LD_{50} each of Stx2 (10 ng, positive control), Stx2c, BT-Stx2dact, or ET-Stx2dact via i.p. inoculation with a 26 gauge needle connected to a tuberculin syringe. Twice in the first 96 hrs post-intoxication, 1 µg 11E10 will be administered via i.v. injection with a 26 gauge needle connected to a tuberculin syringe. In previous studies, we have found that the time points for administration of 11E10 are dependent upon the toxin or strain used as the inoculum. As such, the time points will be determined through experimentation. Mice will be monitored for morbidity and mortality, and any mice that exhibit 2 or more symptoms of morbidity will be euthanized by isoflurane overdose followed by cervical dislocation.

Group 1: 10 ng Stx2, no 11E10
Group 2: 10 ng Stx2, 11E10 administered twice
Group 3: 5 LD_{50} Stx2c, no 11E10
Group 4: 5 LD_{50} Stx2c, 11E10 administered twice
Group 5: 5 LD_{50} BT-Stx2dact, no 11E10
Group 6: 5 LD_{50} BT-Stx2dact, 11E10 administered twice
Group 7: 5 LD_{50} ET-Stx2dact, no 11E10
Group 8: 5 LD_{50} ET-Stx2dact, 11E10 administered twice

5 mice/group x 8 groups x 10 iterations = 400 male CD-1 mice

V.1.6.1.2: Monoclonal antibody-mediated rescue from mortality of male CD-1 mice infected with STEC. Groups of ten male CD-1 mice will be orally infected with 10 LD_{50} each of B2F1 (positive control), K3995, or C227-11 as described in V.1.B. Twice in the first 96 hrs post-infection, 1 µg 11E10 will be administered via i.v. injection with a 26 gauge needle connected to a tuberculin syringe. In previous studies, we have found that the time points for administration of 11E10

are dependent upon the toxin or strain used as the inoculum. As such, the time points will be determined through experimentation. Mice will be monitored for morbidity and mortality, and any mice that exhibit 2 or more symptoms of morbidity will be euthanized by isoflurane overdose followed by cervical dislocation.

Group 1: 10 mice infected with B2F1, no 11E10
Group 2: 10 mice infected with B2F1, 11E10 administered twice
Group 3: 10 mice infected with K3995, no 11E10
Group 4: 10 mice infected with K3995, 11E10 administered twice
Group 5: 10 mice infected with C227-11, no 11E10
Group 6: 10 mice infected with C227-11, 11E10 administered twice

10 mice/group x 6 groups x 10 iterations = 600 male CD-1 mice

Total number of mice (V.1.6.1) = 400 + 600 = 1000 male CD-1 mice

V.1.6.2: Assessment of efficacy of liposome-encapsulated iRNAs. Groups of 5 male CD-1 mice will be administered 5xLD_{50} of Stx2 (i.p. using a 26 gauge needle attached to a tuberculin syringe) followed by or given simultaneously with a dose of the therapeutic compound that neutralizes the toxin for tissue culture cells administered by i.v. injection. Control groups will receive a similar dose of toxin and diluent, or control liposomes, as appropriate. An additional control group will have the toxin and therapeutic agent mixed prior to i.p. injection. In previous studies, we have found that the time points for administration of anti-Stx therapeutics are dependent upon the toxin used as the inoculum. As such, the doses and time points will be determined through experimentation. The mice will be monitored for signs of extreme morbidity (see V.4.5 for definition) for 14 days post-intoxication. Any mice that exhibit signs of extreme morbidity will be euthanized immediately by inhalational isoflurane overdose and cervical dislocation.

Group 1: 5 mice, 10 ng Stx2, sterile saline
Group 2: 5 mice, 10 ng Stx2, liposome-iRNA, low tissue culture dose
Group 3: 5 mice, 10 ng Stx2, liposome-iRNA, high tissue culture dose
Group 4: 5 mice, sterile saline, liposome-iRNA at low tissue culture dose
Group 5: 5 mice, sterile saline, liposome-iRNA at high tissue culture dose
Group 6: 5 mice, 10 ng Stx2 pre-mixed with liposome-iRNA at low tissue culture dose
Group 7: 5 mice, 10 ng Stx2 pre-mixed with liposome-iRNA at high tissue culture dose

5 mice/group x 7 groups x 10 iterations = ***350 male CD-1 mice***

V.1.6.3: Determination of capacity of morpholinos to rescue infected mice from STEC disease. Groups of 5 mice will be challenged orally with STEC as described in V.1.A. Morpholinos will be administered orally by gavage (0.1-0.2 ml in sterile saline) and/or pipet feeding (25-50 µl in sterile 20% sucrose) or systemically by intravenous injection (0.1 ml in sterile saline) two to five times after infection. The optimal number of doses

and time points for morpholino administration will be determined experimentally. Previous studies with other types of *E. coli* strains showed that the effective and nontoxic dose range is 50-300 µg administered two to four times in the first 48 hrs after inoculation. Our preliminary studies with B2F1-infected CD-1 mice suggest that a minimum of two daily doses of therapeutic will be needed and that three to five doses may be more optimal. The mice will be monitored for signs of extreme morbidity (see V.4.5 for definition) for 14 days post-intoxication. Any mice that exhibit signs of extreme morbidity will be euthanized immediately by inhalational isoflurane overdose and cervical dislocation.

V.1.6.3.1: Infection of str-treated CD-1 mice with B2F1 and treatment with morpholinos.

Group 1: 5 mice, B2F1, sterile saline
Group 2: 5 mice, B2F1, 200 µg morpholino #1 administered twice
Group 3: 5 mice, B2F1, 200 µg morpholino #2 administered twice
Group 4: 5 mice, B2F1, 200 µg morpholino #3 administered twice
Group 5: 5 mice, B2F1, 200 µg morpholino #4 administered twice
Group 6: 5 mice, B2F1, 200 µg morpholino #1 administered four times
Group 7: 5 mice, B2F1, 200 µg morpholino #2 administered four times
Group 8: 5 mice, B2F1, 200 µg morpholino #3 administered four times
Group 9: 5 mice, B2F1, 200 µg morpholino #4 administered four times

5 mice/group x 9 groups x 10 iterations = 450 male CD-1 mice

V.1.6.3.2: Infection of BALB/c mice with 86-24 and treatment with morpholinos.

Group 1: 5 mice, 86-24, sterile saline
Group 2: 5 mice, 86-24, 200 µg morpholino #1 administered twice
Group 3: 5 mice, 86-24, 200 µg morpholino #2 administered twice
Group 4: 5 mice, 86-24, 200 µg morpholino #3 administered twice
Group 5: 5 mice, 86-24, 200 µg morpholino #4 administered twice
Group 6: 5 mice, 86-24, 200 µg morpholino #1 administered four times
Group 7: 5 mice, 86-24, 200 µg morpholino #2 administered four times
Group 8: 5 mice, 86-24, 200 µg morpholino #3 administered four times
Group 9: 5 mice, 86-24, 200 µg morpholino #4 administered four times

5 mice/group x 9 groups x 10 iterations = 450 female BALB/c mice

V.1.6.3.3: Infection of C57BL/6 mice with C227-11 and treatment with morpholinos.

Group 1: 5 mice, C227-11, sterile saline
Group 2: 5 mice, C227-11, 200 µg morpholino #1 administered twice
Group 3: 5 mice, C227-11, 200 µg morpholino #2 administered twice
Group 4: 5 mice, C227-11, 200 µg morpholino #3 administered twice
Group 5: 5 mice, C227-11, 200 µg morpholino #4 administered twice
Group 6: 5 mice, C227-11, 200 µg morpholino #1 administered four times
Group 7: 5 mice, C227-11, 200 µg morpholino #2 administered four times
Group 8: 5 mice, C227-11, 200 µg morpholino #3 administered four times
Group 9: 5 mice, C227-11, 200 µg morpholino #4 administered four times

5 mice/group x 9 groups x 10 iterations = 450 female C57BL/6 mice

V.1.6.3.4. Systemic inoculation and therapeutic delivery. If we fail to observe a therapeutic effect of the morpholinos in our STEC infection models, we will determine if the morpholinos have the capacity to reduce the effects of systemic infection with STEC. To do so, we will first need to do three pilot studies to determine the LD_{50}s for B2F1, 86-24, and C227-11 administered i.p to male CD-1 mice. Each strain will be diluted to the appropriate density in sterile saline, and the inoculum (in 200 µl) will be administered i.p. with a 26 gauge, ½" needle. The first experiment with each strain will utilize 3 mice/group and multiple doses that are predicted to fall on either side of the LD_{50}. We will then refine the experiment to utilize 5 mice/group and a narrower range of doses; this experiment will be done twice to confirm the results. Although the endpoint of this assay is mortality, mice that are extremely moribund, as defined in Section V.4.5, will be euthanized by laboratory personnel using isoflurane inhalation overdose and cervical dislocation. Surviving mice will be euthanized by LAM personnel with CO_2 inhalation followed by cervical dislocation.

V.1.6.3.4.1. Pilot experiment: estimation of LD_{50} for STEC strains administered i.p.
Group 1: 3 mice, 10^2 CFU
Group 2: 3 mice, 10^3 CFU
Group 3: 3 mice, 10^4 CFU
Group 4: 3 mice, 10^5 CFU
Group 5: 3 mice, 10^6 CFU
Group 6: 3 mice, 10^7 CFU

6 doses x 3 mice/dose x 3 strains: 54 CD-1 mice

V.1.6.3.4.2. Dose response: i.p. inoculation with STEC strains
Group 1: 5 mice, 100X predicted LD_{50}
Group 2: 5 mice, 10X predicted LD_{50}
Group 3: 5 mice, 1X predicted LD_{50}
Group 4: 5 mice, 0.1X predicted LD_{50}

4 doses x 5 mice/dose x 3 strains x 2 iterations: 120 CD-1 mice

V.1.6.3.4.3. Infection of CD-1 mice with STEC strains and treatment with morpholinos. CD-1 mice will be challenged via i.p. inoculation with 10xLD_{50} of B2F1, 86-24, or C227-11 and treated via i.p. or i.v. injection with 0.1 ml morpholino in sterile saline at various times post-infection. The optimal number of doses and time points for morpholino administration will be determined experimentally.

Group 1: 5 mice, sterile saline
Group 2: 5 mice, 50 µg morpholino 1 twice
Group 3: 5 mice, 50 µg morpholino 2 twice
Group 4: 5 mice, 50 µg morpholino 3 twice
Group 5: 5 mice, 50 µg morpholino 4 twice
Group 6: 5 mice, 300 µg morpholino 1 twice
Group 7: 5 mice, 300 µg morpholino 2 twice
Group 8: 5 mice, 300 µg morpholino 3 twice
Group 9: 5 mice, 300 µg morpholino 4 twice

5 mice/group x 9 groups x 10 iterations x 3 strains = 1350 male CD-1

Total number of mice (V.1.6.3):
450 + 54 + 120 + 1350 = 1974 male CD-1 mice
450 female BALB/c mice
450 female C57BL/6 mice

Total number of mice (V.1.6):
1000 + 350 + 1974 = 3324 male CD-1 mice
450 female BALB/c mice
450 female C57BL/6 mice

Experiment 1.7: Isolation of mouse intestinal mucus. The focus of aim #2 of the NIH grant is the mucus-activatable Stx2dact toxin found in several non-O157 strains. We will address why that particular Stx2 variant is so toxic compared to Stx2. For these studies, we will need mouse mucus to demonstrate the activatable phenotype of this toxin.

Intestinal mucus will be isolated from groups of 5 adult male CD-1 mice. Mice will be administered str in their drinking water, and, simultaneously, food will be withheld from the mice for about 18 h prior to mucus isolation. Although we have found that treatment of mice with str is not required for mouse mucus to activate Stx2dact, we have shown that such treatment reduces bacterial contamination of the mucus sample. Moreover, mucus obtained from animals administered the antibiotic, in principle, more closely resembles the mucus in the gut of the str-treated, orally-infected mouse than mucus from untreated mice. Food is withheld to reduce the amount of fecal matter in the intestines and also so that the mucus is isolated from mice treated in the same way as the orally-infected mouse model. Within 18 hours after str administration and food removal, mice will be euthanized by inhalational isoflurane overdose followed by cervical dislocation. The intestines will be removed and fecal material expelled from intestinal segment. The mucus will be pushed out of the intestines into HEPES buffer, pH 7.4, diluted in HEPES buffer and used immediately or frozen at -70°C for future use. Based on current rates of mucus usage, we anticipate the need for 20 CD-1 mice for this purpose per year.

20 mice/year x 3 years = ***60 male CD-1 mice***

Experiment 1.8. Studies to assess ricin intoxication, transit of ricin throughout the body, and anti-ricin therapeutics. In these experiments, we will identify the LD_{50} for a new lot of ricin, use *in vivo* imaging of mice to visualize transit of fluorescently-labeled ricin throughout the body, and test the efficacy of therapeutics to rescue mice from acute ricin intoxication. All of these experiments will be conducted in six to eight week-old male CD-1 mice according to V.1.C above.

V.1.8.1: Identification of LD_{50} of ricin administered via oral gavage or i.p. injection. We will use historical data from previous lots of ricin and current data on Vero cell cytotoxicity of the new lot of ricin to determine the dose range of ricin to administer to identify the LD_{50}s for new lots of ricin. We anticipate that we will produce up to three new lots of toxin in the three years of this protocol.

V.1.8.1.1: Oral gavage
Group 1: 5 mice, 5 LD_{50}s old lot of ricin in sterile saline
Groups 2-7: 5 mice, five two-fold dilutions of ricin in sterile saline

5 mice/group x 7 groups x 2 iterations x 3 lots of ricin = 210 male CD-1 mice

V.1.8.1.2: Intraperitoneal injection
Group 1: 5 mice, 5 LD_{50}s old lot of ricin in sterile saline
Groups 2-7: 5 mice, five two-fold dilutions of ricin in sterile saline

5 mice/group x 7 groups x 2 iterations x 3 lots of ricin = 210 male CD-1 mice

Total number of mice (V.1.8.1) = 210 + 210 = 420 male CD-1 mice

V.1.8.2: *in vivo imaging of CD-1 mice orally intoxicated with ricin.* Our previous studies demonstrated that, after oral gavage into the stomach, ricin travels through the gastrointestinal tract and is excreted in the feces or across the gastrointestinal tract and into the bloodstream. We have observed ricin in the kidneys, spleen, liver and brain in these preliminary studies. Further studies are needed to investigate the sites in the body to which ricin travels and the mechanism by which ricin binds to and transits through the gastrointestinal epithelium. Mice will be orally intoxicated and imaged throughout the course of the study in our Carestream Multispectral FX Pro *in vivo* imaging system. At specific time points, mice will be euthanized and organs will be imaged *ex vivo* prior to harvest and fixation for immunohistochemistry and immunofluorescence.

Group 1: 5 mice, 1 LD_{50} ricin, euthanize 4 h post-intoxication
Group 2: 5 mice, 1 LD_{50} ricin, euthanize 8 h post-intoxication
Group 3: 5 mice, 1 LD_{50} ricin, euthanize 12 h post-intoxication
Group 4: 5 mice, 1 LD_{50} ricin, euthanize 24 h post-intoxication
Group 5: 5 mice, 1 LD_{50} ricin, euthanize 48 h post-intoxication
Group 6: 5 mice, 1 LD_{50} ricin, euthanize 72 h post-intoxication

5 mice/group x 6 groups x 3 iterations = ***90 male CD-1 mice***

V.1.8.3: Measurement of the efficacy of therapeutic compounds designed to ablate the lethal effects of ricin. The first approach involves administration of toxin-neutralizing monoclonal antibodies after intoxication. We will use a well-characterized monoclonal antibody, R70, that neutralizes the activity of the A (catalytic) subunit of ricin (RtxA) *in vitro*. In our second approach, we will administer liposome-encapsulated R70 anti-ricin antibody after intoxication. The cell binding domain of ricin (RtxB) will be conjugated to the exterior of the liposomes, which are vesicles composed of an outer lipid bilayer that forms around an aqueous solution (anti-RtxA antibody in sterile saline) when the two components are mixed together. We theorize that conjugation of RtxB to the exterior of liposomes will target and deliver liposome-encapsulated R70 anti-ricin antibody to the cytoplasm of toxin-sensitive cells. We have shown that treatment of ricin-intoxicated tissue culture cells with R70 anti-ricin alone or liposome-encapsulated R70 anti-ricin neutralizes the cytotoxicity of ricin in a dose-dependent manner.

V.1.8.3.1: Monoclonal antibody-mediated rescue from mortality of male CD-1 mice intoxicated with ricin. Groups of five male CD-1 mice will be intoxicated with 5 LD_{50} each of ricin in sterile saline via oral gavage. Twice in the first 96 hrs post-intoxication, 1 µg R70 anti-ricin in sterile saline will be administered via i.v. injection with a 26 gauge needle connected to a tuberculin syringe In previous studies, we have found that the time points for administration of monoclonal antibodies are dependent upon the toxin used as the inoculum. As such, the time points will be determined through experimentation. Mice will be monitored for morbidity and mortality, and any mice that exhibit 2 or more symptoms of morbidity will be euthanized by isoflurane overdose followed by cervical dislocation.

Group 1: 5 mice, 5 LD_{50} ricin, no R70
Group 2: 5 mice, 5 LD_{50} ricin, low dose R70 administered twice
Group 3: 5 mice, 5 LD_{50} ricin, high dose R70 administered twice

5 mice/group x 3 groups x 10 iterations = 150 male CD-1 mice

V.1.8.3.2: Assessment of efficacy of liposome-encapsulated R70 anti-ricin. Groups of 5 male CD-1 mice will be administered 5xLD_{50} of ricin in sterile saline by oral gavage, followed by or given simultaneously with a dose of the liposome-encapsulated R70 anti-ricin that neutralizes the toxin for tissue culture cells; the liposome-antibody conjugated will be diluted in sterile saline and administered by i.v. or i.p. injection. Control groups will receive a similar dose of toxin and diluent, or control liposomes without antibody and/or RtxB, as appropriate. An additional control group will have the toxin and therapeutic agent mixed prior to i.v. or i.p. injection. In previous studies, we have found that the time points for administration of anti-ricin therapeutics are dependent upon the route of ricin inoculation and the route of therapeutic administration. As such, the doses and time points will be determined through experimentation. The mice will be monitored for signs of extreme morbidity (see V.4.5 for definition) for 14 days post-intoxication. Any mice that exhibit signs of extreme morbidity will be

euthanized immediately by inhalational isoflurane overdose and cervical dislocation.

Group 1: 5 mice, 5xLD_{50} ricin, sterile saline
Group 2: 5 mice, 5xLD_{50} ricin, liposome-R70 anti-ricin, low tissue culture dose
Group 3: 5 mice, 5xLD_{50} ricin, liposome-R70 anti-ricin, high tissue culture dose
Group 4: 5 mice, sterile saline, liposome-R70 anti-ricin at low tissue culture dose
Group 5: 5 mice, sterile saline, liposome-R70 anti-ricin at high tissue culture dose
Group 6: 5 mice, 5xLD_{50} ricin pre-mixed with liposome-R70 anti-ricin at low tissue culture dose
Group 7: 5 mice, 5xLD_{50} ricin pre-mixed with liposome-R70 anti-ricin at high tissue culture dose

5 mice/group x 7 groups x 10 iterations = 350 male CD-1 mice

Total number of mice (V.1.8.3) = 150 + 350 = 500 male CD-1 mice

Total number of mice (V.1.8) = 420 + 90 + 500 = 1010 male CD-1 mice

V.2. Data Analysis: A sample size of 5-10 animals per condition will have 80% power to detect a difference of 1.3 - 1.8 standard deviations based on a t test for independent samples with a 5%, two-sided significance level. A sample size of 15 animals per group will have 80% power to compare two proportions (i.e. survival data) based on a t test for independent samples with a 5%, two-sided significance level. These are the smallest differences that would be considered clinically meaningful for this type of study. Lethal dose 50% will be estimated by the method of Reed and Muench (5) or calculated by Probit analysis using 95% confidence intervals if the data allow it. For protection studies, ANOVA will be used to compare the mean time-to-death between groups and Chi-square tests with 95% confidence intervals will be used to compare the percentages of deaths between groups.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: Whenever possible, we will test the toxicity of Stx preparations on Vero cells rather than in animals. All of our initial assessments and comparative assessments of cytotoxicity are done on Vero cells rather than mice. The use of the Vero cell assays does allow us to reduce the range of toxin doses that we subsequently administer to mice. Additionally, historical data allow us to approximate an LD_{50} rather than the more usual range of at least 10 doses. Unfortunately, some of the differences in toxicity among Stx family members are only evident in the whole animal (*e.g.*, Stx2c versus Stx2dact). Further, the proposed studies to assess the efficacy of therapeutics require systemic responses that are not possible to achieve in tissue culture systems. No other alternatives to the use of animals were identified for this proposal.

V.3.2. Animal Model and Species Justification: The long term goals of this project are to define the pathogenic mechanisms by which STEC and ricin cause disease and to develop strategies for the prevention and treatment of STEC-mediated HUS and ricin intoxication. Humans are the most appropriate model with which to assess the virulence of STEC. However, humans cannot be used for logistical as well as ethical reasons, *i.e.*, a volunteer fed an STEC strain might develop HUS, a life-threatening condition. Instead, we use mice in four types of mouse assays developed in my laboratory. All of the mouse strains are used for specific experiments for historical reasons. We use male CD-1 mice for our str-treated model of STEC-mediated lethality because they are sensitive to infection with STECs and they exhibit toxin-mediated renal tubular necrosis when fed those STEC strains. In our initial studies more than twenty years ago, we found that outbred male CD-1 mice were susceptible to infection with STECs in the presence of str; their continued susceptibility to STEC and their low cost make them the strain of choice for our ongoing studies. We use female BALB/c for our intact commensal flora (ICF) model because we found that these mice are highly likely to be colonized by STEC when normal gastrointestinal flora are present. Female mice are used in this model because they are highly colonized more consistently than are males. We use female C57BL/6 mice for our amp-treated model, which is specific for C227-11. C227-11 was isolated from an outbreak in Germany in May 2011 and is a hybrid between enteroaggregative (FAEC) and enterohemorrhagic *E. coli* strains; the only published mouse model for EAEC utilized C57BL/6 mice. Females are used because they are more susceptible to infection than males. We use BALB/c and CD-1 mice for toxicity assays because we have found that these strains of mice are susceptible to Sbxs (BALB/c, CD-1) and ricin (ricin) administered orally or systemically in these strains of mice. Some Stxs exhibit greater toxicity in inbred mice (BALB/c) and some in outbred mice (CD-1). In addition, other groups routinely use CD-1 mice for ricin toxicity studies. Our observations that ricin and some of the Sbxs are toxic by oral and systemic routes have important implications for ricin and STEC pathogenesis.

All of the mouse assays will be used in conjunction with tissue culture assays to further dissect the virulence characteristics of these organisms and toxins. While *in vitro* cell-based assays can provide an estimate of toxin potency, they do not accurately reflect the multitude of effects of toxins on complex living systems and so cannot be used to replace the testing in animals. The interactions between EHEC and the plethora of cell types within the gastrointestinal tract, circulation, and kidneys cannot be mimicked in tissue culture systems. Unfortunately mice do not completely mimic STEC-mediated disease because diarrhea and glomerular damage are notably absent in infected animals. The most accurate endpoint to assess the virulence of an EHEC strain or the potency of Stxs and ricin is lethality. All published studies of EHEC, Stx, and ricin pathogenesis of which we are aware utilize mice, rabbits, or primates to determine the local and systemic effects of the organisms and toxins. At this time mice are the least sentient species we can use to test certain aspects of STEC and ricin pathogenesis and to evaluate potential therapies. With respect to the number of animals required for each experiment, we have used the minimal number of animals required for biological and statistical significance. If we establish in our preliminary protection studies that the therapeutic(s) under investigation do in fact reduce or prevent morbidity or mortality, the pain category for these animals will be changed from category E to category C.

V.3.3. Laboratory Animals

	Species #1	Species #2	Species #3	Species #4	Species #5	Species #6
V.3.3.1. Genus & Species	*Mus musculus*	*Mus musculus*	*Mus musculus*	*Mus musculus*	*Mus musculus*	*Mus musculus*
V.3.3.2. Strain/ Stock	CD-1	BALB/c	C57BL/6J	DBA/2J	BXD	CEA Tg
V.3.3.3. Source/ Vendor	(b)(4)					
V.3.3.4. Age	5-6 weeks	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks	6-8 weeks
V.3.3.5. Weight:	14-25 grams	12-25 grams	10-20 grams	10-20 grams	10-20 grams	10-20 grams
V.3.3.6. Sex	Male	Female	Both	Both	Both	Both
V.3.3.7. Special Considerations	N/A	N/A	N/A	N/A	N/A	N/A
V.3.4. Number of Animals Required (by Species)	7674	4719	1117	57	255	50

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: For certain experiments death will be the study endpoint; however, we will carefully monitor the animals and euthanize those that appear moribund because it is our experience that these animals will ultimately succumb to Stx intoxication after inoculation or infection with a virulent strain. Investigators will carefully monitor intoxicated animals and euthanize those that appear to be extremely moribund. We define extremely moribund as when a mouse exhibits 2 or more of the following symptoms: ruffled fur, lethargy, hunched posture, impaired ambulation that prevents the animals from reaching food and water, >25% weight loss, difficult or labored breathing, and the inability to remain upright. Death due to intoxication typically occurs 2-3 days post-injection and death due to infection typically occurs 3-6 days post-inoculation. Mice will be inspected every 4 h during normal working hours (0600-1900) and every 7-8 h during non-business hours. All inspections will be noted on cage cards so that LAM personnel are aware that the mice are being monitored.

As part of (b)(6) we investigated the possible use of weight loss as a surrogate endpoint for the Stx or ricin intoxication and STEC infection experiments. Our studies

demonstrated that a positive correlation between ≥ 25% weight loss at 4 days post-challenge and lethality exists. However, only about 50% of the mice that lost 25% of their body weight by day 4 succumbed to intoxication or infection. All mice that lost 30% of their body weight by day 4 were humanely euthanized prior to death because we found that the mice would have succumbed within another 24 hrs. We will continue to monitor weight loss in intoxicated or infected animals, and animals will be humanely euthanized when they are extremely moribund.

V.3.5.2. **Reduction**: As many of our experiments involve infected animals or tissues, we do not participate in any shared tissue and or control groups with other investigators. To reduce the number of mice required, the studies with BXD mice will be done using a single dose of toxin or STEC, and only one iteration will be used for the first round of screening. In theory, we could use up-down dosing to determine the oral LD_{50} for Stxs and ricin. However, the amount of toxin that is needed for these studies prohibits this type of study. Instead, we will do one to two traditional LD_{50} studies with small numbers of mice in each group so as to reduce the amount of the Select Agent toxins that are needed.

V.3.5.3. **Replacement**: Whenever possible, we will test the toxicity of Shiga toxin preparations/constructs on Vero cells rather than in animals. Unfortunately, some of the differences in toxicity among Stx family members are only evident in the whole animal. We will also routinely assess the capacity of anti-intimin sera to block STEC adherence to tissue culture cells rather than test the sera for blocking activity in animals.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	CD-1	BALB/c	C57BL/6J	DBA/2J	BXD	CEA Tg
V.4.1.1.1.1. Column C:	260	32	-	-	-	-
V.4.1.1.1.2. Column D:	-	900	-	-	-	-
V.4.1.1.1.3. Column E:	5890	3787	1117	57	255	50

Table V.4.1.1.2. Breakdown by study number of experimental animals for pain category assignment.

Study number	Pain category C	Pain Category D	Pain Category E
V.1.1.1.1			200
V.1.1.1.2			140
V.1.1.2			300
V.1.1.3.1			1440
V.1.1.3.2			720
V1.1.3.3			720
V1.1.3.4			720
V1.1.4.1			480
V1.1.4.2			480
V1.1.4.3			240
V1.2.1.1			120
V1.2.1.2			560
V1.2.1.3	32		32
V1.2.2.1		180	
V1.2.2.2		180	
V1.2.2.3		180	
V1.2.2.4		360	
V1.3.1.1			80
V1.3.1.2			110
1.3.2.1			60
1.3.2.2			110
1.3.3			105
1.4.1			80
1.4.2			120
1.4.3.1			120
1.4.3.2			120
1.4.3.3			120
1.5.1.1			105
1.5.1.2			168
1.5.2			96
1.5.3			100
1.6.1.1			400
1.6.1.2			600
1.6.2	100		250
1.6.3.1			450
1.6.3.2			450
1.6.3.3			450
1.6.3.4.1			54
1.6.3.4.2			120
1.6.4.3.3	150		1200
1.7	60		
1.8.1.1			210
1.8.1.2			210
1.8.2			90
1.8.3.1			150
1.8.3.2	100		250

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: Administration of isoflurane gas prior to intoxication or infection will be done in one of two ways: 1) via a Gas Anesthesia System that delivers isoflurane gas to a 5-port anesthesia manifold, or 2) by soaking gauze with liquid isoflurane in the bottom of a bell jar in a chemical hood and placing the mice on a metal rack above the gauze. When using the Gas Anesthesia System, animals will be placed into an induction chamber and anesthetized with isoflurane (3-4%) with oxygen as the carrier gas. The induction chamber will be set to deliver isoflurane at a constant rate. The manifold also provides waste gas scavenging ports to help prevent gas from entering the surrounding work environment. A built-in vacuum system helps ventilate waste gas away from researchers and into disposable isoflurane-absorbing filters. We use the bell jar method rather than the gas anesthesia system when mice are extremely moribund because 1) the fume hood is closer to our animal room than is the anesthesia system, and 2) the bell jar can be set up more quickly than the gas anesthesia system, which results in less suffering for moribund mice. Once anesthetized using either method, the depth of anesthesia will be assessed by the onset and maintenance of slow, shallow breathing as well as the loss of toe pinch response in the animals.

For experiment V.1.2.2 (intestinal loop model), mice will be anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) immediately before the surgical procedure. Once anesthetized, the depth of anesthesia will be assessed by the onset and maintenance of slow, shallow breathing as well as the loss of toe pinch response in the animals. At the end of the survival surgery, a few drops of liquid bupivacaine (0.5%) will be placed on the incision site to provide post-surgical wound anesthesia. In addition, buprenorphine (0.1 mg/kg) will be administered once by s.c. injection to provide post-surgical systemic analgesia. Mice will be euthanized 6 hrs after surgery, which is prior to the time at which the bupivacaine and buprenorphine effects are expected to wear off, and mice will be monitored during this period.

For *in vivo* imaging studies, isoflurane has the advantage of maintaining the mice under anesthesia during the imaging procedure while permitting the animals a quick recovery time. The anesthetized mice will be placed on a warm platform and fluorescence and x-ray images will be captured over a 5 min period at 0.5, 1, 3, 6, 12, 24, 48, and/or 72 h post intoxication. To anesthetize the mice and secure their immobility during imaging with the Carestream Multispectral *in vivo* imaging system, we will follow the following procedures. Administration of isoflurane gas will be done via a Gas Anesthesia System that delivers isoflurane gas to a 5-port anesthesia manifold housed in the imaging chamber of the *in vivo* imaging system. Animals will be initially placed into an induction chamber and anesthetized with isoflurane (3-4%) with oxygen as the carrier gas. Once the animals are properly sedated, they will be swiftly moved to nose cones on the 5-port anesthesia manifold housed in the imaging chamber and maintained at 1.5-3.0% isoflurane. Any unused manifold ports will be closed off. Sufficient time will be given to allow the anesthetic gas to reach the manifold nose cones prior to removing the animals from the induction chamber. Both the induction chamber and manifold will be set to deliver isoflurane at a constant rate. The manifold also provides waste gas scavenging

ports to help prevent gas from entering the surrounding work environment. A built-in vacuum system helps ventilate waste gas away from researchers and into disposable isoflurane-absorbing filters.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Only mice that appear to be in good health will undergo anesthesia. We define good health as clean unruffled fur, alert demeanor and no obvious signs of sickness. Mice recovering from anesthesia will be placed into a clean cage with external heat applied, through either the use of an overhead heat lamp or a heating pad placed under the recovery cage, to prevent mice from undergoing a rapid loss in body temperature. Once the mice have roused from anesthesia and appear to be moving in an alert manner, they will be placed back into a clean cage with their cagemates. Mice injected with toxin or infected with *E. coli* will be monitored by laboratory personnel up to 6 times daily following the procedure as well as twice daily observation by LAM staff.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: Pubmed and AGRICOLA

V.4.1.3.2. Date of Search: 16 May 2012

V.4.1.3.3. Period of Search: All available

V.4.1.3.4. Key Words of Search: Pain AND (alleviation OR alleviate OR alternative OR reduction) AND (mouse OR mice); add AND (toxin OR bacteria) for Pubmed search

V.4.1.3.5. Results of Search:

PubMed: Search of Pain AND (alleviation OR alleviate OR alternative OR reduction) yielded 39252 hits. Limiting these to (mouse OR mice) yielded 1015 hits. Further limiting these results to (toxin OR bacteria) yielded 76 hits. Of those 76 references, only three dealt with alternatives to mouse assay for intoxication or infection. The three references suggest the use of hen egg approaches that would not work with Shiga toxins or STEC. One additional article conducted and reviewed studies that used opioids as analgesics in *in vivo* immunological experiments (4). (b)(5) *et al.* concluded that the opioid analgesics fentanyl and buprenorphine affected the immune system in such a way as to alter the outcome of the experiments.

AGRICOLA: Search Request. Search = (Pain OR alleviation OR reduction OR alternatives) AND (mouse OR mice) yielded 25 hits. Only one article dealt with alternatives to or reduction of pain in mouse intoxication or infection experiments. The investigators assessed the effect of buprenorphine on *Toxoplasma gondii* studies in mice, and concluded that buprenorphine does not affect the outcome of 28 day *T. gondii* infections in mice (1).

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: Our justification for the use of the mouse model to study toxin mediated STEC disease is

that tissue culture systems used to evaluate toxin production cannot always discriminate between animal-virulent and animal-attenuated strains. Examples of the importance of using animals rather than Vero cells for assessing relative toxicity of Stxs or virulence of STEC strains are as follows. First, we discovered that Stx2 is at least 100-fold more toxic than Stx1 for mice and that observation led to an experiment by Dr. Tom Obrig in which he demonstrated that Stx2 is a 1000-fold more toxic for human renal capillary endothelial cells than is Stx1. Moreover, with the recognition that animals and primary human renal cells (that are extremely difficult to obtain and maintain) are more susceptible to Stx2 than Stx1 came an understanding of the basis for the epidemiological observation that infection with Stx2-producing STEC strains more frequently leads to HUS in children than does infection with an Stx1-only producing strain. Secondly, we would not have recognized the fact that Stx2dact is activated by intestinal mucus without feeding the strain producing the toxin to str-treated CD-1 mice. Lastly, the str-treated mouse model (renal tubular necrosis) is one of two (the rabbit is the other) small animal models available for studying the systemic delivery of Stx from the intestinal tract and for identifying methods to prevent or neutralize the effect of that toxin and/or STEC-mediated disease. We further minimize distress during the studies by careful cage-side monitoring of the animals to determine if an animal should be euthanized. Signs indicating that euthanasia should be implemented would include an inability to reach food and water, labored breathing, excessive weight loss, and inability to remain upright.

No analgesics will be given to STEC-infected animals for the following two reasons. First, inflammation and/or the inflammatory response are key components of both STEC-mediated hemorrhagic colitis (inflammation of the colon and neutrophilic infiltrate) and STEC-mediated HUS. Pro-inflammatory cytokines are believed to play a role in HUS potentially by up-regulating toxin receptor expression or by exacerbating Stx-mediated damage. Because we will be using these animal models to study both the pathogenesis of disease and possible treatment therapies, we believe that the use of non-steroidal anti-inflammatory drugs (NSAIDs) could confound or possibly mask the extent of STEC-mediated damage that we will be evaluating by histological examination or toxicity. Secondly, the use of opioids as analgesics has the potential to exacerbate symptoms rather than eliminate pain since peristalsis would be reduced. Opium compounds have been used in several animal models for gastroenteritis to **increase** the likelihood of establishing and/or maintaining bacterial infection. Additionally, anti-motility agents are not recommended for patients with suspected STEC-mediated diarrhea because this treatment may increase the risk of progression to HUS.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: Intestinal Loop Model

V.4.3.1. Pre-surgical Provisions: Only mice that appear to be in good health will undergo anesthesia. We define good health as clean unruffled fur, alert demeanor and no obvious signs of sickness. The mice will be fasted for 18 h prior to surgery. Mice will be anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) immediately prior to the surgical procedure.

V.4.3.2. **Procedure:** The procedure that we will use is a modification of a protocol that was described by (b)(6) *et al.* for analysis of the effects of *Clostridium perfringens* epsilon toxin on different segments of the intestines (2). BALB/c mice will used in this study. All procedures will be conducted in a certified Biosafety Cabinet in our satellite animal room, (b)(6)

The mice will be fasted for 18 h prior to surgery. An overnight fast is needed to reduce the volume of the stomach contents so that the entire inoculum can be administered. In addition, we have found that mice fed by Pipetman or weigh boat are more likely to ingest the inoculum if they have been fasted. Mice will be anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) immediately prior to the surgical procedure. The abdominal area will be shaved with clippers. A 3-4 cm midline abdominal incision will be performed with a pointed scalpel and the stomach, the small intestine or the large intestine will be partially externalized. Prior to ligation, the intestinal segments will be flushed with 1ml of warm sterile saline to remove the lumenal contents. For the proximal portion of the small intestine, a segment of 4 cm of intestine including duodenum and jejunum will be ligated with Prolene 3-0 just below the pylorus. The middle portion of the small intestine will be ligated 1 cm distal the ligament of Treitz and 5 cm down to the ileum. For the distal portion, the ileum will be ligated approximately 5 and 1 cm before the ileocecal valve, respectively. For the colonic loops, a ligature will be placed at the cecocolonic junction and a second ligature will be placed on the colon immediately before the opening to the rectum. Care will be taken that the ligatures are tight enough to prevent the flow of liquids into and from the ligated intestinal segments but not too tight to cause damage to the tissues involved. *E. coli* O157:H7 (enterohemorrhagic *E. coli* or EHEC) will be diluted in warm sterile saline, which will also serve as the control. Bacterial or saline preparations will be administered using 1-ml tuberculin syringes with a 27-gauge needle by oblique insertion of the needle in the intestinal segment, always in distal direction, with care being taken to avoid intestinal blood vessels. After administration of test solutions, the organs will be gently repositioned and the abdominal incisions will be sutured in two planes using 3-0 Prolene to close the abdominal incision and tissue glue to close the skin layer. Topical bupivacaine ointment will be placed on the incision site after suturing to provide post-surgical wound analgesia. Buprenorphine (0.1 mg/kg, subcutaneous) will be used as a post-surgical analgesic. Injection volumes will be kept constant at 300 µl per animal regardless of the site of administration. The surgical procedure is predicted to last approximately 7-10 min per animal. Mice will be euthanized by isoflurane overdose and cervical dislocation 6 hrs after inoculation and tissues will be collected for bacterial enumeration and histological examination.

V.4.3.3. **Post-surgical Provisions:** Topical bupivacaine drops will be placed on the incision site after suturing to provide post-surgical wound analgesia. Buprenorphine (0.1 mg/kg, subcutaneous) will be used as a post-surgical analgesic and will be administered by s.c. injection prior to recovery from anesthesia. Mice will be observed hourly after the survival surgery. Any mice that appear to be extremely moribund, as described in V.3.5 above, will be euthanized by isoflurane overdose and cervical

dislocation. In addition, all mice will be euthanized 6 hrs after inoculation and tissues will be collected for bacterial enumeration and histological examination.

V.4.3.4. Location: (b)(6)

V.4.3.5. Surgeon: The procedure will be performed by Dr. (b)(6) who is a postdoctoral fellow in our laboratory. Dr. (b)(6) was trained by MAJ (b)(6) (b)(6) DVM.

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

Intraperitoneal:
V.1.C, V.1.2.2, V.1.4.3, V.1.6.2, V.1.8.3: Mice will receive i.p. injections of Stxs, ricin, ketamine/xylazine, or anti-Stx or anti-ricin therapeutics diluted in 0.1-0.2 ml sterile saline or sterile PBS. All will be injected i.p. using a 26 gauge needle attached to a tuberculin syringe. The dose of toxins and therapeutics will be determined in the experiments. Doses of 100 mg/kg ketamine and 10 mg/kg xylazine diluted in 0.1-0.2 ml sterile saline will be administered for anesthesia in V.1.2.2. In V.1.4.3, a dose of 40 µg of cipro will be diluted in 0.1 ml sterile saline and administered by i.p. injection on days 2, 3, 4, and 5 post-inoculation.

Intravenous:
V.1.6.1, V.1.6.3., V.1.8.3: Mice will receive anti-Stx or anti-ricin therapeutics i.v. via the tail vein or retro-orbital sinus in a volume of 100 or 25 µl PBS, respectively, using a 30 gauge 0.5 inch needle attached to a tuberculin syringe.

Intestinal injection:
V.1.2.2: Mice will receive an injection of STEC or Stx diluted in 0.3 ml sterile saline directly into an intestinal loop with a 27 gauge needle attached to a tuberculin syringe.

Subcutaneous:
V.1.2.2: Mice will receive 0.1 mg/kg buprenorphine in 0.1 ml sterile saline via s.c. injection into the right flank with a 26 gauge, ½" needle attached to a tuberculin syringe. Buprenorphine will be administered only to mice that have undergone survival surgery.

V.4.4.2. Biosamples:
Mucus: We collect mucus from the small intestine of mice treated with str and fasted for 18 h. An overnight fast is needed to reduce the volume of the stomach contents so that the entire inoculum can be administered. In addition, we have found that mice fed by Pipetman or weigh boat are more likely to ingest the inoculum if they have been fasted.

The mice are euthanized by inhalational isoflurane overdose and cervical dislocation prior to mucus collection.

Sera: Blood will be collected from the mice in sections V.1.A, V.1.B and V.1.C by tail vein bleed or retro-orbital bleed immediately prior to or by exsanguination upon euthanasia. Retro-orbital bleeds will be performed using sterile fine-bore Pasteur pipets or micro-capillary tubes. Mice will be lightly anesthetized with isoflurane. No more than 0.1 ml of blood will be collected by placing the pipet or micro-capillary tube against the capillary bed immediately to the right or left of the eye and applying slight pressure to stimulate blood flow. A single mouse will be bled no more frequently than once every 3-4 days and mice will be bled from alternating eyes if they are bled more than once.

Feces: approximately 1 gram obtained by pellet collection.

Tissues: Intestines, kidneys, livers, spleens, hearts, brains will be collected after inhalational isoflurane overdose and cervical dislocation.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Mice will be identified by cage cards and ear tags or ear punch.

V.4.4.6. Behavioral Studies:

V.4.4.7. Other Procedures:
Streptomycin and ampicillin in V.1.A will be administered orally in the drinking water at a dose of 5 g/L str or 10 g/L amp. Antibiotic-containing water will be changed every 1-2 days.
Oral gavage of 0.1-0.3 ml STEC, Shiga toxin, or ricin in sterile saline will be performed using a 18-20 gauge feeding needle. Anesthesia is not required for this procedure.
Custom diets that contain 2% or 10% guar gum as the sole source of fiber will be used in V.1.2. We have fed mice these diets in the past and have observed no negative impact.

V.4.4.8. Tissue Sharing: N/A

V.4.5. Study Endpoint: The experimental question is answered by determining the number of mice that survived or succumbed to STEC or toxin. See Table 4.5.1 below for a description of the study outcome for each experiment. Investigators will carefully monitor intoxicated animals and euthanize those that appear extremely moribund because it is our experience that these animals will ultimately succumb to intoxication. We define extremely moribund as when a mouse exhibits 2 or more of the following symptoms: ruffled fur, lethargy, hunched posture, impaired ambulation that prevents the animals from reaching food and water, excessive weight loss (>25% of initial weight), difficult or labored breathing, and the inability to remain upright.

Mice will be weighed daily and those that lose more than 25% of their initial body weight by day 4 post-inoculation will be euthanized. During the first 5-10 days after infection or first 2-5 days after intoxication (range depends upon the model and agent), mice will be inspected every 4 h during normal working hours (0600-1900) and every 7-8 h during non-business hours. After day 5-14 (endpoint of increased monitoring depends upon the model and the agent), mice will be inspected twice per day because few additional deaths are expected. We carry out the experiments for 7-28 days because we occasionally observe symptoms that range from mild morbidity to death until the endpoint noted in Table 4.5.1 below. All inspections will be noted on cage cards so that LAM personnel are aware that the mice are being monitored. Surviving mice will be euthanized by LAM personnel by CO_2 overdose and cervical dislocation.

Table 4.5.1. Study endpoints and predicted outcomes

Study number	Pain Category C	Pain Category D	Pain Category E	Study Outcome
V.1.1.1.1			200	Death or euthanasia on day 21
V.1.1.1.2			140	Death or euthanasia on day 21
V.1.1.2			300	Death or euthanasia on day 21
V.1.1.3.1			1440	Death or euthanasia on day 21
V.1.1.3.2			720	Death or euthanasia on day 21
V1.1.3.3			720	Death or euthanasia on day 7 or 14
V1.1.3.4			720	Death or euthanasia on day 7 or 14
V1.1.4.1			480	Death or euthanasia on day 7 or 14
V1.1.4.2			480	Death or euthanasia on day 7 or 14
V1.1.4.3			240	Death or euthanasia on day 7 or 14
V1.2.1.1			120	Death or euthanasia on day 7 or 14
V1.2.1.2			560	Death or euthanasia on day 7 or 14
V1.2.1.3	32		32	Death or euthanasia on day 14
V1.2.2.1		180		Death or euthanasia at hr 6
V1.2.2.2		180		Death or euthanasia at hr 6
V1.2.2.3		180		Death or euthanasia at hr 6
V1.2.2.4		360		Death or euthanasia at hr 6
V1.3.1.1			80	Death or euthanasia on day 14
V1.3.1.2			110	Death or euthanasia on day 14
1.3.2.1			60	Death or euthanasia on day 14
1.3.2.2			110	Death or euthanasia on day 14
1.3.3			105	Death or euthanasia at 4, 8, 12, 24, 48, 72, 96 hrs
1.4.1			80	Death or euthanasia at 3, 6, 12, 24, 48, 72, 96, 120 hrs
1.4.2			120	Death or euthanasia on day 28
1.4.3.1			120	Death or euthanasia on day 28
1.4.3.2			120	Death or euthanasia on day 28
1.4.3.3			120	Death or euthanasia on day 28
1.5.1.1			105	Death or euthanasia on day 14
1.5.1.2			168	Death or euthanasia on day 14
1.5.2			96	Death or euthanasia on day 14
1.5.3			100	Death or euthanasia on day 14
1.6.1.1			400	Death or euthanasia on day 14
1.6.1.2			600	Death or euthanasia on day 14
1.6.2	100		250	Death or euthanasia on day 14

Study number	Pain Category C	Pain Category D	Pain Category E	Study Outcome
1.6.3.1			450	Death or euthanasia on day 21
1.6.3.2			450	Death or euthanasia on day 21
1.6.3.3			450	Death or euthanasia on day 28
1.6.3.4.1			54	Death or euthanasia on day 14
1.6.3.4.2			120	Death or euthanasia on day 14
1.6.3.4.3	150		1200	Death or euthanasia on day 14
1.7	60			Euthanize immediately
1.8.1.1			210	Death or euthanasia on day 14
1.8.1.2			210	Death or euthanasia on day 14
1.8.2			90	Death or euthanasia at 4, 8, 12, 24, 48, 72, 96 hrs
1.8.3.1			150	Death or euthanasia on day 14
1.8.3.2	100		250	Death or euthanasia on day 14

V.4.6. Euthanasia: Mice will be euthanized by either isoflurane overdose (3-4% concentration) within the isoflurane induction chamber of our Gas Anesthesia System, which is connected to a waste disposal system, or within a bell jar containing isoflurane-saturated gauze, or by CO_2 overdose. Cervical dislocation will be done on deeply anesthetized mice to confirm euthanasia. CO_2 will be delivered from a compressed gas cylinder via a regulated rate of flow in accordance with the 2007 AVMA Guidelines on Euthanasia. For euthanasia using CO_2, all procedures will be performed by LAM personnel. Investigative staff will perform isoflurane euthanasia followed by cervical dislocation.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. Mice will be housed in USUHS room (b)(6) that is maintained as a dedicated ABSL2 room. Before the start of the experiment, LAM personnel will be responsible for routine animal observations and cage maintenance and provide food and water as detailed in the LAM Husbandry SOP. During the experiment the investigator will be responsible for animal observations (up to 4 times daily) and provide all care for the animals. For animals in Experiment V.1.1.1 that are housed in the ABSL-3 facility once it is completed, all husbandry will be performed by the investigator.

V.5.1.1. Study Room:

Building(s)	(b)(6)	Room Number(s)	(b)(6)	

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes x (some mice will be fasted overnight)
Fluid Restriction:	No

V.5.1.3. Exceptions: None

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
Untreated animals will be observed and cared for by the Center for LAM personnel according to USUHS Center for LAM SOPs. Immunized, infected or intoxicated animals will be checked regularly by laboratory personnel working on these projects. Moribund mice will be euthanized.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions: None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING: Dr (b)(6) who will not do these "hands on" experiments herself, has 35 years experience working with mice in microbicidal assays and has been involved with the mouse anthrax model since 2002.

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Oral inoculations	(b)(6)	Senior technician, 20 yrs experience	Investigator training, 1995, Trained by Dr. (b)(6) rodent handling class, Walter Reed, 2000
Oral inoculations		Research Associate Professor, 20 yrs experience	Investigator training, 1995, Trained by Dr. (b)(6)
Oral inoculations		Associate Professor, 17 yrs experience	Investigator training, 1995, Trained by Dr. (b)(6)
Oral inoculations		Research Technician, 7 yrs experience	Investigator training, 2004, Trained by Dr (b)(6)
Oral Inoculations		Graduate student, 5 yrs experience	Investigator training, 2007, Rodent handling course, 2007, Trained by Dr (b)(6)
Oral Inoculations		Graduate student, 3 yrs experience	Investigator training, 2008, Rodent handling course, 2008; trained by Dr (b)(6)
Oral Inoculations		Research Technician, 1 yr experience	Investigator training, 2011, trained by M (b)(6)

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Oral Inoculations	(b)(6)	Research technician, 1 yr experience	Investigator training, 2012, will be trained by Mr (b)(6)
Oral Inoculations		Postdoctoral fellow, 2 yrs experience	Investigator training, 2009, Rodent handling course, 2009; trained by Mr (b)(6)
Oral Inoculations		Senior research associate, 1 yr experience	Investigator training, 2009, Rodent handling course, 2009; trained by Mr (b)(6)
Oral Inoculations		Graduate student, 3 yrs experience	Investigator training, 2008, Rodent handling course, 2009, trained by Mr (b)(6)
Oral Inoculations		Postdoctoral Fellow, 1 yr experience	Investigator training, 2008; trained by Mr (b)(6)
Iv, ip, sc injections		Senior technician, 20 yrs experience	Investigator training, 1995, Trained by Dr. (b)(6) rodent handling class, Walter Reed, 2000
Iv, ip, sc injections		Research Associate Professor, 20 yrs experience	Investigator training, 1995, Trained by Dr. (b)(6)
Iv, ip, sc injections		Associate Professor, 17 yrs experience	Investigator training, 1995, Trained by Dr. (b)(6)
Iv, ip, sc injections		Research Technician, 7 yrs experience	Investigator training, 2004, Trained by Drs. (b)(6)
Iv, ip, sc injections		Graduate student, 5 yrs experience	Investigator training, 2007, Rodent handling course, 2007; Trained by Dr (b)(6)
Iv, ip, sc injections		Graduate student, 3 yrs experience	Investigator training, 2008, Rodent handling course, 2008, trained by Mr (b)(6)
Iv, ip, sc injections		Research Technician, 1 yr experience	Investigator training, 2011, trained by Mr (b)(6)
Iv, ip, sc injections		Research technician, 1 yr experience	Investigator training, 2012, will be trained by Mr (b)(6) and Mr (b)(6)
Iv, ip, sc, intestinal injections		Postdoctoral fellow, 2 yrs experience	Investigator training, 2009, Rodent handling course, 2009; trained by Mr (b)(6) and MAJ (b)(6)
Iv, ip, sc injections		Postdoctoral Fellow, 1 yr experience	Investigator training, 2008; trained by Mr (b)(6)

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Iv, ip, sc injections	(b)(6)	Senior research associate, 1 yr experience	Investigator training, 2009, Rodent handling course, 2009; trained by Mr. (b)(6)
Iv, ip, sc injections		Graduate student, 3 yrs experience	Investigator training, 2008, Rodent handling course, 2009; trained by Mr. (b)(6)
Iv, ip, sc injections		Senior Scientist, 16 yr experience	Investigator training, 2008, Rodent Handling Course UAB, 1997, External training, UAB, 1997; UW 2004, NIH 2007
Tail vein, retro-orbital, terminal bleeds	(b)(6)	Senior technician, 20 yrs experience	Investigator training, 1995, Trained by Dr. (b)(6) Rodent handling class, Walter Reed, 2000
Tail vein, retro-orbital, terminal bleeds		Research Associate Professor, 20 yrs experience	Investigator training, 1995, Trained by Dr. (b)(6)
Tail vein, retro-orbital, terminal bleeds		Associate Professor, 17 yrs experience	Investigator training, 1995, Trained by Dr. (b)(6)
Tail vein, retro-orbital, terminal bleeds		Research Technician, 7 yrs experience	Investigator training, 2004, Trained by Drs. (b)(6)
Tail vein, retro-orbital, terminal bleeds		Graduate student, 5 yrs experience	Investigator training, 2007, Rodent handling course, 2007; Trained by Dr. (b)(6)
Tail vein, retro-orbital, terminal bleeds		Graduate student, 3 yrs experience	Investigator training, 2008, Rodent handling course, 2008; trained by Mr. (b)(6)
Tail vein, retro-orbital, terminal bleeds		Research Technician, 1 yr experience	Investigator training, 2011, trained by Mr. (b)(6)
Tail vein, retro-orbital, terminal bleeds		Research technician, 1 yr experience	Investigator training, 2012, will be trained by Mr. (b)(6) and Mr. (b)(6)
Tail vein, retro-orbital, terminal bleeds		Postdoctoral fellow, 2 yrs experience	Investigator training, 2009, Rodent handling course, 2009; trained by Mr. (b)(6)
Tail vein, retro-orbital, terminal bleeds		Senior research associate, 1 yr experience	Investigator training, 2009, Rodent handling course, 2009; trained by Mr. (b)(6)
Tail vein, retro-orbital, terminal bleeds		Graduate student, 3 yrs experience	Investigator training, 2008, Rodent handling course, 2009; trained by Mr. (b)(6)

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Tail vein, retro-orbital, terminal bleeds	(b)(6)	Senior Scientist, 16 yr experience	Investigator training, 2008, Rodent Handling Course UAB, 1997, External training: UAB, 1997; UW 2004; NIH 2007
Tail vein, retro-orbital, terminal bleeds		Postdoctoral Fellow, 1 yr experience	Investigator training, 2008; trained by Mr. (b)(6)
Euthanasia by isoflurane overdose	(b)(6)	Senior technician, 20 yrs experience	Investigator training, 1995, Trained by Dr (b)(6) rodent handling class, Walter Reed, 2000
Euthanasia by isoflurane overdose		Research Associate Professor, 20 yrs experience	Investigator training, 1995, Trained by Dr (b)(6)
Euthanasia by isoflurane overdose		Associate Professor, 17 yrs experience	Investigator training, 1995, Trained by Dr (b)(6)
Euthanasia by isoflurane overdose		Research Technician, 7yrs experience	Investigator training, 2004, Drs (b)(6) (b)(6)
Euthanasia by isoflurane overdose		Graduate student, 5 yrs experience	Investigator training, 2007, Rodent handling course, 2007, Trained by Dr (b)(6)
Euthanasia by isoflurane overdose		Graduate student, 3 yrs experience	Investigator training, 2008, Rodent handling course, 2008; trained by Mr (b)(6)
Euthanasia by isoflurane overdose		Research Technician, 1 yr experience	Investigator training, 2011, trained by Mr (b)(6)
Euthanasia by isoflurane overdose		Research technician, 1 yr experience	Investigator training, 2012, will be trained by Mr (b)(6) and Mr (b)(6)
Euthanasia by isoflurane overdose		Postdoctoral fellow, 2 yrs experience	Investigator training, 2009, Rodent handling course, 2009; trained by Mr (b)(6)
Euthanasia by isoflurane overdose		Senior research associate, 1 yr experience	Investigator training, 2009, Rodent handling course, 2009; trained by Mr (b)(6)
Euthanasia by isoflurane overdose		Graduate student, 3 yrs experience	Investigator training, 2008, Rodent handling course, 2009; trained by Dr (b)(6)
Euthanasia by isoflurane overdose		Senior Scientist, 16 yr experience	Investigator training, 2008, Rodent Handling Course UAB, 1997, External training: UAB, 1997; UW 2004; NIH 2007

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Euthanasia by isoflurane overdose	(b)(6)	Postdoctoral Fellow, 1 yr experience	Investigator training, 2008, trained by Mr. (b)(6)
Ear tag / Ear punch	(b)(6)	Senior technician, 20 yrs experience	Investigator training, 1995, Trained by Dr. (b)(6) rodent handling class, Walter Reed, 2000
Ear tag / Ear punch		Research Associate Professor, 20 yrs experience	Investigator training, 1995, Trained by Dr. (b)(6)
Ear tag / Ear punch		Associate Professor, 17 yrs experience	Investigator training, 1995, Trained by Dr. (b)(6)
Ear tag / Ear punch		Research Technician, 7 yrs experience	Investigator training, 2004, Trained by Drs. (b)(6)
Ear tag / Ear punch		Graduate student, 5 yrs experience	Investigator training, 2007, Rodent handling course, 2007; Trained by Dr. (b)(6)
Ear tag / Ear punch		Graduate student, 3 yrs experience	Investigator training, 2008, Rodent handling course, 2008; trained by Mr. (b)(6)
Ear tag / Ear punch		Research Technician, 1 yr experience	Investigator training, 2011, trained by Mr. (b)(6)
Ear tag / Ear punch		Research technician, 1 yr experience	Investigator training, 2012, will be trained by Mr. (b)(6) and Mr. (b)(6)
Ear tag / Ear punch		Postdoctoral fellow, 2 yrs experience	Investigator training, 2009, Rodent handling course, 2009, trained by Mr. (b)(6)
Ear tag / Ear punch		Senior research associate, 1 yr experience	Investigator training, 2009, Rodent handling course, 2009; trained by Mr. (b)(6)
Ear tag / Ear punch		Graduate student, 3 yrs experience	Investigator training, 2008, Rodent handling course, 2009; trained by Dr. (b)(6)
Ear tag / Ear punch		Senior Scientist, 16 yr experience	Investigator training, 2008, Rodent Handling Course UAB, 1997, External training: UAB, 1997; UW 2004; NIH 2007
Ear tag / Ear punch		Postdoctoral Fellow, 1 yr experience	Investigator training, 2008, trained by Mr. (b)(6)

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Intestinal Loop surgery	(b)(6)	Postdoctoral fellow, 2 yrs experience	Investigator training, 2009. Rodent handling course, 2009, trained by MAJ (b)(6)

VII. **BIOHAZARDS/SAFETY**: A detailed risk assessment was completed at the request of the Division of Select Agents and Toxins (DSAT) at the Centers for Disease Control and Prevention. All animal handling procedures meet or exceed the requirements of the DSAT and the Biosafety in Microbiological and Biomedical Laboratories, Fifth Edition, for ABSL2 experiments. The mice are housed under biocontainment conditions (ABSL2). Mice infected with STEC or intoxicated with Stx or ricin will be isolated from non-infected animals. Gloves, lab coats, and surgical masks must be worn by all personnel when handling mice. N95 masks are required when mice are inoculated with ricin, Stxs, or C227-11. In addition, all inoculations with C227-11 will be conducted in a certified Type II Biosafety Cabinet. Survival surgery will be conducted in a certified Type II Biosafety Cabinet. Bedding may be contaminated with fecal pellets that contain STEC; therefore, all bedding and all animal carcasses should be treated as infectious and must be autoclaved and/or burned. All cages will be changed in a certified Biosafety Cabinet in Room (b)(6). All needles and scalpels will be disposed of in dedicated Sharps containers that are decontaminated and incinerated as Regulated Medical Waste.

All procedures that involve the use of isoflurane will be performed in either a chemical fume hood (bell jar) or with a gas anesthesia system with waste-scavenging charcoal filters (both located in Dr (b)(6) laboratory). All personnel who use isoflurane are aware of its associated safety hazards and have been properly trained in its transport, use, and storage.

VIII. **ENCLOSURES**:

1. Registration Certificate for use of Select Agents from the Division of Select Agents and Toxins, Centers for Disease Control and Prevention

2. Letter of approval for Experiment V.1.1.1 from Division of Select Agents and Toxins, Centers for Disease Control and Prevention

3. (b)(6) from NIAID, "Pathogenicity of Shiga Toxin-Producing *E. coli*"

4. (b)(6) Administrative Supplement to (b)(6) from NIAID, "Pathogenicity of Shiga Toxin-Producing *E. coli*"

5. (b)(6) from NIAID, "Shiga toxin and ricin interaction with enterocytes and rescue of target cells"

6. (b)(6) from DTRA, "Antibacterial and Anti-Shiga Toxin Expression Activities of Morpholino Compounds against EHECO104:H4 and Related Prokaryotes"

7. (b)(6) from USUHS, "Susceptibility of Recombinant Inbred Mice to Shiga Toxins and *E. coli* O157:H7"

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1. **Lindsay, D. S., T. Kaur, S. M. Mitchell, D. G. Goodwin, J. Strobl, and J. P. Dubey.** 2005. Buprenorphine does not affect acute murine toxoplasmosis and is recommended as an analgesic in *Toxoplasma gondii* studies in mice. The Journal of parasitology **91**:1488-1490.

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3. **Myhal, M. L., D. C. Laux, and P. S. Cohen.** 1982. Relative colonizing abilities of human fecal and K-12 strains of *Escherichia coli* in the large intestines of streptomycin-treated mice. Eur.J Clin.Microbiol. **1**:186-192.

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5. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am.J.Hyg. **27**:493-497.

6. **Schaedler, R. W., and G. H. Warren.** 1980. Effect of cyclacillin and ampicillin on the gut flora of mice. Chemotherapy **26**:289-296.

7. (b)(6)

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

A. **Training:** I verify that I have attended the USUHS Investigator/Animal User Training Course

B. (b)(6) [redacted]

Principal Investigator Signature | Date

A. **Training:** The following personnel will attend the next USUHS Investigator/Animal User Training Course: All personnel are current on their training

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6)	
Principal Investigator Signature	Date 5/17/2022

I. Painful Procedure(s): I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)	
Principal Investigator Signature	Date 5/17/22

X. PROTOCOL ABSTRACT:

A. **Animal Protocol Number:** (b)(6)

B. **Animal Protocol Title:** Pathogenicity of Shiga toxin-producing *E. coli* and ricin intoxication (mice)

C. **Principal Investigator:** (b)(6) Ph.D.

D. **Performing Organization:** USUHS

E. **Funding:** NIAID, DTRA, USUHS

F. **Objective and Approach:** Shiga toxin (Stx)-producing Escherichia coli (STEC) are known as the causative agent of "hamburger disease" because they are often transmitted through undercooked hamburgers. STEC are food- and water-borne pathogens that are the most comroon infectious cause of bloody diarrhea in the United States. The hemolytic uremic syndrome (HUS), which can occur as a result of STEC infection, is the most frequent basis for acute kidney failure in U.S. children. The STEC make one or more Stxs (Stx1, Stx2 or variant of those) that are key factors in the development of the HUS. The Stxs are generally encoded within bacteriophage genomes that lysogenize *E. coli*, and can therefore be transmitted among susceptible bacteria. Evidence that such transmission may occur became evident in 2011, when a large food-borne outbreak of bloody diarrhea and HUS due to an unusual O104:H4 *E. coli* strain that produced Stx2 occurred in Germany. The outbreak isolate was an enteroaggregative *E. coli* that had most likely acquired an *stx*$_2$-encoding phage from a more typical STEC. The mechanism of action of the Stxs is the same of that of the plant toxin ricin: they both halt protein synthesis in the target cell. The specific aims of this project are to: 1.) evaluate the properties of certain Stx family members either in purified form or as produced from STEC strains that make them more toxic for mice and human kidney cells than other family members; 2.) determine if an O157:H7 strain that produces Stx2dact is virulent in mice, and, conversely, if the Stx2dact-producing mouse virulent strain B2F1 is rendered less virulent when Stx2dact is mutated to Stx2; 3.) determine the effect of alterations in dietary fiber on the susceptibility of mice to the Stxs or to STEC; 4.) assess if Stx1 or Stx2 enhances the colonization capacity of STEC strains; 5.) evaluate the protective capacity of anti-bacterial or anti-toxin morpholino compounds in STEC-infected mice; 6.) measure the protective properties of anti-Stx or anti-ricin therapeutic conjugates in tissue culture or in mice; 7.) evaluate the susceptibility of advanced recombinant inbred (ARI) mice to Stx1 or Stx2; and, 8.) measure differences in the capacity of STEC strains that produce Stx1 or Stx2 to colonize BXD mice.

G. **Indexing Terms (Descriptors):** STEC, Shiga toxin-producing *E. coli*, Shiga toxin, animal model, mice, hemorrhagic colitis, hemolytic uremic syndrome, ricin, morpholino, antibody

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January 25, 2012

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF MICROBIOLOGY

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via designated member review on January 25, 2012:

Animal Protocol Title: "New Therapies and Vaccines Against Bacillus Anthracis"

USUHS Protocol Number: (b)(6)

Expiration Date: January 24, 2015

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

original signatures

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: New Therapies and Vaccines against *Bacillus anthracis* (mice)

GRANT TITLE (if different from above): New Therapies and Vaccines against *Bacillus anthracis*

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Biological Defense Research Directorate, Naval Medical Research Center

EARLIEST ANTICIPATED FUNDING START DATE: 1 October 2011

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6)		(b)(6)	
Principal Investigator Signature	MIC Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	Professor	(b)(6)	12/15/11
Research Unit Chief / Dept. Head Signature	Title	Telephone	Date

Typed Name: (b)(6) Ph.D.

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)	MIC	(b)(6)	
Statistician Signature	Department	Telephone	Date

Typed Name (b)(6) Ph.D.

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)	LAM	(b)(6)	29 Dec 2011
Attending/Consulting Veterinarian Signature	Department	Telephone	Date

Typed Name (b)(6) DVM

~~PRINCIPAL INVESTIGATOR:~~ (b)(6) Ph.D.

Revised 1-25-12

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

ANIMAL PROTOCOL TITLE: New Therapies and Vaccines against *Bacillus anthracis* (mice and guinea pigs)

GRANT TITLE (if different from above): New Therapies and Vaccines against *Bacillus anthracis*

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S) (b)(6) Ph.D. (corresponding) (b)(6) Ph.D., (b)(6) Ph.D., LTC (b)(6) VMD

TECHNICIANS(S): Mr. (b)(6) Mr. (b)(6) Mr. (b)(6)

I. **NON-TECHNICAL SYNOPSIS:** *Bacillus anthracis*, the bacterium responsible for anthrax, persists in the environment as a dormant, well-protected spore that is resistant to damage and drying. Disease occurs when the spores are inhaled, ingested, or enter wounds in the skin. Once in the body, the dormant spores germinate, or lose their protective coating, and expand into rod-shaped bacteria that multiply and produce toxins that lead to often fatal disease in the patient. Spores of *B. anthracis* have long been considered potential biological weapons. During October, 2001 in the US there were 22 cases of anthrax that resulted from such a deliberate exposure, 11 of which had the most lethal inhalational disease form. Indeed, the aggressive use of quinolones and other antibiotics coupled with the early recognition of disease resulted in the survival of 6 of the 11 patients. Nonetheless, hundreds of other individuals potentially exposed to anthrax spores required an extended course of antibiotic therapy due to the concern that they still carried spores in their lungs that could germinate after the conclusion of their antibiotic treatment. Our overarching goals are to 1) develop a vaccine that is safe and effective for prevention of anthrax disease, and 2) develop therapies that prevent the first stage of disease, specifically germination of the spores. Two ways to block such germination are to 1) administer antibodies against the spores before or immediately after exposure; or, 2) immunize at-risk individuals (such as soldiers) with a vaccine that contains spore-surface (also known as exosporium) proteins. Our experiments will be done with *B. anthracis* Sterne, a strain that does not cause disease in humans and is not considered a bioweapon. We will determine if mice immunized with exosporium proteins of *B. anthracis* or inactivated spores from an avirulent *B. cereus* strain are protected against an otherwise lethal dose of spores introduced into the nose, the skin, or the bloodstream. In addition, we will test chemical compounds that alter germination of spores in lab cultures for their capacity to prevent disease in mice infected with spores. Another aim is to study how certain traits of *B. anthracis* influence disease production. Identification of these traits, or virulence factors, will illuminate which are important targets for antimicrobials and vaccines. We will measure the outcome of infection in mice exposed to the Sterne strain versus that seen when mice are infected with strains mutated (deficient) in those traits. Likewise, we will investigate the very closely-related bacterium *B. cereus* that causes self-limiting

gastrointestinal or ocular diseases in healthy people but which is responsible for illnesses like sepsis, meningitis, and endocarditis in immunocompromised persons. We hope to determine the bacterial properties (some likely shared with *B. anthracis*) that lead to disease with *B. cereus*.

II. BACKGROUND:

II.1. Background: Our studies will continue to investigate two highly related species from the genus *Bacillus*: *B. anthracis* and *B. cereus*. The infectious form of *B. anthracis* and *B. cereus* for animals and humans is the spore. To cause systemic disease, the spore must germinate and undergo outgrowth to the vegetative rod. Although the pathogenesis of inhalational, cutaneous, and gastrointestinal anthrax likely involves the same general steps, the morbidities and mortalities differ significantly. After introduction of spores into the host via inhalation, ingestion, or wound contamination, the spores are rapidly phagocytosed by resident macrophages (MΦs). We and others have shown through *in vivo* imaging studies that the spores germinate in the MΦs, either at the site of inoculation or after the MΦs have migrated to the regional lymph nodes (7, 9, 10, 19). The spores germinate to form encapsulated vegetative rods, which replicate in the MΦs and produce toxins that kill the MΦs. The extracellular bacilli then disseminate within the host and express the full constellation of virulence factors.

The two principal toxins produced by the vegetative bacteria are Edema Toxin (ET) and Lethal Toxin (LT). Edema Factor (EF) and Lethal Factor (LF) interact with and are delivered to the cell interior by the same binding subunit, called the Protective Antigen (PA). PA is the principal component of the licensed anthrax vaccine (Biothrax®). ET is comprised of PA and EF and LT is comprised of LF and PA. ET is a calmodulin-dependent adenylate cyclase that converts ATP to cAMP within its target cells. Lethal toxin is a zinc-metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPKK) within MΦs, which can lead to apoptosis of the MΦ and release of vegetative cells and toxins into the surrounding milieu. In addition to the toxins, all strains of *B. anthracis* and some strains of *B. cereus* that cause disease in humans produce one or more capsules. The capsules protect the vegetative bacteria from phagocytosis by macrophages and neutrophils, particularly during dissemination through the lymph and blood to distal sites.

The majority of animal models of anthrax disease mimic inhalational or subcutaneous anthrax in humans [see (20) for review of animal models of anthrax disease]. Inhalational models have been used most often, despite the relatively low frequency of inhalational anthrax (compared to the other routes of inoculation), because aerosolized spores have the potential to be used as bioweapons. Like humans, non-human primates and rabbits are particularly susceptible to the effects of LT and ET, a characteristic that makes these animal models particularly useful for studies of late stage anthrax disease as well as vaccines and therapeutics. Mice and guinea pigs can be used to model both the infection (early) and toxemia (late) stages of disease. Mice have been used most extensively due to their low cost, ease of handling, and genetic diversity. Mouse models of anthrax disease are used to mimic inhalational (aerosol, intranasal, intratracheal inoculation), cutaneous (subcutaneous inoculation), and gastrointestinal (oral inoculation) forms of human anthrax, and all of these models are useful for studies of infection and toxemia. In addition, challenge by intraperitoneal or

intravenous routes can be useful for investigations of the roles of capsules and toxins in *Bacillus* pathogenesis.

All of our *B. anthracis* animal studies are conducted with *B. anthracis* Sterne, an attenuated strain that does not produce capsule and that is used as a vaccine for cattle. *B. anthracis* Sterne is fully virulent in A/J mouse models of anthrax disease. The genes that encode the toxin components in *B. anthracis* (*pag, lef, cya*) are located on the pXO1 megaplasmid. The pXO2 megaplasmid, which is not present in *B. anthracis* Sterne, encodes the genes required for production of a poly-D-glutamic acid capsule. Our studies of *B. cereus* virulence are conducted with *B. cereus* G9241, a strain that was isolated from the blood and sputum of a welder who had anthrax-like pneumonia (11). *B. cereus* G9241 is unique among *B. cereus* strains for the following reasons: 1) *B. cereus* typically causes mild gastroenteritis rather than anthrax-like pneumonia in humans; and 2) the strain contains two megaplasmids, pBCXO1 and pBC218. The pBCXO1 megaplasmid is 99% identical to pXO1 in *B. anthracis*. One key difference between the two megaplasmids is that the *hasACB* operon on pBCXO1 is intact, which provides *B. cereus* G9241 with the tools necessary to produce a hyaluronic acid capsule (16). The *hasACB* operon on the *B. anthracis* pXO1 contains a frameshift mutation that renders the operon non-functional. In addition, pBC218 contains a second functional capsule operon and a homolog of the gene that encodes PA (*pag*). A third toxin, Certhrax, that functions as an ADP-ribosyltransferase is encoded on pBC218 as well (8); the role of this toxin in *B. cereus* disease will be investigated in the studies described in this protocol.

Despite the prominent role of the toxins in anthrax disease, we propose that if spores can be blocked from germination, the vegetative cells that express these toxins will not be formed. We hypothesize that antibodies against spore surface components could prevent germination, possibly via opsonization of the spores to facilitate phagocytic cell uptake and subsequent killing. We have previously demonstrated that intraperitoneal (i.p.) immunization of A/J mice with a sublethal dose of PA followed by the spore proteins BclA, BxpB, and/or p5303 protects mice from subsequent subcutaneous (s.c.) challenge with *B. anthracis* Sterne (6). We also vaccinated A/J and BALB/c mice with formalin-inactivated *B. cereus* G9241 pBCXO1- pBC218- spores (DC FIS); DC FIS are non-viable due to formalin-inactivation and do not contain toxin components. All A/J mice vaccinated intranasally (i.n.) or s.c. with DC FIS were protected from subsequent *B. anthracis* Sterne challenge via i.n. or s.c. routes. When our collaborators at USAMRIID vaccinated BALB/c mice with DC FIS i.p., they saw 60% protection from i.n. challenge and 80% protection from i.p. challenge with the fully virulent *B. anthracis* Ames. These data provide strong evidence for our hypothesis that spore surface components are effective immunogens for generation of antibodies that protect against *B. anthracis* infection.

We also theorize that administration of a small molecule that inhibits germination as a post-challenge therapeutic will protect mice from anthrax disease. The small molecule 6-thioguanine (6-TG), an analog of known *in vitro* germinants inosine and alanine, was previously shown to inhibit germination of *B. anthracis* spores *in vitro* and in MΦs (1). We have screened 3722 small molecules to identify compounds that have the capacity to alter germination of *B. anthracis* Sterne spores in a high-throughput *in vitro* assay. We have discovered 20 potential lead compounds (molecules that inhibit the germination rate by at least 2/3), as well as 65 additional compounds classified as "moderate inhibitors" that reduce the germination rate to between 1/3 and 2/3 of the

positive control. These 85 compounds will be further tested *in vitro* and in MΦ phagocytosis and killing assays. Small molecules that inhibit germination in all of the *in vitro* assays will be tested in our A/J mouse models of *B. anthracis* disease.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: DoD Biomedical Research Database (BRD), NIH Research Portfolio Online Reporting Tools (RePORT), and PUBMED

II.2.2. Date of Search: 12 December 2011

II.2.3. Period of Search: BRD Database [FY1998 to FY2009 (most recent)], NIH RePORT (FY2002 - FY2012), PUBMED (Available – December 2011, English only)

II.2.4. Key Words and Search Strategy: *Bacillus* AND (spores OR capsule OR germination OR dissemination OR immunization) AND (mouse OR mice)

II.2.5. Results of Search:
RESULTS FROM BRD: Search of the BRD database using *Bacillus* AND *anthracis* AND spores resulted in 102 hits, of which 40 were unique and 7 were from my laboratory. Other studies utilized guinea pigs, rabbits, and monkeys to ascertain *B. anthracis* virulence properties. Fourteen documents were from our collaborators at USAMRIID and outlined our joint studies to investigate spore proteins as vaccine candidates and the roles of antibodies against spores in inhibition of germination and growth. An additional document was from another collaborator and resulted in a joint publication between our laboratories (23).
Five of the studies were focused on development of vaccines against anthrax disease that are based on PA and LF and five were focused on multivalent vaccines against two or more organisms considered to be high bioterrorism threats (*B. anthracis*, *Yersinia pestis*, *Burkholderia spp*). Finally, the use of antibodies against PA and/or LF as post-exposure prophylaxis was the subject of two studies. None of the studies identified in the BRD database overlap with the studies proposed here, except those outlined in proposals from my laboratory.

RESULTS FROM NIH RePORT: A total of 102 unique awards were identified in NIH RePORT using *Bacillus* AND (spores OR capsule OR germination OR dissemination OR immunization) AND mouse, of which two were from my laboratory. Awards to my collaborators include two to Dr. (b)(6) two to Dr. (b)(6) and one was a program project on which I was an investigator (awarded to Dr. (b)(6) (b)(6) Of these, 5 proposed the use of various spore coat components as vaccine antigens; however, they were focused on different spore antigens than those we propose to study. A search using *Bacillus* and mouse and germination resulted in 28 awards, including 1 from my laboratory, 1 from the Middle Atlantic Regional Center of Excellence (b)(6) and 2 from our collaborator Dr. (b)(6) Of these, 11 were focused on various vaccine strategies using spores or spore components; again, none directly overlap with our studies. A search of NIH RePORT for *Bacillus* and immunization and mouse yielded 203 hits. Most immunization strategies are focused on PA and/or LF. Two focused on the use of capsule as a

vaccine candidate. Three others targeted proteins on the surface of the *Bacillus* spore coat, but the focus of those awards was different than our focus in Experiment 5 below.

RESULTS FROM PUBMED: A search of the Pubmed database using *Bacillus* AND (spores OR capsule OR germination OR dissemination OR immunization) AND mouse resulted in 318 relevant articles, of which 6 were published by our laboratory. Fifteen of these articles describe vaccination strategies using various antigens from the spore surface, but none directly overlap with the studies proposed here because we are using different antigens and/or different immunization or challenge methods. An additional 75 articles include those that describe the use of *Bacillus* spores as vaccine platforms (21), the efficacy of vaccines against PA (26), multivalent vaccines that target multiple organisms (2), and vaccines that use other toxin components than PA as the primary immunogen (4). A total of 33 papers describe the development of therapeutics against *B. anthracis* infection; 11 discuss the use of post-exposure antibody administration, 12 present possible antibiotic therapies, and 10 use alternate approaches. None of these papers demonstrates the utility of small molecule inhibitors of germination. A total of 92 papers focus on the host response to *B. anthracis* infection; 36 focus on macrophages, neutrophils or epithelial cells and 22 investigate the contributions of components of the inflammasome.

III. **OBJECTIVE/ HYPOTHESIS**: One way to increase the likelihood that individuals who are exposed to aerosolized *B. anthracis* spores remain disease free is to prevent germination of the infectious dormant spores into active vegetative cells that can produce potentially lethal toxins. Our findings that rabbit antiserum against *B. anthracis* spores or spore proteins decreases spore germination *in vitro*, taken with the fact that FIS can serve as a protective vaccine against anthrax in guinea pigs and mice, led us to the following **hypothesis**. Vaccine candidates that contain spore surface components will elicit antibodies that block or reduce spore germination *in vivo* or render spores more susceptible to phagocytosis and ultimately killing by macrophages. Based on this theory, our **goals** are to characterize spore surface components as potential targets for incorporation into a second-generation PA-based vaccine and to identify small molecules that inhibit germination *in vivo* for post-exposure prophylaxis. The specific aims are to:

1. Assess the virulence of *B. anthracis* Sterne or *B. cereus* G9241 strains in which a particular virulence trait has been deleted;
2. Administer *B. cereus* G9241 toxin components to assess their activity *in vivo*;
3. Monitor the location and kinetics of germination, vegetative outgrowth, and dissemination of fluorescent derivatives of *B. anthracis* Sterne and *B. cereus* G9241 in the mouse with an *in vivo* imaging system;
4. Characterize the *in vivo* efficacy of small molecules to inhibit germination of *B. anthracis* Sterne spores; and
5. Immunize A/J mice with a sublethal dose of recombinant PA (rPA) followed by spore antigens alone or in combination and/or with exosporium protein preparations, then challenge with *B. anthracis* to assess the protective efficacy of the vaccine candidates.

IV. **MILITARY RELEVANCE**: The potential use of *B. anthracis* as a weapon of biowarfare was brought to the forefront in the aftermath of the terrorist attacks of September 11th, 2001. From October to December 2001, a total of 22 cases of

confirmed or suspected bioterrorism-related anthrax occurred in the United States. Eleven of these cases were inhalational (the most lethal form of the infection) and resulted in the death of 6 individuals. In light of these events, the development of therapeutic agents against *B. anthracis* infection is paramount for the protection of both deployed military personnel and civilians who may come into contact with sabotaged contaminated materials.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

1. Three general types of experiments will be conducted in the studies outlined in this protocol.

A. Mice will be inoculated with *B. anthracis* or *B. cereus* spores or with purified toxin components by intranasal (i.n.), intratracheal (i.t.), subcutaneous (s.c.), or intraperitoneal (i.p.) administration and monitored for morbidity and mortality with death as an endpoint. The purpose of these studies is to determine 1) if isogenic mutant strains that lack a particular virulence factor are attenuated for virulence when compared to the parent strain, 2) if the toxins produced by *B. cereus* G9241 exhibit similar activity *in vivo* as do their *B. anthracis* counterparts, or 3) the *in vivo* location(s) of *B. anthracis* and *B. cereus* strains that fluoresce at a particular stage in growth (germination, vegetative outgrowth, dissemination).

B. Mice will be vaccinated with formalin-inactivated spores (FIS), viable spores of an avirulent *B. cereus* strain, purified spore proteins (alone or in combination), or crude exosporium protein preparations, then they will be challenged with a lethal dose of *B. anthracis* Sterne, *B. cereus* G9241, or fluorescent derivatives thereof. The purpose of these studies is to further develop a potential second-generation vaccine strategy to prevent anthrax disease.

C. Mice will be challenged with *B. anthracis* Sterne spores and treated one hour post-challenge with small molecules known to inhibit germination *in vitro*. The purpose of these studies is to determine whether the small molecule(s) could be used as post-exposure prophylaxis to prevent germination and subsequent vegetative outgrowth in the host.

2. For all of the mouse studies outlined in this protocol, 6-8 week old female A/J or C57BL/6 mice will be challenged by one of four routes (i.n., i.t., s.c., i.p.) with *Bacillus anthracis* Sterne spores, *B. cereus* G9241 spores, or purified toxin components (i.p. only). Moribund mice will be anesthetized by isoflurane overdose and sacrificed by cervical dislocation. We define moribund as when the mouse is crouched down or lying in the bottom of the cage with little movement, even when gently prodded. Other nonspecific signs that will be used are ruffled fur, lethargy, hunched posture, decreased alertness, weight loss, and difficulty breathing.

A. For i.n. inoculation, mice will be anesthetized with inhaled isoflurane delivered via a Xenogen XGI-8 gas anesthesia system or with i.p. ketamine (100 mg/kg) and xylazine

nostril). The mice will be held upright until the inoculum is inhaled (~1 min), then

(10 mg/kg). Once anesthesia is confirmed by non-response to toe pinch, mice will be inoculated i.n. by manually pipetting 50 µl spores in H_2O onto the nares (25 µl/per nostril). The mice will be held upright until the inoculum is inhaled (~1 min), then returned to their cages to recover from the anesthesia. Mice will remain in the laboratory for one hour after inoculation to permit careful monitoring of mice. The study endpoint will be either death (see section V.4.5) or euthanasia 14 days post-challenge. Obviously moribund animals will be euthanized using CO_2 inhalation or isoflurane inhalation followed by cervical dislocation (see section V.4.6).

B. For i.t. inoculation (conducted as described in Brown *et al.* (4)), A/J mice will be anesthetized by i.p. administration of ketamine (100 mg/kg) and xylazine (10 mg/kg). Once anesthetized (defined as no response to toe pinch), each mouse will be placed on its back on a flat surface that can be elevated 75°. Paper tape will be applied to each arm to gently adhere the arms to the flat surface. A hypodermic needle will be inserted into the flat surface ~5 cm from the top of the mouse's head. A rubber band will be looped over the needle and under the upper incisors of the open mouth of the mouse to tilt the neck backward and to hold the mouth open. The flat surface will be elevated to 75° such that the mouse's nose will face upward. The two flexible fiber-optic arms of a halogen light source will be placed on either side of the mouse's neck to provide transillumination of the trachea. The tongue will be held to one side of the mouth with forceps. A sterile 68.6 mm gel-loading pipette tip, which is tapered for 25 mm to a narrow tip, will be attached to a P-100 pipettor and the 50 µl inoculum will be aspirated into the tip. The tip will be inserted into the trachea and the inoculum will be delivered by gentle and constant depression of the pipettor plunger. The tip will be removed from the mouse, the rubber band will be removed from the teeth, and the mouse will remain elevated for ~1 min to allow the inoculum to flow into the lungs by gravity. The tape will then be removed from the arms and the mouse will be returned to its cage to recover from anesthesia. Mice will remain in the laboratory for one hour after inoculation to permit careful monitoring of mice. The study endpoint will be either death (see section V.4.5) or euthanasia 14 days post-challenge. Obviously moribund animals will be euthanized using CO_2 inhalation or isoflurane inhalation followed by cervical dislocation (see section V.4.6).

C. For s.c. inoculation, 100-200 µl heat-activated *Bacillus* spores will be administered to each mouse behind the foreleg, between the scapulae, or at the base of the tail using a tuberculin syringe attached to a 26 gauge 0.5 inch needle. Mice will remain in the laboratory for one hour after inoculation to permit careful monitoring of mice. The study endpoint will be either death (see section V.4.5) or euthanasia 14 days post-challenge. Obviously moribund animals will be euthanized using CO_2 inhalation or isoflurane inhalation followed by cervical dislocation (see section V.4.6).

D. For i.p. inoculation, 100-200 µl heat-activated *Bacillus* spores or purified toxin components will be administered to each mouse into the peritoneal cavity through the ventral abdominal wall using a tuberculin syringe attached to a 26 gauge 0.5 inch needle. Mice will remain in the laboratory for one hour after inoculation to permit careful monitoring of mice. The study endpoint will be either death (see section V.4.5) or euthanasia 14 days post-challenge. Obviously moribund animals will be euthanized

using CO_2 inhalation or isoflurane inhalation followed by cervical dislocation (see section V.4.6).

V.1.1. Assessment of virulence of *B. anthracis* Sterne and *B. cereus* G9241 and mutants. In these studies, we will address the contributions of several virulence factors to the pathogenesis of *B. anthracis* Sterne and/or *B. cereus* G9241 disease. The 50% lethal doses (LD_{50}) for *B. anthracis* Sterne administered i.n. and s.c. to A/J mice are 6.3×10^4 and 1.6×10^5, respectively and the LD_{50}s for *B. cereus* G9241 administered to A/J mice via i.n. and s.c. routes are 3.2×10^5 spores and 1.3×10^3 spores, respectively (23). Further, we and others have previously demonstrated that the *B. cereus* G9241 megaplasmids that encode the capsule operons and the toxin genes are required for virulence (23). We propose to investigate the virulence of *B. anthracis* Sterne or *B. cereus* G9241 mutants that do not produce certain capsules, toxins, spore proteins, or virulence regulators. We will test the virulence of these strains via s.c., i.t., and/or i.p. routes.

V.1.1.1. Identification of LD_{50} of *B. anthracis* Sterne and *B. cereus* G9241 delivered via i.t. or i.p. routes. We first need to determine the LD_{50}s of *B. anthracis* Sterne and *B. cereus* G9241 after delivery by each route because we have not used these routes of inoculation to any significant degree in the past. *B. anthracis* Sterne or *B. cereus* G9241 spores will be administered via i.t. or i.p. routes as described above. The i.t. experiment will be done twice with each strain, with a wide range of doses used in the first experiment and a narrow range of doses used in the second experiment to more accurately pinpoint the LD_{50}. A previous study with *B. anthracis* Ames showed that the LD_{50}s for spores administered i.n. and i.t. were 3.7×10^4 and 8.7×10^2, respectively (14), so we anticipate that the LD_{50}s for *B. anthracis* Sterne and *B. cereus* G9241 spores delivered i.t. will be lower than the i.n. LD_{50}s (6.3×10^4 and 3.2×10^5 spores, respectively). In a preliminary i.p. study, we found that a dose of 10^3 spores was lethal to all mice. However, another group determined that the LD_{50} for *B. cereus* G9241 delivered i.p. was 3.8×10^2 spores (16). Thus, we will do one additional study with a narrow range of doses that should permit us to calculate the i.p. LD_{50} for *B. cereus* G9241.

Intratracheal experiment 1
Group 1: 5 mice: 10^1 *Bacillus* spores in H_2O
Group 2: 5 mice: 10^2 *Bacillus* spores in H_2O
Group 3: 5 mice: 10^3 *Bacillus* spores in H_2O
Group 4: 5 mice: 10^4 *Bacillus* spores in H_2O
Group 5: 5 mice: 10^5 *Bacillus* spores in H_2O
Group 6: 5 mice: 10^6 *Bacillus* spores in H_2O
Group 7: 3 mice: H_2O
33 A/J mice

Intratracheal experiment 2
Remove two doses farthest from LD_{50} and increase to 10 mice/group = **43 A/J mice**

Total for intratracheal experiments: (33 mice + 43 mice) x 2 bacterial strains = 152 A/J mice

Intraperitoneal experiment 1 for B. anthracis Sterne
Group 1: 5 mice: 10^1 *Bacillus* spores in H_2O
Group 2: 5 mice: 10^2 *Bacillus* spores in H_2O
Group 3: 5 mice: 10^3 *Bacillus* spores in H_2O
Group 4: 5 mice: 10^4 *Bacillus* spores in H_2O
Group 5: 5 mice: 10^5 *Bacillus* spores in H_2O
Group 6: 3 mice: H_2O
28 A/J mice

Intraperitoneal experiment 2 for B. anthracis Sterne
Remove two doses farthest from LD_{50} and increase to 10 mice/group = **33 A/J mice**

Intraperitoneal experiment for B. cereus G9241
Group 1: 10 mice: 10^1 *B. cereus* spores in H_2O
Group 2: 10 mice: 10^2 *B. cereus* spores in H_2O
Group 3: 10 mice: 10^3 *B. cereus* spores in H_2O
Group 4: 10 mice: 10^4 *B. cereus* spores in H_2O
Group 7: 3 mice: H_2O
43 mice

Total for Intraperitoneal experiments = 28 + 33 + 43 = 104 A/J mice

Total mice for V.1.1.1 = 152 + 104 = 256 A/J mice

V.1.1.2. Identification of LD_{50} of *B. anthracis* Sterne or *B. cereus* G9241 mutants delivered via s.c. and i.t./i.n. routes. We will assess the contribution of several virulence factors to the pathogenesis of *B. anthracis* Sterne or *B. cereus* G9241 disease. These experiments are designed similarly to the LD_{50} analyses in V.1.1.1 above, except that a positive control group of mice that are infected with $10xLD_{50}$ of the parent strain (*B. anthracis* Sterne or *B. cereus* G9241) will be included for comparison purposes. Inasmuch as possible, more than one mutant strain will be tested in a given experiment so that negative (H_2O) and positive ($10xLD_{50}$ of the parent strain) control groups can be shared. The LD_{50} for each mutant strain will be determined as above with two experiments.

Intratracheal/intranasal experiment 1 for Bacillus mutants
Group 1: 5 mice: 10^1 *Bacillus* mutant spores in H_2O
Group 2: 5 mice: 10^2 *Bacillus* mutant spores in H_2O
Group 3: 5 mice: 10^3 *Bacillus* mutant spores in H_2O
Group 4: 5 mice: 10^4 *Bacillus* mutant spores in H_2O
Group 5: 5 mice: 10^5 *Bacillus* mutant spores in H_2O
Group 6: 5 mice: 10^6 *Bacillus* mutant spores in H_2O
Group 7: 5 mice: $10xLD_{50}$ *Bacillus* parent strain spores in H_2O
Group 8: 3 mice: H_2O
38 A/J mice

Intratracheal/intranasal experiment 2
Remove two doses farthest from LD_{50} and increase to 10 mice/group for mutant strains only = **48 A/J mice**

Total for intratracheal experiments: (38 mice + 48 mice) x 20 mutant strains = 1720 A/J mice

Subcutaneous experiment 1 for Bacillus mutants
Group 1: 5 mice: 10^2 *Bacillus* mutant spores in H_2O
Group 2: 5 mice: 10^3 *Bacillus* mutant spores in H_2O
Group 3: 5 mice: 10^4 *Bacillus* mutant spores in H_2O
Group 4: 5 mice: 10^5 *Bacillus* mutant spores in H_2O
Group 5: 5 mice: 10^6 *Bacillus* mutant spores in H_2O
Group 6: 5 mice: 10^7 *Bacillus* mutant spores in H_2O
Group 7: 5 mice: 10xLD_{50} *Bacillus* parent strain spores in H_2O
Group 8: 3 mice: H_2O
38 A/J mice

Subcutaneous experiment 2
Remove two doses farthest from LD_{50} and increase to 10 mice/group for mutant strains only = **48 A/J mice**

Total for subcutaneous experiments: (38 mice + 48 mice) x 20 mutant strains = 1720 A/J mice

Total mice for V.1.1.2 = 1720 + 1720 = 3440 A/J mice

Total mice for V.1.1 = 256 + 3440 A/J mice = 3696 A/J mice

V.1.2. Experiment 2: Determination of toxicity of putative *B. cereus* G9241 lethal toxin (LT), edema toxin (ET), and Certhrax. The purpose of these studies is to investigate the potential for each of these toxins to contribute to *B. cereus* G9241 disease. As mentioned in the Background, LT is composed of PA + LF and ET is composed of PA + EF. The activity of these toxins in *B. anthracis* has been characterized; in these studies, we propose to confirm that the *B. cereus* G9241 toxins exhibit the same *in vivo* activities as their *B. anthracis* homologs. Additionally, Certhrax is a *B. cereus* G9241-specific toxin that contains a PA binding domain and a functional ADP-ribosyltransferase domain; however, we do not yet know if PA is required for Certhrax activity *in vitro* or *in vivo*. Prior to initiation of LD_{50} studies in mice, we will confirm that each toxin is functional in *in vitro* systems.

The LD_{50} studies will be conducted in C57BL/6J mice according to established models of LT and ET toxicity. Female C57BL/6J mice will be injected i.p. with PA + LF (1:1 ratio) or with PA + EF (1:1 ratio) in sterile saline as described in V.1, section 2D above. Previous studies by other groups have shown that C57BL/6 mice inoculated i.p. with

100 µg PA +100 µg LF from *B. anthracis* typically die within 3 days after injection (13) and those inoculated i.v. with 25 µg PA + 25 µg EF from *B. anthracis* also die within 3 days after injection (12). Mice will be weighed daily to determine if a correlation between weight loss and eventual death exists. Mice will be carefully monitored as described in V.4.5 and euthanized as in V.4.6 if they are extremely moribund.

The published lethal doses for the *B. anthracis* toxins and the relative *in vitro* toxicities of the *B. cereus* G9241 toxins compared to the *B. anthracis* toxins will guide the dosing strategy for the first LD_{50} study for each *B. cereus* G9241 toxin.

Lethal Toxin Experiment 1
Groups 1-7 will be intoxicated with doses of the B. cereus G9241 toxin components that correspond with the published LD_{50}s for each B. anthracis toxin (i.e. for Group 1, mice will be intoxicated with doses of the B. cereus G9241 PA and LF that correspond to 20 LD_{50}s of the B. anthracis toxin components)
Group 8 will be intoxicated with B. anthracis Sterne toxin components (control)

Group 1: 5 mice: 20xLD_{50} *B. anthracis* PA + 20xLD_{50} *B. anthracis* LF
Group 2: 5 mice: 10xLD_{50} *B. anthracis* PA + 10xLD_{50} *B. anthracis* LF
Group 3: 5 mice: 5xLD_{50} *B. anthracis* PA + 5xLD_{50} *B. anthracis* LF
Group 4: 5 mice: 1xLD_{50} *B. anthracis* PA + 1xLD_{50} *B. anthracis* LF
Group 5: 5 mice: 0.5xLD_{50} *B. anthracis* PA + 0.5xLD_{50} *B. anthracis* LF
Group 6: 5 mice: 10xLD_{50} *B. anthracis* PA
Group 7: 5 mice: 10xLD_{50} *B. anthracis* LF
Group 8: 5 mice: 10xLD_{50} *B. anthracis* PA + 10xLD_{50} *B. anthracis* LF
40 C57BL/6J mice

Lethal Toxin Experiment 2
Groups 1-5 will be intoxicated with B. cereus G9241 toxin components
Group 6 will be intoxicated with B. anthracis Sterne toxin components (control)

Group 1: 5 mice: 20xLD_{50} *B. cereus* PA + 20xLD_{50} *B. cereus* LF
Group 2: 5 mice: 10xLD_{50} *B. cereus* PA + 10xLD_{50} *B. cereus* LF
Group 3: 5 mice: 5xLD_{50} *B. cereus* PA + 5xLD_{50} *B. cereus* LF
Group 4: 5 mice: 1xLD_{50} *B. cereus* PA + 1xLD_{50} *B. cereus* LF
Group 5: 5 mice: 0.5xLD_{50} *B. cereus* PA + 0.5xLD_{50} *B. cereus* LF
Group 6: 5 mice: 10xLD_{50} *B. anthracis* PA + 10xLD_{50} *B. anthracis* LF
30 C57BL/6J mice

Total for Lethal Toxin experiments: 40 mice + 30 mice = 70 C57BL/6J mice

Edema Toxin Experiment 1
Groups 1-7 will be intoxicated with doses of the B. cereus G9241 toxin components that correspond with the published LD_{50}s for each B. anthracis toxin (i.e. for Group 1, mice will be intoxicated with doses of the B. cereus G9241 PA and EF that correspond to 20 LD_{50}s of the B. anthracis toxin components)
Group 8 will be intoxicated with B. anthracis Sterne toxin components (control)

Group 1: 5 mice: 20xLD$_{50}$ *B. anthracis* PA + 20xLD$_{50}$ *B. anthracis* EF
Group 2: 5 mice: 10xLD$_{50}$ *B. anthracis* PA + 10xLD$_{50}$ *B. anthracis* EF
Group 3: 5 mice: 5xLD$_{50}$ *B. anthracis* PA + 5xLD$_{50}$ *B. anthracis* EF
Group 4: 5 mice: 1xLD$_{50}$ *B. anthracis* PA + 1xLD$_{50}$ *B. anthracis* EF
Group 5: 5 mice: 0.5xLD$_{50}$ *B. anthracis* PA + 0.5xLD$_{50}$ *B. anthracis* EF
Group 6: 5 mice: 10xLD$_{50}$ *B. anthracis* PA
Group 7: 5 mice: 10xLD$_{50}$ *B. anthracis* EF
Group 8: 5 mice: 10xLD$_{50}$ *B. anthracis* PA + 10xLD$_{50}$ *B. anthracis* EF
40 C57BL/6J mice

Edema Toxin Experiment 2
Groups 1-5 will be intoxicated with B. cereus G9241 toxin components
Group 6 will be intoxicated with B. anthracis Sterne toxin components (control)

Group 1: 5 mice: 20xLD$_{50}$ *B. cereus* PA + 20xLD$_{50}$ *B. cereus* EF
Group 2: 5 mice: 10xLD$_{50}$ *B. cereus* PA + 10xLD$_{50}$ *B. cereus* EF
Group 3: 5 mice: 5xLD$_{50}$ *B. cereus* PA + 5xLD$_{50}$ *B. cereus* EF
Group 4: 5 mice: 1xLD$_{50}$ *B. cereus* PA + 1xLD$_{50}$ *B. cereus* EF
Group 5: 5 mice: 0.5xLD$_{50}$ *B. cereus* PA + 0.5xLD$_{50}$ *B. cereus* EF
Group 6: 5 mice: 10xLD$_{50}$ *B. anthracis* PA + 10xLD$_{50}$ *B. anthracis* EF
30 C57BL/6J mice

Total for Edema Toxin experiments: 40 mice + 30 mice = 70 C57BL/6J mice

Certhrax Experiment 1

Group 1: 5 mice: 500 µg PA + 500 µg Certhrax
Group 2: 5 mice: 100 µg PA + 100 µg Certhrax
Group 3: 5 mice: 50 µg PA + 50 µg Certhrax
Group 4: 5 mice: 5 µg PA + 5 µg Certhrax
Group 5: 5 mice: 1 µg PA + 1 µg Certhrax
Group 6: 5 mice: 100 µg PA
Group 7: 5 mice: 100 µg Certhrax
35 C57BL/6J mice

Certhrax Experiment 2

Group 1: 5 mice: 20xLD$_{50}$ *B. cereus* PA + 20xLD$_{50}$ Certhrax
Group 2: 5 mice: 10xLD$_{50}$ *B. cereus* PA + 10xLD$_{50}$ Certhrax
Group 3: 5 mice: 5xLD$_{50}$ *B. cereus* PA + 5xLD$_{50}$ Certhrax
Group 4: 5 mice: 1xLD$_{50}$ *B. cereus* PA + 1xLD$_{50}$ Certhrax
Group 5: 5 mice: 0.5xLD$_{50}$ *B. cereus* PA + 0.5xLD$_{50}$ Certhrax
Group 6: 5 mice: 10xLD$_{50}$ PA
Group 7: 5 mice: 10xLD$_{50}$ Certhrax
35 C57BL/6J mice

Total for Certhrax experiments: 35 mice + 35 mice = 70 C57BL/6J mice

Total mice for V.1.2 = 70 + 70 + 70 mice = 210 C57BL/6J mice

V.1.3. Experiment 3: *In vivo* imaging of *B. anthracis* Sterne germination, outgrowth, and dissemination. Our previous *in vivo* imaging studies used *B. anthracis* Sterne derivatives that produced a luminescent signal when spores germinated or grew vegetatively in response to host signals (19). The luminescent signal was sufficient to provide information about the general location *in vivo* of a large bolus of spores or vegetative bacilli, but we were unable to visualize the less intense signal *in vivo*; instead, we used *ex vivo* imaging of organs post-necropsy at specific time points post-inoculation. To more accurately study the stages of *Bacillus* growth and the locations within the host in which these events occur, we will use a new *in vivo* imaging system that we recently purchased.

The Carestream Multispectral FX Pro has a larger dynamic range and a higher signal:noise ratio for fluorescence; in addition, it has x-ray capability that permits more accurate signal localization. Construction of fluorescent *B. anthracis* Sterne reporter strains that contain one of the Red Fluorescent Proteins (RFPs) mKate2, E2-Crimson, and FP650 is underway. These reporters will be controlled by germination, vegetative growth, or sporulation promoters and will be expressed episomally. In addition, we plan to label *B. anthracis* Sterne-RFP spores with an AlexaFluor label, which will allow us to monitor deposition and uptake of spores in the airway in a manner that does not require bacterial transcription, which is required for expression of RFP. The choice of Alexa-Fluor label and RFPs allow for simultaneous measurement of multiple fluorophores at once with the Multispectral unmixing functionality of our *in vivo* imaging system.

V.1.3.1. Virulence assessment of *B. anthracis* Sterne-RFP strains. Our earlier studies showed that the LD_{50} for *B. anthracis* Sterne-*sspB::lux* spores administered s.c. to A/J mice is higher than that of the wild-type *B. anthracis* Sterne spores ($1.5x10^4$ CFU vs. $2.3x10^3$ CFU, respectively). Similarly, the LD_{50} for the *B. anthracis* Sterne-*sspB::lux* administered i.n. to A/J mice is $1.4x10^6$ CFU, while the LD_{50} for wild-type *B. anthracis* Sterne is $6.7x10^4$ CFU. Since fluorescence is more intense than luminescence, complete deposition of the spores into the lungs is critical when mimicking inhalational anthrax. In previous studies, we found that i.n. inoculation under isoflurane anesthesia can lead to deposition of the spores into both the airways and the gastrointestinal tract. For this reason we will inoculate mice via the i.t. route to ensure that spores are deposited directly into the airway. In order to use the *B. anthracis* Sterne-RFP constructs to monitor the different stages of infection, we first need to establish the LD_{50} for these strains when delivered s.c. or i.t. As mentioned above, we will test the s.c. and i.t. virulence of three different *B. anthracis* Sterne-RFP constructs (mKate2, E2-Crimson, FP650) that are expressed by promoters that are active during germination, vegetative outgrowth, or sporulation.

Subcutaneous Inoculation Experiment 1
Group 1: 5 mice: H_2O or sterile saline
Group 2: 10 mice: 10^3 *B. anthracis* Sterne-RFP spores
Group 3: 10 mice: 10^4 *B. anthracis* Sterne-RFP spores
Group 4: 10 mice: 10^5 *B. anthracis* Sterne-RFP spores
Group 5: 10 mice: 10^6 *B. anthracis* Sterne-RFP spores
Group 6: 10 mice: 10^7 *B. anthracis* Sterne-RFP spores
Group 7: 10 mice: 10^8 *B. anthracis* Sterne-RFP spores
Group 8: 10 mice: 10xLD_{50} *B. anthracis* Sterne spores
75 A/J mice

Subcutaneous experiment 2
Remove two doses farthest from LD_{50} = **55 A/J mice**

Total for s.c. experiments = (75 + 55) x 3 RFP strains = 390 A/J mice

Intratracheal Inoculation Experiment 1
Group 1: 5 mice: H_2O or sterile saline
Group 2: 10 mice: 10^4 *B. anthracis* Sterne-RFP spores
Group 3: 10 mice: 10^5 *B. anthracis* Sterne-RFP spores
Group 4: 10 mice: 10^6 *B. anthracis* Sterne-RFP spores
Group 5: 10 mice: 10^7 *B. anthracis* Sterne-RFP spores
Group 6: 10 mice: 10^8 *B. anthracis* Sterne-RFP spores
Group 7: 10 mice: 10xLD_{50} *B. anthracis* Sterne spores
65 A/J mice

Intratracheal experiment 2
Remove two doses farthest from LD_{50} = **45 A/J mice**

Total for i.t. experiments = (65 + 45) x 3 RFP strains = 330 A/J mice

Total mice for V.1.3.1 = 390 + 330 = 720 A/J mice

V1.3.2. Assessment of deposition, uptake and dissemination using Alexa Fluor-labeled live or dead spores. We will label *B. anthracis* Sterne-RFP spores with an Alexa-Fluor (AF) conjugate, a fluorescent molecule that emits fluorescence when the spore is intact (before germination occurs), so that we can monitor deposition and uptake with the *in vivo* imaging system. We will monitor germination, outgrowth, dissemination, and sporulation through detection of the RFP signal (different excitation and emission wavelengths than the AF). Thus, we will be able to observe each step of inhalational *B. anthracis* disease. In addition, mice will be inoculated with non-viable spores to observe deposition and uptake of spores by host cells in the absence of germination and to determine the length of time that spores can persist in the airway. Epidemiological data from the human anthrax outbreak in Sverdlovsk, USSR in 1979 suggest that viable spores can persist in the airway for up to 45 days after exposure (15).

Female A/J mice will be inoculated i.t. or s.c. (described in V.1, sections 2B and 2C) with 10xLD_{50} AF-labeled *B. anthracis* Sterne-RFP spores (determined in V1.3.1) in H_2O. At 0.5, 1, 6, 12, 24, 48, 72 hrs, and 2 weeks post-inoculation, 5 mice will be

anesthetized with isoflurane and placed on a heated platform within the imaging box of the Carestream Multispectral FX Pro for imaging(~ 5 min). After whole animal imaging, each mouse will be sacrificed by isoflurane overdose and the body cavity will be opened and the organs exposed for *ex vivo* imaging, then the organs will be harvested and homogenized for enumeration of spores and vegetative bacilli. This experiment will be done twice with each of the three AF-*B. anthracis* Sterne-RFP strains.

V.1.3.2.1: Dissemination of Alexa Fluor-labeled B. anthracis Sterne-RFP spores after i.t. inoculation

Group 1: 5 mice sacrificed at 0.5 hr: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 2: 5 mice sacrificed at 1 hr: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 3: 5 mice sacrificed at 6 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 4: 5 mice sacrificed at 12 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 5: 5 mice sacrificed at 24 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 6: 5 mice sacrificed at 48 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 7: 5 mice sacrificed at 72 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 8: 5 mice sacrificed at 14 days: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
40 mice x 3 *B. anthracis* strains x 2 iterations = 240 A/J mice

V.1.3.2.2: Dissemination of Alexa Fluor-labeled B. anthracis Sterne-RFP spores after s.c. inoculation

Group 1: 5 mice sacrificed at 0.5 hr: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 2: 5 mice sacrificed at 1 hr: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 3: 5 mice sacrificed at 6 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 4: 5 mice sacrificed at 12 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 5: 5 mice sacrificed at 24 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 6: 5 mice sacrificed at 48 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 7: 5 mice sacrificed at 72 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 8: 5 mice sacrificed at 14 days: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
40 mice x 3 *B. anthracis* strains x 2 iterations = 240 A/J mice

V1.3.2.3. Persistence of AF-B. anthracis spores in the airway. We will investigate the number of days that non-viable AF-*B. anthracis* Sterne spores persist in the airways by daily imaging of mice inoculated with these spores. We predict that the spores will remain fluorescent until they are cleared by the mouse. The AF-*B. anthracis* Sterne spores will be inactivated by ultraviolet irradiation or fixation in 10% formalin for one week. Loss of viability will be confirmed by bacterial enumeration on agar plates. A/J mice will be inoculated i.t. with non-viable AF-*B. anthracis* Sterne spores and monitored by whole body *in vivo* and organ *ex vivo* imaging as described in V.1.3.2.1.

Group 1: 5 mice sacrificed at 0.5 hr: 10^7 AF-*B. anthracis* Sterne spores
Group 2: 5 mice sacrificed at 2 days: 10^7 AF-*B. anthracis* Sterne spores
Group 3: 5 mice sacrificed at 4 days: 10^7 AF-*B. anthracis* Sterne spores
Group 4: 5 mice sacrificed at 7 days: 10^7 AF-*B. anthracis* Sterne spores
Group 5: 5 mice sacrificed at 14 days: 10^7 AF-*B. anthracis* Sterne spores
Group 6: 5 mice sacrificed at 21 days: 10^7 AF-*B. anthracis* Sterne spores
Group 7: 5 mice sacrificed at 28 days: 10^7 AF-*B. anthracis* Sterne spores
Group 8: 5 mice sacrificed at 35 days: 10^7 AF-*B. anthracis* Sterne spores
Group 9: 5 mice sacrificed at 42 days: 10^7 AF-*B. anthracis* Sterne spores
Group 10: 5 mice sacrificed at 49 days: 10^7 AF-*B. anthracis* Sterne spores
Group 11 5 mice sacrificed at 56 days: 10^7 AF-*B. anthracis* Sterne spores
55 mice x 2 iterations = 110 A/J mice

Total mice for V.1.3.2 = 240 + 240 + 110 = 590 A/J mice

Total mice for V.1.3 = 720 + 590 = 1310 C57BL/6J mice

V.1.4. Experiment 4. Characterization of efficacy of small molecule inhibitors of germination. Small molecules that prevent both *B. anthracis* spore germination and macrophage killing will be tested in A/J mice for the capacity to prevent disease progression and death of animals challenged i.t. or s.c. with otherwise lethal doses of *B. anthracis* Sterne spores. Additionally, the capacity of selected compounds to block, reduce the extent, or delay spore germination *in vivo* will be evaluated i.t. or s.c. inoculation with *B. anthracis* Sterne-RFP and treatment with the compound of interest.

V.1.4.1. Assessment of efficacy of 6-Thioguanine (6-TG) in preventing anthrax disease. The small molecule 6-TG has been shown to inhibit spore germination *in vitro* (1). The studies described here are designed to 1) test the efficacy of 6-TG in prevention of spore germination and/or anthrax disease *in vivo*, and 2) act as a pilot study that allows us to investigate routes of inoculation with inhibitor and spores as well as the timing of inhibitor administration in preparation for the studies outlined in V.1.4.2 below. These kinetics studies should allow us to focus future studies involving additional inhibitors so that we use fewer mice.

V.1.4.1.1. Pilot study to identify a safe dose of 6-TG to use for subsequent experiments. The LD_{50} of 6-TG in mice is 50 mg/kg (2). In a very small pilot study approved under MIC-06-148, we injected A/J mice s.c. with 5 µg/kg of 6-TG and challenged with *B. anthracis* Sterne-*lux* spores. This dose was chosen because it inhibited spore germination following uptake by macrophages *in vitro*. We determined that this dose did not cause harm to the mice in the absence of spores, but, in a very small group of mice (n=3), it did not protect the mice from mortality. As such, we now propose a pilot dose study to identify a higher dose of 6-TG that is not toxic to the mice. To identify a safe dose of 6-TG for use in subsequent studies, 5 A/J mice per group with be injected s.c. with 0.1 ml of 6-TG in ≤5% DMSO as in V.1.2.C. The study endpoint for this experiment will be either a moribund state (see section V4.5) or euthanasia approximately 1 week post-injection. Thus, it is not an

LD_{50} study, so we will not use death as an endpoint. This experiment will be done once.

Group 1: 5 mice: ≤5% DMSO
Group 2: 5 mice: 5 µg/kg 6-TG in ≤5% DMSO
Group 3: 5 mice: 50 µg/kg6-TG in ≤5% DMSO
Group 4: 5 mice: 500 µg/kg 6-TG in ≤5% DMSO
Group 5: 5 mice: 5 mg/kg 6-TG in <5% DMSO
25 A/J mice

V.1.4.1.2. Assessment of efficacy of 6-TG treatment for inhibition of germination. Mice will be treated s.c. or i.n./i.t. with 6-TG in ≤5% DMSO 1 hr prior to, concurrent with, or 1 hr after inoculation with 10x LD_{50} *B. anthracis* Sterne spores (see Table 1.4.1 below for a description of each treatment condition and Section V.1.2 for description of the inoculation methods). For s.c. delivery of 6-TG, the 6-TG will be diluted in sterile ≤5% DMSO in sterile saline and 0.1 ml of the dose identified in V1.4.1.1 will be delivered s.c. with a 26 gauge, 0.5 inch needle attached to a tuberculin syringe 9 V.1, section 2C). For i.n. delivery of 6-TG, mice will be anesthetized by isoflurane inhalation and 0.05 ml of the dose identified in V1.4.1.1 of 6-TG will be delivered i.n. using a pipet tip attached to a pipette (V.1.2.A). For mice that are inoculated into the airway with *B. anthracis* Sterne spores and 6-TG concurrently, the 6-TG will be administered i.t. with the spores as in V.1.2.B. Mice will be imaged twice daily with the Carestream *in vivo* imaging system, and they will be monitored for morbidity and mortality for 2 weeks. In addition, mice will be weighed daily to determine if a correlation between weight loss and eventual death exists. Mice will be euthanized by isoflurane overdose followed by cervical dislocation to terminate the experiment.

Table 1.4.1. Groups of A/J mice to be challenged with germination inhibitor 6-TG

Number of mice	*Time of inhibitor delivery*	*Inhibitor*	*Challenge*
10	1 hr pre-challenge	6-TG	Water
10		6-TG	*B. anthracis* Sterne-RFP spores
10		≤5% DMSO	*B. anthracis* Sterne-RFP spores
10	Simultaneous with challenge	6-TG	Water
10		6-TG	*B. anthracis* Sterne-RFP spores
10		≤5% DMSO	*B. anthracis* Sterne-RFP spores
10	1 hr post-challenge	6-TG	Water
10		6-TG	*B. anthracis* Sterne-RFP spores
10		≤5% DMSO	*B. anthracis* Sterne-RFP spores

V.1.4.1.2a: Subcutaneous delivery of inhibitor and challenge with B. anthracis Sterne-RFP spores

10 mice/group x 9 groups x 2 iterations: 180 mice

V.1.4.1.2a: Intranasal delivery of inhibitor and challenge with B. anthracis Sterne-RFP spores

10 mice/group x 9 groups x 2 iterations: 180 mice

Total number of mice for V.1.4.1: 25 + 180 + 180 = 385 mice

V.1.4.2. Assessment of efficacy of additional small molecule inhibitors in prevention of spore germination and/or anthrax disease. The small molecule inhibitors of germination to be screened in this group of experiments will be identified for *in vivo* analysis as a result of *in vitro* screening of libraries of small molecule inhibitors. Each compound in the small molecule libraries will be screened *in vitro* for the ability to inhibit germination of *B. anthracis* Sterne in culture as well as in tissue culture. Up to 10 of the most effective germination inhibitors *in vitro* will be screened for efficacy *in vivo* as described below. The identity and composition of each molecule are known. As potential germination inhibitors are identified, we will conduct literature searches to identify studies in which the molecule has been used in mice to guide our further experimentation. If the results obtained with 6-TG (V1.4.1) give a clear idea of the most effective experimental conditions under which inhibitory effects can be seen, we will limit our screening experiments to those parameters. However, it may be necessary to screen inhibitors by all of the following experimental parameters: i.) s.c. administration before inoculation, ii.) simultaneous s.c. administration, iii.) i.n. administration before inoculation and iv.) simultaneous i.t. administration. A minor modification will be made to this protocol to describe the identity, potential toxicity, proposed solvent, route(s) of administration, and dose (or range of doses, see V1.4.2.1 below) of each compound prior to commencing *in vivo* studies.

V.1.4.2.1. Pilot study to identify a safe dose of each small molecule inhibitor to use for subsequent experiments. The purpose of this experiment is to identify the safest dose of each small molecule that can be given to the mouse without causing apparent morbidity. Thus, it is not an LD_{50} study, so we will not use death as an endpoint. We will use the outcome of the pilot study in V.1.4.1.1 above and a thorough search of the literature about each compound to guide the pilot dosing study for each new compound to be tested. To identify a safe dose of each small molecule inhibitor for use in subsequent studies, 5 A/J mice per group with be injected s.c. with 0.1 ml of 1 of 4 different doses of inhibitor in solvent or solvent alone using a 0.5 inch, 26 gauge needle. The study endpoint for this experiment will be either a moribund state (see section V4.5) or euthanasia approximately 1 week post-injection. Mice exhibiting symptoms of toxicity, including ruffled fur, lethargy, decreased activity, or unresponsiveness to external stimuli will be euthanized by isoflurane overdose followed by cervical dislocation (see section V.4.1.4.). Note: when possible, the safety of multiple inhibitors will be tested simultaneously (in

different mice) so that a single group of control animals (solvent injection only) can be used so as to reduce the number of mice needed.

Group 1: 5 mice: solvent
Groups 2-5: 5 mice each: 10-fold dilutions of molecule in solvent

5 mice/group x 5 groups/inhibitor x 10 inhibitors = 250 A/J mice

V1.4.2.2 Assessment of efficacy of small molecule inhibitors in prevention of spore germination and/or anthrax disease. All procedures in V.1.4.2.2. will be conducted as above for V.1.4.2.1.

Table 1.4.2.2A. Groups of mice to be challenged with small molecule inhibitors of germination.

Stage	*Control or experiment*	*Number of mice*	*Number of inhibitors*	*Challenge*	*Total mice*
First screen of 5 inhibitors	Control	5/inhibitor	5	Water	25
		10 total	Solvent	*B. anthracis* Sterne-RFP spores	10
	Experiment	10/inhibitor	5	*B. anthracis* Sterne-RFP spores	50
					85
Second screen of 5 inhibitors	Control	5/inhibitor	5	Water	25
		10 total	Solvent	*B. anthracis* Sterne-RFP spores	10
	Experiment	10/inhibitor	5	*B. anthracis* Sterne-RFP spores	50
					85
Confirmation of 2 best inhibitors	Control	5/inhibitor	2	Water	10
		10 total	Solvent	*B. anthracis* Sterne-RFP spores	10
	Experiment	10/inhibitor	2	*B. anthracis* Sterne-RFP spores	20
					40

Each inhibitor will be tested under 4 conditions:
Experiment 4.2a: i.n. administration of inhibitor 1 hr pre-challenge
Experiment 4.2b: i.t. administration of inhibitor simultaneous with challenge
Experiment 4.2c: s.c. administration of inhibitor 1 hr pre-challenge
Experiment 4.2d: s.c. administration of inhibitor simultaneous with challenge

Table 1.4.2.2B. Calculation of total number of mice for Experiment 1.4.2.2			
Stage	*Experiment*	*Number of mice*	*Total number of mice*
Screening of up to 10 candidate inhibitors (5 per experiment)	Expt 4.2.2a: s.c. inhibitor pre-challenge	170	680 200 Pain C 480 Pain E
	Expt 4.2.2b: s.c.inhibitor simultaneous with challenge	170	
	Expt 4.2.2c: i.n. inhibitor pre-challenge	170	
	Expt 4.2.2d: i.t. inhibitor simultaneous to challenge	170	
Confirmation of 2 best candidates	Expt 4.2.2e: s.c. inhibitor pre-challenge	40	160 40 Pain C 120 Pain E
	Expt 4.2.2f: s.c. inhibitor simultaneous to challenge	40	
	Expt 4.2.2g: i.n. inhibitor pre-challenge	40	
	Expt 4.2.2h: i.t. inhibitor simultaneous to challenge	40	
Grand total number of mice			840 240 Pain C 600 Pain E

Total number of mice for V.1.4.2 = 250 + 840 = 1090 mice

Total number of mice for V.1.4 = 385 + 1090 = 1475 A/J mice

V.1.5. Experiment 5. Characterization of vaccine candidates to protect against *B. anthracis* disease. In our prior animal protocols, we assessed the immunogenicity of intact *B. cereus* G9241 spores and *B. anthracis* Sterne exosporium proteins, as well as the protective efficacy of antibodies against these exosporium components in mouse models of s.c. and i.n. anthrax disease. Our previous immunization studies and our proposed studies comprise a single immunization with a suboptimal dose of of the toxin component PA expressed recombinantly (rPA), followed by immunization with the antigen or antigens under investigation (3, 6). We previously demonstrated that a sublethal dose of rPA enhances the protective efficacy of vaccines comprised of spore proteins (3, 6). In the proposed studies, we will immunize A/J mice with rPA followed by rBclA, glycosylated BclA (Gly-BclA), BxpA, ExsE, and/or p2138. BclA is a glycoprotein of the exosporium that will be used as a control in these studies as (b)(6) *et al* previously demonstrated that immunization with rBclA (not glycosylated) in conjunction with rPA provides protection against murine anthrax disease (3). We will also test the protection afforded by immunization with the native glycosylated form of BclA, Gly-BclA. After immunization, mice will be challenged subcutaneously with *B. anthracis* Sterne spores.

V.1.5.1. Immunization of A/J and BALB/c mice with formalin-inactivated spores (FIS) to generate antisera for *in vitro* studies. As mentioned above, we showed that s.c. or i.n. immunization of A/J mice with a single dose of *B. cereus* G9241 pBCXO1- pBC218- (double-cured G9241 or DC-9241) live spores or FIS conferred 100% protection from subsequent s.c. or i.n. *B. anthracis* Sterne challenge. In addition, our collaborators at

USAMRIID demonstrated that immunization of BALB/c mice with a single dose of DC-G9241 FIS i.p. or i.n. protected 55-60% of the mice from subsequent challenge with the fully virulent *B. anthracis* Ames. Studies are underway in our laboratory to identify the protective immunogens on the surface of DC-G9241 using immunoproteomics methods. To conduct these studies, we will use antisera from A/J and BALB/c mice immunized with DC-G9241 or *B. cereus* 569, a related but avirulent environmental isolate of *B. cereus* that, when administered in FIS form i.p. or i.n., protects BALB/c mice from *B. anthracis* Ames challenge slightly less effectively than does DC-G9241.

To generate antisera against DC-G9241 or *B. cereus* 569, we will inject FIS of either strain into groups of 10 A/J and 10 BALB/c mice. Spores will be inactivated by fixation in 10% formalin for one week and extensive washing with water. Loss of viability will be confirmed by bacterial enumeration on agar plates. A dose of 10^7 spores (as enumerated prior to fixation) will be injected s.c. with or without Alhydrogel (139 µg aluminum/dose, Section 4.4.3) twice 14 days apart. On day 29, mice will be deeply anesthetized with inhaled isoflurane and exsanguinated by cardiac puncture to collect blood, then sacrificed by cervical dislocation. In previous studies approved under MIC-09-418, the mice exhibited no signs of morbidity after FIS administration. However, if two or more signs of morbidity, as defined in V.4.5, are observed, mice will be euthanized by isoflurane overdose and cervical dislocation as in V.4.6.

Group 1: 10 A/J mice immunized with DC-G9241 FIS
Group 2: 10 A/J mice immunized with *B. cereus* 569 FIS
Group 3: 10 BALB/c mice immunized with DC-G9241 FIS
Group 4: 10 BALB/c mice immunized with *B. cereus* 569 FIS
(20 A/J mice + 20 BALB/c mice) x 2 iterations = 40 A/J and 40 BALB/c mice

V.1.5.2. Dissemination of AF-*B. anthracis* Sterne spores after immunization of A/J mice with DC-G9241 spores. As mentioned above, a AF-*B. anthracis* Sterne spore emits a fluorescent signal only until germination occurs; as the spore germinates into a vegetative bacillus, the labeled spore coat is shed and the fluorescent signal is quenched. In a preliminary *in vivo* imaging experiment with only a few mice, we observed that AF-*B. anthracis* Sterne spores remained fluorescent and in the lungs of A/J mice that were immunized with DC-G9241, while the fluorescent signal from AF-*B. anthracis* Sterne spores introduced to naïve mice was quenched rapidly. We propose to repeat this experiment with additional mice in an effort to understand the mechanism behind the inhibition and germination and dissemination observed in immunized mice.

Groups of 18 A/J mice will be immunized with 10^7 DC-G9241 viable or formalin inactivated spores or sham-immunized with sterile saline by i.p. injection or i.n./i.t. administration as in V.1.5.1 above. On day 29, we will inoculate mice with $10xLD_{50}$ AF-*B. anthracis*-Sterne spores (determined in V.1.3.1) via s.c. or i.t. administration. We will lightly anesthetize mice with inhaled isoflurane as in V.1.4.2.1 below and image them 0.5, 2, 6, and 12 hrs, and 1, 2, 3, 4, and 7 days post-inoculation. Two mice per time point will be sacrificed by isoflurane overdose and the organs will be imaged *ex vivo*. Organs will be fixed in 10% formalin and processed to determine the location of spores and vegetative bacilli. The experiment will be conducted twice and the time points will be adjusted for the second experiment if necessary.

Group 1: 18 mice immunized with viable 10^7 DC-G9241 spores in sterile saline
Group 2: 18 mice immunized with 10^7 DC-G9241 FIS in sterile saline
Group 3: 18 mice sham immunized with sterile saline

54 mice x 2 iterations = 108 A/J mice

V.1.5.3. Immunization of A/J mice with candidate spore antigens and subsequent challenge with *B. anthracis* Sterne spores. A/J mice will receive 1 dose of either PBS, 10-25 µg exosporium antigen, or 50 ng rPA on day 1, then a dose of 10-25 µg of each exosporium antigen (alone or in combination with other exosporium antigens) on day 15. Antigens will be mixed with TiterMax Gold or Alhydrogel (139 µg aluminum/dose) (Section 4.4.3) in a final volume of 0.1 ml. We previously used TiterMax Gold with success. We may use Alhydrogel instead of TiterMax Gold as the adjuvant so as to better mimic the currently licensed Anthrax vaccine.

Blood will be collected by tail venipuncture every two weeks beginning with day 0 and processed to determine the specific antibody titer. Immunogens emulsified in adjuvant will be injected i.p. with a tuberculin syringe attached to a 26 gauge 0.5 inch needle on days 1, 15, 29, and 43 or until a specific antibody response is detected in the sera. On the 14th day after the last boost, mice will be challenged s.c. with 10-20xLD_{50} of *B. anthracis* Sterne spores delivered from a tuberculin syringe attached to a 26 gauge 0.5 inch needle. Mice will be monitored as described in section V4.5 following infection. Mice will be weighed daily to determine if a correlation between weight loss and eventual death exists. The study endpoint for this virulence experiment will be either death (see section V4.5) or euthanasia 2 weeks post-infection. Obviously moribund animals will be euthanized by CO_2 inhalation or isoflurane inhalation followed by cervical dislocation (see section V.4.1.4.).

The immunogens that we plan to test are listed in Table V.1.5.1 below. These proteins were shown to react with antisera from mice that had been immunized with a derivative of *B. cereus* G9241 that was cured of both virulence plasmids (double-cured *B. cereus* G9241 or DC-G9241). Each experiment will be done twice. Additional antigens will be added to the protocol as they are identified in *in vitro* studies.

Table V.1.5.1. Candidate antigens and antigen combinations				
Experiment	*rPA*	*Immunogen(s)*	*Challenge*	*Number of mice per group*
A	-	Gly-BclA	*B. anthracis* Sterne	10
	+	Gly-BclA	*B. anthracis* Sterne	10
	-	BxpA	*B. anthracis* Sterne	10
	+	BxpA	*B. anthracis* Sterne	10
	-	ExsE	*B. anthracis* Sterne	10
	+	ExsE	*B. anthracis* Sterne	10
	-	P2138	*B. anthracis* Sterne	10
	+	P2138	*B. anthracis* Sterne	10
	-	PBS + adjuvant	*B. anthracis* Sterne	10
	+	PBS + adjuvant	*B. anthracis* Sterne	10
	-	PBS + adjuvant	water	5
			Experiment A total	**105**
B	-	Gly-BclA + BxpA	*B. anthracis* Sterne	10
	+	Gly-BclA + BxpA	*B. anthracis* Sterne	10
	-	Gly-BclA + ExsE	*B. anthracis* Sterne	10
	+	Gly-BclA + ExsE	*B. anthracis* Sterne	10
	-	Gly-BclA +P2138	*B. anthracis* Sterne	10
	+	Gly-BclA +P2138	*B. anthracis* Sterne	10
	-	BxpA + ExsE	*B. anthracis* Sterne	10
	+	BxpA + ExsE	*B. anthracis* Sterne	10
	-	BxpA + P2138	*B. anthracis* Sterne	10
	+	BxpA + P2138	*B. anthracis* Sterne	10
	-	ExsE + P2138	*B. anthracis* Sterne	10
	+	ExsE + P2138	*B. anthracis* Sterne	10
	-	PBS + adjuvant	*B. anthracis* Sterne	10
	+	PBS + adjuvant	*B. anthracis* Sterne	10
	-	PBS + adjuvant	water	5
			Experiment B total	**145**
C	-	Gly-BclA + BxpA + ExsE	*B. anthracis* Sterne	10
	+	Gly-BclA + BxpA + ExsE	*B. anthracis* Sterne	10
	-	Gly-BclA + BxpA +P2138	*B. anthracis* Sterne	10
	+	Gly-BclA + BxpA +P2138	*B. anthracis* Sterne	10
	-	Gly-BclA + ExsE + P2138	*B. anthracis* Sterne	10
	+	Gly-BclA + ExsE + P2138	*B. anthracis* Sterne	10
	-	BxpA + ExsE + P2138	*B. anthracis* Sterne	10
	+	BxpA + ExsE + P2138	*B. anthracis* Sterne	10
	-	Gly-BclA + BxpA + ExsE + P2138	*B. anthracis* Sterne	10
	+	Gly-BclA + BxpA + ExsE + P2138	*B. anthracis* Sterne	10
	-	PBS + adjuvant	*B. anthracis* Sterne	10
	+	PBS + adjuvant	*B. anthracis* Sterne	10
	-	PBS + adjuvant	water	5
			Experiment C total	**125**
V.1.5. Grand total for 2 iterations of each experiment				***750 A/J mice***

Total number of mice for V.1.5 = 385 + 1090 = 1475 A/J mice

V.2. Data Analysis: A sample size of 5-10 animals per condition will have 80% power to detect a difference of 1.3 - 1.8 standard deviations based on a *t* test for independent samples with a 5%, two-sided significance level. These are the smallest differences that would be considered clinically meaningful for this type of study. Because mice have not been extensively used for work with *Bacillus* species, we need to establish the baseline virulence of the strains and the derivatives of those strains with which we are working. Furthermore, we are testing several vaccine candidates for protective efficacy in treatments that may eventually be used in humans.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: At this time, there are no non-animal alternatives for animal use that would achieve the objectives outlined in these studies. Our justification for the use of the mouse model to study *Bacillus* virulence is that tissue culture systems challenged with *Bacillus* spores cannot always discriminate between animal-virulent and animal-attenuated strains. *In silico* simulations and *In vitro* cell culture studies were considered but rejected because they cannot address the objectives of these studies.

V.3.2. Animal Model and Species Justification: Animal use will help develop prophylactic and therapeutic measures to counter *B. anthracis* spore exposure in humans. The major goals of this project are to identify *B. anthracis* spore antigens and small molecule compounds that act as anti-germination targets and to identify key virulence determinants in *B. cereus*. The use of murine animal models is essential to the evaluation of therapeutic and preventative strategies against disease caused by *Bacillus* spp. since no *In vitro* systems or models are currently available to predict the efficacy of such reagents. The mouse is one of three small animal models (rabbit and guinea pigs are others) available for studying *B. anthracis* and *B. cereus* virulence and the efficacy of agents that may mitigate the effects of that disease. Mice are the lowest animal on the evolutionary scale with which to perform virulence studies for *B. anthracis* and *B. cereus*.

Studies of pathogenesis and virulence of *Bacillus* spp. have historically been performed in female mice (3, 5, 9, 10, 17, 19, 21-23). Our laboratory has always used female mice for these studies, as have other laboratories who study *Bacillus* virulence. In addition, female mice are used to reduce the impact of fighting among the mice. Further, all of the mice in an experimental group can be housed in the same cage when female mice are used.

V.3.3. Laboratory Animals

		Species #1
V.3.3.1.	**Genus & Species:**	*Mus musculus*
V.3.3.2.	**Strain/Stock:**	A/J, C57BL/6J, BALB/c
V.3.3.3.	**Source/Vendor:**	(b)(4)
V.3.3.4.	**Age:**	6-8 weeks
V.3.3.5.	**Weight:**	15-25 g
V.3.3.6.	**Sex:**	*Female
V.3.3.7.	**Special Considerations:**	-

V.3.4. Number of Animals Required (by Species): 7629

V.3.3.6: Studies of pathogenesis and virulence of *Bacillus* spp. have historically been performed in female mice (3, 5, 9, 10, 17, 19, 21-23). Our laboratory has always used female mice for these studies, as have other laboratories who study *Bacillus* virulence. In addition, female mice are used to reduce the impact of fighting among the mice. Further, all of the mice in an experimental group can be housed in the same cage when female mice are used.

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: Titermax Gold™ or Alhydrogel will be used instead of Freund's adjuvant (complete and incomplete). TiterMax Gold™ and Alhydrogel are adjuvants known to be less reactogenic than Freund's. Alhydrogel is the adjuvant in the currently FDA-approved human anthrax vaccine AVA® In addition, we will use small pilot studies to rapidly narrow the number of bacteria or amount of therapeutic to be administered in subsequent LD_{50} studies. These pilot studies will be done with the smallest number of mice that will provide the necessary information in a statistically significant manner. We further minimize distress during the studies by careful cage-side monitoring of the animals to determine if an animal should be euthanized as described in V4.1.4 and V4.5. Signs indicating that euthanasia should be implemented would include an inability to reach food and water, labored breathing, excessive weight loss, and inability to remain upright.

V.3.5.2. Reduction: As many of our experiments involve infected/spore-treated animals or tissues, we do not participate in any shared tissue and/or control group studies with other investigators. We have taken into consideration all information we have gleaned from 9 years of experience working with this anthrax model when proposing subsequent experiments as well as in the refinement of the total number of animals required for the proposed experiments. With respect to the number of animals

required for each experiment, we have used the minimal number of animals required for biological and statistical significance (generally 5 to 10 animals per group). Most experiments will be performed in duplicate to confirm the data. In these experiments, the two spore doses that are farthest from the LD_{50} of the strain in question will be eliminated in the second experiment in order to reduce the number of mice used.

V.3.5.3. Replacement: At this time, there are no replacements for animal use that would achieve the objectives outlined in these studies. Our justification for the use of the mouse model to study *Bacillus* virulence is that tissue culture systems challenged with *Bacillus* spores cannot always discriminate between animal-virulent and animal-attenuated strains. *In silico* simulations and *in vitro* cell culture studies were considered but rejected because they cannot address the objectives of these studies.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals: Animals are assigned to pain category E because we use death as an endpoint for the majority of our experiments. The symptoms on *Bacillus* infection, which will not be relieved using analgesics or antibiotics, are as follows: ruffled fur, lethargy, hunched posture, decreased alertness, weight loss, difficulty breathing, unresponsiveness to external stimuli, and trembling. Animals that are used as controls in each experiment (no spore inoculation) and animals that will be injected with small molecules during the studies outlined in V1.4 are assigned to pain category C because we do not expect them to suffer in any way from the control treatments.

Table V.4.1.1.1. Breakdown by study number of experimental and control animals for pain category assignment.

Experiment	*Total number of mice*	*Pain Category C*	*Pain Category E*
V.1.1.1	256	21	235
V.1.1.2	3440	240	3200
V.1.2	210	30	180
V.1.3.1	720	60	660
V.1.3.2.1	240	0	240
V.1.3.2.2	240	0	240
V.1.3.2.3	110	110	0
V.1.4.1.1	25	25	0
V.1.4.1.2	360	120	240
V.1.4.2.1	250	250	0
V.1.4.2.2	840	240	600
V.1.5.1	80	80	0
V.1.5.2	108	108	0
V.1.5.3	750	30	720
Total	**7629**	**1314**	**6315**

	Species #1 A/J mice	Species #2 C57BL/6J mice	Species #2 BALB/c mice
V.4.1.1.1.1. Column C:	1244	30	40
V.4.1.1.1.2. Column D:	-	-	-
V.4.1.1.1.3. Column E:	6135	180	0

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: We will use inhalation of isoflurane gas (1.5-3.0% in O_2) to anesthetize animals for *in vivo* imaging studies. We will use either an i.p. injection of ketamine-xylazine (100 mg/kg ketamine and 25 mg/kg xylazine) or inhalation of isoflurane gas (1.5-3.0% in O_2) to anesthetize animals for i.t. and i.n. inoculation. Animals used in s.c and i.p. challenge are not expected to endure anything more than momentary pain, so no analgesics will be necessary.

For imaging studies, isoflurane has the advantage of maintaining the mice under anesthesia during the imaging procedure while permitting the animals a quick recovery time. The anesthetized mice will be placed on a warm platform and monitored for bioluminescence for 5 min at 0.5, 1, 3, 6, 12, 24, 48, and/or 72 h post infection. To anesthetize the mice and secure their immobility during imaging with the Carestream Multispectral *in vivo* imaging system, we will follow the following procedures. Administration of isoflurane gas will be done via a Gas Anesthesia System that delivers isoflurane gas to a 5-port anesthesia manifold housed in the imaging chamber of the *in vivo* imaging system. Animals will be initially placed into an induction chamber and anesthetized with isoflurane (3-4%) with oxygen as the carrier gas. Once the animals are properly sedated, they will be swiftly moved to nose cones on the 5-port anesthesia manifold housed in the imaging chamber and maintained at 1.5-3.0% isoflurane. Any unused manifold ports will be closed off. Sufficient time will be given to allow the anesthetic gas to reach the manifold nose cones prior to removing the animals from the induction chamber. Both the induction chamber and manifold will be set to deliver isoflurane at a constant rate. The manifold also provides waste gas scavenging ports to help prevent gas from entering the surrounding work environment. A built-in vacuum system helps ventilate waste gas away from researchers and into disposable isoflurane-absorbing filters. In both anesthesia procedures, depth of anesthesia will be assessed by the onset and maintenance of slow, shallow breathing as well as the loss of toe pinch response in the animals.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Only mice that appear to be in good health will undergo anesthesia. We define good health as clean unruffled fur, alert demeanor and no obvious signs of sickness. Mice recovering from anesthesia will be placed into a clean cage with external heat applied (a heating pad placed under the recovery cage) to prevent mice from undergoing a rapid loss in body temperature. Once the mice have roused from anesthesia and appear to be moving in an alert manner, they will be placed back into a clean cage with their cage mates. All mice will be monitored in the laboratory for at least one hour prior to their return to (b)(6)

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: AGRICOLA and PUBMED databases were searched

V.4.1.3.2. Date of Search: 12 December 2011

V.4.1.3.3. Period of Search: AGRICOLA Database (1970 – December 2011), PUBMED Database (1966 – December 2011, English only)

V.4.1.3.4. Key Words of Search: Pain AND (alleviation OR alleviate OR alternative OR reduction) AND (mouse OR mice) AND (*Bacillus*)

V.4.1.3.5. Results of Search: The search for *Bacillus* and mouse and pain and alternative yielded no documents in AGRICOLA and two documents in PUBMED. The documents did not provide any methods for alleviation of pain or distress in mouse models that we use.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: No analgesics will be given to *Bacillus*-infected animals for the following reasons. Inflammation and/or the inflammatory response are key components of *Bacillus*-mediated disease (macrophages play a key role in the dissemination of *Bacillus* spp). Because we will be using the mouse as a model to study both the pathogenesis of disease and possible treatment therapies, we believe that the use of non-steroidal anti-inflammatory drugs and opioids could confound or possibly mask the extent of *Bacillus*-mediated damage that we will evaluate by histological examination or toxicity (18).

Mice will be weighed daily to determine if a correlation between weight loss and eventual death exists. Investigator and staff will carefully monitor *Bacillus*-infected animals and euthanize those that appear moribund because it is our experience that these animals will ultimately succumb to *Bacillus* infection after inoculation. We define extremely moribund as when a mouse exhibits 2 or more of the following symptoms: ruffled fur, lethargy, hunched posture, impaired ambulation that prevents the animals from reaching food and water, excessive weight loss/emaciation, difficult or labored breathing, and the inability to remain upright. Death due to infection after i.n., i.t., s.c., or i.p. inoculation with *B. anthracis* and *B. cereus* typically occurs 3-5 days post-inoculation. During this time period, mice will be inspected every 4 h during normal working hours and every 8 h during non-business hours. All inspections will be noted on cage cards so that LAM personnel are aware that the mice are being monitored.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: N/A

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: We will be giving i.p. and s.c. injections. All mouse injections are given with a 26 gauge 0.5 inch needle attached to a tuberculin syringe. All doses are given in a total volume of 0.1-0.2 ml.

For Immunization – for each mouse in this set of experiments, each recombinant exosporium protein or combination of proteins will be emulsified 1:1 with TiterMax (TiterMax, Norcross, Georgia 30092) or Alhydrogel and will be administered at a dosage of 25 µg protein (antigen) per animal. For spore immunization, 10^7 DC-G9241 viable or formalin inactivated spores will be administered by i.p. injection with or without Alhydrogel. Antigen/adjuvant will be administered by i.p. injection through the ventral abdominal wall with a tuberculin syringe attached to a 26 gauge 0.5 inch needle. Doses of antigen/adjuvant will be in a volume of 100 µl/animal. Control animals will receive 100 µl of saline- or PBS-adjuvant emulsion minus antigen. The injections will be done 10-14 days apart.

For Small Molecule Treatment – each mouse in the pilot experiments (to identify a non-toxic dose) will be injected s.c. between the scapulae, at the base of the tail, or behind the right foreleg with 0.1 ml of 1 of 4 different doses of the small molecule in water using a 0.5 inch, 26 gauge needle. Once a non-toxic dose of the small molecule is identified, each mouse in these experiments will be injected s.c. in the same location with 0.1 ml of that dose of small molecule in water using a 0.5 inch, 26 gauge needle.

For Spore Challenge or Toxin Administration – for each mouse in this set of experiments, heat-activated *Bacillus* spores will be administered via s.c. injection between the scapulae, at the base of the tail, or behind the right foreleg or via i.p. injection through the ventral abdominal wall using a tuberculin syringe attached to a 26 gauge 0.5 inch needle. Spores will be in a volume of 100-200 µl/animal. Toxin components will be injected i.p. through the ventral abdominal wall using a tuberculin syringe attached to a 26 gauge 0.5 inch needle.

For Anesthesia – for each mouse in this set of experiments, anesthesia will be induced by either by i.p. injection through the ventral abdominal wall of ketamine-xylazine (100 mg/kg ketamine, 25 mg/kg xylazine) in sterile phosphate buffered saline (PBS) using a tuberculin syringe attached to a 26 gauge 0.5 inch needle or inhalation of

isoflurane gas (1.5-3.0% in O_2) to anesthetize animals prior to imaging (see anesthesia section V.4.1.2.1).

V.4.4.2. **Biosamples:**

Blood samples from immunized mice will be obtained from the tail vein of mice using either a 26 gauge ½" needle or by nicking the tail with a sterile razor blade and collecting about 0.1 ml of whole blood into a sterile 1 ml tube. This will be done will be done 10-14 days apart prior to each subsequent boost. Exsanguination by terminal cardiac puncture will be conducted under deep isoflurane anesthesia. Once anesthetized, as confirmed by lack of toe-pinch response, each mouse will be placed on its back and a sterile 23 gauge ½" needle will be introduced directly into the heart through the chest cavity. The syringe plunger will be gently pulled to remove as much blood as possible from the mouse.

Lungs and other organs will be removed from the mice post-euthanasia as needed.

V.4.4.3. **Adjuvants**: TiterMax Gold contains the block copolymer CRL-8300, the metabolizable oil squalene, and a unique microparticulate stabilizer. Alhydrogel 2% is provided as a ready-to-use, sterile aluminium hydroxide wet gel (colloidal) suspension. TiterMax [1:1 (v/v)] will be resuspended with antigen in a total volume of 0.1 ml and will be given by i.p. injection. Alhydrogel 2% (139 µg Aluminum/dose) will be resuspended with antigen in a final volume of 0.1 ml and will be given by i.p. injection. The injections will be done 10-14 days apart with a tuberculin syringe attached to a 26 gauge 0.5 inch needle. Mice will be monitored daily after immunization. In the event of an adverse reaction to the adjuvant (injection site reaction), LAM veterinary staff will be consulted as to the proper course of action (alternative endpoint or veterinary treatment). Immunized mice that appear moribund will be sacrificed by isoflurane overdose and cervical dislocation.

V.4.4.4. **Monoclonal Antibody (MAbs) Production**: N/A

V.4.4.5. **Animal Identification:** Mice will be identified by cage cards and/or ear punch. Ear punches will be administered by (b)(6) staff (e.g. models EP-901 or EP-900, Braintree Scientific Inc., Braintree, MA 02185). (also see Section VI for staff qualifications).

V.4.4.6. **Behavioral Studies**: N/A

V.4.4.7. **Other Procedures:**

Inoculation or immunization by the intranasal route will take place under isoflurane anesthesia by the droplet method. Approximately 50 µl will be administered onto the nose of each mouse using a previously sterile pipet tip attached to a manual pipetman. The mouse will be allowed to inhale the droplet at will and then be returned to its cage.

Inoculation by the intratracheal route will take place under ketamine (100 mg/kg)-xylazine (10 mg/kg) anesthesia. Once anesthetized (defined as no response to toe pinch), each mouse will be placed on its back on a flat surface that can be elevated 75°. Paper tape will be applied to each arm to gently adhere the arms to the flat

surface. A hypodermic needle will be inserted into the flat surface ~5 cm from the top of the mouse's head. A rubber band will be looped over the needle and under the upper incisors of the open mouth of the mouse to tilt the neck backward and to hold the mouth open. The flat surface will be elevated to 75° such that the mouse's nose will face upward. The two flexible fiber-optic arms of a halogen light source will be placed on either side of the mouse's neck to provide transillumination of the trachea. The tongue will be held to one side of the mouth with forceps. A sterile 68.6 mm gel-loading pipette tip, which is tapered for 25 mm to a narrow tip, will be attached to a P-100 pipettor and the 50 µl inoculum will be aspirated into the tip. The tip will be inserted into the trachea and the inoculum will be delivered by gentle and constant depression of the pipettor plunger. The tip will be removed from the mouse, the rubber band will be removed from the teeth, and the mouse will remain elevated for ~1 min to allow the inoculum to flow into the lungs by gravity. The tape will then be removed from the arms and the mouse will be returned to its cage to recover from anesthesia.

Imaging of mice: Anesthetized mice will be placed on a warm platform within the Carestream Multispectral *in vivo* FX Pro imaging system and monitored for fluorescence for 5 min at a time at 0.5, 1, 3, 6, 12, 24, 48, and/or 72 h post-inoculation.

Photography: Mice may be photographed during inoculation procedures, during *in vivo* imaging, and post-mortem. Photos of inoculation procedures will only be used for teaching rodent handling techniques to new members of the laboratory. Photos from *in vivo* imaging will be used in seminars presented by approved users on this protocol and may be published in research articles. No photos will be taken of mice that are visibly moribund. The photos will be stored only on password-protected DoD computers at USUHS. Photos will be disposed of 5 years after the end of the project.

V.4.4.8. Tissue Sharing: As many of our experiments involve infected/spore-treated animals or tissues, we do not participate in any shared tissue and/or control group studies with other investigators.

V.4.5. Study Endpoint: Table V.4.5.1 below summarizes the endpoint for each proposed experiment. The study endpoint for virulence and challenge studies with infected mice will be death or euthanasia approximately 14 days post-inoculation with spores. The study endpoint for mice immunized with various exosporium protein preparations or capsule preparations and inoculated with *B. anthracis* or *B. cereus* spores will occur 43-70 days post immunization (14 days post-inoculation). The study endpoint for mice monitored for fluorescence will be 72 h or 2 weeks post-infection. For all studies, mice will be weighed daily to determine if a correlation between weight loss and eventual death exists. Investigator and staff will carefully monitor *Bacillus*-infected animals and euthanize those that appear moribund because it is our experience that these animals will ultimately succumb to *Bacillus* infection after inoculation. We define extremely moribund as when a mouse exhibits 2 or more of the following symptoms: ruffled fur, lethargy, hunched posture, impaired ambulation that prevents the animals from reaching food and water, excessive weight loss/emaciation,

difficult or labored breathing, and the inability to remain upright. Death due to infection after i.n., i.t., s.c., or i.p. inoculation with *B. anthracis* and *B. cereus* typically occurs 3-5 days post-inoculation. During this time period, mice will be inspected every 4 h during normal working hours and every 10-12 h during non-business hours. All inspections will be noted on cage cards so that LAM personnel are aware that the mice are being monitored.

Table V4.5.1. Endpoint and time to endpoint for each study in proposal			
Experiment	*Number of mice*	*Endpoint*	*Time to endpoint*
V.1.1.1	256	Death or sacrifice when moribund	14 days
V.1.1.2	3440	Death or sacrifice when moribund	14 days
V.1.2	210	Death or sacrifice when moribund	14 days
V.1.3.1	720	Death or sacrifice when moribund	14 days
V.1.3.2.1	240	Euthanasia	0.5, 1, 6, 12, 24, 48, 72 hrs, 14 days
V.1.3.2.2	240	Euthanasia	0.5, 1, 6, 12, 24, 48, 72 hrs, 14 days
V.1.3.2.3	110	Euthanasia	0.5 hr, 2, 4, 7, 14, 21, 28, 35, 42, 49, 56 days
V.1.4.1.1	25	Euthanasia	7 days
V.1.4.1.2	360	Death or sacrifice when moribund	14 days
V.1.4.2.1	250	Euthanasia	7 days
V.1.4.2.2	840	Death or sacrifice when moribund	14 days
V.1.5.1	80	Euthanasia	29 days
V.1.5.2	108	Euthanasia	0.5, 2, 6, 12, 24, 48, 72, 96 hrs, 7 days
V.1.5.3	750	Death or sacrifice when moribund	14 days

V.4.6. Euthanasia: Mice will be euthanized by either isoflurane overdose (4-5% concentration) within the isoflurane induction chamber located inside a fume hood, followed by cervical dislocation, or by CO_2 overdose. CO_2 will be delivered from a compressed gas cylinder via a regulated rate of flow in accordance with the 2007 AVMA Committee on Euthanasia. For euthanasia using CO_2, all procedures will be performed by LAM personnel in LAM facilities. Investigative staff will perform isoflurane and cervical dislocation euthanasia procedures; death will be confirmed by performing bilateral thoracotomy (as listed in the 2007 AVMA Guidelines on Euthanasia).

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6) (b)(6)

V.5.1.2. Special Husbandry Provisions: Care must be taken to avoid spread of the *Bacillus* among mice in other cages. Mice infected with *Bacillus* will be left in filter-top cages in (b)(6) To minimize spore dissemination, our laboratory staff will be responsible for changing cages and feeding/watering our mice once they have been inoculated. With respect to infected animals, all bedding, animal carcasses and wastes should be treated as infectious and autoclaved. Mouse carcasses will be treated as biohazard waste and will be double bagged and labeled with a burn tag. Gloves, lab coats and masks should be worn by all personnel when handling potentially infectious material.

Food Restriction: Yes _____ No X

Fluid Restriction: Yes _____ No X

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be observed and cared for by the Center for LAM personnel according to USUHS Center for LAM SOPs. In addition, infected mice will be cared for and checked twice daily or more frequently as needed by members of Dr. (b)(6) laboratory staff working on this project.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING: Dr. (b)(6) who will not do these "hands on" experiments herself, has 35 years experience working with mice in microbicidal assays and has been involved with the mouse anthrax model since 2002. Dr. (b)(6) is a senior scientist who has worked on the anthrax project since 2009 and has taken the Investigator Training Course. Dr. (b)(6) has 2 years experience working with rodents. Dr. (b)(6) is a postdoctoral fellow with 7 years of experience working with rodents and has taken the Investigator Training Course. Dr. (b)(6) has 15 years experience using animal models of infection with various organisms; she has taken the USUHS Investigator Training Course and a rodent handling course at another institution. LTC (b)(6) VMD, is a veterinary pathologist in the United States Army. He has been a veterinarian for 16 years and has 2.5 years of experience with mouse

models of infection. Mr. (b)(6) is a senior technician who has worked for 20years with EHEC/EPEC infection in mice and 7 years with anthrax models. Mr. (b)(6) has 6 years of experience working in our laboratory with mouse models of anthrax disease. Finally, Mr. (b)(6) has no experience working with rodents but has taken the Investigator Training Course. He will be trained by Mr. (b)(6) and Mr. (b)(6) Please see the table below for a specific breakdown of the procedures that each individual will perform and each person's training and experience.

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Intranasal inoculation	(b)(6)	Senior Scientist, 15 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007)
Intranasal inoculation		Senior Scientist, 2 yr experience	1 (2009), 4 (2009-2011, Mr. (b)(6)
Intranasal inoculation		Postdoctoral Fellow, 6.5 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr. (b)(6) 5 (UVA, 2005)
Intranasal inoculation		Graduate Student, 2 yrs experience	1 (2009), 3 & 5 (University of Pennsylvania School of Veterinary Medicine, 1995)
Intranasal inoculation		Senior Research Technician, 20 yrs experience	1, 4 (FDA (b)(6) 2004)
Intranasal inoculation		Research Technician, 7 yr experience	1, 4 (Drs. (b)(6) (b)(6) 2004)
Intranasal inoculation		Research Technician, 1 yr experience	1 (2010), 4 (2011, Mr. (b)(6)
Subcutaneous injection		Senior Scientist, 15 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007)
Subcutaneous injection		Senior Scientist, 2 yr experience	1 (2009), 4 (2009-2011, Mr (b)(6)
Subcutaneous injection		Postdoctoral Fellow, 6.5 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr. (b)(6) 5 (UVA, 2005)
Subcutaneous injection		Graduate Student, 2 yrs experience	1 (2009), 3 & 5 (University of Pennsylvania School of Veterinary Medicine, 1995)
Subcutaneous injection		Senior Research Technician, 20 yrs experience	1 (1992), 4 (Dr (b)(6) (b)(6) 1995)
Subcutaneous injection		Research Technician, 7 yr experience	1, 4 (Drs. (b)(6) (b)(6) 2004)
Subcutaneous injection		Research Technician, 1 yr experience	1 (2010), 4 (2011, Mr. (b)(6)
Intratracheal inoculation		Senior Scientist, 15 yr experience	1 (2008), 3 (1997), 5 (UW, 2004)
Intratracheal inoculation		Senior Scientist, 2 yr experience	1 (2009), 4 (2011, Mr. (b)(6)

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Intratracheal inoculation	(b)(6)	Postdoctoral Fellow, 6.5 yr experience	1 (2011), 3 (UVA, 2005), 4 (2012, Mr. (b)(6) 5 (UVA, 2005)
Intratracheal inoculation		Graduate Student, 2 yrs experience	1 (2009), 3 & 5 (University of Pennsylvania School of Veterinary Medicine, 1995)
Intratracheal inoculation		Senior Research Technician, 20 yrs experience	1, 4 (Mr. (b)(6) 2012)
Intratracheal inoculation		Research Technician, 7 yr experience	1, 4 (Dr. (b)(6) 2011)
Intratracheal inoculation		Research Technician, 1 yr experience	1 (2010), 4 (2012, Mr. (b)(6)
Intraperitoneal injection		Senior Scientist, 15 yr experience	1 (2006), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007)
Intraperitoneal injection		Senior Scientist, 2 yr experience	1 (2009), 4 (2009-2011, Mr. (b)(6)
Intraperitoneal injection		Postdoctoral Fellow, 6.5 yr experience	1 (2011), 3 (UVA, 2005), 5 (UVA, 2006)
Intraperitoneal injection		Graduate Student, 2 yrs experience	1 (2009), 3 & 5 (University of Pennsylvania School of Veterinary Medicine, 1995)
Intraperitoneal injection		Senior Research Technician, 20 yrs experience	1 (1992), 4 (Dr. (b)(6) (b)(6) 1995)
Intraperitoneal injection		Research Technician, 7 yr experience	1, 4 (Drs. (b)(6) (b)(6) 2004)
Intraperitoneal injection		Research Technician, 1 yr experience	1 (2010), 4 (2011, Mr. (b)(6)
Isoflurane anesthesia		Senior Scientist, 15 yr experience	1 (2006), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007)
Isoflurane anesthesia		Senior Scientist, 2 yr experience	1 (2009), 4 (2009-2011, Mr. (b)(6)
Isoflurane anesthesia		Postdoctoral Fellow, 6.5 yr experience	1 (2011), 3 (UVA, 2005), 5 (UVA, 2006)
Isoflurane anesthesia		Graduate Student, 2 yrs experience	1 (2009), 3 & 5 (University of Pennsylvania School of Veterinary Medicine, 1995)
Isoflurane anesthesia		Senior Research Technician, 20 yrs experience	1 (1992), 4 (Dr. (b)(6) (b)(6) 1995)
Isoflurane anesthesia		Research Technician, 7 yr experience	1, 4 (Drs. (b)(6) (b)(6) 2004)
Isoflurane anesthesia		Research Technician, 1 yr experience	1 (2010), 4 (2011, Mr. (b)(6)

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
In vivo imaging	(b)(6)	Senior Scientist, 2 yr experience	1 (2009), 4 (2010, Carestream Healthcare)
In vivo imaging		Postdoctoral Fellow, 6.5 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Dr. (b)(6) 5 (UVA, 2005)
In vivo imaging		Graduate Student, 2 yrs experience	1 (2009), 3 & 5 (University of Pennsylvania School of Veterinary Medicine, 1995), 4 (2011, Dr. (b)(6)
In vivo imaging		Senior Research Technician, 20 yrs experience	1 (1992), 4 (4 (2011, Dr. (b)(6)
In vivo imaging		Research Technician, 7 yr experience	1, 4 (2011, Dr. (b)(6)
In vivo imaging		Research Technician, 1 yr experience	1 (2010), 4 (2011, Dr. (b)(6)
Blood collection: tail venipuncture or nick		Senior Scientist, 15 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007)
Blood collection: tail venipuncture or nick		Senior Scientist, 2 yr experience	1 (2009), 4 (2009-2011, Mr. (b)(6)
Blood collection: tail venipuncture or nick		Postdoctoral Fellow, 6.5 yr experience	1 (2011), 3 (UVA, 2005), 5 (UVA, 2005)
Blood collection: tail venipuncture or nick		Graduate Student, 2 yrs experience	1 (2009), 3 & 5 (University of Pennsylvania School of Veterinary Medicine, 1995)
Blood collection: tail venipuncture or nick		Senior Research Technician, 20 yrs experience	1 (1992), 4 (Dr. (b)(6) (b)(6) 1995)
Blood collection: tail venipuncture or nick		Research Technician, 7 yr experience	1, 4 (Drs. (b)(6) (b)(6) 2004)
Blood collection: tail venipuncture or nick		Research Technician, 1 yr experience	1 (2010), 4 (2011, Mr. (b)(6)
Exsanguination by cardiac puncture		Senior Scientist, 15 yr experience	1 (2008), 3 (1997), 5 (UW 2004)
Exsanguination by cardiac puncture		Senior Scientist, 2 yr experience	1 (2009), 4 (2009-2011, Mr. (b)(6)
Exsanguination by cardiac puncture		Postdoctoral Fellow, 6.5 yr experience	1 (2011), 3 (UVA, 2005), 5 (UVA, 2005)
Exsanguination by cardiac puncture		Graduate Student, 2 yrs experience	1 (2009), 3 & 5 (University of Pennsylvania School of Veterinary Medicine, 1995), 4 (Mr. (b)(6) 2010)
Exsanguination by cardiac puncture		Senior Research Technician, 20 yrs experience	1 (1992), 4 (Mr. (b)(6) 2007)

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Exsanguination by cardiac puncture	(b)(6)	Research Technician, 7 yr experience	1, 4 (Drs. (b)(6) (b)(6) 2004)
Exsanguination by cardiac puncture		Research Technician, 1 yr experience	1 (2010), 4 (2011, Mr. (b)(6)
Euthanasia with isoflurane and cervical dislocation		Senior Scientist, 15 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007)
Euthanasia with isoflurane and cervical dislocation		Senior Scientist, 2 yr experience	1 (2009), 4 (2009-2011, Mr. (b)(6)
Euthanasia with isoflurane and cervical dislocation		Postdoctoral Fellow, 6.5 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr. (b)(6) 5 (UVA, 2005)
Euthanasia with isoflurane and cervical dislocation		Graduate Student, 2 yrs experience	1 (2009), 3 & 5 (University of Pennsylvania School of Veterinary Medicine, 1995)
Euthanasia with isoflurane and cervical dislocation		Senior Research Technician, 20 yrs experience	1 (1992), 4 (Dr. (b)(6) (b)(6) 1995)
Euthanasia with isoflurane and cervical dislocation		Research Technician, 7 yr experience	1, 4 (Drs. (b)(6) (b)(6) 2004)
Euthanasia with isoflurane and cervical dislocation		Research Technician, 1 yr experience	1 (2010), 4 (2011, Mr. (b)(6)
Ear punch		Senior Scientist, 15 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007)
Ear punch		Senior Scientist, 2 yr experience	1 (2009), 4 (2009-2011, Mr (b)(6)
Ear punch		Postdoctoral Fellow, 6.5 yr experience	1 (2011), 3 (UVA, 2005), 5 (UVA, 2005)
Ear punch		Graduate Student, 2 yrs experience	1 (2009), 3 & 5 (University of Pennsylvania School of Veterinary Medicine, 1995)
Ear punch		Senior Research Technician, 20 yrs experience	1 (1992), 4 (Dr (b)(6) (b)(6) 1995)
Ear punch		Research Technician, 7 yr experience	1, 4 (Drs. (b)(6) (b)(6) 2004)
Ear punch		Research Technician, 1 yr experience	1 (2010), 4 (2011, Mr. (b)(6)

1 USUHS Investigator Training Course
2 USUHS Rodent Handling Course
3 Other Rodent Handling Course
4 Site Specific Training
5 External Course Training

VII. **BIOHAZARDS/SAFETY:** With respect to mice used in the anthrax model, bedding may be contaminated with *Bacillus* spores. The bedding and animal carcasses should be treated as infectious and autoclaved. Infected animals will be maintained under ABSL-2 conditions in filter-top cages. All procedures that involve the use of isoflurane will be conducted in either a chemical fume hood (euthanasia) or in an anesthesia system with waste-scavenging charcoal filters (both located in Dr. (b)(6) laboratory). In addition, all personnel who will use isoflurane will be made aware of its associated safety hazards and will be properly trained in its use.

All personnel will wear a disposable gown, gloves, and mask at all times when handling mice. A N95 respirator mask will be worn during inoculation procedures and inoculation procedures will be conducted in a Type II Biosafety Cabinet (in (b)(6) when possible. Sharps will be disposed of in approved Sharps containers located in (b)(6) and Sharps containers will be disposed of as Regulated Medical Waste through the EHS office. All cage changes will be done in a Type II Biosafety Cabinet (in (b)(6)

VIII. **ENCLOSURES:** Funding document

1. **Akoachere, M., R. C. Squires, A. M. Nour, L. Angelov, J. Brojatsch, and E. Abel-Santos.** 2007. Identification of an *in vivo* inhibitor of *Bacillus anthracis* spore germination. J Biol Chem **282**:12112-12118.
2. **Aubrecht, J., M. E. Goad, and R. H. Schiestl.** 1997. Tissue specific toxicities of the anticancer drug 6-thioguanine is dependent on the Hprt status in transgenic mice. J Pharmacol Exp Ther **282**:1102-1108.
3. (b)(6)
4. **Brown, R. H., D. M. Walters, R. S. Greenberg, and W. Mitzner.** 1999. A method of endotracheal intubation and pulmonary functional assessment for repeated studies in mice. J Appl Physiol **87**:2362-2365.
5. (b)(6)
6.
7. **Drysdale, M., S. Heninger, J. Hutt, Y. Chen, C. R. Lyons, and T. M. Koehler.** 2005. Capsule synthesis by *Bacillus anthracis* is required for dissemination in murine inhalation anthrax. EMBO J **24**:221-227.
8. **Fieldhouse, R. J., Z. Turgeon, D. White, and A. R. Merrill.** 2010. Cholera- and anthrax-like toxins are among several new ADP-ribosyltransferases. PLoS Comput Biol **6**:e1001029.
9. **Glomski, I. J., F. Dumetz, G. Jouvion, M. R. Huerre, M. Mock, and P. L. Goossens.** 2008. Inhaled non-capsulated *Bacillus anthracis* in A/J mice: nasopharynx and alveolar space as dual portals of entry, delayed dissemination, and specific organ targeting. Microbes Infect **10**:1396-1404.
10. **Glomski, I. J., A. Piris-Gimenez, M. Huerre, M. Mock, and P. L. Goossens.** 2007. Primary involvement of pharynx and peyer's patch in inhalational and intestinal anthrax. PLoS Pathog **3**:e76.
11. **Hoffmaster, A. R., J. Ravel, D. A. Rasko, G. D. Chapman, M. D. Chute, C. K. Marston, B. K. De, C. T. Sacchi, C. Fitzgerald, L. W. Mayer, M. C. Maiden, F. G. Priest, M. Barker, L. Jiang, R. Z. Cer, J. Rilstone, S. N. Peterson, R. S. Weyant, D. R. Galloway, T. D. Read, T. Popovic,**

and C. M. Fraser. 2004. Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. Proc Natl Acad Sci U S A 101:8449-8454.

12. Leysath, C. E., K. H. Chen, M. Moayeri, D. Crown, R. Fattah, Z. Chen, S. R. Das, R. H. Purcell, and S. H. Leppla. 2011. Mouse monoclonal antibodies to anthrax edema factor protect against infection. Infect Immun 79:4609-4616.
13. Liu, S., S. Miller-Randolph, D. Crown, M. Moayeri, I. Sastalla, S. Okugawa, and S. H. Leppla. 2010. Anthrax toxin targeting of myeloid cells through the CMG2 receptor is essential for establishment of *Bacillus anthracis* infections in mice. Cell Host Microbe 8:455-462.
14. Lyons, C. R., J. Lovchik, J. Hutt, M. F. Lipscomb, E. Wang, S. Heninger, L. Berliba, and K. Garrison. 2004. Murine model of pulmonary anthrax: kinetics of dissemination, histopathology, and mouse strain susceptibility. Infect Immun. 72:4801-4809.
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19. (b)(6)
20.
21. Welkos, S. L., and A. M. Friedlander. 1988. Pathogenesis and genetic control of resistance to the Sterne strain of *Bacillus anthracis*. Microb Pathog 4:53-69.
22. Welkos, S. L., T. J. Keener, and P. H. Gibbs. 1986. Differences in susceptibility of inbred mice to *Bacillus anthracis*. Infect Immun. 51:795-800.
23 (b)(6)

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 12/15/11

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 12/15/11

Principal Investigator Signature Date

I. **Painful Procedure(s):**

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) _____ 9/15/11

Principal Investigator Signature Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number: [redacted]

B. Animal Protocol Title: New Therapies and Vaccines against *Bacillus anthracis* (mice)

C. Principal Investigator: [redacted] Ph.D.

D. Performing Organization: USUHS

E. Funding: Biological Defense Research Directorate, Naval Medical Research Center

F. Objective and Approach: *Bacillus anthracis*, a Gram-positive spore-forming rod, is the causative agent of human anthrax, which can manifest as cutaneous, gastrointestinal or inhalational disease depending on the route of inoculation. Although it has long been recognized that the spores of *B. anthracis* could be used as a weapon of biowarfare and/or bioterrorism, no successful nefarious application of the microbe in the United States was reported until October 4th, 2001. From then until December 2001, 22 cases of confirmed or suspected bioterrorism-related anthrax occurred, 11 of which were inhalational. A prevailing health concern is that the people who receive antibiotic prophylaxis, as was the case for hundreds of people who were exposed in 2001, may present with inhalational anthrax after the conclusion of their therapy as dormant viable spores germinate. One way to increase the likelihood of patient survival as well as enhance the probability of protection for those exposed to *B. anthracis* spores is to prevent the germination of the infectious dormant spores into active vegetative cells. In the proposed studies, we will characterize the protective efficacy of immunizing mice with *B. anthracis* spores or spore surface (called the exosporium) proteins prior to inoculation with *B. anthracis* Sterne spores (avirulent for humans but virulent for mice) via intratracheal or subcutaneous routes. In addition, we will assess the efficacy of small molecule inhibitors of spore germination that we have identified and characterized *in vitro*. The proposed immunization and small molecule inhibitor studies will be enhanced by our ability to monitor germination, outgrowth and dissemination in the mouse through the application of an *in vivo* imaging system using spores that are fluorescent. Further, we will broaden the scope of our studies to investigate the factors of *B. cereus* that allow it to cause human disease using approaches similar to those described above for *B. anthracis*. *B. cereus* causes gastroenteritis and eye infections in otherwise healthy individuals and can cause sepsis, meningitis, and endocarditis in immunocompromised persons. The studies proposed here utilize the minimum number of mice, which are the best small animal model for *Bacillus* studies, while maintaining sufficient power to accurately assess the stages of *B. anthracis* and *B. cereus* infection.

G. Indexing Terms (Descriptors): animals, mice, *Bacillus anthracis*, *Bacillus cereus*, small molecule inhibitors, immunization, exosporium, capsule, *in vivo* imaging, fluorescence, toxin, anthrax

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

December 19, 2014

MEMORANDUM FOR DR (b)(6)
(b)(6)

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on December 19, 2014:

Title of Application: "Use of Salmonella Typhimurium Enterobacterial Common Antigen Mutants as a Vaccine and Vehicle for Heterologous Antigen Delivery (Mice/Mus musculus))"

USUHS Protocol Number: (b)(6)

Expiration Date: December 18, 2017

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc: Office of Research

Revised

IACUC Date Stamp

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Use of *Salmonella* Typhimurium Enterobacterial Common Antigen Mutants as a Vaccine and Vehicle for Heterologous Antigen Delivery (Mice/*Mus musculus*)

GRANT TITLE (if different from above): *Salmonella* Typhimurium Mutants as a Vaccine and Heterologous Antigen Delivery System

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: USUHS

EARLIEST ANTICIPATED FUNDING START DATE: 10/1/2014

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6) _____ (b)(6) _____ 10/29/14

Principal Investigator Signature | Department Office/Lab Telephone | Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) _____ (b)(6) _____ 10/29/14

Research Unit Chief / Dept. Head Signature | Title Telephone | Date

Typed Name (b)(6) Ph.D.

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) _____ (b)(6) _____ 10/29/14

Statistician Signature | Department Telephone | Date

Typed Name (b)(6) Ph.D.

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) _____ LAM (b)(6) _____ 10/31/14

an Signature | Department | Telephone | Date

DVM

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

ANIMAL PROTOCOL TITLE: Use of *Salmonella* Typhimurium Enterobacterial Common Antigen Mutants as a Vaccine and Vehicle for Heterologous Antigen Delivery (Mice/*Mus musculus*)

GRANT TITLE (if different from above): *Salmonella* Typhimurium Mutants as a Vaccine and Heterologous Antigen Delivery System

USUHS PROJECT NUMBER (b)(6)

CO-INVESTIGATOR(S):

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS: Most infectious diseases initiate at mucus membrane surfaces and 15-20 million adults and children acquire or die of these types of infections annually. *Salmonella* spp. are a group of bacteria that causes diverse mucosal infections. Among this group, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is one of the most prominent strains to cause infection. As we assumed that this knowledge could help with the design of effective vaccines to prevent *Salmonella* infections, our previous studies sought to identify genes of the bacterium that were important for surviving in the host and causing disease. We identified a glycolipid known as ECA, which is ubiquitously found on the surface of *S.* Typhimurium as well as other closely related bacteria. We showed that ECA negative mutant strains did not cause disease in mice; however rather than being cleared from the animals, these mutant strains set up a persistent infection that lasts for long periods of time. Excitingly, immunization of mice with ECA negative mutant strains provided protection against a subsequent lethal challenge with wild type *S.* Typhimurium. Thus, ECA negative strains of *Salmonella* may be useful as live-attenuated vaccine strains, or as vehicles for delivery of vaccine candidates from other bacteria. The goal of the current proposal is to further analyze the potential usefulness of these strains. Specifically, we anticipate that our studies will: 1) provide insights into the efficacy of these vaccines to induce protection to other closely related bacterial species, and 2) provide functional data that support our hypothesis that these ECA mutants will be effective carriers for antigens from other bacteria.

II. BACKGROUND:

II.1. Background: Given that the human gastrointestinal mucosa is very large and that the acquisition of most infectious diseases occurs at mucosal surfaces, it is not surprising that 15-20 million adults and children acquire or die of mucosal infections annually. *Salmonella* is one bacterial genus that causes diverse mucosal infections. In

the United States, non-typhoidal *Salmonella spp.* cause an estimated 1.4 million cases of salmonellosis annually and account for greater than 500 deaths per year. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is one of the most prominent strains. Economically, it has been estimated that Salmonellosis costs up to $50 million per year in the U.S. as a result of medical expenses and work absences. This issue is particularly relevant to the military because of the necessity to safely feed our troops; many of our troops are stationed in countries with underdeveloped health and hygiene practices so that food preparation is a particular health issue, as is a source of clean water.

To understand the dynamic and complex interactions between the human host and *Salmonella* so that treatment modalities and therapeutic strategies can be developed, both virulence determinants of the pathogen and the relevant host factors must be identified and analyzed. One experimental approach to resolving and combating *Salmonella* infections is to develop efficacious vaccines. Effective vaccines can be generated by identification of virulence factors on the bacterium and constructing mutants that do not express these molecules.

We have identified mutants of a *Salmonella* cell surface virulence factor that appear to be efficacious live vaccine candidates. Specifically, *S.* Typhimurium, a Gram-negative enteric bacterium and a member of the Enterobacteriacae family, expresses the cell surface glycolipid that is ubiquitous among all family members: the phophoglyceride-linked Enterobacterial common antigen (ECA_{PG}). We have shown that ECA negative mutant strains were severely attenuated for virulence when inoculated into mice. However, rather than being cleared from the animal, these mutant strains set up a persistent infection that is maintained for up to 70 days post infection, which was the latest day we monitored colonization. Excitingly, immunization of mice with ECA negative mutant strains provided protection against a subsequent lethal oral or intraperitoneal challenge with wild type *S.* Typhimurium. Thus, our goal is to investigate usefulness of ECA negative strains of *Salmonella* as live-attenuated vaccine strains, or as vehicles for heterologous antigen expression.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: The DoD Biomedical Research Database (BRD), NIH Research Portfolio Online Reporting Tools (RePORTER), and PubMed (includes Medline) were searched.

II.2.2. Date of Search: 2 September 2014

II.2.3. Period of Search: DoD BRD – 1998-2009; NIH RePORTER – 1986-2014; Pubmed 1950-2014

II.2.4. Key Words and Search Strategy: Included *Salmonella*, *S.* Typhimurium, mice, vaccine, ECA

II.2.5. Results of Search: From the BRD database, the search terms "*Salmonella* and vaccine", "*S.* Typhimurium and vaccine", and "*S.* Typhimurium and mice" retrieved 28,

13, and 11 studies, respectively. Several of the retrieved studies were performed at USUHS and provided the foundational research for the work proposed herein. The remaining studies did not duplicate this research protocol.

A search of the NIH RePORTER database using the terms "*Salmonella* and vaccine", "*S.* Typhimurium and vaccine", and "*Salmonella* and mice" returned 77, 20, and 137 studies, respectively. Several of these studies investigate the varying vaccine capabilities of *Salmonella*; however, none of the studies employ ECA mutants nor do they overlap the experiments proposed herein.

A PubMed (Medline) search using the search terms "*Salmonella* and vaccine", "*S.* Typhimurium and vaccine", and "*Salmonella* and mice" returned thousands of articles. However, none appear to be investigating the potential of ECA negative *S.* Typhimurium strains as vaccine candidates. Adding the term "ECA" to the "Salmonella and vaccine" search narrowed the results to 5 articles. One of these was a study published by our lab about our ECA negative strain as a vaccine candidate. The other 4 articles did not use ECA negative strains as potential vaccines.

III. OBJECTIVE\HYPOTHESIS: The goal of this protocol is to analyze the potential usefulness of ECA negative *S.* Typhimurium strains as vaccine candidates. Specifically, we anticipate that our studies will: 1) provide insights into the efficacy of these vaccines to induce protection to other Enterobacteriaceae family members, and 2)

IV. MILITARY RELEVANCE: *S.* Typhimurium, as well as other enteric bacterial infections, are and have been responsible for morbidity and mortality of troops stationed in countries with underdeveloped health and hygiene practices. Thus, the development of efficacious vaccines is vital to resolving and combating *Salmonella* infections, thereby reducing the morbidity and mortality associated with these infections.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1: Determination of cross-protection conferred by vaccination with ECA negative strains of *S.* Typhimurium.

Rationale: To date, our studies show that ECA negative *S.* Typhimurium strains provide protection against lethal challenge with the wildtype strain from which the mutants were derived. However, it is currently unclear if this protection extends across strains or species. Therefore, groups of C57BL/6 mice will be vaccinated with ECA negative derivatives of TML or SL1344, each of which are well-characterized clinical isolates. Vaccinated animals will subsequently be challenged with a typically lethal dose of wildtype derivatives of the strain not used for vaccination. Additionally, we will test the ability of the ECA negative strains to protect against challenge with *S.* Enteritidis, which is able to infect mice but expresses a different O-antigen.

Procedure: Single strain vaccination assays of female C57BL/6 mice will be performed according to published procedures using orogastric and intraperitoneal (IP) administration of ECA negative *S.* Typhimurium strains (Michetti, P *et al. Infect. Immun.* 60(5): 1786, 1992; Schmitt, CK *et al. J. Bacteriol.* 176(2): 368, 1994; Schmitt, CK *et al. J. Bacteriol.* 178(10): 2911, 1996; Lawley, TD *et al. PLoS Pathog.* 2(2): e11, 2006; Lawley, TD *et al. Infect. Immun.* 76(1):403, 2008). Vaccinated animals will subsequently be challenged with a lethal dose of wildtype derivatives of the *S.* Typhimurium strain not used for vaccination or a wildtype *S.* Enteritidis strain. The animals will be grouped into three immunization and challenge combinations: oral immunization with oral challenge, IP immunization with oral challenge, and IP immunization with IP challenge. Animals will be immunized with either the ECA$^-$ TML strain, the ECA$^-$ SL1344 strain, or PBS, and then subsequently challenged with either wildtype TML, wildtype SL1344, or wildtype *S.* Enteritidis.

Prior to orogastric administration of bacteria, animals will be fasted (food withheld but not water) for 2 hours prior to infection and then infected orogastrically with either PBS or a dose of one of the ECA mutant strains that was previously shown to provide protection against lethal challenge. Orogastric infection will be accomplished by passing a straight or curved stainless steel atraumatic ball-tipped gavage needle (20 gauge x 1-1/2) into the terminal esophagus or the stomach. This can be done quickly and safely under manual restraint by trained individuals. Because the entire path from the open mouth of the rodent to the nonglandular portion of the stomach is covered by a thick and resistant keratinized epithelium, injury is very rare. Gavage guarantees that the fluid suspension of bacteria is delivered to the stomach rather than being spit out or being inhaled (which could cause aspiration pneumonia). This is an important point since aspiration could lead to pulmonary infection. Dosing volumes of 0.1-0.2 ml (based on animal weight not to exceed 10mL/kg) will be used in the mice as this is well-tolerated. Animals will be observed immediately after dosing and then periodically over the next few minutes to ascertain that there is no sign of aspiration or trauma before they are returned to their cages. Dosed animals will be maintained without food or water for a period of at least 1 hour but not more than 2 hours to permit gastric processing of the PBS or bacterial suspension, but then will be returned to a normal diet thereafter.

A separate group of animals will be vaccinated via intraperitoneal injection with either PBS or a dose of one of the ECA mutant strains that was previously shown to provide protection against lethal challenge. The animals will receive 50-200 µL of PBS or bacterial suspension using 23-25 gauge needles following alcohol preparation of the site for injection. Animals will be monitored for at least 1 hour but not more than 2 hours after dosing for any signs of distress.

Fecal pellets will be collected on a weekly basis for PBS and ECA- vaccinated animals. Briefly, mice will be placed individually in a clean, empty cage until a fecal pellet is passed. The fecal pellet will be collected into a clean microcentrifuge tube for processing. The mouse will be returned to its cage, minimizing its disruption and discomfort.

Blood samples will be collected via standard tail vein bleeds from each group of vaccinated animals 1 day prior to challenge without the use of anesthesia. For these bleeds, a 26 gauge needle will be used. Mice will be weighed prior to the bleed to calculate the total circulating volume based on the approximation of 58.5mL total blood volume per kg, and the blood volume collected will not exceed 10% of the calculated total blood volume of the animal. Serum will then be collected from these samples and

used to monitor antibody titers using ELISA assays.

The orogastrically and intraperitoneally vaccinated animals will be challenged 30 days post-immunization using the oral gavage and IP injection methods as described above. The animals will be challenged with either wild-type *S.* Typhimurium TML or SL1344 strains or wildtype *S.* Enteritidis. The challenged animals will be initially monitored every 8 hours during the first week for mortality and onset of end-stage disease symptoms, including hunched poster, ruffled fur, weight loss, and poor mobility. The monitoring will continue once per day for an additional 23 days until the 30-day post-challenge time point is reached.Animals surviving at the 30-day timepoint will undergo terminal cardiac bleeding to assess antibody titers. For this, the animals will be placed in a compressed gas CO_2 inhalation chamber and observed. The chamber will not be overcrowded. Cylinderized carbon dioxide with a pressure gauge and flowmeter will be used as the source of carbon dioxide. With animals in the chamber, the flow rate of CO_2 will displace at least 20% of the cage volume per minute as indicated on the flowmeter. Animals will be watched to ensure that they are lying down and that their purposeful movements have stopped. The anesthesia cage will be opened and the animal will be checked for response to toe pinch. If animal responds to toe pinch, the lid of the chamber will be closed and carbon dioxide flow will continue until the animal is adequately anesthetized as indicated by lack of response to toe pinch. If there is no response to toe pinch then the carbon dioxide flow will be stopped. Animals will then be quickly removed from the chamber and a 22 or 23 gauge needle on a syringe will be used to pierce the thoracic wall and the chamber of the heart. At this point, the animal is narcotized (anesthetized) by CO_2. After sufficient blood is obtained, the needle will be removed and the animal will immediately be euthanized by cervical dislocation. In most cases, death will already have occurred by exsanguination.

As detailed below, we will need 216 mice to complete the experiment outlined above, and the study will be repeated once to verify obtained results. In addition, we request 10% extra animals for unforeseen problems.

Table 1 Vaccination Strategy to Determine Cross Protection Conferred by ECA- *S.* Typhimurium

Immunization		Mice		Challenge	
Strain	Route	Expt 1	Expt 2	Strain	Route
PBS	Oral	8	8	TML WT	Oral
PBS	Oral	8	8	SL1344 WT	Oral
PBS	Oral	8	8	S. Enteritidis WT	Oral
ECA- TML	Oral	8	8	TML WT	Oral
ECA- TML	Oral	8	8	SL1344 WT	Oral
ECA- TML	Oral	8	8	S. Enteritidis WT	Oral
ECA-SL1344	Oral	8	8	TML WT	Oral
ECA-SL1344	Oral	8	8	SL1344 WT	Oral
ECA-SL1344	Oral	8	8	S. Enteritidis WT	Oral
PBS	IP	8	8	TML WT	Oral
PBS	IP	8	8	SL1344 WT	Oral
PBS	IP	8	8	S. Enteritidis WT	Oral
ECA- TML	IP	8	8	TML WT	Oral
ECA- TML	IP	8	8	SL1344 WT	Oral
ECA- TML	IP	8	8	S. Enteritidis WT	Oral
ECA-SL1344	IP	8	8	TML WT	Oral
ECA-SL1344	IP	8	8	SL1344 WT	Oral
ECA-SL1344	IP	8	8	S. Enteritidis WT	Oral
PBS	IP	8	8	TML WT	IP
PBS	IP	8	8	SL1344 WT	IP
PBS	IP	8	8	S. Enteritidis WT	IP
ECA- TML	IP	8	8	TML WT	IP
ECA- TML	IP	8	8	SL1344 WT	IP
ECA- TML	IP	8	8	S. Enteritidis WT	IP
ECA-SL1344	IP	8	8	TML WT	IP
ECA-SL1344	IP	8	8	SL1344 WT	IP
ECA-SL1344	IP	8	8	S. Enteritidis WT	IP
	Total:	216	216		

Experiment totals:
[2 immunization strains X 3 immunization/challenge route combinations X 3 challenge strains X 8 animals per group = 144 animals, 1 mock-immunized group X 3 immunization/challenge route combinations X 3 challenge strains X 8 animals per group = 72 animals; 144 + 72 = 216 animals x 2 (biological repeats) = 432 animals]
Additional animals (~10%) = 43 mice
Grand total of animals requested for Experiment #1 = 475 mice

V.1.2. <u>Experiment #2:</u> Determine whether ECA negative strains of *S.* Typhimurium can be utilized as a vehicle for heterologous antigen delivery.

Rationale: Numerous lines of research suggest that the ability to utilize attenuated bacterial strains as vehicles for delivery of foreign antigens is an attractive means of developing novel vaccines. Moreover, regulated delayed antigen synthesis (RDAS) systems for use in recombinant attenuated *Salmonella* vaccine (RASV) strains have been shown to enhance downstream immune responses due to a reduction in the adverse effects of high-levels of antigen synthesis. To determine whether or not ECA negative strains can be utilized to this end, we will investigate the delivery of two potential heterologous antigens. Groups of BALB/c animals will be vaccinated with ECA negative strains bearing RDAS vectors encoding the *Streptococcus pneumoniae* PspA protein (carried on pPspA) or expressing the O antigen portion *of Pseudomonas*

aeruginosa LPS (carried on pLPS2). Importantly, these two antigens were chosen because prior studies in different RASV strains have shown that appropriate delivery of these antigens provides protection against *S. pneumoniae* and *P. aeruginosa* challenge, respectively.

Procedure:

Single strain vaccination assays of female BALB/c mice will be performed according to published procedures using orogastric and intraperitoneal (IP) administration of the ECA negative SL1344 *S.* Typhimurium strain, an ECA negative SL1344 strain carrying either pLPS2 or pPspA, or PBS. Vaccinated animals will subsequently be challenged with a lethal dose of wildtype SL1344 *S.* Typhimurium, wildtype *S. pneumoniae*, or wildtype *P. aeruginosa*.

Prior to orogastric administration of bacteria, animals will be fasted (food withheld but not water) for 2 hours prior to infection and then infected orogastrically with either the ECA negative SL1344 *S.* Typhimurium strain, an ECA negative SL1344 strain carrying either pLPS2 or pPspA, or PBS. Orogastric infection will be accomplished by passing a straight or curved stainless steel atraumatic ball-tipped gavage needle (20 gauge x 1-1/2) into the terminal esophagus or the stomach. This can be done quickly and safely under manual restraint by trained individuals. Because the entire path from the open mouth of the rodent to the nonglandular portion of the stomach is covered by a thick and resistant keratinized epithelium, injury is very rare. Gavage guarantees that the fluid suspension of bacteria is delivered to the stomach rather than being spit out or being inhaled (which could cause aspiration pneumonia). Dosing volumes of 0.1-0.2 ml (based on animal weight not to exceed 10mL/kg) will be used in the mice as this is well-tolerated. Animals will be observed immediately after dosing and then periodically over the next few minutes to ascertain that there is no sign of aspiration or trauma before they are returned to their cages. Dosed animals will be maintained without food or water for a period of at least 1 hour but not more than 2 hours to permit gastric processing of the PBS or bacterial suspension, but then will be returned to a normal diet thereafter.

In addition, a separate group of animals will be vaccinated via intraperitoneal injection with either the ECA negative SL1344 *S.* Typhimurium strain, an ECA negative SL1344 strain carrying either pLPS2 or pPspA, or PBS. The animals will receive 50-200 µL of PBS or bacterial suspension using 23-25 gauge needles following alcohol preparation of the site for injection. Animals will be monitored for at least 1 hour but not more than 2 hours after dosing for any signs of distress.

The orogastrically and intraperitoneally vaccinated animals will be challenged 30 days post-immunization. The animals will be challenged with either wildtype SL1344 *S.* Typhimurium, wildtype *S. pneumoniae*, or wildtype *P. aeruginosa*. *S.* typhimurium will be delivered via oral gavage, while *S. pneumoniae* and *P. aeruginosa* will be delivered as a bacterial suspension that is placed into the external nares of the animals. For these infections, mice will be anesthetized by intraperitoneal administration of a freshly prepared mixture of ketamine hydrochloride (65 mg/kg) and xylazine (10 mg/kg) in a 200uL volume. After anesthesia, mice will be held upright and 25uls of bacterial suspension will be placed within each nostril (50 uls total). This suspension is subsequently inhaled by the animals and results in inhalational pneumonia. Following anesthesia, the mice are placed on their backs next to each other and monitored until all have recovered from the anesthesia.

Fecal pellets will be collected on a weekly basis for PBS and ECA- vaccinated animals. Briefly, mice will be placed individually in a clean, empty cage until a fecal pellet is passed. The fecal pellet will be collected into a clean microcentrifuge tube for processing. The mouse will be returned to its cage, minimizing its disruption and discomfort.

Blood samples will be collected via standard tail vein bleeds from each group of vaccinated animals 1 day prior to challenge. For these bleeds, a 26 gauge needle will be used. Mice will be weighed prior to the bleed to calculate the total circulating volume based on the approximation of 58.5mL total blood volume per kg, and the blood volume collected will not exceed 10% of the calculated total blood volume of the animal. Serum will then be collected from these samples and used to monitor antibody titers against *S. pneumoniae* PspA and *P. aeruginosa* O antigen using ELISA assays. The challenged animals will be initially monitored every 8 hours during the first week for mortality and onset of end-stage disease symptoms, including hunched poster, ruffled fur, weight loss, and poor mobility. The monitoring will continue once per day for an additional 23 days until the 30-day post-challenge time point is reached. Animals surviving at the 30-day timepoint will undergo terminal cardiac bleeding to assess antibody titers. For this, the animals will be placed in a compressed gas CO_2 inhalation chamber and observed. The chamber will not be overcrowded. Cylinderized carbon dioxide with a pressure gauge and flowmeter will be used as the source of carbon dioxide. With animals in the chamber, the flow rate of CO_2 will displace at least 20% of the cage volume per minute as indicated on the flowmeter. Animals will be watched to ensure that they are lying down and that their purposeful movements have stopped. The anesthesia cage will be opened and the animal will be checked for response to toe pinch. If animal responds to toe pinch, the lid of the chamber will be closed and carbon dioxide flow will continue until the animal is adequately anesthetized as indicated by lack of response to toe pinch. If there is no response to toe pinch then the carbon dioxide flow will be stopped. Animals will then be quickly removed from the chamber and a 22 or 23 gauge needle on a syringe will be used to pierce the thoracic wall and the chamber of the heart. At this point, the animal is narcotized (anesthetized) by CO_2. After sufficient blood is obtained, the needle will be removed and the animal will immediately be euthanized by cervical dislocation. In most cases, death will already have occurred by exsanguination.

As detailed below, we will need 128 mice to complete the experiment outlined above, and the study will be repeated once to verify obtained results. In addition, we request 10% extra animals for unforeseen problems.

Table 2

Strategy for use of ECA- *S.* Typhimurium as a Vehicle for Heterologous Antigen Delivery

Immunization				Challenge
		Mice		
Strain	Route	Expt 1	Expt 2	Strain
PBS	Oral	8	8	SL1344 WT
PBS	Oral	8	8	*S. pneumoniae* WT
PBS	Oral	8	8	*P. aeruginosa* WT
ECA-SL1344	Oral	8	8	SL1344 WT
ECA-SL1344	Oral	8	8	*S. pneumoniae* WT
ECA-SL1344	Oral	8	8	*P. aeruginosa* WT
ECA-SL1344 carrying pPspA	Oral	8	8	*S. pneumoniae* WT
ECA-SL1344 carrying pLPS2	Oral	8	8	*P. aeruginosa* WT
PBS	IP	8	8	SL1344 WT
PBS	IP	8	8	*S. pneumoniae* WT
PBS	IP	8	8	*P. aeruginosa* WT
ECA-SL1344	IP	8	8	SL1344 WT
ECA-SL1344	IP	8	8	*S. pneumoniae* WT
ECA-SL1344	IP	8	8	*P. aeruginosa* WT
ECA-SL1344 carrying pPspA	IP	8	8	*S. pneumoniae* WT
ECA-SL1344 carrying pLPS2	IP	8	8	*P. aeruginosa* WT
	Total:	128	128	

Experiment totals:
[1 immunization strain X 2 immunization routes X 3 challenge strains X 8 animals per group = 48 animals, 1 mock-immunized group X 2 immunization routes X 3 challenge strains X 8 animals per group = 48 animals, 2 heterologous delivery strains X 2 immunization routes X 8 animals per group = 32 animals; 48 + 48 + 32 = 128 animals x 2 (biological repeats) = 256 animals]
Additional animals (~10%) = 26 mice
Grand total of animals requested for Experiment #2 = 282 mice

V.1.3 Experiment #3: Test the ability of an ECA- strain of *S.* Typhimurium to protect against lethal challenge with other *S.* Typhimurium strains.

Rationale: To date, our data indicate that intraperitoneal or orogastric vaccination with an SL1344 ECA- strain is able to provide complete protection from subsequent oral challenge with the wildtype *S.* Typhimurium TML strain. The dose of TML used for the challenge was 5.00E+04, which represents the approximate oral LD50 for this strain. Therefore, we need to validate our preliminary findings and determine the limits of protection. Therefore, groups of C57BL/6 mice will be inoculated with PBS or vaccinated with an ECA negative derivative of *S.* Typhimurium strain SL1344. The vaccinated animals will subsequently be challenged intaperitoneally with a dose equivalent to 1, 10, 100 or 1000 times the LD50 of the wildtype SL1344, TML or LT2 strains of *S.* Typhimurium. Extending our studies to higher doses of SL1344 and TML and including another well-studied virulent *S.* Typhimurium strain will allow us to make broader statements about the protection that we observe.

Procedure: Single strain vaccination assays of female C57BL/6 mice will be performed according to published procedures using orogastric and intraperitoneal

(IP) administration of ECA negative *S.* Typhimurium strains (Michetti, P *et al. Infect. Immun.* 60(5): 1786, 1992; Schmitt, CK *et al. J. Bacteriol.* 176(2): 368, 1994; Schmitt, CK *et al. J. Bacteriol.* 178(10): 2911, 1996; Lawley, TD *et al. PLoS Pathog.* 2(2): e11, 2006; Lawley, TD *et al. Infect. Immun.* 76(1):403, 2008). Vaccinated animals will subsequently be challenged with varying doses of wildtype derivatives of *S.* Typhimurium SL1344, TML or LT2. The animals will be grouped into two immunization and challenge combinations: oral immunization with IP challenge and IP immunization with IP challenge. Animals will be immunized with either the ECA$^-$ SL1344 strain or PBS, and then subsequently challenged with either wildtype SL1344, TML or LT2.

Prior to orogastric administration of bacteria, animals will be fasted (food withheld but not water) for 2 hours prior to infection and then infected orogastrically with either PBS or a dose of the ECA- SL1344 strain that was previously shown to provide protection against lethal challenge. Orogastric infection will be accomplished by passing a straight or curved stainless steel atraumatic ball-tipped 20 gauge gavage needle into the terminal esophagus or the stomach. This can be done quickly and safely under manual restraint by trained individuals without the use of anesthesia. Because the entire path from the open mouth of the rodent to the nonglandular portion of the stomach is covered by a thick and resistant keratinized epithelium, injury is very rare. Gavage guarantees that the fluid suspension of bacteria is delivered to the stomach rather than being spit out or being inhaled (which could cause aspiration pneumonia). This is an important point since aspiration could lead to pulmonary infection. Dosing volumes of 0.1-0.2 ml (based on animal weight not to exceed 10mL/kg) will be used in the mice as this is well-tolerated. Animals will be observed immediately after dosing and then periodically over the next few minutes to ascertain that there is no sign of aspiration or trauma before they are returned to their cages. Dosed animals will be maintained without food or water for a period of at least 1 hour but not more than 2 hours to permit gastric processing of the PBS or bacterial suspension, but then will be returned to a normal diet thereafter.

A separate group of animals will be vaccinated via intraperitoneal injection with either PBS or a dose of one of the ECA mutant strains that was previously shown to provide protection against lethal challenge. The animals will receive 50-200 μL of PBS or bacterial suspension using 23-25 gauge needles following alcohol preparation of the site for injection. Animals will be monitored for at least 1 hour but not more than 2 hours after dosing for any signs of distress.

Fecal pellets will be collected on a weekly basis for PBS and ECA- vaccinated animals. Briefly, mice will be placed individually in a clean, empty cage until a fecal pellet is passed. The fecal pellet will be collected into a clean microcentrifuge tube for processing. The mouse will be returned to its cage, minimizing its disruption and discomfort. Blood samples will be collected via standard tail vein bleeds from each group of vaccinated animals 1 day prior to challenge without the use of anesthesia. For these bleeds, a 26 gauge needle will be used. Mice will be weighed prior to the bleed to calculate the total circulating volume based on the approximation of 58.5mL total blood volume per kg, and the blood volume collected will not exceed 10% of the calculated total blood volume of the animal. Serum will then be collected from these samples and used to monitor antibody titers using ELISA assays.

The orogastrically and intraperitoneally vaccinated animals will be challenged 30 days post-immunization using the IP injection methods as described above. The animals will be challenged with wild-type *S.* Typhimurium SL1344, TML or

LT2. The challenged animals will be initially monitored every 8 hours during the first week for mortality and onset of end-stage disease symptoms, including hunched poster, ruffled fur, weight loss, and poor mobility. The monitoring will continue once per day for an additional 23 days until the 30-day post-challenge time point is reached. Animals surviving at the 30-day timepoint will undergo terminal cardiac bleeding to assess antibody titers. For this, the animals will be placed in a compressed gas CO_2 inhalation chamber and observed. The chamber will not be overcrowded. Cylinderized carbon dioxide with a pressure gauge and flowmeter will be used as the source of carbon dioxide. With animals in the chamber, the flow rate of CO_2 will displace at least 20% of the cage volume per minute as indicated on the flowmeter. Animals will be watched to ensure that they are lying down and that their purposeful movements have stopped. The anesthesia cage will be opened and the animal will be checked for response to toe pinch. If animal responds to toe pinch, the lid of the chamber will be closed and carbon dioxide flow will continue until the animal is adequately anesthetized as indicated by lack of response to toe pinch. If there is no response to toe pinch then the carbon dioxide flow will be stopped. Animals will then be quickly removed from the chamber and a 22 or 23 gauge needle on a syringe will be used to pierce the thoracic wall and the chamber of the heart. At this point, the animal is narcotized (anesthetized) by CO_2. After sufficient blood is obtained, the needle will be removed and the animal will immediately be euthanized by cervical dislocation. In most cases, death will already have occurred by exsanguination.

As detailed below, we will need 480 mice to complete the experiment outlined above, and the study will be repeated once to verify obtained results. In addition, we request 10% extra animals for unforeseen problems.

Immunization		Challenge		
Strain	Route	S. Typhimurium	Dose x LD_{50}	Mice
PBS	Oral	SL1344 WT	1, 10, 100, 1000	10/dose
ECA-SL1344	Oral	SL1344 WT	1, 10, 100, 1000	10/dose
PBS	Oral	TML	1, 10, 100, 1000	10/dose
ECA-SL1344	Oral	TML	1, 10, 100, 1000	10/dose
PBS	Oral	LT2	1, 10, 100, 1000	10/dose
ECA-SL1344	Oral	LT2	1, 10, 100, 1000	10/dose
Strain	Route	S. Typhimurium	Dose x LD_{50}	Mice
PBS	[illegible]	SL1344 WT	1, 10, 100, 1000	10/dose
ECA-SL1344	[illegible]	SL1344 WT	1, 10, 100, 1000	10/dose
PBS	[illegible]	TML	1, 10, 100, 1000	10/dose
ECA-SL1344	[illegible]	TML	1, 10, 100, 1000	10/dose
PBS	[illegible]	LT2	1, 10, 100, 1000	10/dose
ECA-SL1344	[illegible]	LT2	1, 10, 100, 1000	10/dose

Experiment totals:
[1 immunization strain X 2 immunization/challenge route combinations X 3 challenge strains X 4 doses X 10 animals per group = 240 animals, 1 mock-vaccinated group X 2 immunization/challenge route combinations X 3 challenge strains X 4 doses X 10 animals per group = 240 animals; 240 + 240 = 480 animals X 2 (biological repeats) = 960 animals]
Additional animals (~10%) = 96 mice
Grand total of animals requested for Experiment #3 = 1,056 mice

V.1.4. Experiment #4: Test the ability of a ECA- strain of *S.* Typhimurium to protect against lethal challenge with other *Salmonella* serovars.

Rationale: To date, our data indicate that intraperitoneal vaccination with the ECA- strain SL1344 is able to provide partial protection against challenge with greater than 10,000 LD50 of the *S.* Enteriditis R11 strain. While no protection was seen from animals that were vaccinated orally, we did observe a slight but insignificant increase in the MTD (mean time to death) of the vaccinated animals. To validate our preliminary IP findings and determine whether higher levels of protection can be obtained using decreased doses of the R11 challenge strain, we will repeat these studies. Additionally we will explore cross protection against two other common *Salmonella* serovars. Therefore, groups of C57BL/6 mice will be inoculated with PBS or vaccinated with an ECA negative derivative of SL1344 and subsequently challenged intaperitoneally with a dose equivalent to 1, 10, 100 or 1000 times the LD50 of wildtype R11 *S.* Enteriditis, *S.* Stanleyville strain J65 or the *S.* Dublin strain R17. This expansion of our studies will allow us to make broader statements about the protection that we observe.

Procedure: Single strain vaccination assays of female C57BL/6 mice will be performed according to published procedures using orogastric and intraperitoneal (IP) administration of PBS and ECA negative *S.* Typhimurium strains (Michetti, P *et al. Infect. Immun.* 60(5): 1786, 1992; Schmitt, CK *et al. J. Bacteriol.* 176(2): 368, 1994; Schmitt, CK *et al. J. Bacteriol.* 178(10): 2911, 1996; Lawley, TD *et al. PLoS Pathog.* 2(2): e11, 2006; Lawley, TD *et al. Infect. Immun.* 76(1):403, 2008). Vaccinated animals will subsequently be challenged with varying doses of wildtype derivatives of R11 *S.* Enteriditis, J65 *S.* Stanleyville or R17 *S.* Dublin. The animals will be grouped into two immunization and challenge combinations: oral immunization with IP challenge and IP immunization with IP challenge. Animals will be immunized with either the ECA- SL1344 strain or PBS, and then subsequently challenged with either R11 *S.* Enteriditis, J65 *S.* Stanleyville or R17 *S.* Dublin.

Prior to orogastric administration of bacteria, animals will be fasted (food withheld but not water) for 2 hours prior to infection and then infected orogastrically with either PBS or a dose of the ECA- SL1344 strain that was previously shown to provide protection against lethal challenge. Orogastric infection will be accomplished by passing a straight or curved stainless steel atraumatic ball-tipped 20 gauge gavage needle into the terminal esophagus or the stomach. This can be done quickly and safely under manual restraint by trained individuals without the use of anesthesia. Because the entire path from the open mouth of the rodent to the nonglandular portion of the stomach is covered by a thick and resistant keratinized epithelium, injury is very rare. Gavage guarantees that the fluid suspension of bacteria is delivered to the stomach rather than being spit out or being inhaled (which could cause aspiration pneumonia). This is an important point since aspiration could lead to pulmonary infection. Dosing volumes of 0.1-0.2 ml (based on animal weight not to exceed 10mL/kg) will be used in the mice as this is well-tolerated. Animals will be observed immediately after dosing and then periodically over the next few minutes to ascertain that there is no sign of aspiration or trauma before they are retumed to their cages. Dosed animals will be maintained without food or water for a period of at least 1 hour but not more than 2 hours to permit gastric processing of the PBS or bacterial suspension, but then will be returned to a normal diet thereafter.

A separate group of animals will be vaccinated via intraperitoneal injection with either PBS or a dose of one of the ECA- SL1344 strain that was previously shown to provide protection against lethal challenge. The animals will receive 50-200 μL of PBS

or bacterial suspension using 23-25 gauge needles following alcohol preparation of the site for injection. Animals will be monitored for at least 1 hour but not more than 2 hours after dosing for any signs of distress.

Fecal pellets will be collected on a weekly basis for PBS and ECA- vaccinated animals. Briefly, mice will be placed individually in a clean, empty cage until a fecal pellet is passed. The fecal pellet will be collected into a clean microcentrifuge tube for processing. The mouse will be returned to its cage, minimizing its disruption and discomfort. Blood samples will be collected via standard tail vein bleeds from each group of vaccinated animals 1 day prior to challenge without the use of anesthesia. For these bleeds, a 26 gauge needle will be used.Mice will be weighed prior to the bleed to calculate the total circulating volume based on the approximation of 58.5mL total blood volume per kg, and the blood volume collected will not exceed 10% of the calculated total blood volume of the animal. Serum will then be collected from these samples and used to monitor antibody titers using ELISA assays.

The orogastrically and intraperitoneally vaccinated animals will be challenged 30 days post-immunization using the IP injection methods as described above. The animals will be challenged with wild-type R11 *S.* Enteriditis, J65 *S.* Stanleyville or R17 *S.* Dublin. The challenged animals will be initially monitored every 8 hours during the first week for mortality and onset of end-stage disease symptoms, including hunched poster, ruffled fur, weight loss, and poor mobility. The monitoring will continue once per day for an additional 23 days until the 30-day post-challenge time point is reached. Animals surviving at the 30-day timepoint will undergo terminal cardiac bleeding to assess antibody titers. For this, the animals will be placed in a compressed gas CO_2 inhalation chamber and observed. The chamber will not be overcrowded. Cylinderized carbon dioxide with a pressure gauge and flowmeter will be used as the source of carbon dioxide. With animals in the chamber, the flow rate of CO_2 will displace at least 20% of the cage volume per minute as indicated on the flowmeter. Animals will be watched to ensure that they are lying down and that their purposeful movements have stopped. The anesthesia cage will be opened and the animal will be checked for response to toe pinch. If animal responds to toe pinch, the lid of the chamber will be closed and carbon dioxide flow will continue until the animal is adequately anesthetized as indicated by lack of response to toe pinch. If there is no response to toe pinch then the carbon dioxide flow will be stopped. Animals will then be quickly removed from the chamber and a 22 or 23 gauge needle on a syringe will be used to pierce the thoracic wall and the chamber of the heart. At this point, the animal is narcotized (anesthetized) by CO_2. After sufficient blood is obtained, the needle will be removed and the animal will immediately be euthanized by cervical dislocation. In most cases, death will already have occurred by exsanguination.

As detailed below, we will need 480 mice to complete the experiment outlined above, and the study will be repeated once to verify obtained results. In addition, we request 10% extra animals for unforeseen problems.

Immunization		Challenge		
Strain	Route	Salmonella Serovar	Dose x LD_{50}	Mice
PBS	Oral	S. Enteritidis	1, 10, 100, 1000	10/dose
ECA-SL1344	Oral	S. Enteritidis	1, 10, 100, 1000	10/dose
PBS	Oral	S. Stanleyville	1, 10, 100, 1000	10/dose
ECA-SL1344	Oral	S. Stanleyville	1, 10, 100, 1000	10/dose
PBS	Oral	S. Dublin	1, 10, 100, 1000	10/dose
ECA-SL1344	Oral	S. Dublin	1, 10, 100, 1000	10/dose
Strain	Route	Salmonella Serovar	Dose x LD_{50}	Mice
PBS	IP	S. Enteritidis	1, 10, 100, 1000	10/dose
ECA-SL1344	IP	S. Enteritidis	1, 10, 100, 1000	10/dose
PBS	IP	S. Stanleyville	1, 10, 100, 1000	10/dose
ECA-SL1344	IP	S. Stanleyville	1, 10, 100, 1000	10/dose
PBS	IP	S. Dublin	1, 10, 100, 1000	10/dose
ECA-SL1344	IP	S. Dublin	1, 10, 100, 1000	10/dose
			*total:	480

Experiment totals:
[1 immunization strain X 2 immunization/challenge route combinations X 3 challenge strains X 4 doses X 10 animals per group = 240 animals, 1 mock-vaccinated group X 2 immunization/challenge route combinations X 3 challenge strains X 4 doses X 10 animals per group = 240 animals; 240 + 240 = 480 animals X 2 (biological repeats) = 960 animals]
Additional animals (~10%) = 96 mice
Grand total of animals requested for Experiment #4 = 1,056 mice

V.1.5. Experiment #5: Determine if protection is induced in immunodeficient animals

Rationale: The data generated through Experiments #1, #2, #3, and #4 investigate the contribution of *Salmonella* specific antibodies to protection. Given that *Salmonella* is an intracellular pathogen, there is a good chance that both humoral and cell mediated responses may be involved in protection. Thus, we will also utilize animals deficient in various immunological components to determine whether both humoral and cell-mediated immunity is involved in protection. To this end, we will vaccinate animals that are deficient for these components with the ECA- SL1344 strain and then determine whether protection is lost in the absence of the respective component. For animals that are deficient in T-cells we will utilize B6.129P2-*Tcrb*^{tm1Mom} *Tcrd*^{tm1Mom}/J mice, which carry double mutations in T-cell receptor (TCR) beta and delta. For B-cell deficient animals we will utilize B6.129S2-*Ighm*^{tm1Cgn}/J mice, which do not develop mature B-cells. Finally, since no macrophage deficient animals currently are available, B6.FVB-Tg(ITGAM-DTR/EGFP)34Lan/J mice, which carry a transgenic diphtheria toxin receptor from a "macrophage-specific" promoter will be used to deplete macrophages via administration of diphtheria toxin via established protocols. Groups of 4 animals from each immunodeficient group will be vaccinated orally with PBS or the ECA-SL1344 strain and followed as described above. 30 days post-vaccination, animals will challenged intraperitoneally with a dose equivalent to 10 LD50 of wildtype SL1344. Mortality and morbidity will be monitored for 30 days.

Procedure: Single strain vaccination assays of the immunodeficient mice will be

performed according to published procedures using orogastric administration of our ECA negative SL1344 *S.* Typhimurium strain or PBS (Michetti, P *et al. Infect. Immun.* 60(5): 1786, 1992; Schmitt, CK *et al. J. Bacteriol.* 176(2): 368, 1994; Schmitt, CK *et al. J. Bacteriol.* 178(10): 2911, 1996; Lawley, TD *et al. PLoS Pathog.* 2(2): e11, 2006; Lawley, TD *et al. Infect. Immun.* 76(1):403, 2008). Vaccinated animals will subsequently be challenged with a dose equivalent to 10 LD50 of wildtype SL1344.

Prior to orogastric administration of bacteria, animals will be fasted (food withheld but not water) for 2 hours prior to infection and then infected orogastrically with either PBS or a dose of the ECA- SL1344 strain that was previously shown to provide protection against lethal challenge. Orogastric infection will be accomplished by passing a straight or curved stainless steel atraumatic ball-tipped 20 gauge gavage needle into the terminal esophagus or the stomach. This can be done quickly and safely under manual restraint by trained individuals without the use of anesthesia. Because the entire path from the open mouth of the rodent to the nonglandular portion of the stomach is covered by a thick and resistant keratinized epithelium, injury is very rare. Gavage guarantees that the fluid suspension of bacteria is delivered to the stomach rather than being spit out or being inhaled (which could cause aspiration pneumonia). This is an important point since aspiration could lead to pulmonary infection. Dosing volumes of 0.1-0.2 ml (based on animal weight not to exceed 10mL/kg) will be used in the mice as this is well-tolerated. Animals will be observed immediately after dosing and then periodically over the next few minutes to ascertain that there is no sign of aspiration or trauma before they are returned to their cages. Dosed animals will be maintained without food or water for a period of at least 1 hour but not more than 2 hours to permit gastric processing of the PBS or bacterial suspension, but then will be returned to a normal diet thereafter. To deplete the macrophages, B6.FVB-Tg(ITGAM-DTR/EGFP)34Lan/J mice will be given diphtheria toxin via IP injection according to published procedures (Cailheir, JF *et al. J. Immunol.* 174(4):2336, 2005). Briefly, twenty-four hours prior to vaccination, the animals will receive *a* dose of diphtheria toxin at 25ng/g using 23-25 gauge needles following alcohol preparation of the site for injection. The diphtheria toxin will be diluted in PBS at a concentration of 2.5ng/ul such that the mice will be dosed with approximately 100-200ul. Animals will be monitored for at least 1 hour but not more than 2 hours after dosing for any signs of distress.

Fecal pellets will be collected on a weekly basis for PBS and ECA- vaccinated animals. Briefly, mice will be placed individually in a clean, empty cage until a fecal pellet is passed. The fecal pellet will be collected into a clean microcentrifuge tube for processing. The mouse will be returned to its cage, minimizing its disruption and discomfort. Blood samples will be collected via standard tail vein bleeds from each group of vaccinated animals 1 day prior to challenge without the use of anesthesia. For these bleeds, a 26 gauge needle will be used. Mice will be weighed prior to the bleed to calculate the total circulating volume based on the approximation of 58.5mL total blood volume per kg, and the blood volume collected will not exceed 10% of the calculated total blood volume of the animal. Serum will then be collected from these samples and used to monitor antibody titers using ELISA assays.

The orogastrically vaccinated animals will be challenged 30 days post-immunization via IP injection. Briefly, the animals will receive 50-200 µL of the bacterial suspension using 23-25 gauge needles following alcohol preparation of the site for injection. Animals will be monitored for at least 1 hour but not more than 2 hours after dosing for any signs of distress. The animals will be challenged with a dose equivalent

to 10 times the LD50 of wildtype *S.* Typhimurium SL1344. The challenged animals will be initially monitored every 8 hours during the first week for mortality and onset of end-stage disease symptoms, including hunched poster, ruffled fur, weight loss, and poor mobility. The monitoring will continue once per day for an additional 23 days until the 30-day post-challenge time point is reached. Animals surviving at the 30-day timepoint will undergo terminal cardiac bleeding to assess antibody titers. For this, the animals will be placed in a compressed gas CO_2 inhalation chamber and observed. The chamber will not be overcrowded. Cylinderized carbon dioxide with a pressure gauge and flowmeter will be used as the source of carbon dioxide. With animals in the chamber, the flow rate of CO_2 will displace at least 20% of the cage volume per minute as indicated on the flowmeter. Animals will be watched to ensure that they are lying down and that their purposeful movements have stopped. The anesthesia cage will be opened and the animal will be checked for response to toe pinch. If animal responds to toe pinch, the lid of the chamber will be closed and carbon dioxide flow will continue until the animal is adequately anesthetized as indicated by lack of response to toe pinch. If there is no response to toe pinch then the carbon dioxide flow will be stopped. Animals will then be quickly removed from the chamber and a 22 or 23 gauge needle on a syringe will be used to pierce the thoracic wall and the chamber of the heart. At this point, the animal is narcotized (anesthetized) by CO_2. After sufficient blood is obtained, the needle will be removed and the animal will immediately be euthanized by cervical dislocation. In most cases, death will already have occurred by exsanguination.

As detailed below, we will need 24 mice to complete the experiment outlined above, and the study will be repeated once to verify obtained results. In addition, we request 10% extra animals for unforeseen problems.

Experiment totals:
[1 immunization strain X 3 types of immunodeficient mice X 4 animals per group = 12 animals, 1 mock-vaccinated group X 3 types of immunodeficient mice X 4 animals per group = 12 animals; 12 + 12 = 24 animals X 2 (biological repeats) = 48 animals]
Additional animals (~10%) = 5 mice
Grand total of animals requested for Experiment #5 = 53 mice

V.2. Data Analysis: The percent protection will be calculated as well as meantime to death (MTD): MTD = the sum of all X values divided by the total number of mice dead, where X is the days post-infection multiplied by the number of mice that died that day.

The biological replicates will be pooled for data analysis such that n=16/group. Pooling the survival data allows us to detect a difference of 80% vs. 25% survival when employing a log-rank test with a 5%, two-sided significance level and 80% power. (Power calculations were performed using STATA version 12 software (stpower procedure) and were based on a method described in: Freedman, L. S. 1982. Tables of the number of patients required in clinical trials using the logrank test. *Statistics in Medicine* 1: 121–129.)

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: Whenever possible, we test our *Salmonella* strains on tissue culture cells. Unfortunately, *in vitro* tissue culture models are not appropriate for investigating the effectiveness of potential vaccine candidates. Additionally, computer modeling programs are not suitable for predicting the immunization ability of bacterial strains.

V.3.2. Animal Model and Species Justification:
Mice were chosen for these studies for a variety of reasons: 1) genetically inbred animals are required to effectively study immune responses and vaccine efficacy; 2) many animals are necessary to control the studies in a satisfactory manner; 3) mice are the least sentient species we can use to evaluate potential therapies. It is necessary, in this case to use *in vivo* models in order to effectively determine the usefulness of our strains in vaccination strategies.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	*Mus musculus*	
V.3.3.2. Strain/Stock:	C57BL/6; BALB/c; B6.129P2-*Tcrb*^{tm1Mom} *Tcrd*^{tm1Mom}/J; B6.129S2-*Ighm*^{tm1Cgn}/J; B6.FVB-Tg(ITGAM-DTR/EGFP)34Lan/J	
V.3.3.3. Source/Vendor:	(b)(4)	
V.3.3.4. Age:	6-8 weeks	
V.3.3.5. Weight:	18-20 grams	
V.3.3.6. Sex:	Female	
V.3.3.7. Special Considerations:		

V.3.4. Number of Animals Required (by Species): Mice = 2,922

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: Animals are sedated and anesthetized by CO_2 inhalation to alleviate pain and distress prior to cervical dislocation. Additionally, we use the gavage procedure to infect the animals to minimize the possibility of aspiration pneumonia.

Whenever possible, visibly moribund mice will be euthanized rather than being monitored until the end of the observation period.

V.3.5.2. Reduction: As many of our experiments involve infected animals or tissues, we do not participate in any shared tissue and/or control groups with other investigators. To reduce the number of animals necessary for the study proposed here, we have used the minimal number of animals required for biological and statistical significance.

V.3.5.3. Replacement: Whenever possible, we will test our strains on tissue culture cells. Unfortunately, *in vitro* tissue culture models are not appropriate for revealing vaccine efficacy and are limited in the amount of information that they provide concerning host response to infection.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:		
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	2,922	

Malaise is often observed in mice injected with wildtype *S.* Typhimurium, while acute fatal pneumonia may occur in mice infected with *S. pneumoniae* or *P. aeruginosa*. The administration of our potential vaccine strains may prevent such response in animals challenged with wildtype strains. However, it is not possible to know how many animals will respond to the variables in the studies designed in this proposal, and thus, it is not possible to know how many mice will experience pain or distress. Thus, it is formally possible that all 2,922 mice could experience pain or distress. When animals become moribund indicated by severe changes in body weight (>20% weight loss (assessed daily) compared to controls or to the baseline), behavior, appetite, excretion, hair coat, etc., they will be euthanized. No analgesics will be given to mice post-challenge in these studies, since administration of analgesics confounds the results of virulence assessment assays. First, studies have shown that analgesics directly affect the immune response in various ways, including the inhibition of antibody production and the enhancement of bacterial killing (Bancos, S. *et al. Cell Immunol.* 258(1):18–28, 2009; Stables, M.J. *et al. Blood.* 116(16):2950-2959, 2010). Second, NSAID analgesics, including ibuprofen and diclofenac, have displayed anti-microbial activity against *Salmonella* (Al-Janabi, A.A. *J Glob Infect Dis.* 2(2):105–108, 2010; Dastidar, S.G. *et al. Int J Antimicrob Agents.* 14(3):249-51, 2000). In fact, the administration of diclofenac provided protection in mice against *Salmonella* challenge (Dastidar, S.G. *et al. Int J*

Antimicrob Agents. 14(3):249-51, 2000). Third, morphine administration increases the bacterial load in *Salmonella*-infected mice (MacFarlane, A.S. *et al. J Infect Dis*. 181(4):1350-8, 2000; Breslow, J.M. *et al. Microb Pathog*. 49(6):330-5, 2010). Finally, opioids have the ability to alter the virulence of pathogenic microbes (Babrowski,T. *et al. Ann Surg*. 255:386–393, 2012; reviewed in Moss, J. *Mayo Clin Proc*. 83(10):1116-30, 2008). Thus, analgesics will not be administered in an effort to eliminate any factors that would alter the normal course of our challenge infection and impede the interpretation of data relating to the efficacy of our vaccine candidate.

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: For challenge studies described in experiment 2, animals that will be intranasal infected with *S. pneumoniae* or *P. aeuriginosa* will be anesthetized by intraperitoneal administration of a freshly prepared mixture of ketamine hydrochloride (65 mg/kg) and xylazine (10 mg/kg) in a 200uL volume. Following infection, the mice are placed on their backs next to each other and monitored until all have recovered from the anesthesia.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: We have not observed any overt complications from any of the procedures described other than occasional oral bleeding from the gavage. In our hands, this is rare and has occurred only when training a new person in this technique. There are extremely rare instances in which mice are severely injured during gavage. Laboratory personnel will monitor the behavior and general health of the animals following infection before returning them to LAM. Animals showing signs of injury due to gavage (as evidenced by shaking, sometimes accompanied by circling and other signs of distress) will be humanely euthanized as described in the euthanasia section (V.4.6). We will also frequently check all animals that are infected and/or dosed until it is time for the study to end; if evidence of signs of distress is noted, they will be humanely euthanized immediately.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: Toxline and PubMed

V.4.1.3.2. Date of Search: 2 September 2014

V.4.1.3.3. Period of Search: Pubmed (1950-Current) Toxline (1950's-Current)

V.4.1.3.4. Key Words of Search: *S.* Typhimurium, alleviate, mice, vaccine, survival, alternative

V.4.1.3.5. Results of Search: The search terms "*S.* Typhimurium and alleviate" and "*S.* Typhimurium and alleviate and mice" returned 6 and 1 articles, respectively, on Toxline. Unfortunately, none of these articles describes methods of pain alleviation during *S.* Typhimurium infection. The additional search terms "*S.* Typhimurium and

vaccine and survival and alternative" returned 1 article, which did not present alternative models for the proposed studies.

Searches on PubMed with "*S.* Typhimurium and alleviate" returned 22 articles, while "*S.* Typhimurium and alleviate and mice" returned 4 articles; however, none of these discusses a method of alleviating the potential pain in mice infected with *S.* Typhimurium. Additionally, searching with the terms "*S.* Typhimurium and vaccine and survival and alternative" returned 8 articles, none of which provided an alternative to survival studies of vaccine candidates.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

We believe that these experiments are justified because animals serve as surrogates for volunteer studies, and our long-range goal is to develop vaccines to specifically prevent salmenollosis. Since in the US alone, 1.4 million cases of salmonellosis occur annually causing greater than 500 deaths per year, it is important to develop efficacious vaccines to help prevent recurrence of this disease. Additionally, the development of a live-attenuated vaccine capable of heterologous delivery of foreign antigens would potentially provide a resource for the prevention of a multitude of infectious diseases. Unfortunately, to effectively determine the usefulness of a vaccine strain, it is imperative to perform survival studies in mice such as those we have proposed herein.

No analgesics will be given to mice post-challenge in these studies, since administration of analgesics confounds the results of virulence assessment assays. First, studies have shown that analgesics directly affect the immune response in various ways, including the inhibition of antibody production and the enhancement of bacterial killing (Bancos, S. *et al. Cell Immunol.* 258(1):18–28, 2009; Stables, M.J. *et al. Blood.* 116(16):2950-2959, 2010). Second, NSAID analgesics, including ibuprofen and diclofenac, have displayed anti-microbial activity against *Salmonella* (Al-Janabi, A.A. *J Glob Infect Dis.* 2(2):105–108, 2010; Dastidar, S.G. *et al. Int J Antimicrob Agents.* 14(3):249-51, 2000). In fact, the administration of diclofenac provided protection in mice against *Salmonella* challenge (Dastidar, S.G. *et al. Int J Antimicrob Agents.* 14(3):249-51, 2000). Third, morphine administration increases the bacterial load in *Salmonella*-infected mice (MacFarlane, A.S. *et al. J Infect Dis.* 181(4):1350-8, 2000; Breslow, J.M. *et al. Microb Pathog.* 49(6):330-5, 2010). Finally, opioids have the ability to alter the virulence of pathogenic microbes (Babrowski,T. *et al. Ann Surg.* 255:386–393, 2012; reviewed in Moss, J. *Mayo Clin Proc.* 83(10):1116-30, 2008). Thus, analgesics will not be administered in an effort to eliminate any factors that would alter the normal course of our challenge infection and impede the interpretation of data relating to the efficacy of our vaccine candidate.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: N/A

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. **Procedure**: N/A

V.4.3.3. **Post-surgical Provisions**: N/A

V.4.3.4. **Location**: N/A

V.4.3.5. **Surgeon**: N/A

V.4.3.6. **Multiple Major Survival Operative Procedures**: N/A

V.4.3.6.1. **Procedures**: N/A

V.4.3.6.2. **Scientific Justification**: N/A

V.4.4. **Animal Manipulations**:

V.4.4.1. **Injections**: Groups of animals will receive 50-200 µL intraperitoneal (IP) injections of either PBS or a bacterial suspension for vaccination or challenge. These injections will be performed with 23-25 gauge needles following alcohol preparation of the site for injection.

For challenge studies described in experiment 2, animals that will be intranasally infected with *S. pneumoniae* or *P. aeuriginosa* will be anesthetized by intraperitoneal administration of a freshly prepared mixture of ketamine hydrochloride (65 mg/kg) and xylazine (10 mg/kg) in a 200uL volume.

V.4.4.2. **Biosamples**:

Blood:
1) Blood samples will be collected via standard tail vein bleeds from each group of vaccinated animals 1 day prior to challenge without the use of anesthesia. For these bleeds, a 26 gauge needle will be used. Mice will be weighed prior to the bleed to calculate the total circulating volume based on the approximation of 58.5mL total blood volume per kg, and the blood volume collected will not exceed 10% of the calculated total blood volume of the animal. Serum will then be collected from these samples and used to monitor antibody titers using ELISA assays.

2) Animals surviving at the 30-day timepoint will undergo terminal cardiac bleeding to assess antibody titers. For this, the animals will be placed in a compressed gas CO_2 inhalation chamber and observed. When respiratory arrest occurs, they will be quickly removed and a 22 or 23 gauge needle on a syringe will be used to pierce the thoracic wall and the chamber of the heart. At this point the animal is narcotized (anesthetized) by CO_2. After sufficient blood is obtained, the needle will be removed and the animal will immediately be euthanized by cervical dislocation. In most cases, death will already have occurred by exsanguination. Serum will then be collected from these blood samples and used to monitor antibody titers using ELISA assays.

Fecal Pellets: Mice will be placed individually in a clean, empty cage until a fecal pellet is passed. The fecal pellet will be collected into a clean microcentrifuge tube for processing. The mouse will be returned to its cage, minimizing its disruption and discomfort. We propose to perform the fecal collections on a weekly basis for challenged mice and PBS-inoculated animals as a negative control.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: cage cards will be used

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: Manual restraint of animals will be used during oral inoculation of mice: 0.1-0.2 ml of bacteria solution will be inoculated by **gavage** using a straight or curved stainless steel atraumatic ball-tipped gavage needle (20 gauge x 1-1/ 2) into the terminal esophagus and the stomach.

V.4.4.8. Tissue Sharing: As many of our experiments involve infected animals or tissues, we do not participate in any shared tissue and/or control groups with other investigators.

V.4.5. Study Endpoint: Maximum survival of animals will be 30 days post-challenge, after which animals will be euthanized. Since animals will be dosed with a normally lethal amount of bacteria to test the efficacy of our vaccine strains, we will be initially watching the animals every 8 hours during the first week for mortality and onset of end-stage disease symptoms. The monitoring will continue once per day for an additional 23 days until the 30-day post-challenge time point is reached. Therefore, an early endpoint for moribund animals will be considered in the event of severe changes in body weight (>20% weight loss (assessed daily) compared to controls or to the baseline), behavior, appetite, excretion, hair coat, etc. in accordance with USU IACUC Policy 020. Moribund (dying) animals will be euthanized. These animals will undergo terminal cardiac bleeding to assess antibody titers. For this, the animals will be placed in a compressed gas CO_2 inhalation chamber and observed. The chamber will not be overcrowded. Cylinderized carbon dioxide with a pressure gauge and flowmeter will be used as the source of carbon dioxide. With animals in the chamber, the flow rate of CO_2 will displace at least 20% of the cage volume per minute as indicated on the flowmeter. Animals will be watched to ensure that they are lying down and that their purposeful movements have stopped. The anesthesia cage will be opened and the animal will be checked for response to toe pinch. If animal responds to toe pinch, the lid of the chamber will be closed and carbon dioxide flow will continue until the animal is adequately anesthetized as indicated by lack of response to toe pinch. If there is no response to toe pinch then the carbon dioxide flow will be stopped. Animals will then be quickly removed from the chamber and a 22 or 23 gauge needle on a syringe will be used to pierce the thoracic wall and the chamber of the heart. At this point, the animal is narcotized (anesthetized) by CO_2. After sufficient blood is obtained, the needle will be

removed and the animal will immediately be euthanized by cervical dislocation. In most cases, death will already have occurred by exsanguination.

V.4.6. Euthanasia: The PI and other research staff as assigned to the protocol will perform these procedures. Euthanasia will be performed using terminal cardiac bleeds following CO_2 inhalation in a compressed gas CO_2 inhalation chamber according to the most recent AVMA Guidelines on Euthanasia. The chamber will not be overcrowded. Cylinderized carbon dioxide with a pressure gauge and flowmeter will be used as the source of carbon dioxide. With animals in the chamber, the flow rate of CO_2 will displace at least 20% of the cage volume per minute as indicated on the flowmeter. Animals will be watched to ensure that they are lying down and that their purposeful movements have stopped. The anesthesia cage will be opened and the animal will be checked for response to toe pinch. If animal responds to toe pinch, the lid of the chamber will be closed and carbon dioxide flow will continue until the animal is adequately anesthetized as indicated by lack of response to toe pinch. If there is no response to toe pinch then the carbon dioxide flow will be stopped. Animals will then be quickly removed from the chamber and a 22 or 23 gauge needle on a syringe will be used to pierce the thoracic wall and the chamber of the heart. At this point, the animal is narcotized (anesthetized) by CO_2. After sufficient blood is obtained, the needle will be removed and the animal will immediately be euthanized by cervical dislocation. In most cases, death will already have occurred by exsanguination.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions: As a precaution animals will be housed in cages with micro-isolater lids or in cages kept on individually vented racks (Thoren units). Animals will be placed on **floor grids** during fasting to keep them dry and to prevent coprophagia. Cages containing fasting animals will be clearly labeled to prevent inadvertent feeding by LAM personnel. Additionally, if LAM personnel identify dead animals, they will alert laboratory personnel to remove the carcass.

Food Restriction: Yes _____ No X

Fluid Restriction: Yes _____ No X

Animals will be fasted (food withheld but not water) for 2 hours prior to all gavage procedures, including the administration of bacterial suspensions. Additionally, dosed animals will be maintained without food or water for a period of at least 1 hour but not more than 2 hours to facilitate gastric processing of the PBS or bacterial suspensions.

This fasting and fluid restriction period provides limited distress to the animal and increases the likelihood of achieving successful infection, thus decreasing the total number of animals. Afterwards, the animals will be returned to a normal diet for the remainder of the study.

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: LAM personnel will provide routine checks for health and general husbandry at least once/day. Infected animals will be checked daily by myself or other laboratory personnel. Any animals exhibiting signs of illness will be evaluated by a LAM veterinarian. Animals will typically be monitored for changes in body weight, behavior, appetite, excretion, hair coat, etc. After consulting with the PI (if available), moribund animals will be euthanized as described.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions: Animals will be placed on floor grids during fasting (prior to orogastric procedures) to keep them dry and to prevent coprophagia.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING: Dr. (b)(6) has considerable experience working with animal models of bacterial pathogenesis. His previous work includes utilization of infant mice and rabbits for the study of *V. cholerae* (training and experience since 1996 at (b)(6) He has been working with gerbil and mice models of *H. pylori* infection since 2001 (training at (b)(6) (b)(6) Any other personnel recruited to this project will be appropriately trained and added to all current protocols.

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Gavage	(b)(6)	Animal handling experience with mice and gerbils since 1996	(b)(6) Training, 1996; (b)(6) (b)(6) Training, 2001; Rodent handling class and Investigator Training, 2004
Gavage		Research Technician, 14	Rodent handling class,

		years experience with mice and gerbils	2000; Investigator Training, 2005; Lab-specific training with Dr. (b)(6) 2005
Gavage	(b)(6)	Postdoctoral Fellow with gerbil & mouse handling experience since 2012	Investigator Training, Jan 2012; Lab-specific training with Dr (b)(6) 2012
IP Injections		Animal handling experience with mice and gerbils since 1996	(b)(6) Training, 1996; (b)(6) (b)(6) Training, 2001; Rodent handling class and Investigator Training, 2004
IP Injections		Research Technician, 14 years experience with mice and gerbils	Rodent handling class, 2000; Investigator Training, 2005; Lab-specific training with Dr. (b)(6) 2005
IP Injections		Postdoctoral Fellow with gerbil & mouse handling experience since 2012	Investigator Training, Jan 2012; Lab-specific training with Dr (b)(6) 2012
Euthanasia		Animal handling experience with mice and gerbils since 1996	(b)(6) Training, 1996; (b)(6) (b)(6) Training, 2001; Rodent handling class and Investigator Training, 2004
Euthanasia		Research Technician, 14 years experience with mice and gerbils	Rodent handling class, 2000; Investigator Training, 2005; Lab-specific training with Dr. (b)(6) 2005
Euthanasia		Postdoctoral Fellow with gerbil & mouse handling experience since 2012	Investigator Training, Jan 2012; Lab-specific training with Dr (b)(6) 2012
Tail Vein Bleeds		Animal handling experience with mice and gerbils since 1996	(b)(6) Training, 1996; (b)(6) (b)(6) Training, 2001; Rodent handling class and Investigator Training, 2004
Tail Vein Bleeds		Research Technician, 14 years experience with mice and gerbils	Rodent handling class, 2000; Investigator Training, 2005; Lab-specific training with Dr. (b)(6) 2005
Tail Vein Bleeds		Postdoctoral Fellow with gerbil & mouse handling experience since 2012	Investigator Training, Jan 2012; Lab-specific training with Dr (b)(6) 2012

VII. BIOHAZARDS/SAFETY: Since S. Typhimurium, S. Enteritidis, *S. pneumonia*, and *P. aeruginosa* are classified as BSL 2/ABSL 2 agents by the CDC, animals will be housed at the ABSL 2 level. Gloves, face-masks and lab coats will be worn by all personnel handling infected animals or infected tissues within the animal rooms and laboratory when interacting with the animals. Cages that housed infected animals will be double-bagged, sprayed with disinfectant, and autoclaved.

VIII. ENCLOSURES: N/A

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 10/29/14

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 10/29/14

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) _____ 10/29/14

Principal Investigator Signature Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number: (b)(6)

B. Animal Protocol Title: Use of *Salmonella* Typhimurium Enterobacterial Common Antigen Mutants as a Vaccine and Vehicle for Heterologous Antigen Delivery (Mice/*Mus musculus*)

C. Principal Investigator: (b)(6) Ph.D.

D. Performing Organization: Uniformed Services University (USU)

E. Funding: Uniformed Services University (USU)

F. Objective and Approach: Given that the human gastrointestinal mucosa is very large and that the acquisition of most infectious diseases occurs at mucosal surfaces, it is not surprising that 15-20 million adults and children acquire or die of mucosal infections annually. *Salmonella* is one bacterial genus that causes diverse mucosal infections. In the United States, non-typhoidal *Salmonella* spp. cause an estimated 1.4 million cases of salmonellosis annually and account for greater than 500 deaths per year. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is one of the most prominent strains.

We have identified mutants of a *Salmonella* cell surface virulence factor that appear to be efficacious live vaccine candidates. Specifically, *S.* Typhimurium, a Gram-negative enteric bacterium and a member of the Enterobacteriacae family, expresses the cell surface glycolipid that is ubiquitous among all family members: the phophoglyceride-linked Enterobacterial common antigen (ECA_{PG}). We have shown that ECA negative mutant strains were severely attenuated for virulence when inoculated into mice. However, rather than being cleared from the animal, these mutant strains set up a persistent infection that was maintained for up to 70 days post infection. Excitingly, immunization of mice with ECA negative mutant strains provided protection against a subsequent lethal oral or intraperitoneal challenge with wild type *S.* Typhimurium. Thus, ECA negative strains of *Salmonella* may be useful as live-attenuated vaccine strains, or as vehicles for heterologous antigen expression.

To this end, we will use a mouse model of *S.* Typhimurium infection to determine whether vaccination with ECA negative strains of *S.* Typhimurium provides protection against other strains and species of *Salmonella*. Mice will be first vaccinated with ECA negative derivatives of TML or SL1344 strains and then subsequently challenged with a lethal dose of wildtype derivatives of the *S.* Typhimurium strain not used for vaccination or *S.* Enteritidis, which expresses a different O antigen. Additionally, we will determine whether ECA negative strains of *S.* Typhimurium can be utilized as a vehicle for heterologous antigen delivery. Mice will be vaccinated with ECA negative strains bearing vectors encoding the *Streptococcus pneumoniae* PspA protein or expressing the O antigen portion of *Pseudomonas aeruginosa*. These animals will then we challenged with virulent *S. pneumoniae* or *P. aeruginosa* to determine the

protective efficacy of the vaccine. Together, these studies will reveal the ability of ECA negative strains of *Salmonella* to act as live-attenuated vaccine strains or vehicles for heterologous antigen delivery.

G. Indexing Terms (Descriptors): *Salmonella*, *S.* Typhimurium, vaccine, mice, ECA, heterologous antigen delivery

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

January 13, 2015

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF MICROBIOLOGY

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on January 13, 2015:

Animal Protocol Title: "Pathogenesis of Bacillus Sterne and Bacillus cereus G9241 in Mice"

USUHS Protocol Number: (b)(6)

Expiration Date: January 12, 2018

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

Revised

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Pathogenesis of *Bacillus anthracis* Sterne and *Bacillus cereus* G9241 in mice

GRANT TITLE (if different from above): Identification, Countermeasures, and New Therapies Toward Biological Threat Agents; Protective Measures, Surveillance, and New Therapies toward Infectious Disease of Global Health Concerns; Contribution of Certhrax to Virulence of Anthrax-like *Bacillus cereus* Strain

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Biological Defense Research Directorate, Naval Medical Research Center (b)(6) National Institute of Allergy and Infectious Diseases (b)(6)

EARLIEST ANTICIPATED FUNDING START DATE: ongoing

PRINCIPAL INVESTIGATOR:

(b)(6)	MIC	(b)(6)	11/24/14
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	Professor and Chair	(b)(6)	11/24/14
Research Unit Chief / Dept. Head Signature Typed Name:	Title	Telephone	Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)	MIC	(b)(6)	11/21/14
Statistician Signature Typed Name:	Department	Telephone	Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) LAM (b)(6) 12/2/14

Attending/Consulting Veterinarian Signature | Department | Telephone | Date

Typed Name: (b)(6) VMO

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) PhD

ANIMAL PROTOCOL TITLE: Pathogenesis of *Bacillus anthracis* Sterne and *Bacillus cereus* G9241 in mice

GRANT TITLE (if different from above): Identification, Countermeasures, and New Therapies Toward Biological Threat Agents; Protective Measures, Surveillance, and New Therapies toward Infectious Disease of Global Health Concerns; Contribution of Certhrax to Virulence of Anthrax-like *Bacillus cereus* Strain

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6) PhD; (b)(6) PhD; (b)(6) MPH

TECHNICIANS(S): (b)(6) MS; (b)(6) BS

I. NON-TECHNICAL SYNOPSIS: *Bacillus anthracis*, the bacterium responsible for anthrax, persists in the environment as a dormant, well-protected spore that is resistant to damage and drying. Disease occurs when the spores are inhaled, ingested, or enter wounds in the skin. Once in the body, the dormant spores germinate, or lose their protective coating, and expand into rod-shaped bacteria that multiply and produce toxins that lead to often fatal disease in the patient. Spores of *B. anthracis* have long been considered potential biological weapons. In October 2001, 22 cases of anthrax occurred as a result of deliberate exposure, including 11 patients who suffered the most lethal inhalational disease form. The aggressive use of quinolones and other antibiotics coupled with the early recognition of disease resulted in the survival of 6 of the 11 patients. Hundreds of other individuals potentially exposed to anthrax spores required an extended course of antibiotic therapy due to the concern that they still carried spores in their lungs that could germinate after the conclusion of their antibiotic treatment. Our overarching goals are to 1) determine how *Bacillus* species cause anthrax(-like) disease, and 2) develop therapies that prevent the first stage of disease, specifically germination of the spores. Our experiments will be done with *B. anthracis* Sterne, a strain that does not cause disease in humans and that is used as a vaccine in cattle, and in *Bacillus cereus* G9241, a strain that caused anthrax-like lung disease in a welder but that is attenuated like *B. anthracis* Sterne in mice. A major aim is to study how certain traits of *B. anthracis* and *B. cereus* influence disease progression. Identification of these traits, or virulence factors, will illuminate which are important targets for antimicrobials and vaccines. We will measure the outcome of infection in mice exposed to Sterne or G9241 versus that seen when mice are infected with strains mutated (deficient) in those traits. Further, we will test chemical compounds that alter germination of spores in lab cultures for their capacity to prevent disease in mice infected with spores.

II. BACKGROUND:

II.1. Background: Our studies will continue to investigate two highly related species from the genus *Bacillus*: *B. anthracis* and *B. cereus*. The infectious form of *B. anthracis* and *B. cereus* for animals and humans is the spore. To cause systemic disease, the spore must germinate and undergo outgrowth as a vegetative rod-shaped bacterium. Although the pathogenesis of inhalational, cutaneous, and gastrointestinal anthrax likely involves the same general steps, the morbidities and mortalities differ significantly. After introduction of spores into the host via inhalation, ingestion, or wound contamination, the spores are rapidly phagocytosed by resident macrophages (MΦs). We and others have shown through *in vivo* imaging studies that the spores germinate in the MΦs, either at the site of inoculation or after the MΦs have migrated to the regional lymph nodes (1-4). The spores germinate to form encapsulated vegetative rods, which replicate in the MΦs and produce toxins that kill the MΦs. The extracellular bacilli then disseminate within the host and express the full constellation of virulence factors.

The two principal toxins produced by the vegetative bacteria are Edema Toxin (ET) and Lethal Toxin (LT). Edema Factor (EF) and Lethal Factor (LF) interact with and are delivered to the cell interior by the shared binding subunit Protective Antigen (PA). PA is the principal component of the licensed anthrax vaccine (Biothrax®). ET is comprised of PA and EF and LT is comprised of PA and LF. ET is a calmodulin-dependent adenylate cyclase that converts ATP to cAMP within its target cells. Lethal toxin is a zinc-metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPKK) within MΦs, which can lead to apoptosis of the MΦ and release of vegetative cells and toxins into the surrounding milieu. In addition to the toxins, all strains of *B. anthracis* and some strains of *B. cereus* that cause disease in humans produce one or more capsules. The capsules protect the vegetative bacteria from phagocytosis by macrophages and neutrophils, particularly during dissemination through the lymph and blood to distal sites.

The majority of animal models of anthrax disease mimic inhalational or subcutaneous anthrax in humans [see (5) for review of animal models of anthrax disease]. Inhalational models have been used most often, despite the relatively low frequency of inhalational anthrax (compared to the other routes of inoculation), because aerosolized spores have the potential to be used as bioweapons. Like humans, non-human primates and rabbits are particularly susceptible to the effects of LT and ET, a characteristic that makes these animal models particularly useful for studies of late stage anthrax disease as well as for vaccine and therapeutic development. Mice and guinea pigs can be used to model both the infection (early) and toxemia (late) stages of disease. Mice have been used most extensively due to their low cost, ease of handling, and genetic diversity. Mouse models of anthrax disease are used to mimic inhalational [aerosol, intranasal (i.n.), intratracheal inoculation], cutaneous [subcutaneous (s.c.) inoculation], and gastrointestinal [oral/intragastric (i.g.) inoculation] forms of human anthrax, and all of these models are useful for studies of infection and toxemia. In addition, intravenous (i.v.) or intraperitoneal (i.p.) injection of spores or purified toxins can be useful for investigations of the roles of capsules and toxins in *Bacillus* pathogenesis.

All of our *B. anthracis* animal studies are conducted with *B. anthracis* Sterne, an attenuated strain that does not produce capsule and that is used as a vaccine for cattle.

B. anthracis Sterne is fully virulent in A/J mouse models of anthrax disease; A/J mice are C5-deficient and are defective in neutrophil recruitment, so they are more susceptible to infections caused by *Bacillus* species. The genes that encode the toxin components in *B. anthracis* (*pag, lef, cya*) are located on the pXO1 megaplasmid. The pXO2 megaplasmid, which is not present in *B. anthracis* Sterne, encodes the genes required for production of a poly-D-glutamic acid capsule. Our studies of *B. cereus* virulence are conducted with *B. cereus* G9241, a strain that was isolated from the blood and sputum of a welder who had anthrax-like lung disease (6). *B. cereus* G9241 is unique among *B. cereus* strains for the following reasons: 1) *B. cereus* typically causes mild gastroenteritis rather than anthrax-like pneumonia in humans; and 2) the strain contains two megaplasmids, pBCXO1 and pBC210. The pBCXO1 megaplasmid is 99% identical to pXO1 in *B. anthracis*. One key difference between the two megaplasmids is that the *hasACB* operon on pBCXO1 is intact, which provides *B. cereus* G9241 with the tools necessary to produce a hyaluronic acid capsule (7). The *hasACB* operon on the *B. anthracis* pXO1 contains a frameshift mutation that renders the operon non-functional. In addition, pBC210 contains a second functional polysaccharide capsule operon and a homolog of the gene that encodes PA (*pagA2*). A third toxin, Certhrax, which functions as an ADP-ribosyltransferase, is encoded on pBC210 as well (8); the role of Certhrax in *B. cereus* G9241 pathogenesis will be investigated in the studies described in this protocol. Our preliminary *in vivo* experiments with G9241 and G9241 Δ*certhrax* suggest that Certhrax may reduce virulence of G9241 in subcutaneous and intranasal models. Further *in silico* analysis and literature reports of related toxins have led us to speculate that Certhrax may play a role in gastrointestinal disease.

Despite the prominent role that the toxins play in anthrax disease, we propose that if spore germination can be blocked, the vegetative cells that express these toxins will not be formed. We hypothesize that administration of a small molecule that inhibits germination as a therapeutic will protect mice from anthrax disease. The small molecule 6-thioguanine (6-TG), an analog of known *in vitro* germinants inosine and alanine, was previously shown to inhibit germination of *B. anthracis* spores *in vitro* and in MΦs (9). In preliminary mouse studies under (b)(6) we determined that two s.c. injections (-4, -1 hr) of 6-TG prior to s.c. inoculation with *B. anthracis* Sterne spores extends the median time-to-death by 1-2 days and reduces the mortality rate from 100% to ~50% (unpublished). We screened 3722 small molecules to identify compounds that have the capacity to alter germination of *B. anthracis* Sterne spores in a high-throughput *in vitro* assay. We discovered 20 potential lead compounds that inhibit the spore germination rate by at least 2/3, and we found that 2 of the lead compounds exert sporicidal activity *in vitro* and inhibit spore germination in MΦs. In very preliminary analyses, we found that both compounds have the capacity to reduce mortality in the same animal model system described above for 6-TG.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:

II.2.2. Date of Search: 3 October 2014

II.2.3. Period of Search: BRD: 1998-2009 (most recent); RePORT: 2004-2014, Active Projects; PubMed

II.2.4. Key Words and Search Strategy: *Bacillus* AND (spores OR capsule OR germination OR dissemination OR immunization OR Certhrax) AND (mouse OR mice)

II.2.5. Results of Search:
RESULTS FROM BRD: Search of the BRD database resulted in 102 hits, of which 42 were unique and 9 were from my laboratory. Other studies utilized guinea pigs and rabbits to ascertain *B. anthracis* virulence properties. Sixteen documents were from our collaborators at (b)(6) and outlined our joint studies to investigate spore proteins as vaccine candidates and the roles of antibodies against spores in inhibition of germination and growth. An additional document was from another collaborator and resulted in a joint publication between our laboratories. The remaining studies were focused on the following topics: development of vaccines against anthrax disease that are based on PA and LF; development of multivalent vaccines against two bioterrorism threats (*B. anthracis*, *Yersinia pestis*, *Burkholderia spp*, ricin); the use of hyperbaric oxygen, antimicrobials, or antibodies against PA and/or LF as post-exposure prophylaxis; the role of the unique poly-D-glutamic acid capsule of *B. anthracis* in disease; the contribution of different spore structures on pathogenesis; and methods for detection of *B. anthracis* spores. None of the studies identified in the BRD database overlap with the studies proposed here, except those outlined in proposals from my laboratory.

RESULTS FROM NIH RePORT: The search parameters included *Bacillus* and mouse/mice and [spore (24) OR capsule (5) OR dissemination (3) OR immunization (55) OR Certhrax (0)], where the number in parentheses indicates the number of awards that were identified by the search parameters. An award to my collaborator Dr. (b)(6) is to investigate the regulation of *B. anthracis* virulence factors; we are working with the (b)(6) lab on similar studies in *B. cereus*. Of the 24 awards that matched with "spore", 6 focused on the use of spore proteins as vaccine candidates in the context of PA, 4 on treatment, 2 on toxins, 7 on pathogenesis, and 6 on irrelevant organisms (bacillus can be used to define rod-shaped bacteria). Two of the 5 capsule-oriented awards focused on multi-valent vaccine development, two on pathogenesis, and one was irrelevant. Only one of the 3 dissemination-focused awards was targeted toward *B. anthracis* vaccine development; the others were irrelevant. Finally, 55 awards involved *Bacillus* immunization in mice, but 37 of them were focused on other organisms [mostly the use of the bacillus Calmette-Guerin (BCG) vaccine against *Mycobacterium tuberculosis*]. Twelve focused on *B. anthracis* vaccine development and/or *B. anthracis* toxins, one was directed toward treatment, and four focused on pathogenesis. None of the studies described in this protocol is the focus of any of the awards identified by NIH RePORT.

RESULTS FROM PUBMED: The search parameters were as follows: *Bacillus* AND (spores OR capsule OR germination OR dissemination OR immunization OR Certhrax) AND (mouse OR mice) NOT *Mycobacterium* NOT mycobacteria filtered by "English only". The search yielded 730 results, of which 213 were deemed irrelevant because they focused on BCG as a vaccine or adjuvant, other *Bacillus spp.*, other organisms (*Listeria monocytogenes*, *Streptococcus pneumoniae*), sterilization techniques (*Bacillus* spores are used to determine sterility), immunization with *Bacillus thuringiensis* Cry

toxins, the use of *Bacillus spp.* as probiotics, or cancer. A further 71 articles described the use of spores from various *Bacillus spp.* as a platform for vaccine delivery of heterologous antigens, and three reports showed the efficacy of lethal toxin variants as vaccine adjuvants. A total of 119 articles focused on the pathogenesis of anthrax disease in mice. These studies focused on the interactions of *Bacillus anthracis* spores and/or vegetative cells with various cells in the host, including macrophages and dendritic cells. In addition, many of the papers described the progression of anthrax disease in mice, guinea pigs, rabbits, and monkeys. Of the pathogenesis studies, four focused on the development of models of gastrointestinal anthrax disease and eight described intranasal and aerosol models of anthrax lung disease.

Further, a few dozen articles described the characterization of strains that contained mutations in *B. anthracis* genes. An additional 27 studies demonstrate the activities and functions of the anthrax toxins lethal toxin and edema toxin, which are critical virulence factors for *B. anthracis*. The other major virulence factor is the poly-D-glutamic acid capsule, which is the focus of 23 articles; of the 23, 16 focused on the protective capacity of anti-capsular antibodies during *B. anthracis* infection. A total of 143 papers focused on the development of vaccines against anthrax. The vast majority (95) described many different versions of vaccines that target Protective Antigen (PA), while others focused on the use of *B. anthracis* Sterne spores (10), spore proteins (3), lethal factor (17), and other *Bacillus* antigens (20). Most of the papers that demonstrate the use of spore proteins as vaccine candidates are from my laboratory and/or the laboratory of our collaborator Dr. (b)(6) Twenty-two manuscripts described the structure and function of spores, while an additional 19 papers focused on germination. Two of those manuscripts are from the laboratory of our collaborator Dr. (b)(6) (b)(6) they describe the discovery and characterization of 6-thioguanine as a germination inhibitor. A total of 35 papers describe studies of treatments for anthrax disease, including 11 that describe monoclonal antibodies that reduce mortality, 12 that assess the efficacy of various antibiotics, and 12 that describe lesser known treatment approaches. An additional 21 articles focused on different methods to detect *Bacillus* spores in the environment and how to rapidly differentiate non-pathogenic species from *B. anthracis*. One paper provided preliminary characterization of Certhrax, the novel ADP-ribosyltransferase produced by *B. cereus* G9241 that is the focus of some of the experiments in this protocol. Three other manuscripts described *B. cereus* G9241: two studies from our competitor Dr. (b)(6) laboratory described surface structures that play a role in virulence, and the third paper is the first paper to describe the strain.

A total of nine manuscripts were published by my laboratory. The studies described herein are follow-on studies to those previously published. None of our proposed experiments overlap with published research.

III. OBJECTIVE\HYPOTHESIS: Our hypotheses for the studies proposed herein are as follows: 1) deletion of putative or known virulence genes will alter the pathogenesis of *B. anthracis* Sterne and *B. cereus* G9241; 2) Certhrax plays a significant role in gastrointestinal disease caused by *B. cereus* G9241 and antibodies against the toxin will protect against disease progression; and 3) small molecules that inhibit spore germination will reduce mortality caused by *B. anthracis* Sterne. Our **goals** are to

characterize the progression of *B. anthracis* Sterne disease, to characterize the roles of toxins and capsules in *B. cereus* G9241 virulence, and to identify candidate therapeutics to prevent or treat anthrax disease. The specific aims are as follows: **1)** assess the virulence of *B. anthracis* Sterne or *B. cereus* G9241 strains in which a particular virulence trait has been deleted; **2)** administer *B. cereus* G9241 toxin components to assess their activity *in vivo*; **3)** monitor the location and kinetics of germination, vegetative outgrowth, and dissemination of fluorescent derivatives of *B. anthracis* Sterne and *B. cereus* G9241 in the mouse with an *in vivo* imaging system; **4)** characterize the *in vivo* efficacy of small molecules that inhibit germination of *B. anthracis* Sterne spores *in vitro*; and **5)** assess the capacity of antibodies against *B. cereus* G9241 toxins or capsule to protect from disease.

IV. **MILITARY RELEVANCE**: The potential use of *B. anthracis* as a weapon of biowarfare was brought to the forefront in the aftermath of the terrorist attacks of September 11th, 2001. From October to December 2001, a total of 22 cases of confirmed or suspected bioterrorism-related anthrax occurred in the United States. Eleven of these cases were inhalational (the most lethal form of the infection) and resulted in the death of 6 individuals. In light of these events, the development of therapeutic agents against *B. anthracis* infection is paramount for the protection of both deployed military personnel and civilians who may come into contact with sabotaged contaminated materials. Further, a more complete understanding of the progression of disease caused by these two highly related *Bacillus* species will inform future development of more effective vaccine and therapeutic candidates.

V. **MATERIALS AND METHODS**:

V.1. **Experimental Design and General Procedures**:

A. Three general types of experiments will be conducted in the studies outlined in this protocol.

1. Mice will be inoculated with *B. anthracis* or *B. cereus* spores or vegetative cells, or with purified toxin components by intranasal (i.n.), subcutaneous (s.c.), intragastric (i.g.), intraperitoneal (i.p.), or intravenous (i.v.) administration and monitored for morbidity and mortality with death as an endpoint. The purpose of these studies is to determine 1) if isogenic mutant strains that lack a particular virulence factor exhibit a difference virulence profile than the parent strain, 2) if the toxins produced by *B. cereus* G9241 exhibit similar activity *in vivo* as do their *B. anthracis* counterparts, 3) if Certhrax contributes to *B. cereus* G9241 virulence, or 4) the *in vivo* location(s) of *B. anthracis* and *B. cereus* strains that fluoresce at a particular stage in growth (germination, vegetative outgrowth, dissemination). In some studies of specific virulence factors (i.e. Certhrax, pBC210 capsule), rabbit polyclonal antibodies against the virulence factor will be administered before or after spore or toxin challenge to assess the capacity of the antibodies to protect or rescue mice from disease.

2. Mice will be challenged with *B. anthracis* Sterne spores and treated with small molecules known to inhibit germination *in vitro*. The purpose of these studies is to

determine whether the small molecule(s) could be used as post-exposure prophylaxis to prevent germination and subsequent vegetative outgrowth in the host.

3. Mice will be immunized multiple times with viable *B. cereus* G9241 spores to generate convalescent sera to identify proteins and/or polysaccharides that are expressed during infection. The purpose of this study is to determine if and where specific virulence factors (i.e. Certhrax) are produced during the course of disease.

B. For all of the mouse studies outlined in this protocol, 6-8 week old female A/J or C57BL/6 mice will be challenged by one of five routes (i.n., s.c., i.g., i.p., or i.v.) with *Bacillus anthracis* Sterne spores, *B. cereus* G9241 spores or vegetative cells, or purified toxin components (i.g., i.p., or i.v. only). Moribund mice will be euthanized by inhalational isoflurane overdose followed by cervical dislocation. We define moribund as when the mouse is crouched down or lying in the bottom of the cage with little movement, even when gently prodded. Other nonspecific signs that will be used are ruffled fur, lethargy, hunched posture, decreased alertness, weight loss, and difficulty breathing.

1. For i.n. inoculation, mice will be anesthetized with inhaled isoflurane delivered via a Xenogen XGI-8 gas anesthesia system or with i.p. ketamine (100 mg/kg) and xylazine (10 mg/kg). Once anesthesia is confirmed by non-response to toe pinch, mice will be inoculated i.n. by manually pipetting 50 µl heat-activated spores in H_2O onto the nares (25 µl/per nostril). The mice will be held upright until the inoculum is inhaled (~1 min), then returned to their cages to recover from the anesthesia. Mice will remain in the laboratory for one hour after inoculation to permit careful monitoring of mice. The study endpoint will be either death (see section V.4.5) or euthanasia 14 days post-challenge. Obviously moribund animals will be euthanized by inhalational overdose of CO_2 or isoflurane followed by cervical dislocation (see section V.4.6).

2. For s.c. inoculation, 100-200 µl heat-activated *Bacillus* spores will be administered to each mouse behind the foreleg, between the scapulae, or at the base of the tail using a tuberculin syringe attached to a 26 gauge 0.5 inch needle. Mice will remain in the laboratory for one hour after inoculation to permit careful monitoring of mice. The study endpoint will be either death (see section V.4.5) or euthanasia 14 days post-challenge. Obviously moribund animals will be euthanized by inhalational overdose of CO_2 or isoflurane followed by cervical dislocation (see section V.4.6).

3. For i.g. inoculation, six- to eight-week-old mice will be fasted overnight to reduce the volume of the stomach contents so that the entire inoculum can be administered, in addition, water will be removed from the cages 2-4 hrs prior to inoculation. The next morning, groups of 10 mice will be fed 10^2 to 10^9 vegetative cells or heat-activated spores of the *Bacillus* strain to be tested in 50-100 µl PBS with or without 8.5% sodium bicarbonate ($NaHCO_3$) to neutralize the stomach acid (see V.1.1.2 below). The inoculation will be accomplished by oral gavage with a 20 gauge, 1 ½" disposable gavage needle. After inoculation the animals will then be returned to their cages and permitted food and water *ad libitum*. The endpoint for this study is

extreme morbidity or mortality, as defined in Section V.4.5, and the experiment will be continued for up to 28 days. Mice will be monitored for signs of extreme morbidity (see Section V.4.5 below for definition of extreme morbidity), and obviously moribund animals will be euthanized by inhalational overdose of CO_2 or isoflurane followed by cervical dislocation (see section V.4.6). In some experiments, mice will be euthanized by the same method at earlier time points to permit postmortem tissue harvest followed by histology and/or bacterial/spore enumeration.

4. For i.p. inoculation, 100-200 µl heat-activated *Bacillus* spores or purified toxin components will be administered into the peritoneal cavity through the ventral abdominal wall using a tuberculin syringe attached to a 26 gauge 0.5 inch needle. For i.v. inoculation, 100-200 µl heat-activated *Bacillus* spores or purified toxin components will be administered to each mouse by tail venipuncture or retro-orbital injection using a tuberculin syringe attached to a 26 gauge 0.5 inch needle. Mice will remain in the laboratory for one hour after inoculation to permit careful monitoring of mice. The study endpoint will be either death (see section V.4.5) or euthanasia 14 days post-challenge. Obviously moribund animals will be euthanized by inhalational overdose of CO_2 or isoflurane followed by cervical dislocation (see section V.4.6).

V.1.1. Assessment of virulence of *B. anthracis* Sterne and *B. cereus* G9241 and mutants. In these studies, we will address the contributions of several virulence factors to the pathogenesis of *B. anthracis* Sterne and/or *B. cereus* G9241 disease. The 50% lethal doses (LD_{50}) for *B. anthracis* Sterne administered i.n. and s.c. to A/J mice are 6.3×10^4 spores and 1.6×10^3 spores, respectively and the LD_{50}s for *B. cereus* G9241 administered to A/J mice via i.n. and s.c. routes are 3.2×10^5 spores and 1.3×10^3 spores, respectively (10). We and others have previously demonstrated that the *B. cereus* G9241 megaplasmids that encode the capsule operons and the toxin genes are required for virulence (10). We propose to investigate the virulence of *B. anthracis* Sterne or *B. cereus* G9241 mutants that do not produce certain capsules, toxins, spore proteins, or virulence regulators. We will test the virulence of these strains via s.c., i.n., and/or i.g. routes.

V.1.1.1. Identification of LD_{50} of *B. anthracis* Sterne or *B. cereus* G9241 mutants delivered via s.c. or i.n. routes. We will assess the contribution of several virulence factors to the pathogenesis of *B. anthracis* Sterne or *B. cereus* G9241 disease. These experiments will be done twice with each bacterial strain in immunocompromised A/J mice, with a wide range of doses used in the first experiment and a narrow range of doses used in the second experiment to more accurately pinpoint the LD_{50}. In addition, a positive control group of mice infected with $10 \times LD_{50}$ of the parent strain (*B. anthracis* Sterne or *B. cereus* G9241) will be included for comparison purposes. If we find that a mutant is as virulent as or more virulent that the parent strain, we will repeat the LD_{50} analysis in C57BL/6 mice, which are more resistant to infection with both parent strains (higher LD_{50}s) because the mice are fully immunocompetent. In some experiments, we will challenge mice with a complemented mutant strain in which the gene of interest is expressed episomally on a plasmid; for these studies, we will add to the drinking water 5-10 g/L spectinomycin to maintain the plasmid and 25 mM isopropyl β-D-thiogalactoside to induce expression of the gene. Inasmuch as possible, more than one mutant strain will be tested in a given experiment so that negative (H_2O) and positive

(10xLD_{50} of the parent strain) control groups can be shared. The LD_{50} for each mutant strain will be determined with two experiments.

V.1.1.1.1. Subcutaneous experiment 1 for Bacillus mutants
Group 1: 5 mice: 10^1 *Bacillus* mutant spores in H_2O
Group 2: 5 mice: 10^2 *Bacillus* mutant spores in H_2O
Group 3: 5 mice: 10^3 *Bacillus* mutant spores in H_2O
Group 4: 5 mice: 10^4 *Bacillus* mutant spores in H_2O
Group 5: 5 mice: 10^5 *Bacillus* mutant spores in H_2O
Group 6: 5 mice: 10^6 *Bacillus* mutant spores in H_2O
Group 7: 5 mice: 10xLD_{50} *Bacillus* parent strain spores in H_2O
Group 8: 3 mice: H_2O
38 A/J mice and 38 C57BL/6 mice

V.1.1.1.2. Subcutaneous experiment 2
Remove two doses farthest from LD_{50} and increase to 10 mice/group for mutant strains only = **48 A/J mice and 48 C57BL/6 mice**

Total for subcutaneous experiments
(38 A/J mice + 48 A/J mice) x 10 mutant strains = **860 A/J mice**
(38 C57BL/6mice + 48 C57BL/6 mice) x 10 mutant strains = **860 C57BL/6 mice** (if needed)

V.1.1.1.3. Intranasal experiment 1 for Bacillus mutants
Group 1: 5 mice: 10^2 *Bacillus* mutant spores in H_2O
Group 2: 5 mice: 10^3 *Bacillus* mutant spores in H_2O
Group 3: 5 mice: 10^4 *Bacillus* mutant spores in H_2O
Group 4: 5 mice: 10^5 *Bacillus* mutant spores in H_2O
Group 5: 5 mice: 10^6 *Bacillus* mutant spores in H_2O
Group 6: 5 mice: 10^7 *Bacillus* mutant spores in H_2O
Group 7: 5 mice: 10xLD_{50} *Bacillus* parent strain spores in H_2O
Group 8: 3 mice: H_2O
38 A/J mice and 38 C57BL/6 mice

V.1.1.1.4. Intranasal experiment 2
Remove two doses farthest from LD_{50} and increase to 10 mice/group for mutant strains only = **48 A/J mice and 48 C57BL/6 mice**

Total for intranasal experiments:
(38 A/J mice + 48 A/J mice) x 10 mutant strains = **860 A/J mice**
(38 C57BL/6mice + 48 C57BL/6 mice) x 10 mutant strains = **860 C57BL/6 mice** (if needed)

Total for V.1.1.1:
860 A/J mice + 860 A/J mice = 1720 A/J mice
860 C57BL/6 mice + 860 C57BL/6 mice mice = 1720 C57BL/6 mice

V.1.1.2. Determination of LD_{50}s of *B. cereus* G9241 and isogenic mutants delivered via i.g. inoculation. Because we have not used the i.g. animal model previously for *Bacillus spp.*, a pilot study is needed to determine whether $NaHCO_3$-mediated stomach acid neutralization is required to promote colonization of the GI tract and whether spores or vegetative cells colonize the GI tract more efficiently. We have many years of experience with oral inoculation studies with Shiga toxin-producing *E. coli*, Shiga toxins and ricin (b)(6) and previous versions thereof), so we are confident that we will identify the optimal inoculation conditions quickly and with as few mice as possible. In their studies of *Bacillus* GI infection, Rolny *et al.* inoculated C57BL/6 mice with 10^8-10^9 *B. cereus* vegetative cells (food isolate) suspended in PBS (11), while Glomski *et al.* inoculated BALB/c mice with 10^6 spores of a non-toxigenic but capsulated and luminescent *B. anthracis* strain suspended in PBS (12). In contrast, Xie *et al.* first administered $NaHCO_3$ by gavage, then immediately delivered $2.3x10^6$-$2.3x10^9$ *B. anthracis* Sterne vegetative cells suspended in culture medium; the LD_{50} was determined to be $2.3x10^7$ Sterne cells (13). For these studies, *B. cereus* G9241 spores or vegetative cells will be administered to female A/J mice via i.g. gavage as described in V.1.B.3 above. In the pilot study, fecal samples will be collected every day for 4-7 days to enumerate the bacterial load, which will allow us to determine the efficiency of intestinal colonization. In subsequent studies, fecal collection and bacterial enumeration will be done as needed to assess colonization. In addition, intestines, stomach, spleen, liver, and lungs will be collected postmortem to assess histology and bacterial loads in those organs. Mice will be monitored for morbidity and mortality several times each day, and extremely moribund mice will be euthanized by inhalational isoflurane overdose followed by cervical dislocation.

V.1.1.2.1. Pilot study to determine the appropriate inoculum (spores vs. vegetative cells) and the appropriate medium (PBS ± $NaHCO_3$) for inoculum delivery. Some form of the pilot study will likely need to be conducted 2-3 times until we determine that the conditions are optimized.

Group 1: 10 mice: 10^9 G9241 spores in 0.1 ml PBS
Group 2: 10 mice: 10^9 G9241 vegetative cells in 0.1 ml PBS
Group 3: 10 mice: 10^9 G9241 spores in 0.1 ml PBS, pretreated with 0.05 ml 8.5% (w/v) $NaHCO_3$
Group 4: 10 mice: 10^9 G9241 vegetative cells in 0.1 ml PBS, pretreated with 0.05 ml 8.5% (w/v) $NaHCO_3$
Group 5: 5 mice: PBS only
Group 6: 5 mice: PBS only, pretreated with 0.05 ml 8.5% (w/v) $NaHCO_3$
50 A/J mice x 3 iterations = 150 A/J mice

V.1.1.2.2. Determination of LD_{50} for G9241 delivered by i.g. gavage. *B. cereus* G9241 spores or vegetative cells suspended in PBS ± $NaHCO_3$ (as determined in V.1.1.2.1) will be administered via i.g. gavage as described in V.1.B.3 above. The i.g. LD_{50} experiment will be done twice with G9241, with a wide range of doses used in the first experiment and a narrow range of doses used in the second experiment to more accurately pinpoint the LD_{50}.

V.1.1.2.2.1 Intragastric experiment 1

Group 1: 5 mice: 10^3 G9241
Group 2: 5 mice: 10^4 G9241
Group 3: 5 mice: 10^5 G9241
Group 4: 5 mice: 10^6 G9241
Group 5: 5 mice: 10^7 G9241
Group 6: 5 mice: 10^8 G9241
Group 7: 5 mice: 10^9 G9241
Group 8: 3 mice: H_2O
38 A/J mice

V.1.1.2.2.2 Intragastric experiment 2

Remove three doses farthest from LD_{50} and increase to 10 mice/group = **43 A/J mice**

V.1.1.2.3. Determination of LD_{50}s for G9241 mutants delivered by i.g. gavage.

These studies will be conducted exactly as in V.1.1.2.2 above, except that one additional group of 5 mice inoculated with 10xLD_{50} G9241 will be added to each study as a positive control group.

V.1.1.2.3.1 Intragastric G9241 mutant LD_{50} experiment 1

Group 1: 5 mice: 10^3 G9241 mutant
Group 2: 5 mice: 10^4 G9241 mutant
Group 3: 5 mice: 10^5 G9241 mutant
Group 4: 5 mice: 10^6 G9241 mutant
Group 5: 5 mice: 10^7 G9241 mutant
Group 6: 5 mice: 10^8 G9241 mutant
Group 7: 5 mice: 10^9 G9241 mutant
Group 8: 5 mice: 10xLD_{50} G9241
Group 9: 3 mice: H_2O
43 A/J mice x 6 mutant strains = 258 A/J mice

V.1.1.2.3.2 Intragastric G9241 mutant LD_{50} experiment 2

Remove three doses of the mutant farthest from LD_{50} and increase to 10 mice/group = **48 A/J mice x 6 mutant strains = 288 A/J mice**

Total for V.1.1.2: 777 A/J mice

Total for V.1.1:
1720 A/J mice + 777 A/J mice = **2497 A/J mice**
1720 C57BL/6 mice (if needed)

V.1.2. Experiment 2: Determination of toxicity of putative *B. cereus* G9241 lethal toxin (LT), edema toxin (ET), and Certhrax. The purpose of these studies is to investigate the potential for each of these toxins to contribute to *B. cereus* G9241 disease. As mentioned in the Background, LT is composed of PA + LF and ET is composed of PA + EF. The activity of these toxins in *B. anthracis* has been characterized; in these studies, we propose to confirm that the *B. cereus* G9241 toxins exhibit the same *in vivo* activities as their *B. anthracis* homologs. Additionally, Certhrax

is a *B. cereus* G9241-specific toxin that contains a PA binding domain and a functional ADP-ribosyltransferase domain; however, we do not yet know if PA is required for Certhrax activity *in vitro* or *in vivo*. Prior to initiation of LD_{50} studies in mice, we will confirm that each toxin is functional in *in vitro* systems.

The LD_{50} studies will be conducted in C57BL/6J mice according to established models of LT and ET toxicity. Female C57BL/6J mice will be injected i.p. with PA + LF (1:1 ratio) or with PA + EF (1:1 ratio) in sterile saline as described in V.1, section B4 above. Previous studies by other groups have shown that C57BL/6 mice inoculated i.p. with 100 µg PA +100 µg LF from *B. anthracis* typically die within 3 days after injection (14) and those inoculated i.v. with 25 µg PA + 25 µg EF from *B. anthracis* also die within 3 days after injection (15). Mice will be weighed daily to determine if a correlation between weight loss and eventual death exists. Mice will be carefully monitored as described in V.4.5 and euthanized as in V.4.6 if they are extremely moribund.

The published lethal doses for the *B. anthracis* toxins and the relative *in vitro* toxicities of the *B. cereus* G9241 toxins compared to the *B. anthracis* toxins will guide the dosing strategy for the first LD_{50} study for each *B. cereus* G9241 toxin.

V.1.2.1 Lethal Toxin Experiment 1: Groups 1-7 will be intoxicated with doses of the *B. cereus* G9241 toxin components that correspond with the published LD_{50}s for each *B. anthracis* toxin (i.e. for Group 1, mice will be intoxicated with doses of the *B. cereus* G9241 PA and LF that correspond to 20 LD_{50}s of the *B. anthracis* toxin components). Group 8 will be intoxicated with *B. anthracis* Sterne toxin components (control).

Group 1: 5 mice: 20xLD_{50} *B. anthracis* PA + 20xLD_{50} *B. anthracis* LF
Group 2: 5 mice: 10xLD_{50} *B. anthracis* PA + 10xLD_{50} *B. anthracis* LF
Group 3: 5 mice: 5xLD_{50} *B. anthracis* PA + 5xLD_{50} *B. anthracis* LF
Group 4: 5 mice: 1xLD_{50} *B. anthracis* PA + 1xLD_{50} *B. anthracis* LF
Group 5: 5 mice: 0.5xLD_{50} *B. anthracis* PA + 0.5xLD_{50} *B. anthracis* LF
Group 6: 5 mice: 10xLD_{50} *B. anthracis* PA
Group 7: 5 mice: 10xLD_{50} *B. anthracis* LF
Group 8: 5 mice: 10xLD_{50} *B. anthracis* PA + 10xLD_{50} *B. anthracis* LF
40 C57BL/6J mice

V.1.2.2. Lethal Toxin Experiment 2: Groups 1-5 will be intoxicated with *B. cereus* G9241 toxin components. Group 6 will be intoxicated with *B. anthracis* Sterne toxin components (control).

Group 1: 5 mice: 20xLD_{50} *B. cereus* PA + 20xLD_{50} *B. cereus* LF
Group 2: 5 mice: 10xLD_{50} *B. cereus* PA + 10xLD_{50} *B. cereus* LF
Group 3: 5 mice: 5xLD_{50} *B. cereus* PA + 5xLD_{50} *B. cereus* LF
Group 4: 5 mice: 1xLD_{50} *B. cereus* PA + 1xLD_{50} *B. cereus* LF
Group 5: 5 mice: 0.5xLD_{50} *B. cereus* PA + 0.5xLD_{50} *B. cereus* LF
Group 6: 5 mice: 10xLD_{50} *B. anthracis* PA + 10xLD_{50} *B. anthracis* LF
30 C57BL/6J mice

Total for V.1.2.1 and V.1.2.2: 40 mice + 30 mice = 70 C57BL/6J mice

V.1.2.3. Edema Toxin Experiment 1: Groups 1-7 will be intoxicated with doses of the *B. cereus G9241* toxin components that correspond with the published LD_{50}s for each B. anthracis toxin (i.e. for Group 1, mice will be intoxicated with doses of the *B. cereus* G9241 PA and EF that correspond to 20 LD_{50}s of the *B. anthracis* toxin components). Group 8 will be intoxicated with *B. anthracis* Sterne toxin components (control).

Group 1: 5 mice: 20xLD_{50} *B. anthracis* PA + 20xLD_{50} *B. anthracis* EF
Group 2: 5 mice: 10xLD_{50} *B. anthracis* PA + 10xLD_{50} *B. anthracis* EF
Group 3: 5 mice: 5xLD_{50} *B. anthracis* PA + 5xLD_{50} *B. anthracis* EF
Group 4: 5 mice: 1xLD_{50} *B. anthracis* PA + 1xLD_{50} *B. anthracis* EF
Group 5: 5 mice: 0.5xLD_{50} *B. anthracis* PA + 0.5xLD_{50} *B. anthracis* EF
Group 6: 5 mice: 10xLD_{50} *B. anthracis* PA
Group 7: 5 mice: 10xLD_{50} *B. anthracis* EF
Group 8: 5 mice: 10xLD_{50} *B. anthracis* PA + 10xLD_{50} *B. anthracis* EF
40 C57BL/6J mice

V.1.2.4. Edema Toxin Experiment 2: Groups 1-5 will be intoxicated with *B. cereus* G9241 toxin components. Group 6 will be intoxicated with *B. anthracis* Sterne toxin components (control).

Group 1: 5 mice: 20xLD_{50} *B. cereus* PA + 20xLD_{50} *B. cereus* EF
Group 2: 5 mice: 10xLD_{50} *B. cereus* PA + 10xLD_{50} *B. cereus* EF
Group 3: 5 mice: 5xLD_{50} *B. cereus* PA + 5xLD_{50} *B. cereus* EF
Group 4: 5 mice: 1xLD_{50} *B. cereus* PA + 1xLD_{50} *B. cereus* EF
Group 5: 5 mice: 0.5xLD_{50} *B. cereus* PA + 0.5xLD_{50} *B. cereus* EF
Group 6: 5 mice: 10xLD_{50} *B. anthracis* PA + 10xLD_{50} *B. anthracis* EF
30 C57BL/6J mice

Total for V.1.2.3 and V.1.2.4: 40 mice + 30 mice = 70 C57BL/6J mice

V.1.2.5. Certhrax Experiment 1: Groups 1-5 will be intoxicated with *B. cereus* G9241 toxin component combinations. Groups 6 and 7 will be intoxicated with the individual *B. cereus* G9241 toxin components.

Group 1: 5 mice: 500 µg PA + 500 µg Certhrax
Group 2: 5 mice: 100 µg PA + 100 µg Certhrax
Group 3: 5 mice: 50 µg PA + 50 µg Certhrax
Group 4: 5 mice: 5 µg PA + 5 µg Certhrax
Group 5: 5 mice: 1 µg PA + 1 µg Certhrax
Group 6: 5 mice: 100 µg PA
Group 7: 5 mice: 100 µg Certhrax
35 C57BL/6J mice x 2 forms of PA = 70 C57BL/6 mice

V.1.2.6. Certhrax Experiment 2: Groups 1-5 will be intoxicated with *B. cereus* G9241 toxin component combinations. Groups 6 and 7 will be intoxicated with the individual *B. cereus* G9241 toxin components.

Group 1: 5 mice: 20xLD_{50} *B. cereus* PA + 20xLD_{50} Certhrax
Group 2: 5 mice: 10xLD_{50} *B. cereus* PA + 10xLD_{50} Certhrax
Group 3: 5 mice: 5xLD_{50} *B. cereus* PA + 5xLD_{50} Certhrax
Group 4: 5 mice: 1xLD_{50} *B. cereus* PA + 1xLD_{50} Certhrax

Group 5: 5 mice: 0.5xLD_{50} *B. cereus* PA + 0.5xLD_{50} Certhrax
Group 6: 5 mice: 10xLD_{50} PA
Group 7: 5 mice: 10xLD_{50} Certhrax
35 C57BL/6J mice x 2 forms of PA = 70 C57BL/6 mice

Total for V.1.2.5 and V.1.2.6: 70 mice + 70 mice = 140 C57BL/6J mice

V.1.2.7: Certhrax and Lethal Toxin Synergy: If we determine that Certhrax and LF act synergistically to elicit increased cytopathicity or cytotoxicity *in vitro*, we will test the synergy hypothesis *in vivo*. For these studies, we will first identify an appropriate sublethal dose of PA1 + LF and, if PA2 acts as a binding partner for LF *in vitro* and/or for Certhrax *in vitro* and *in vivo*, of PA2 + LF. The published LD_{50} for PA1 + LF administered i.v. is ~50 µg of each component (16), so we will conduct a dose response experiment with 4 doses of PA1 + LF and of PA2 and LF. The 4 doses for the PA2 and LF dose response will be determined based on the relative toxicity of PA1 + Certhrax and PA2 + Certhrax (i.e. if the LD_{50} for PA2 + Certhrax is 4-fold lower than the LD_{50} for PA1 + Certhrax, we will use doses of PA2 + LF that are 4-fold lower than the doses for PA1 + LF).

V.1.2.7.1: LD_{50} determination for PA1 + LF to identify a sublethal dose.
5 mice: 10 µg PA1 + 10 µg LF
5 mice: 25 µg PA1 + 25 µg LF
5 mice: 50 µg PA1 + 50 µg LF
5 mice: 500 µg PA1 + 500 µg LF
20 C57BL/6 mice

V.1.2.7.2: LD_{50} determination for PA2 + LF to identify a sublethal dose. For PA2 + LF dose response, doses will be adjusted based on the relative potency of PA2-Certhrax (from V.1.2.5 and V.1.2.6 above)
5 mice: 10 µg PA2 + 10 µg LF
5 mice: 25 µg PA2 + 25 µg LF
5 mice: 50 µg PA2 + 50 µg LF
5 mice: 500 µg PA2 + 500 µg LF
20 C57BL/6 mice

V.1.2.7.3: Assessment of synergy between Certhrax and LF. The doses to be used in these studies will be extrapolated from a comparison of the *in vitro* 50% cytotoxic doses (CD_{50}) and the *in vivo* LD_{50}s. That is, if we find *in vitro* that PA + 0.25 CD_{50} LF + 0.25 CD_{50} Certhrax causes cytotoxicity to 100% of the cells, we will begin our *in vivo* studies with an estimated LD_{50} of PA + 0.25 LD_{50} LF + 0.25 LD_{50} Certhrax.
5 mice: 10 LD_{50} PA1 + 10 LD_{50} LF (positive control for LT-mediated lethality)
5 mice: 10 LD_{50} PA1/PA2 + Certhrax (positive control for Certhrax-mediated lethality)
5 mice: *PA1 + 5.0 LD_{50} LF + 5.0 LD_{50} Certhrax
5 mice: *PA1 + 1.0 LD_{50} LF + 1.0 LD_{50} Certhrax
5 mice: *PA1 + 0.5 LD_{50} LF + 0.5 LD_{50} Certhrax
5 mice: *PA1 + 0.25 LD_{50} LF + 0.25 LD_{50} Certhrax

5 mice: *PA1 + 0.1 LD_{50} LF + 0.1 LD_{50} Certhrax
5 mice: *PA1 + 0.025 LD_{50} LF + 0.025 LD_{50} Certhrax
40 C57BL/6 mice x 4 iterations = 160 C57BL/6 mice

*PA2 will be included in these experiments if we find that PA2 is the preferred *in vivo* binding partner for Certhrax in V.1.2.5 and V.1.2.6 above.

Total for V.1.2.5, V.1.2.6, and V.1.2.7: 70 mice + 70 mice + 200 mice = 340 C57BL/6J mice

Total mice for V.1.2 = 70 + 70 + 340 mice = 480 C57BL/6J mice

V.1.3. Experiment 3: *in vivo* imaging of *B. anthracis* Sterne germination, outgrowth, and dissemination. Our previous *in vivo* imaging studies used the IVIS 100 *in vivo* imaging system to visualize *B. anthracis* Sterne derivatives that produced a luminescent signal when spores germinated or grew vegetatively in response to host signals (17). The luminescent signal was sufficient to provide information about the general location *in vivo* of a large bolus of spores or vegetative bacilli, but we were unable to visualize the less intense signal *in vivo*; instead, we used *ex vivo* imaging of organs post-necropsy at specific time points post-inoculation. To more accurately study the stages of *Bacillus* growth and the locations within the host in which these events occur, we will use our Carestream Multispectral FX Pro (MS FX Pro). The MS FX Pro has a larger dynamic range and a higher signal:noise ratio for fluorescence; in addition, it has x-ray capability that permits more accurate signal localization. Construction of fluorescent *B. anthracis* Sterne reporter strains that contain one of the Red Fluorescent Proteins (RFPs) mKate2, E2-Crimson, and FP650 is underway. These reporters will be controlled by germination, vegetative growth, or sporulation promoters and will be expressed episomally. In addition, we plan to label *B. anthracis* Sterne-RFP spores with an AlexaFluor label, which will allow us to monitor deposition and uptake of spores in the airway in a manner that does not require bacterial transcription, which is required for expression of RFP. The choice of Alexa-Fluor label and RFPs allow for simultaneous measurement of multiple fluorophores at once with the MS FX Pro unmixing functionality of our *in vivo* imaging system.

V.1.3.1. Virulence assessment of *B. anthracis* Sterne-RFP strains. Our earlier studies showed that the LD_{50} for *B. anthracis* Sterne-*sspB::lux* spores administered s.c. to A/J mice is higher than that of the wild-type *B. anthracis* Sterne spores ($1.5x10^4$ CFU vs. $2.3x10^3$ CFU, respectively). Similarly, the LD_{50} for the *B. anthracis* Sterne-*sspB::lux* administered i.n. to A/J mice is $1.4x10^6$ CFU, while the LD_{50} for wild-type *B. anthracis* Sterne is $6.7x10^4$ CFU. In order to use the *B. anthracis* Sterne-RFP constructs to monitor the different stages of infection, we first need to establish the LD_{50} for these strains when delivered s.c. or i.n. As mentioned above, we will test the s.c. and i.n. virulence of three different *B. anthracis* Sterne-RFP constructs (mKate2, E2-Crimson, FP650) that are expressed by promoters that are active during germination, vegetative outgrowth, or sporulation.

V.1.3.1.1. Subcutaneous Inoculation Experiment 1
Group 1: 5 mice: H_2O or sterile saline
Group 2: 10 mice: 10^3 *B. anthracis* Sterne-RFP spores
Group 3: 10 mice: 10^4 *B. anthracis* Sterne-RFP spores
Group 4: 10 mice: 10^5 *B. anthracis* Sterne-RFP spores
Group 5: 10 mice: 10^6 *B. anthracis* Sterne-RFP spores
Group 6: 10 mice: 10^7 *B. anthracis* Sterne-RFP spores
Group 7: 10 mice: 10^8 *B. anthracis* Sterne-RFP spores
Group 8: 10 mice: 10xLD_{50} *B. anthracis* Sterne spores
75 A/J mice x 3 RFP strains = 225 A/J mice

V.1.3.1.2. Subcutaneous experiment 2
Remove two doses farthest from LD_{50} = **55 A/J mice x 3 RFP strains = 165 mice**

Total for s.c. experiments = (75 + 55) x 3 RFP strains = 390 A/J mice

V.1.3.1.3. Intranasal Inoculation Experiment 1
Group 1: 5 mice: H_2O or sterile saline
Group 2: 10 mice: 10^4 *B. anthracis* Sterne-RFP spores
Group 3: 10 mice: 10^5 *B. anthracis* Sterne-RFP spores
Group 4: 10 mice: 10^6 *B. anthracis* Sterne-RFP spores
Group 5: 10 mice: 10^7 *B. anthracis* Sterne-RFP spores
Group 6: 10 mice: 10^8 *B. anthracis* Sterne-RFP spores
Group 7: 10 mice: 10xLD_{50} *B. anthracis* Sterne spores
65 A/J mice x 3 RFP strains = 195 A/J mice

V.1.3.1.4. Intranasal experiment 2
Remove two doses farthest from LD_{50} = **45 A/J mice x 3 RFP strains = 135 A/J mice**

Total for i.n. experiments = (65 + 45) x 3 RFP strains = 330 A/J mice

Total mice for V.1.3.1 = 390 + 330 = 720 A/J mice

V1.3.2. Assessment of deposition, uptake and dissemination using Alexa Fluor-labeled live or dead spores. We will label *B. anthracis* Sterne-RFP spores with an Alexa-Fluor (AF) conjugate, a fluorescent molecule that emits fluorescence when the spore is intact (before germination occurs), so that we can monitor deposition and uptake with the *in vivo* imaging system. We will monitor germination, outgrowth, dissemination, and sporulation through detection of the RFP signal (different excitation and emission wavelengths than the AF). Thus, we will be able to observe each step of inhalational *B. anthracis* disease. In addition, mice will be inoculated with non-viable spores to observe deposition and uptake of spores by host cells in the absence of germination and to determine the length of time that spores can persist in the airway. Epidemiological data from the human anthrax outbreak in Sverdlovsk, USSR in 1979 suggest that viable spores can persist in the airway for up to 45 days after exposure (18).

Female A/J mice will be inoculated i.n. or s.c. (described in V.1, sections B1 and B2) with 10xLD_{50} AF-labeled *B. anthracis* Sterne-RFP spores (determined in V.1.3.1) in

H_2O. At 0.5, 1, 6, 12, 24, 48, 72 hrs, and 2 weeks post-inoculation, 5 mice will be anesthetized with isoflurane and placed on a heated platform within the imaging box of the MS FX Pro for imaging(~ 5 min). After whole animal imaging, each mouse will be euthanized by inhalational isoflurane overdose and cervical dislocation, the body cavity will be opened and the organs exposed for *ex vivo* imaging, then the organs will be harvested and homogenized for enumeration of spores and vegetative bacilli. This experiment will be done twice with each of the three AF-*B. anthracis* Sterne-RFP strains.

V.1.3.2.1: Dissemination of Alexa Fluor-labeled B. anthracis Sterne-RFP spores after i.n. inoculation

Group 1: 5 mice euthanized at 0.5 hr: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 2: 5 mice euthanized at 1 hr: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 3: 5 mice euthanized at 6 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 4: 5 mice euthanized at 12 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 5: 5 mice euthanized at 24 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 6: 5 mice euthanized at 48 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 7: 5 mice euthanized at 72 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 8: 5 mice euthanized at 14 days: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
40 mice x 3 *B. anthracis* strains x 2 iterations = 240 A/J mice

V.1.3.2.2: Dissemination of Alexa Fluor-labeled B. anthracis Sterne-RFP spores after s.c. inoculation

Group 1: 5 mice euthanized at 0.5 hr: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 2: 5 mice euthanized at 1 hr: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 3: 5 mice euthanized at 6 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 4: 5 mice euthanized at 12 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 5: 5 mice euthanized at 24 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 6: 5 mice euthanized at 48 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 7: 5 mice euthanized at 72 hrs: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
Group 8: 5 mice euthanized at 14 days: 10xLD_{50} AF-*B. anthracis* Sterne-RFP spores
40 mice x 3 *B. anthracis* strains x 2 iterations = 240 A/J mice

V.1.3.2.3. Persistence of AF-B. anthracis spores in the airway. We will investigate the number of days that non-viable AF-*B. anthracis* Sterne spores persist in the airways by daily imaging of mice inoculated i.n. with these spores. We predict that the spores will remain fluorescent until they are cleared by the mouse. The AF-*B. anthracis* Sterne spores will be inactivated by ultraviolet irradiation or fixation in 10% formalin for one week. Loss of viability will be confirmed by bacterial enumeration on agar plates and in liquid growth media. A/J mice will be inoculated i.n. with non-viable AF-*B. anthracis* Sterne spores and monitored by whole body *in vivo* and organ *ex vivo* imaging as described in V.1.3.2.1.

Group 1: 5 mice euthanized at 0.5 hr: 10^7 AF-*B. anthracis* Sterne spores
Group 2: 5 mice euthanized at 2 days: 10^7 AF-*B. anthracis* Sterne spores
Group 3: 5 mice euthanized at 4 days: 10^7 AF-*B. anthracis* Sterne spores
Group 4: 5 mice euthanized at 7 days: 10^7 AF-*B. anthracis* Sterne spores

Group 5: 5 mice euthanized at 14 days: 10^7 AF-*B. anthracis* Sterne spores
Group 6: 5 mice euthanized at 21 days: 10^7 AF-*B. anthracis* Sterne spores
Group 7: 5 mice euthanized at 28 days: 10^7 AF-*B. anthracis* Sterne spores
Group 8: 5 mice euthanized at 35 days: 10^7 AF-*B. anthracis* Sterne spores
Group 9: 5 mice euthanized at 42 days: 10^7 AF-*B. anthracis* Sterne spores
Group 10: 5 mice euthanized at 49 days: 10^7 AF-*B. anthracis* Sterne spores
Group 11 5 mice euthanized at 56 days: 10^7 AF-*B. anthracis* Sterne spores
55 mice x 2 iterations = 110 A/J mice

Total mice for V.1.3.2 = 240 + 240 + 110 = 590 A/J mice

Total mice for V.1.3 = 720 + 590 = 1310 C57BL/6J mice

V.1.4. Experiment 4. Characterization of efficacy of therapeutics to treat *B. anthracis* Sterne and/or *B. cereus* G9241 infections.

V.1.4.1. Characterization of efficacy of small molecule inhibitors of germination. Small molecule compounds (SMC) that prevent both *B. anthracis* spore germination and macrophage killing will be tested in A/J mice for the capacity to prevent disease progression and death of animals challenged i.n. or s.c. with otherwise lethal doses of *B. anthracis* Sterne spores. The SMC 6-Thioguanine (6-TG) has been shown to inhibit spore germination *in vitro* (9). In preliminary *in vivo* studies under (b)(6) we found that two doses of 6-TG administered s.c. 4 hrs and 1 hr prior to s.c. inoculation with 10xLD_{50} *B. anthracis* Sterne spores at the same site protected 10/25 mice from mortality and extended the median time-to-death for those mice that succumbed. We also found that i.v. administration of 6-TG 4 hrs and 1 hr prior to s.c. inoculation with 10xLD_{50} *B. anthracis* Sterne spores protected 4/15 mice from death; however, the median time-to-death was not altered in this group. When we administered 6-TG by i.v. injection 30 min after s.c. spore challenge, we rescued 2/15 mice.

In the current studies, we propose to further assess the therapeutic capacity of 6-TG and to determine the efficacy of two SMC that we identified in an *in vitro* screen as germination inhibitors. The two SMC are (b)(4) and (b)(4) and their use in these studies was approved in a minor modification to (b)(4) see Table 1.4.1.1 for the relevant properties of each SMC. In preliminary toxicity studies, we found that all mice injected s.c. with (b)(4) or with (b)(4) at doses up to 25 mg/kg survived. The solubility of these SMC at 25 mg/kg is poor, so

Table V.1.4.1.1 Small molecule inhibitors of *B. anthracis* Sterne spore germination

Name	**6-thioguanine (6-TG)**	(b)(4)	
Inhibitory Activity	sporistatic	sporicidal	sporicidal
Macrophage IC_{50}	450 µM	28 µM	1.6 µM
Macrophage Toxicity	>450 µM	>100 µM	10-50 µM
Mouse Toxicity	i.p. injection LD_{50} = 50 mg/kg	i.p. injection, day 5 survival 50 mg/kg, 200 mg/kg: 5/6 100 mg/kg: 4/6	i.p. injection, day 5 survival 79-266 mg/kg: 6/6 400 mg/kg: 17/18 600 mg/kg: 2/6

we will use the SMC at 0.5 mg/kg (doses up to 25 mg/kg may be used if we identify a more effective diluent that is safe to administer to mice).

Mice will be treated s.c., i.v. or i.n. with 6-TG, (b)(4) diluted in sterile saline containing ≤5% DMSO (and 0.1% Tween-80 for (b)(4) at various time points prior to or after inoculation with 10x LD_{50} *B. anthracis* Sterne spores. Table 1.4.1.2 shows the SMC dosing and timing strategy for s.c. or i.n. spore inoculation and s.c., i.v., i.n., or i.v. SMC inoculation. Two sets of experiments will be conducted for each timing and dosing strategy (Table V.1.4.1.3); in the first experiment, we will use 10 mice/treatment group, and, in the second experiment, we will use 15 mice/treatment group. Further, each experiment will have 10 control mice: 5 will be inoculated with spores only and 5 will be inoculated with SMC only.

V.1.4.1.1. Assessment of efficacy of 6-TG (b)(4) in prevention of spore germination and/or anthrax disease. As stated above, we have very preliminary data that show that pretreatment with two doses of 6-TG at the site of spore injection protected 40% of the mice and extended the median time-to-death for the rest of the treated mice. Herein, we propose to extend these studies to evaluate different doses as well as different administration times and sites for the small molecule therapeutics. In addition, we propose to determine whether two or three of the compounds have synergistic therapeutic potential when co-administered.

Table V.1.4.1.2 SMC Dosing and Timing Strategy

Spore/ SMC Delivery	SMC Time (hrs)	SMC Dose	Control Mice	
S.C./S.C. *or* S.C./I.V. *or* I.N./I.N. *or* I.N./I.V.	-24, -4	1	5 spores 5 SMC	Expt A
		2		
	-4, -1	1		
		2		
	-24, -1	1	5 spores 5 SMC	Expt B
		2		
	+0.5	1		
		2		
	+0 5, +6	1	5 spores 5 SMC	Expt C
		2		
	+0.5, +24	1		
		2		

For s.c. delivery, 0.1 ml SMC will be delivered s.c. at the same site as the spore inoculation with a 26 gauge, 0.5 inch needle attached to a tuberculin syringe. For i.v. or i.p. delivery, 0.1 ml SMC will be delivered by injection with a 26 gauge, 0.5 inch needle attached to a tuberculin syringe. For i.n. delivery of 6-TG, mice will be anesthetized by isoflurane inhalation and 0.05 ml SMC will be delivered i.n. using

Table 1.4.1.3. Number of Mice for V.1.4.1.1.

Spores	SMC	Experiment 1	Experiment 2	Total
s.c.	s.c.	(40 experimental + 10 control) x 3 expts = 120	(60 experimental + 10 control) x 3 expts = 210	30 C 300 E
s.c.	i.v.	(40 experimental + 10 control) x 3 expts = 120	60 experimental + 10 control) x 3 expts = 210	30 C 300 E
i.n.	i.n.	(40 experimental + 10 control) x 3 expts = 120	60 experimental + 10 control) x 3 expts = 210	30 C 300 E
i.n.	i.v.	(40 experimental + 10 control) x 3 expts = 120	60 experimental + 10 control) x 3 expts = 210	30 C 300 E
			Total Number of Mice per SMC	120 C 1200 E
			Number of SMC	3
			Grand Total Number of Mice	360 C 3600 E

a pipet tip attached to a pipette. Mice will be monitored for morbidity and mortality multiple times daily for 2 weeks. Moribund mice will be euthanized by inhalational isoflurane overdose followed by cervical dislocation.

Total number of mice for V.1.4.1.1 = 3960 A/J mice

V.1.4.1.2. Assessment of efficacy of 6-TG, (b)(4) in combination for the prevention of spore germination and/or anthrax disease. Preliminary *in vitro* studies indicate that (b)(4) can act synergistically with 6-TG to prevent spore germination. To assess whether 6-TG and (b)(4) or 6-TG and (b)(4) can act synergistically *in vivo*, we will conduct studies similar to those described in V.1.4.1.1 above, except that two compounds will be administered simultaneously. These studies will be conducted once the best dose, timing, and route of SMC administration are determined in V.1.4.1.1. However, additional dosing studies may be done to determine whether a lower dose of one or both compounds is sufficient to prevent germination and/or anthrax disease.

Table V.1.4.2.1. Synergy Study Strategy

Spore Delivery	SMC Time (hrs)	6-TG Dose	SMC Dose	Control Mice	Number of Mice Expt A	Number of Mice Expt B	Total Mice
S.C.	As determined in V.1.4.1.1	1	1	5 spores 5 SMC	40 expt'l + 10 control = 50	60 expt'l + 10 control = 70	10 C 110 E
		2	2				
		1	2				
		2	1				
I.N.	As determined in V.1.4.1.1	1	1	5 spores 5 SMC	40 expt'l + 10 control = 50	60 expt'l + 10 control = 70	10 C 110 E
		2	2				
		1					
		2	1				
						Grand Total for V.1.4.2.1	**20 C 220 E**

Total number of mice for V.1.4.1.2 = 240 A/J mice

V.1.4.2. Assessment of therapeutic potential of antibodies against *B. cereus* G9241 virulence factors to rescue from G9241-mediated disease. In these studies, we will determine the capacity of antibodies against Certhrax or against the tetrasaccharide (TS) capsule to rescue mice from s.c. and i.n. challenge with *B. cereus* G9241 spores. We obtained from (b)(4) rabbit polyclonal antisera raised by immunization with the ADP-ribosylation domain (ADP-r) of Certhrax or with the G9241 TS capsule (in process). The antibodies in the antisera will be purified by affinity chromatography. Once we confirm *in vitro* that these antibodies can bind to and, in case the case of Certhrax, neutralize their antigen, we will administer them to mice infected with *B. cereus* G9241 spores. These studies will be completed according to the procedures described above in V.1.1, except that we will administer the antibodies at specific times after spore inoculation. Antibodies will be diluted in sterile saline and administered by i.v. or i.p. injection with a 26 gauge, 0.5 inch sterile needle attached to a tuberculin syringe.

Table V.1.4.2. Strategy to Assess Protective Efficacy of Antibodies						
Spore Delivery	**Antibody Dose**	**Antibody Delivery (hrs post-spore)**	**Control Mice**	**Mice Expt A**	**Mice Expt B**	**Total Mice**
S.C.	100 mg/kg	+24	5 spores + 5 antibody = 10 mice x 2 (A+B)	10 expt'l x 4 = 40 mice	15 expt'l x 4 = 60 mice	10 C 110 E
		+48				
		+72				
		+96				
S.C.	200 mg/kg	+24	5 spores + 5 antibody = 10 mice x 2 (A+B)	10 expt'l x 4 = 40 mice	15 expt'l x 4 = 60 mice	10 C 110 E
		+48				
		+72				
		+96				
I.N.	100 mg/kg	+24	5 spores + 5 antibody = 10 mice x 2 (A+B)	10 expt'l x 4 = 40 mice	15 expt'l x 4 = 60 mice	10 C 110 E
		+48				
		+72				
		+96				
I.N.	200 mg/kg	+24	5 spores + 5 antibody = 10 mice x 2 (A+B)	10 expt'l x 4 = 40 mice	15 expt'l x 4 = 60 mice	10 C 110 E
		+48				
		+72				
		+96				
Total Number of Mice per Antibody						40 C 440 E
Number of Antibodies to Test						2
Grand Total						80 C 880 E

Total number of mice for V.1.4.2 = 960 A/J mice

V.1.5. Experiment 5. Generation of Mouse Antiserum against *B. cereus* G9241. In this experiment, we propose to elicit antibodies that are reactive with *in vivo*-expressed antigens in an effort to define critical virulence factors. To do so, we will challenge A/J mice s.c. with a sublethal dose of G9241 spores, then boost at 2-3 week intervals with 1-2 x LD_{50} G9241 spores. Blood will be collected 2-5 days prior to each boost to measure the antibody response. Once a sufficient antibody titer is reached, mice will be exsanguinated by cardiac puncture under deep anesthesia, then euthanized by cervical dislocation. Mice will be monitored for morbidity and mortality (none expected), and extremely moribund mice will be euthanized by inhalational isoflurane overdose followed by cervical dislocation.

5 mice/iteration x 4 iterations = 20 A/J mice

V.2. Data Analysis: A sample size of 5-10 animals per condition will have 80% power to detect a difference of 1.3 - 1.8 standard deviations based on a *t* test for independent samples with a 5%, two-sided significance level. These are the smallest differences that would be considered clinically meaningful for this type of study. Because mice have not been extensively used for work with *Bacillus* species, we need to establish the baseline virulence of the strains and the derivatives of those strains with

which we are working. Furthermore, we are testing several compounds for therapeutic efficacy.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: At this time, there are no non-animal alternatives for animal use that would achieve the objectives outlined in these studies. Our justification for the use of the mouse model to study *Bacillus* virulence is that tissue culture systems challenged with *Bacillus* spores cannot always discriminate between animal-virulent and animal-attenuated strains. *In silico* simulations and *in vitro* cell culture studies were considered but rejected because they cannot address the objectives of these studies.

V.3.2. Animal Model and Species Justification: Animal use will help us to develop prophylactic and therapeutic measures to counter *B. anthracis* spore exposure in humans. The major goals of this project are to characterize small molecule compounds that inhibit germination of *B. anthracis* spores and to identify key virulence determinants in *B. cereus*. The use of murine animal models is essential to the evaluation of therapeutic and preventative strategies against disease caused by *Bacillus* spp. since no *in vitro* systems or models are currently available to predict the efficacy of such reagents. The mouse is one of three small animal models (rabbit and guinea pigs are others) available for studying *B. anthracis* and *B. cereus* virulence and the efficacy of agents that may mitigate the effects of that disease. Mice are the lowest animal on the evolutionary scale with which to perform virulence studies for *B. anthracis* and *B. cereus*.

Studies of pathogenesis and virulence of *Bacillus* spp. have historically been performed in female mice (2-4, 10, 19-22). Our laboratory has always used female mice for these studies, as have other laboratories who study *Bacillus* virulence. In addition, female mice are used to reduce the impact of fighting among the mice. Further, all of the mice in an experimental group can be housed in the same cage when female mice are used.

V.3.3. Laboratory Animals

		Species #1	Species #2
V.3.3.1.	Genus & Species:	*Mus musculus*	*Mus musculus*
V.3.3.2.	Strain/Stock:	A/J	C57BL/6
V.3.3.3.	Source/Vendor:	(b)(4)	
V.3.3.4.	Age:	5-8 weeks	5-8 weeks
V.3.3.5.	Weight:	15-25 grams	15-25 grams
V.3.3.6.	Sex:	*Female	*Female
V.3.3.7.	Special Considerations:	N/A	N/A
V.3.4.	Number of Animals Required (by Species):	**8987**	**2200**

*V.3.3.6: Studies of pathogenesis and virulence of *Bacillus* spp. have historically been performed in female mice (2-4, 10, 19-22). Our laboratory has always used female mice for these studies, as have other laboratories who study *Bacillus* virulence. In addition, female mice are used to reduce the impact of fighting among the mice. Further, all of the mice in an experimental group can be housed in the same cage when female mice are used.

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: We will use small pilot studies to rapidly narrow the number of bacteria, dose of toxin, or dose of therapeutic to be administered in subsequent LD_{50} studies. These pilot studies will be done with the smallest number of mice that will provide the necessary information in a statistically significant manner. We further minimize distress during the studies by careful cage-side monitoring of the animals to determine if an animal should be euthanized as described in V.4.1.4 and V.4.5. Signs indicating that euthanasia should be implemented would include an inability to reach food and water, labored breathing, excessive weight loss, and inability to remain upright.

V.3.5.2. Reduction: The *in vivo* imaging studies described in Experiment V.1.3 represent a significant reduction in the number of animals that would be used to assess germination, dissemination, and vegetative outgrowth in traditional experiments. Traditionally, one group of 5 mice would be euthanized at each time point (up to 8 time points) to harvest organs and enumerate the spores/bacteria in those organs in order to characterize the kinetics and stages of anthrax infection. In our *in vivo* imaging studies, we can instead inoculate a single group of 5-10 mice with spores that express different fluorescently labeled proteins that are markers of germination and vegetative growth. These mice are then imaged under light isoflurane anesthesia at multiple time points throughout the study; we capture fluorescent light images to detect the general location

and density (based on relative fluorescence) of the spores/bacteria and we focus the location with x-ray imaging. Thus, an experiment that would traditionally require up to 40 mice can be completed with just 5-10 mice in these *in vivo* imaging studies.

As many of our experiments involve infected/spore-treated animals or tissues, we do not participate in any shared tissue and/or control group studies with other investigators. We have taken into consideration all information we have gleaned from 12 years of experience working with these anthrax models when proposing subsequent experiments as well as in the refinement of the total number of animals required for the proposed experiments. With respect to the number of animals required for each experiment, we have used the minimal number of animals required for biological and statistical significance (generally 5 to 10 animals per group). Most experiments will be performed in duplicate to confirm the data. In these experiments, the two spore doses that are farthest from the LD_{50} of the strain in question will be eliminated in the second experiment in order to reduce the number of mice used.

V.3.5.3. Replacement: At this time, there are no replacements for animal use that would achieve the objectives outlined in these studies. Our justification for the use of the mouse model to study *Bacillus* virulence is that tissue culture systems challenged with *Bacillus* spores cannot always discriminate between animal-virulent and animal-attenuated strains. *In silico* simulations and *in vitro* cell culture studies were considered but rejected because they cannot address the objectives of these studies.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals: Animals are assigned to pain category E because we use death/moribund status as an endpoint for the majority of our experiments. The symptoms of *Bacillus* infection, which will not be relieved using analgesics or antibiotics, are as follows: ruffled fur, lethargy, hunched posture, decreased alertness, weight loss, difficulty breathing, unresponsiveness to external stimuli, and trembling. Animals that are used as controls in each experiment (no spore inoculation) and animals that will be injected with small molecules during the studies outlined in V.1.4.1 are assigned to pain category C because we do not expect them to suffer in any way from the control treatments. See Table V.4.1.1.1 on the next page for a breakdown of pain category by experiment.

	Species #1 A/J	Species #2 C57BL/6
V.4.1.1.1.1. Column C:	712	160
V.4.1.1.1.3. Column E:	8275	2040

Table V.4.1.1.1. Breakdown by study number of experimental and control animals for pain category assignment.				
Experiment	*Mouse Strain*	*Total number of mice*	*Pain Category C*	*Pain Category E*
V.1.1.1.1	A/J C57BL/6	380 380	30 30	350 350
V.1.1.1.2	A/J C57BL/6	480 480	30 30	450 450
V.1.1.1.3	A/J C57BL/6	380 380	30 30	350 350
V.1.1.1.4	A/J C57BL/6	480 480	30 30	450 450
V.1.1.2.1	A/J	150	30	120
V.1.1.2.2.1	A/J	38	3	35
V.1.1.2.2.2	A/J	43	3	40
V.1.1.2.3.1	A/J	258	18	240
V.1.1.2.3.2	A/J	288	18	270
V.1.2.1	C57BL/6	40	10	30
V.1.2.2	C57BL/6	30	0	30
V.1.2.3	C57BL/6	40	10	30
V.1.2.4	C57BL/6	30	0	30
V.1.2.5	C57BL/6	70	10	60
V.1.2.6	C57BL/6	70	10	60
V.1.2.7.1	C57BL/6	20	0	20
V.1.2.7.2	C57BL/6	20	0	20
V.1.2.7.3	C57BL/6	160	0	160
V.1.3.1.1	A/J	225	15	210
V.1.3.1.2	A/J	165	15	150
V.1.3.1.3	A/J	195	15	180
V.1.3.1.4	A/J	135	15	120
V.1.3.2.1	A/J	240	0	240
V.1.3.2.2	A/J	240	0	240
V.1.3.2.3	A/J	110	0	110
V.1.4.1.1	A/J	3960	360	3600
V.1.4.1.2	A/J	240	20	220
V.1.4.2	A/J	960	80	880
V.1.5	A/J	20	0	20
Total		**11187**	**872**	**10315**

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: We will use inhalation of isoflurane gas (1.5-3.0% in O_2) or i.p. injection with ketamine (100 mg/kg) and xylazine (10 mg/kg) to anesthetize animals for *in vivo* imaging studies, for i.n. inoculation, and for exsanguination. Animals used in s.c and i.p. challenge are not expected to endure anything more than momentary pain, so no analgesics will be necessary. In all anesthesia procedures, depth of anesthesia will be assessed by the onset and

maintenance of slow, shallow breathing as well as the loss of toe pinch response in the animals.

For imaging studies, isoflurane has the advantage of maintaining the mice under anesthesia during the imaging procedure while permitting the animals a quick recovery time. The anesthetized mice will be placed on a warm platform and monitored for fluorescence for 5 min at 0.5, 1, 3, 6, 12, 24, 48, and/or 72 h post infection. To anesthetize the mice and secure their immobility during imaging with the Multispectral *in vivo* imaging system, we will use the following procedures. Administration of isoflurane gas will be done via a Gas Anesthesia System that delivers isoflurane gas to a 5-port anesthesia manifold housed in the imaging chamber of the *in vivo* imaging system. Animals will be initially placed into an induction chamber and anesthetized with isoflurane (3-4%) with oxygen as the carrier gas. Once the animals are properly sedated, they will be swiftly moved to nose cones on the 5-port anesthesia manifold housed in the imaging chamber and maintained at 1.5-3.0% isoflurane. Any unused manifold ports will be closed off. Sufficient time will be given to allow the anesthetic gas to reach the manifold nose cones prior to removing the animals from the induction chamber. Both the induction chamber and manifold will be set to deliver isoflurane at a constant rate. The manifold also provides waste gas scavenging ports to help prevent gas from entering the surrounding work environment. A built-in vacuum system helps ventilate waste gas away from researchers and into disposable isoflurane-absorbing filters.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Only mice that appear to be in good health will undergo anesthesia. We define good health as clean unruffled fur, alert demeanor and no obvious signs of sickness. Mice recovering from anesthesia will be placed into a clean cage with external heat applied (a heating pad placed under the recovery cage) to prevent mice from undergoing a rapid loss in body temperature. Once the mice have roused from anesthesia and appear to be moving in an alert manner, they will be placed back into a clean cage with their cage mates. All mice will be monitored in the laboratory for at least one hour prior to their return to [(b)(6)]

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: AGRICOLA and PUBMED databases

V.4.1.3.2. Date of Search: 24 November 2014

V.4.1.3.3. Period of Search: AGRICOLA (1970 – November 2014), PUBMED (1966 – November 2014, English only)

V.4.1.3.4. Key Words of Search: *Bacillus* AND pain AND (alleviation OR alleviate OR alternative OR reduction) AND (mouse OR mice)

V.4.1.3.5. Results of Search: The search yielded no documents in the AGRICOLA database and two documents in PUBMED. The two manuscripts did not provide any methods for alleviation of pain or distress in the mouse models that we use.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: No analgesics will be given to *Bacillus*-infected animals for the following reasons. Inflammation and/or the inflammatory response are key components of *Bacillus*-mediated disease (macrophages play a key role in the dissemination of *Bacillus* spp). Because we will be using the mouse as a model to study both the pathogenesis of disease and possible treatment therapies, we believe that the use of non-steroidal anti-inflammatory drugs and opioids could confound or possibly mask the extent of *Bacillus*-mediated damage that we will evaluate by histological examination or toxicity (23).

Investigator and staff will carefully monitor *Bacillus*-infected animals and euthanize those that appear moribund because it is our experience that these animals will ultimately succumb to *Bacillus* infection after inoculation. We define extremely moribund as when a mouse exhibits 2 or more of the following symptoms: ruffled fur, lethargy, hunched posture, impaired ambulation that prevents the animals from reaching food and water, excessive weight loss/emaciation, difficult or labored breathing, and the inability to remain upright. Death due to infection after i.n., s.c., or i.p. inoculation with *B. anthracis* and *B. cereus* spores typically occurs 3-5 days post-inoculation. During this time period, mice will be inspected every 4 h during normal working hours and every 8 h during non-business hours. All inspections will be noted on cage cards so that LAM personnel are aware that the mice are being monitored.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: We will be giving i.p., i.v. and s.c. injections. All mouse injections are given with a 26 gauge 0.5 inch needle attached to a tuberculin syringe. All doses are given in a total volume of 0.1-0.2 ml.

For Small Molecule Compound and Passive Antibody Treatment – each mouse will be injected s.c. between the scapulae, at the base of the tail, or behind the right foreleg or i.v. with 0.1 ml of the small molecule in 5% DMSO in water using a 0.5 inch, 26 gauge needle.
For Spore Challenge or Toxin Administration – for each mouse in this set of experiments, heat-activated *Bacillus* spores will be administered via s.c. injection between the scapulae, at the base of the tail, or behind the right foreleg using a tuberculin syringe attached to a 26 gauge 0.5 inch needle. Spores will be in a volume of 100-200 µl/animal. Toxin components will be injected i.p. through the ventral abdominal wall using a tuberculin syringe attached to a 26 gauge 0.5 inch needle.

V.4.4.2. Biosamples:
Blood samples will be obtained from the tail vein or the venous sinus of mice using either a 26 gauge ½" needle or by nicking the tail with a sterile razor blade and collecting about 0.1 ml of whole blood into a sterile 1 ml tube. Blood collections will occur 10-14 days apart. Exsanguination by terminal cardiac puncture will be conducted under deep isoflurane anesthesia. Once anesthetized, as confirmed by lack of toe-pinch response, each mouse will be placed on its back and a sterile 23 gauge ½" needle will be introduced directly into the heart through the chest cavity. The syringe plunger will be gently pulled to remove as much blood as possible from the mouse.
Lungs and other organs will be removed from the mice post-euthanasia as needed.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Mice will be identified by cage cards and/or ear punch. Ear punches will be administered by (b)(6) staff (e.g. models EP-901 or EP-900, Braintree Scientific Inc., Braintree, MA 02185). (also see Section VI for staff qualifications).

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures:
Inoculation or immunization by the i.n. route will take place under isoflurane anesthesia by the droplet method. Approximately 50 µl will be administered onto the nose of each mouse using a previously sterile pipet tip attached to a manual pipetman. The mouse will be allowed to inhale the droplet at will and then be returned to its cage. Mice will remain in the laboratory for one hour after i.n. inoculation.

Antibiotics will be administered orally in the drinking water at a dose of 5-10 g/L for experiments in which a plasmid needs to be maintained in the infecting strain (V.1.1 and V.1.3). We will use chloramphenicol, ampicillin, spectinomycin, or kanamycin. In addition, we will add 25mM isopropyl β-D-thiogalactoside (IPTG) to the water for experiments in which gene expression needs to be induced. The antibiotics will be

administered 0-16 hrs prior to inoculation and will be maintained throughout the duration of the experiment. Antibiotic/IPTG-containing water will be changed every 1-2 days.

Oral gavage of 0.1-0.2 ml spores or vegetative cells suspended in sterile saline will be performed using a 18-20 gauge feeding needle. Anesthesia is not required for this procedure.

Low fluorescence food and bedding will be provided to mice that will undergo *in vivo* imaging to reduce the background fluorescence caused by standard high-chlorophyll food and bedding. Food and bedding will be purchased from (b)(4) by the laboratory.

Imaging of mice: Anesthetized mice will be placed on a warm platform within the Carestream Multispectral *in vivo* FX Pro imaging system and monitored for fluorescence for 5 min at a time at 0.5, 1, 3, 6, 12, 24, 48, and/or 72 h post-inoculation.

Photography: Mice may be photographed during inoculation procedures, during *in vivo* imaging, and post-mortem. Photos of inoculation procedures will only be used for teaching rodent handling techniques to new members of the laboratory. Photos from *in vivo* imaging will be used in seminars presented by approved users on this protocol and may be published in research articles. No photos will be taken of mice that are visibly moribund. The photos will be stored only on password-protected DoD computers at USUHS. Photos will be disposed of 5 years after the end of the project. All photography procedures will adhere to IACUC Policy #25.

V.4.4.8. Tissue Sharing: As many of our experiments involve infected/spore-treated animals or tissues, we do not participate in any shared tissue and/or control group studies with other investigators.

V.4.5. Study Endpoint: Table V.4.5.1 below summarizes the endpoint for each proposed experiment. The study endpoint for virulence and challenge studies with infected or intoxicated mice will be death or euthanasia when extremely moribund or euthanasia approximately 14 days post-inoculation with spores. The study endpoint for mice monitored for fluorescence will be 72 h or 2 weeks post-infection. For all studies, mice will be weighed daily to determine if a correlation between weight loss and eventual death exists. Investigator and staff will carefully monitor *Bacillus*-infected animals and euthanize those that appear moribund because it is our experience that these animals will ultimately succumb to *Bacillus* infection after inoculation. We define extremely moribund as when a mouse exhibits 2 or more of the following symptoms: ruffled fur, lethargy, hunched posture, impaired ambulation that prevents the animals from reaching food and water, excessive weight loss/emaciation, difficult or labored breathing, and the inability to remain upright. Our definition of extreme moribund status is consistent with USUHS IACUC Policy #20. Death due to infection after i.n., s.c., or i.p. inoculation with *B. anthracis* and *B. cereus* typically occurs 3-5 days post-inoculation. During this time period, mice will be inspected every 4 h during normal

working hours and every 8 h during non-business hours. All inspections will be noted on cage cards so that LAM personnel are aware that the mice are being monitored.

Table V4.5.1. Endpoint and time to endpoint for each study in proposal

Experiment	*Number of mice*	*Endpoint*	*Time to endpoint*
V.1.1.1.1	760	Death or euthanasia when moribund	14 days
V.1.1.1.2	960	Death or euthanasia when moribund	14 days
V.1.1.1.3	760	Death or euthanasia when moribund	14 days
V.1.1.1.4	960	Death or euthanasia when moribund	14 days
V.1.1.2.1	150	Death or euthanasia when moribund	28 days
V.1.1.2.2.1	38	Death or euthanasia when moribund	28 days
V.1.1.2.2.2	43	Death or euthanasia when moribund	28 days
V.1.1.2.3.1	258	Death or euthanasia when moribund	28 days
V.1.1.2.3.2	288	Death or euthanasia when moribund	28 days
V.1.2.1	40	Death or euthanasia when moribund	14 days
V.1.2.2	30	Death or euthanasia when moribund	14 days
V.1.2.3	40	Death or euthanasia when moribund	14 days
V.1.2.4	30	Death or euthanasia when moribund	14 days
V.1.2.5	70	Death or euthanasia when moribund	14 days
V.1.2.6	70	Death or euthanasia when moribund	14 days
V.1.2.7.1	20	Death or euthanasia when moribund	14 days
V.1.2.7.2	20	Death or euthanasia when moribund	14 days
V.1.2.7.3	160	Death or euthanasia when moribund	14 days
V.1.3.1.1	225	Death or euthanasia when moribund	14 days
V.1.3.1.2	165	Death or euthanasia when moribund	14 days
V.1.3.1.3	195	Death or euthanasia when moribund	14 days
V.1.3.1.4	135	Death or euthanasia when moribund	14 days
V.1.3.2.1	240	Euthanasia	0.5, 1, 6, 12, 24, 48, 72 hrs, 14 days
V.1.3.2.2	240	Euthanasia	0.5, 1, 6, 12, 24, 48, 72 hrs, 14 days
V.1.3.2.3	110	Euthanasia	0.5 hr, 2, 4, 7, 14, 21, 28, 35, 42, 49, 56 days
V.1.4.1.1	3960	Death or euthanasia when moribund	14 days
V.1.4.1.2	240	Death or euthanasia when moribund	14 days
V.1.4.2	960	Death or euthanasia when moribund	14 days
V.1.5	20	Euthanasia	Up to 16 weeks

V.4.6. <u>Euthanasia</u>: Mice will be euthanized by either isoflurane overdose (4-5% concentration) within the isoflurane induction chamber located inside a fume hood, followed by cervical dislocation, or by CO_2 overdose. CO_2 will be delivered from a compressed gas cylinder via a regulated rate of flow in accordance with the 2013 AVMA Committee on Euthanasia. For euthanasia using CO_2, all procedures will be performed by LAM personnel in LAM facilities. Investigative staff will perform inhalational isoflurane overdose and cervical dislocation euthanasia procedures; death will be

confirmed by physical examination (as listed in the 2013 AVMA Guidelines on Euthanasia).

V.5. Veterinary Care: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6) (b)(6)

V.5.1.2.

Special Husbandry Provisions:

Food Restriction:	Yes _____	No X
Fluid Restriction:	Yes	No X

V.5.1.3. Exceptions: Mice that undergo oral gavage in Experiments V.1.1.2 will have food removed the night before inoculation and water removed 2-4 hrs before inoculation. Mice that undergo *in vivo* imaging will be provided low-fluorescence food and bedding.

All mice infected with *B. cereus* or *B. anthracis* Sterne or intoxicated with Certhrax or the anthrax toxins will be housed in cages marked with "DO NOT TOUCH" cards. (b)(6) lab personnel will perform all cage changes and routine care of these animals. For safety reasons, LAM personnel should not handle these mice unless a sick call request is made.

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be observed and cared for by the Center for LAM personnel according to USUHS Center for LAM SOPs. In addition, infected mice will be cared for and checked twice daily or more frequently as needed by members of Dr (b)(6) laboratory staff working on this project.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM

SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING: Dr. (b)(6) who will not do these "hands on" experiments herself, has 38 years of experience working with mice in microbicidal assays and has been involved with the mouse anthrax model since 2002. Dr. (b)(6) is a postdoctoral fellow with 10 years of experience working with rodents and has taken the Investigator Training Course. Dr (b)(6) has 18 years of experience using animal models of infection with various organisms; she has taken the USUHS Investigator Training Course and a rodent handling course at another institution. Ms (b)(6) is a graduate student with 3 years of experience working with rodents. Mr (b)(6) s a senior technician who has worked for 23 years with EHEC/EPEC infection in mice and 10 years with anthrax models. Ms (b)(6) is a senior technician who has worked with rodents for 5 years. All personnel listed here completed the CITI Investigators, Staff and Students – Lab Animal Research Training in 2014. Please see the table below for a specific breakdown of the procedures that each individual will perform and each person's training and experience.

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Intranasal inoculation	(b)(6)	Senior Scientist, 18 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007), 6 (2014)
Intranasal inoculation		Postdoctoral Fellow, 10 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr (b)(6) 5 (UVA, 2005), 6 (2014)
Intranasal inoculation		Graduate Student, 3 yrs experience	1 (2011), 4 & 5 (2011-2014, USU), 6 (2014)
Intranasal inoculation		Senior Research Technician, 23 yrs experience	1, 4 (FDA (b)(6) 2004), 6 (2014)
Intranasal inoculation		Senior Research Technician, 5 yrs experience	1 (2014), 4 (Johns Hopkins, 2009; (b)(6) lab, 2014), 5 (JHU, 2009), 6 (2014)
Subcutaneous injection		Senior Scientist, 18 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007), 6 (2014)
Subcutaneous injection		Postdoctoral Fellow, 10 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr (b)(6) 5 (UVA, 2005), 6 (2014)
Subcutaneous injection		Graduate Student, 3 yrs experience	1 (2011), 4 & 5 (2011-2014, USU), 6 (2014)
Subcutaneous injection		Senior Research Technician, 23 yrs experience	1, 4 (FDA (b)(6) 2004), 6 (2014)
Subcutaneous injection		Senior Research Technician, 5 yrs experience	1 (2014), 4 (Johns Hopkins, 2009; (b)(6) lab, 2014), 5 (JHU, 2009), 6 (2014)
Intraperitoneal injection		Senior Scientist, 18 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007), 6 (2014)
Intraperitoneal injection		Postdoctoral Fellow, 10 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr (b)(6) 5 (UVA, 2005), 6 (2014)
Intraperitoneal injection		Graduate Student, 3 yrs experience	1 (2011), 4 & 5 (2011-2014, USU), 6 (2014)

Intraperitoneal injection	(b)(6)	Senior Research Technician, 23 yrs experience	1, 4 (FDA (b)(6) 2004), 6 (2014)
Intraperitoneal injection		Senior Research Technician, 5 yrs experience	1 (2014), 4 (Johns Hopkins, 2009; (b)(6) lab, 2014), 5 (JHU, 2009), 6 (2014)
Isoflurane anesthesia		Senior Scientist, 18 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007), 6 (2014)
Isoflurane anesthesia		Postdoctoral Fellow, 10 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr (b)(6) , 5 (UVA, 2005), 6 (2014)
Isoflurane anesthesia		Graduate Student, 3 yrs experience	1 (2011), 4 & 5 (2011-2014, USU), 6 (2014)
Isoflurane anesthesia		Senior Research Technician, 23 yrs experience	1, 4 (FDA (b)(6) 2004), 6 (2014)
Protocol activity or procedure (e.g., tail vein injections, euthanasia)	**Name of person performing activity**	**Qualifications of person performing activity (e.g., research technician, 2 yrs experience)**	**Specific training in this activity or procedure (e.g., rodent handling class, 1999)**
Isoflurane anesthesia	(b)(6)	Senior Research Technician, 5 yrs experience	1 (2014), 4 (Johns Hopkins, 2009; (b)(6) lab, 2014), 5 (JHU, 2009), 6 (2014)
Oral gavage		Postdoctoral Fellow, 10 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr (b)(6) 5 (UVA, 2005), 6 (2014)
Oral gavage		Graduate Student, 3 yrs experience	1 (2011), 4 & 5 (2011-2014, USU), 6 (2014)
Oral gavage		Senior Research Technician, 23 yrs experience	1, 4 (FDA (b)(6) 2004), 6 (2014)
Oral gavage		Senior Research Technician, 5 yrs experience	1 (2014), 4 (Johns Hopkins, 2009; (b)(6) lab, 2014), 5 (JHU, 2009), 6 (2014)
In vivo imaging		Postdoctoral Fellow, 10 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr (b)(6) 5 (UVA, 2005), 6 (2014)
In vivo imaging		Graduate Student, 3 yrs experience	1 (2011), 4 & 5 (2011-2014, USU), 6 (2014)
In vivo imaging		Senior Research Technician, 23 yrs experience	1, 4 (FDA (b)(6) 2004), 6 (2014)
In vivo imaging		Senior Research Technician, 5 yrs experience	1 (2014), 4 (Johns Hopkins, 2009; (b)(6) lab, 2014), 5 (JHU, 2009), 6 (2014)
Blood collection: retro-orbital or tail venipuncture or nick		Senior Scientist, 18 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007), 6 (2014)
Blood collection: retro-orbital or tail venipuncture or nick		Postdoctoral Fellow, 10 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr (b)(6) 5 (UVA, 2005), 6 (2014)
Blood collection: retro-orbital or tail venipuncture or nick		Graduate Student, 3 yrs experience	1 (2011), 4 & 5 (2011-2014, USU), 6 (2014)
Blood collection: retro-orbital or tail venipuncture or nick		Senior Research Technician, 23 yrs experience	1, 4 (FDA (b)(6) 2004), 6 (2014)
Blood collection: retro-orbital or tail venipuncture or nick		Senior Research Technician, 5 yrs experience	1 (2014), 4 (Johns Hopkins, 2009; (b)(6) lab, 2014), 5 (JHU, 2009), 6 (2014)
Exsanguination by cardiac puncture		Senior Scientist, 18 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007), 6 (2014)

Exsanguination by cardiac puncture	(b)(6)	Postdoctoral Fellow, 10 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr (b)(6) , 5 (UVA, 2005), 6 (2014)
Exsanguination by cardiac puncture		Graduate Student, 3 yrs experience	1 (2011), 4 & 5 (2011-2014, USU), 6 (2014)
Exsanguination by cardiac puncture		Senior Research Technician, 23 yrs experience	1, 4 (FDA (b)(6) 2004), 6 (2014)
Exsanguination by cardiac puncture		Senior Research Technician, 5 yrs experience	1 (2014), 4 (Johns Hopkins, 2009; (b)(6) lab, 2014), 5 (JHU, 2009), 6 (2014)
Euthanasia with isoflurane and cervical dislocation		Senior Scientist, 18 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007), 6 (2014)
Euthanasia with isoflurane and cervical dislocation		Postdoctoral Fellow, 10 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr (b)(6) 5 (UVA, 2005), 6 (2014)
Protocol activity or procedure (e.g., tail vein injections, euthanasia)	**Name of person performing activity**	**Qualifications of person performing activity (e.g., research technician, 2 yrs experience)**	**Specific training in this activity or procedure (e.g., rodent handling class, 1999)**
Euthanasia with isoflurane and cervical dislocation	(b)(6)	Graduate Student, 3 yrs experience	1 (2011), 4 & 5 (2011-2014, USU), 6 (2014)
Euthanasia with isoflurane and cervical dislocation		Senior Research Technician, 23 yrs experience	1, 4 (FDA (b)(6) 2004), 6 (2014)
Euthanasia with isoflurane and cervical dislocation		Senior Research Technician, 5 yrs experience	1 (2014), 4 (Johns Hopkins, 2009; (b)(6) lab, 2014), 5 (JHU, 2009), 6 (2014)
Ear punch		Senior Scientist, 18 yr experience	1 (2008), 3 (1997), 5 (UAB, 1997; UW 2004; NIH 2007), 6 (2014)
Ear punch		Postdoctoral Fellow, 10 yr experience	1 (2011), 3 (UVA, 2005), 4 (2011, Mr (b)(6) 5 (UVA, 2005), 6 (2014)
Ear punch		Graduate Student, 3 yrs experience	1 (2011), 4 & 5 (2011-2014, USU), 6 (2014)
Ear punch		Senior Research Technician, 23 yrs experience	1, 4 (FDA (b)(6) 2004), 6 (2014)
Ear punch		Senior Research Technician, 5 yrs experience	1 (2014), 4 (Johns Hopkins, 2009; (b)(6) lab, 2014), 5 (JHU, 2009), 6 (2014)

1 USUHS Investigator Training Course
2 USUHS Rodent Handling Course
3 Other Rodent Handling Course
4 Site Specific Training
5 External Course Training
6 CITI Training

VII. **BIOHAZARDS/SAFETY**: With respect to mice used in the anthrax model, bedding may be contaminated with *Bacillus* spores. The bedding and animal carcasses should be treated as infectious and autoclaved. Infected animals will be maintained under ABSL-2 conditions in filter-top cages. All procedures that involve the use of isoflurane will be conducted in either a chemical fume hood (euthanasia) or in an anesthesia system with waste-scavenging charcoal filters (both located in Dr. (b)(6) laboratory). In addition, all personnel who will use isoflurane will be made aware of its associated safety hazards and will be properly trained in its use.

All personnel will wear a disposable gown, gloves, and mask at all times when handling mice. A N95 respirator mask will be worn during spore inoculation procedures and spore inoculation procedures will be conducted in a Type II Biosafety Cabinet (in (b)(6) (b)(6) when possible. Sharps will be disposed of in approved Sharps containers located in (b)(6) and Sharps containers will be disposed of as Regulated Medical Waste through the EHS office. All cage changes will be done in a Type II Biosafety Cabinet (in (b)(6)

VIII. **ENCLOSURES**: Funding Document

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IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 11/24/14

Principal Investigator Signature — Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 11/24/14

Principal Investigator Signature — Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) [redacted]

Principal Investigator Signature

11/24/14
Date

X. **PROTOCOL ABSTRACT:**

A. **Animal Protocol Number:** (b)(6)

B. **Animal Protocol Title:** Pathogenesis of *Bacillus anthracis* Sterne and *Bacillus cereus* G9241 in mice

C. **Principal Investigator:** (b)(6) PhD

D. **Performing Organization:** Uniformed Services University of the Health Sciences

E. **Funding:** Biological Defense Research Directorate, National Institute of Allergy and Infectious Diseases

F. **Objective and Approach:** Our hypotheses for the studies proposed herein are as follows: 1) deletion of putative or known virulence genes will alter the pathogenesis of *B. anthracis* Sterne and *B. cereus* G9241; 2) Certhrax plays a significant role in gastrointestinal disease caused by *B. cereus* G9241 and antibodies against the toxin will protect against disease progression; and 3) small molecules that inhibit spore germination will reduce mortality caused by *B. anthracis* Sterne. Our goals are to characterize the progression of *B. anthracis* Sterne disease, to characterize the roles of toxins and capsules in *B. cereus* G9241 virulence, and to identify candidate therapeutics to prevent or treat anthrax disease. The specific aims are as follows: **1)** assess the virulence of *B. anthracis* Sterne or *B. cereus* G9241 strains in which a particular virulence trait has been deleted; **2)** administer *B. cereus* G9241 toxin components to assess their activity *in vivo*; **3)** monitor the location and kinetics of germination, vegetative outgrowth, and dissemination of fluorescent derivatives of *B. anthracis* Sterne and *B. cereus* G9241 in the mouse with an *in vivo* imaging system; 4) characterize the *in vivo* efficacy of small molecules that inhibit germination of *B. anthracis* Sterne spores *in vitro*; and **5)** assess the capacity of antibodies against *B. cereus* G9241 toxins or capsule to protect from disease.

G. **Indexing Terms (Descriptors):** *Bacillus anthracis* Sterne, *Bacillus cereus*, toxin, capsule, virulence, pathogenesis, small molecule compound, germination, therapeutic

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

September 17, 2015

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) by Designated Member Review on September 17, 2015:

Animal Protocol Title: "Axenic Caenorhabditis Elegans Antigen as Treatment for Experimental Autoimmine Encephalomyelitis in Mice (Mus Musculus)"

USUHS Protocol Number: (b)(6)

Expiration Date: September 16, 2018

Supporting Grant(s) Number: TBD

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Axenic *Caenorhabditis elegans* antigen as treatment for experimental autoimmune encephalomyelitis in mice (*Mus musculus*)

GRANT TITLE (if different from above): Pending

Revised

USUHS PROJECT NUMBER:

FUNDING AGENCY:

EARLIEST ANTICIPATED FUNDING START DATE: January 15, 2016

PRINCIPAL INVESTIGATORs: (b)(6) M.D.

(b)(6) _____ MIC (b)(6) 7/29/15

Principal Investigator Signature | Department | Office/Lab Telephone | Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) _____ Chair (b)(6) _____

Research Unit Chief / Dept. Head Signature | Title | Telephone | Date

Typed Name (b)(6) Ph.D.

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the s

(b)(6) _____ MIC (b)(6) 7/29/15

Statistician Signature | Department | Telephone | Date

Typed Name (b)(6) M.D.

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) _____ LAM (b)(6) 29 July 15

n Signature | Department | Telephone | Date

DVM, MPH

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATORs: (b)(6) M.D. and (b)(6) Ph.D.

ANIMAL PROTOCOL TITLE: *Caenorhabditis elegans* antigen as treatment for experimental autoimmune encephalomyelitis in mice (*Mus musculus*)

GRANT TITLE (if different from above): Pharmacokinetics, and neuroprotection of aCeAg (axenic *C. elegans* antigen)

USUHS PROJECT NUMBER:

CO-INVESTIGATOR(S): (b)(6) Ph.D.
(b)(6) Ph.D.
(b)(6) MPH, CPH

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

Autoimmune diseases are characterized by hyper-reactive immune responses to self- antigens expressed on cells. The immune system mistakes a self-antigen in the body as a pathogen and mounts an attack against the cell or tissue expressing the self-antigen. These attacks are typically chronic, and lead to long-term tissue damage. Several autoimmune diseases appear to be due, in large part, to Th1-driven inflammation based on observed cytokine profiles and predominantly high levels of IFN-γ. Examples of autoimmune diseases include, but are not limited to, type-1 insulin-dependent diabetes mellitus, multiple sclerosis, Crohn's disease, inflammatory bowel disease, rheumatoid arthritis, and posterior uveitis.

Multiple sclerosis (MS) is an inflammatory disease of the nervous system in which the insulating myelin sheaths of brain and spinal cord nerve cells are targeted by an autoimmune T cell response. Chronic inflammation leads to scarring of these nerve cells and disrupted communication between the brain and body. With time, irreversible deterioration of the nerves often occurs. MS is thought to affect more than 2.3 million people worldwide. There is no known cure and treatment regimes vary with the severity of clinical symptoms. The major focus of treatment includes recovery from ongoing attacks (anti-inflammatory corticosteroids), symptoms management (physical therapy and muscle relaxants), and inhibition of remittent disease progression (immuno-modulatory drugs).

The prevalence of autoimmune diseases has increased sharply over the past few decades. While genetic factors play a role in susceptibility to these diseases, the dramatic worldwide increase in their prevalence is probably due to changes in environmental factors. One environmental change that may play a part in the recent increase in autoimmune disease is the loss of chronic parasitic worm infections in developed countries. Multiple studies have found that individuals infected with chronic parasitic worm infections have lower rates of autoimmune diseases than others living in the same environment. Experimentally, parasitic worms have been shown to protect against a variety of autoimmune diseases in several animal models. And in human trials, oral administration of porcine whipworm eggs has been shown to protect against inflammatory bowel disease.

A major limitation of working with parasitic worms is the difficulty in producing large

quantities due to their complex lifecycles. For example, *Litomosoides sigmodontis*, the parasitic worm we work with in our laboratory, requires both mite and rodent hosts to complete its lifecycle. Consequently, we tested whether molecules from crushed up *Caenorhabditis elegans*, a free-living non-parasitic worm that is easily maintained in a laboratory setting, can protect against Type 1 diabetes. Data from our lab demonstrates that repeated injections of the soluble fraction of crushed *C. elegans* protects against Type 1 diabetes in NOD mice, and appears to do so utilizing similar immuno-modulatory mechanisms seen in our studies with *L. sigmodontis*. Thus, treatment with *C. elegans* antigen (aCeAg) suggests a plausible new avenue of therapy for Th1-associated autoimmune diseases.

The goal of this project is to analyze the potential for protective and/or suppressive effects of aCeAg treatments in the relapsing/remitting Multiple Sclerosis model of SJL/j mice. Thus, we will determine the potential therapeutic reach of this non-parasitic nematode-derived treatment, and gain a better understanding of the immuno-modulatory responses to such treatments.

II. BACKGROUND:

II.1. Background:

The prevalence of allergies and autoimmune diseases has increased sharply over the past few decades. While genetic factors play a role in susceptibility, the dramatic worldwide increase of these hyperinflammatory diseases is probably due to changes in environmental factors. One environmental change that may play a part in the recent increase in autoimmune disease is the loss of chronic parasitic worm infections in developed countries. Multiple studies have found that individuals infected with chronic parasitic worm infections have lower rates of autoimmune disease than others living in the same environment. Experimentally, parasitic worms have been shown to protect against a variety of autoimmune diseases, including Multiple sclerosis, in several animal models. In humans, oral administration of porcine whipworm eggs has been shown to protect against inflammatory bowel disease.

Until recently, most people had lifelong infections with parasitic worms. As helminths have been identified in Neolithic and pre-Columbian mummies, it is likely that the human immune system evolved in the setting of chronic infection with these parasites. Consequently, it has been posited that the loss of parasitic worm infections is partially responsible for the increased prevalence of autoimmune and allergic diseases in developed countries – the notion being that now, in the absence of the immuno-modulatory responses triggered by helminths, our immune systems have become hyper-responsive.

Unlike most bacterial or viral pathogens, helminth infections induce the production of specific IgE. This IgE binds to basophils and mast cells through the Fc epsilon receptor I (FcεRI), the high affinity IgE receptor. Helminth specific antigens thus activate basophils and mast cells by cross-linking IgE molecules and aggregating FcεRIs. As helminths are large organisms that release substantial amounts of antigen, and as these infections last for years, helminth infections likely induce a state of chronic basophil and mast cell activation. Indeed, recent time course studies in our lab demonstrate that both chronic basophil activation and chronic mast cell activation occur during infection of mice with the filarial nematode *Litomosoides sigmodontis*.

Helminth infections also induce production of the cytokine IL-10. IL-10 is an anti-inflammatory cytokine that was initially described as a Th2-type or Treg-type cytokine

but it is now known that IL-10 is more broadly expressed. IL-10 acts as a crucial feedback regulator of diverse immune responses, including Th1-mediated and Th2-mediated immune responses.

There are at least two likely mechanistic rationales for postulating that chronic activation of basophils and mast cells may protect against Th1-driven autoimmune disease. First, factors released by basophils and mast cells may have direct immuno-modulatory properties that are protective against Th1-mediated autoimmune diseases. Basophils, for example, release large quantities of IL-4 when activated and have been shown to do so in response to parasite antigen in filaria-infected patients as well as in animal models of helminth infection. IL-4 counter regulates Th1 responses and has been shown to improve Th1-driven autoimmune diseases. Similarly, histamine, which is released from both basophils and mast cells, has been shown *in vitro* to suppress Th1 responses by signaling through the H2 receptor on lymphocytes. Second, chronic activation of basophils and mast cells could induce negative feedback pathways that tamp down ongoing autoimmune responses. Interestingly, there is substantial evidence that chronic immunotherapy, in which patients with IgE-mediated allergies are given weekly injections of allergen, augments immune regulatory networks such as the suppressive cytokine IL-10 and natural T-regulatory cells.

To determine whether recapitulation of the IgE-mediated immune responses induced by helminths can afford protection against autoimmunity in the absence of actual infection, non-obese diabetic (NOD) mice were repeatedly administered intraperitoneal injections of axenic *C. elegans* homogenate. Like parasitic helminthes, *C. elegans* is in the phylum Nematoda. NOD mice spontaneously develop Type 1 diabetes (also known as insulin dependent diabetes mellitus), a form of diabetes that develops from the autoimmune destruction of the insulin-producing beta islet cells of the pancreas, and are a recognized animal model for Type 1 diabetes. To mimic chronic helminth infections *in vivo* without using live parasitic worms, NOD mice were treated with twice weekly injections of axenic *C. elegans* homogenate. Treated mice exhibited increases in circulating basophils, eosinophils, total IgE, and *C. elegans*-specific IgE. These immunologic changes are consistent with those observed in chronic helminth infections. Additionally, splenocytes of *C. elegans*-treated mice released substantial quantities of the down-regulatory cytokine IL-10, whereas those from control-treated mice did not. Consistent with our hypothesis, mice given axenic *C. elegans* antigen injections were significantly protected from developing autoimmune diabetes (10% disease rate vs. 80% in controls) and exhibited less inflammation in the pancreatic islets.

These results demonstrate that axenic *C. elegans* homogenate therapy can protect against the onset of Type I diabetes in NOD mice and suggests that repeated administration of axenic *C. elegans* homogenate represents a new avenue of therapy for Th1-associated autoimmune diseases.

The goal of our current project is to analyze protective and/or suppressive effects of aCeAg treatments in the relapsing/remitting Multiple Sclerosis model of SJL/j mice. Additionally, we will investigate possible immuno-modulatory changes to better understand the therapeutic mechanisms of this treatment. In doing so, we will determine the potential reach of this non-parasitic nematode-derived treatment of autoimmune disease.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: DOD BRD, NIH RePORTER, PUBMED

II.2.2. Date of Search: 7/21/2015

II.2.3. Period of Search: DOD BRD 2009-2014, NIH RePORTER (active projects in past 5 years), PUBMED 2009-2014

II.2.4. Key Words and Search Strategy:

DOD BRD search 1: multiple AND sclerosis AND elegans AND mice
DOD BRD search 2: autoimmune AND elegans AND mice

NIH REPORTER search 1: multiple AND sclerosis AND elegans AND mice
NIH REPORTER search 2: autoimmune AND elegans AND mice

PUBMED search 1: multiple AND sclerosis AND elegans AND mice
PUBMED search 2: autoimmune AND elegans AND mice

II.2.5. Results of Search:

DOD BRD search 1: No results
DOD BRD search 2: No results

NIH REPORTER search 1: 6 unique studies, none evaluating protection against multiple sclerosis by *C. elegans* or nematode products.
NIH REPORTER search 2: 4 unique studies, none evaluating protection against multiple sclerosis by *C. elegans* or nematode products

PUBMED search 1: 4 unique studies, none evaluating protection against multiple sclerosis by *C. elegans* or nematode products.
PUBMED search 2: 15 unique studies, none evaluating protection against multiple sclerosis by *C. elegans* or nematode products

III. OBJECTIVE\HYPOTHESIS: The objective of this proposal is to discover potential new treatments for Multiple sclerosis and to better understand the immuno-modulatory mechanisms of helminth/nematode therapy.

IV. MILITARY RELEVANCE:

Multiple sclerosis and other type 1-driven autoimmune processes are common diseases that cause a high degree of morbidity worldwide. Since it is an exclusion criterion for enlistment in the U.S. military (with typical diagnoses of the disease occurring between the ages of 20 and 50), and as it is estimated that 400,000 Americans have been diagnosed, this disease significantly limits the number of Americans able to volunteer for active military duty. Additionally, the research described in this proposal has the potential to lead to new modalities of treatment for other autoimmune diseases that are common in military personnel, including Type 1 diabetes, rheumatoid arthritis, and systemic lupus erythematosis.

V. MATERIALS AND METHODS:

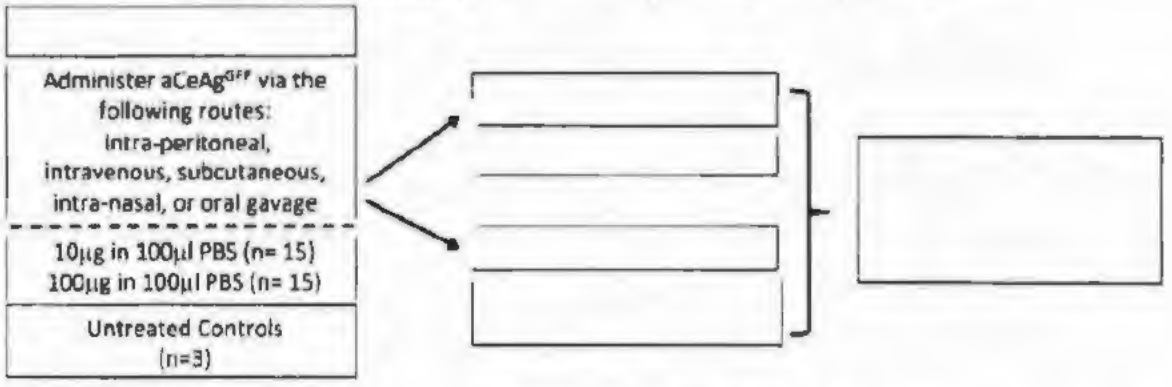
V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1: The purpose of this procedure is to establish a snapshot of the pharmacokinetics (pK) of axenically derived *C. elegans* antigen ($aCeAg^{-/+GFP}$) in SJL/j mice. Pain Category C -- 33 mice

Experimental design: aCeAgG$^{-/+GFP}$ will be processed in the laboratory via methods previously described by C.P. Morris, *et al.* (Experimental Parasitology 135, 2013). Briefly, soluble proteins will be isolated from a homogenate of axenically grown *C. elegans*. This fraction will be sterile filtered and analyzed for endotoxins levels via Limulus Amebocyte Lysate (LAL) assay (Pierce Cat# 88282). Samples measuring at or below 0.2EU/ml will be used for injection.

Mice will be treated with 10 and 100 μg doses of aCeAg$^{-/+GFP}$, in 100μl Phosphate Buffered Saline (PBS) by one of the following routes: intra-peritoneal, intravenous, subcutaneous, intra-nasal, or oral gavage. Blood will be drawn 1 hour post-treatment by tail bleed (~75μl), and 6 hours post -treatment (and following euthanasia) by cardiac puncture (~500 μl). Serum will be processed and analyzed for the amount of aCeAg proteins they contain. Comparisons will be made among the various routes of aCeAg$^{-/+GFP}$ administration.

	Expt 1 - Animal # (33 mice total)			
	Admin. Route	aCeAg (0μg)	aCeAg (10μg)	aCeAg (100μg)
Group 1	N/A	3	---	---
Group 2	IP injection	---	3	3
Group 3	IV. injection	---	3	3
Group 4	SC injection	---	3	3
Group 5	Intra-nasal	---	3	3
Group 6	Oral gavage	---	3	3



V.1.2. Experiment 2: Immunomodulation induced by helminth infections typically takes several weeks to develop. As such, we envision aCeAg (or other worm-derived products) as immuno-modulatory drugs for chronic inflammatory diseases rather than therapeutics for acute exacerbations of autoimmune disease. To test this, we plan to use a remitting/relapsing mouse model of multiple sclerosis.

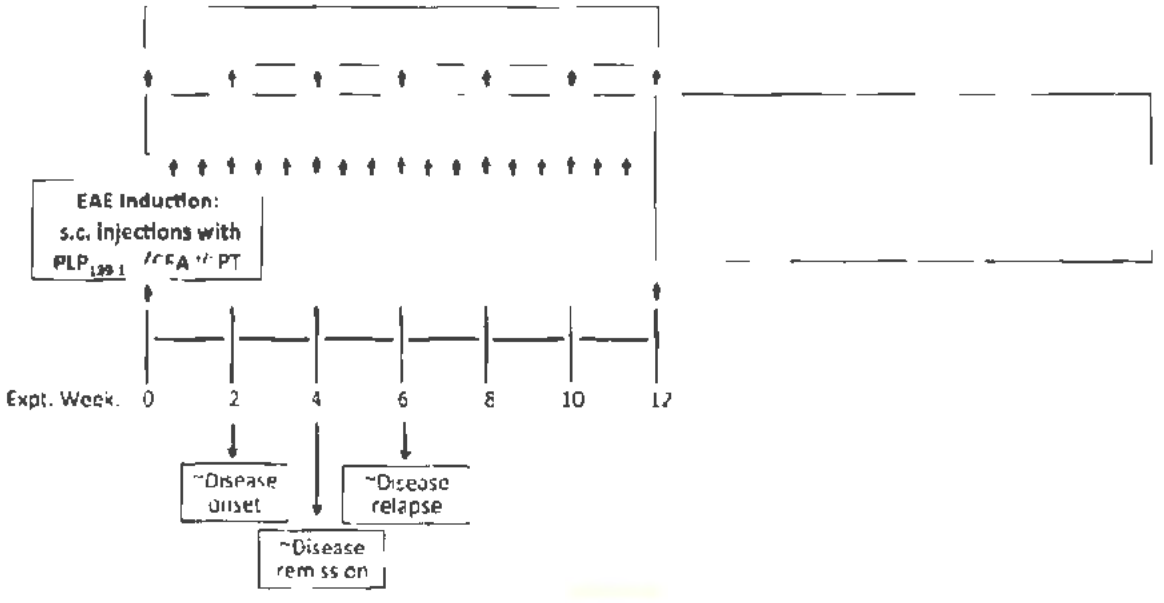
Experiment 2A – The purpose of this experiment is to establish the relapsing EAE model in our laboratory. Pain Category C -- 5-10 mice, Pain Category E -- 10-20 mice

Experimental design: EAE will be induced in SJL/j female mice by immunization with the antigenic Myelin Proteolipid Protein ($PLP_{139-151}$) peptide in complete Freund's adjuvant (CFA), with and without an added Pertussis Toxin (PTX) challenge. Reagents will be obtained from Hooke Laboratories ($PLP_{139-151}$/CFA Emulsion - cat. no. EK-0120), and $PLP_{139-151}$/CFA Emulsion PTX - cat. no. EK-2120). These mice will develop relapsing-remitting paralysis, with typical EAE onset between 9 and 13 days after

immunization (with PTX) and 11 to 15 days after immunization (without PTX), and peak of disease 1 to 2 days after disease onset for each mouse. The peak of disease lasts 1 to 3 days, with mice completely or partially recovering within 7-10 days. Approximately 50 to 80% of mice will show an increase in EAE severity (relapse) after initial partial or complete recovery. This usually occurs 20 to 40 days after immunization. Mice are typically observed for 40 to 45 days after immunization. This is sufficient for at least 50% of mice to relapse.

To determine which regimen best results in a recurrent EAE phenotype, at 6-10 weeks of age mice will be given a total of four subcutaneous injections (in the upper trunk ~2cm from the base of each fore- and hind-limb, consisting of one of the following regimens: 50µl of Phosphate Buffered Saline (PBS) alone, 50µg of $PLP_{139-151}$/CFA emulsion in 50µl PBS, 50µg $PLP_{139-151}$/CFA emulsion in 50µl PBS plus 500ng pertussis toxin in 100µ PBS given by i.p. injection. Mice will be observed daily from experiment Day 7 through study duration for the development of clinical symptoms, which manifest as a peripheral neuropathy ranging from limp tail or weak hind limb to complete hind limb paralysis (detailed description below in section V.4.1.2.1.). We expect disease onset 9 to 15 days after induction, remission of disease within 2 to 3 days after onset, and relapse of disease occurring for 50% to 80% of induced animals. If we do not observe disease onset within 15 days of induction, the experiment will be repeated, in a new set of animals, using 100 µ of $PLP_{139-151}$ in groups 2(a) and 3(a).

	Expt 2A - Animal # (15 - 30 mice total)	
	Treament	# SJL/j
Group 1	PBS treated mice	5 - 10
Group 2	PLP139-151/CFA challenged mice	5 - 10
Group 3	PLP139-151/CFA + Pertussin Toxin challenged mice	5 - 10

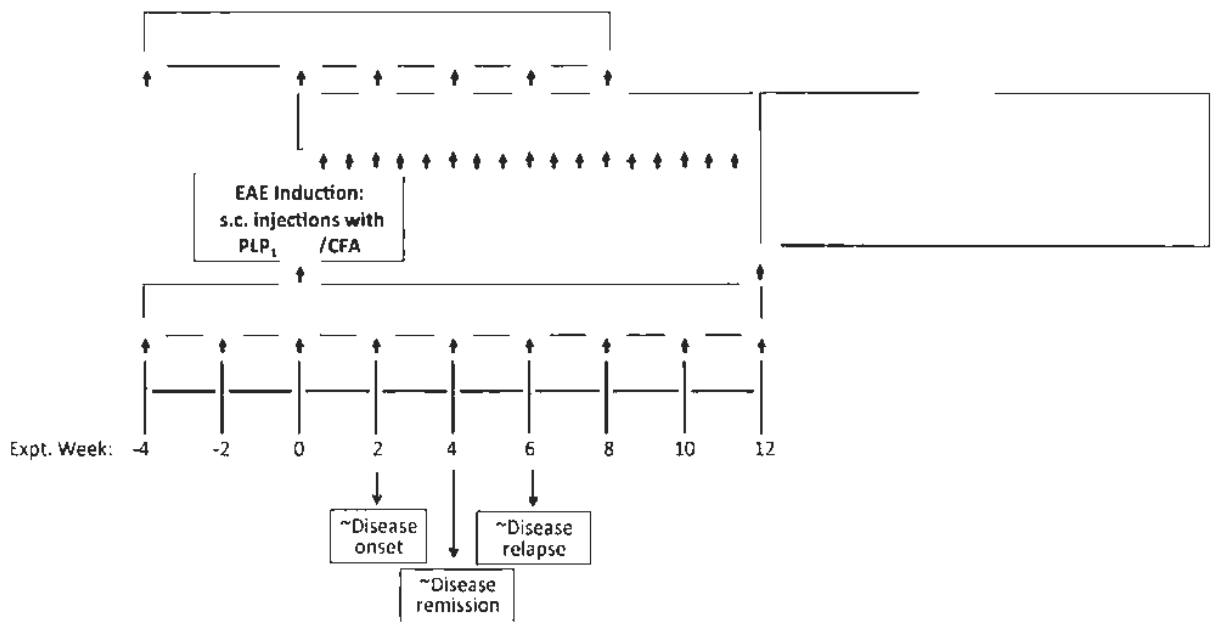


Experiment 2B – The purpose of this procedure is to test whether repeated administration of aCeAg, started before induction of EAE, prevents clinical disease. Pain Category C – 20 mice Pain Category E -- 20 mice

Experimental design: aCeAg$^{-/+GFP}$ immunotherapy, consisting of IP injections of 100μg/mouse in 100μl PBS, will be started 4 weeks prior to induction of EAE, and will continue twice weekly for the duration of the study. EAE will be induced when mice are 8 weeks of age. Specifically, we will use the proteolipid protein (PLP) induction model for experimental autoimmune encephalomyelitis (EAE) in SJL/j mice. SJL/j female mice will receive a total of four subcutaneous injections of a $PLP_{139-151}$/CFA emulsion (50μg in 50μl PBS) in the upper trunk, ~2cm from the base of each fore- and hind-limb) on experiment Day 0. Mice may also receive an intra-peritoneal injection of Pertussis toxin (500ng in 100μl PBS), depending on the results of experiment 2A. Mice will be observed daily from experiment Day 7 through study duration for the development of clinical symptoms, which manifest as a peripheral neuropathy ranging from limp tail or weak hind limb to complete hind limb paralysis (detailed description below in section V.4.1.2.1.). Additionally, ~75 μ of blood will be drawn every 2 to 4 weeks, by tail bleed, and plasma will be frozen, until study's end, for assessment of antibody responses to aCeAg$^{-/+GFP}$ and $PLP_{139-151}$.

At week 12 animals will be euthanized. A final 500μl of blood will be collected for plasma analysis of IgG and IgE levels, the brain and spinal cord will be extracted for histological analysis of inflammation, and the spleen and draining lymph nodes will be collected for analysis of lymphocyte proliferation and cytokine secretion profiles.

	Expt 2B - Animal # (40 mice total)	
	Treament	**# SJL/j**
Group 1	**PBS treated mice**	**10**
Group 2	**PBS treated mice with $PLP_{139-151}$/CFA challenge**	**10**
Group 3	**aCeAg treated mice**	**10**
Group 4	**aCeAg treated mice with $PLP_{139-151}$/CFA challenge**	**10**



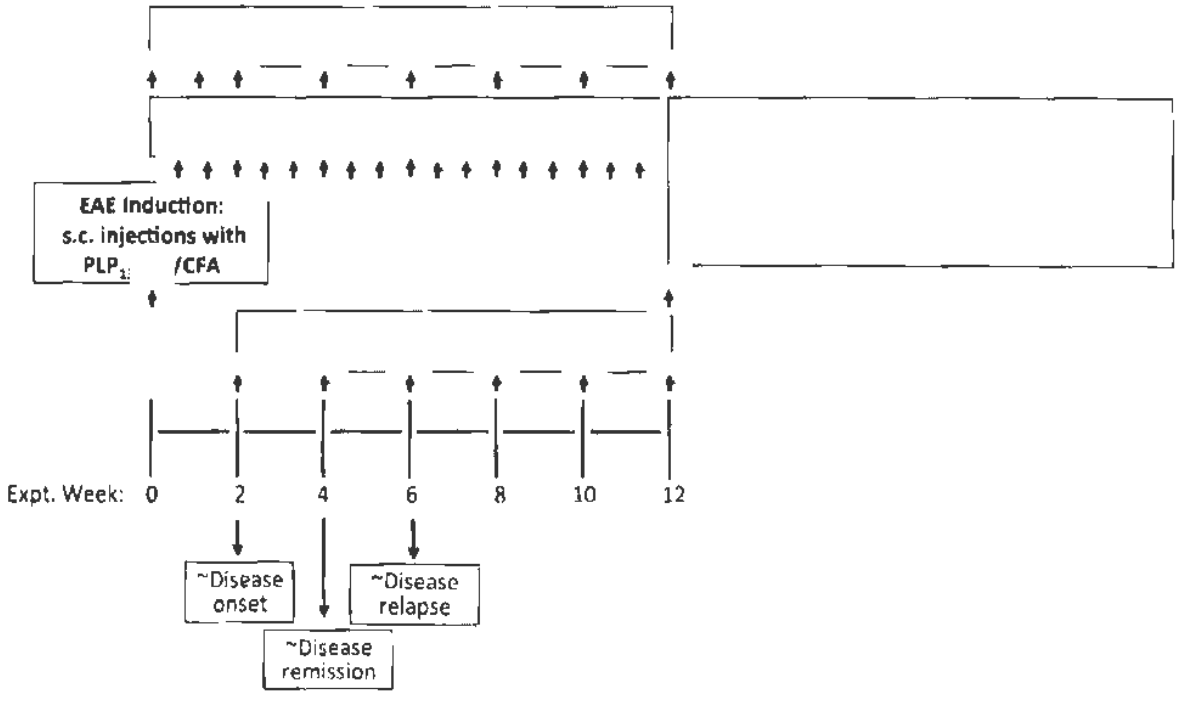
Experiment 2C - In this experiment we will test whether aCeAg injections can ameliorate relapse in animals that have already been sensitized to develop EAE.

Pain Category C -- 40 mice, Pain Category E -- 40 mice

Experimental design: The proteolipid protein (PLP) induction model for EAE (as described in Experiments 2A and 2B) will be induced in 8-week old SJL/j mice. aCeAg immunotherapy, consisting of IP injections of 100μg/mouse in 100μl PBS, will be started 1 week after the induction of EAE, and will continue twice weekly for the duration of the study. Mice will be observed daily from experiment Day 7 through study duration for the development of clinical symptoms, which manifest as a peripheral neuropathy ranging from limp tail or weak hind limb to complete hind limb paralysis (detailed description below in section V.4.1.2.1.). Additionally, ~75 μ of blood will be drawn every 2 to 4 weeks, by tail bleed, and plasma will be frozen, until study's end, for assessment of antibody responses to $aCeAg^{-/+GFP}$ and $PLP_{139-151}$.

At week 12 animals will be euthanized. A final 500μl of blood will be collected for plasma analysis of IgG and IgE levels, the brain and spinal cord will be extracted for histological analysis of inflammation, and the spleen and draining lymph nodes will be collected for analysis of lymphocyte proliferation and cytokine secretion profiles. Because the occurrence of relapsing EAE is somewhat variable, this experiment will require 20 animals per treatment group.

	Expt 2C - Animal # (80 mice total)	
	Treament	**# SJL/j**
Group 1	**PBS treated mice**	**20**
Group 2	**PBS treated mice with $PLP_{139-151}$/CFA challenge**	**20**
Group 3	**aCeAg treated mice**	**20**
Group 4	**aCeAg treated mice with $PLP_{139-151}$/CFA challenge**	**20**



V.2. Data Analysis:

Primary endpoint is clinical EAE disease activity scoring. A sample size of 10 mice in each group will have 80% power to detect a probability of 0.862 that an observation in one group is less than an observation in another group using a Mann-Whitney rank-sum test with a 0.05 two-sided significance level. For experiments 2A and 2B a total of 70 SJL/j mice are required

For analysis of EAE relapse greater variability (and standard deviation) is expected, thus it is recommended that 15-20 animals per groups be analyzed. For experiment 2C a total of 80 SJL/j mice are required.

As experiment 1 is observational, statistical analysis is not required.

Experiment 1: The purpose of this experiment is to obtain a pharmacokentic snapshot of aCeAg metabolism in SJL/j mice based of route of administration.

Five groups of 6 animals each will be used, in which 3 animals will receive10μg of aCeAg, and 3 animals will receive a 100μg dose of aCeAg. Three animals will serve as negative controls with no treatment. Each group will differ in the route of aCeAg administration – intra-peritoneal injection, intra-venous injection, subcutaneous injection, intra-nasal administration, or oral gavage. 200 μl of blood will be drawn at 1 hour by tail bleed. Mice will be euthanized at 6 hours and 500 μl of blood drawn by cardiac puncture. Blood samples will be drawn into plasma separator tubes. Plasma will then be tested for presence of known *C. elegans*$^{-/+GFP}$ antigen proteins, CAPG-1 and -/+GFP, by sandwich ELISA. This experiment requires a total of 5 groups x 6 mice/group plus 3 control animals = 33 mice.

Experiment 2A: The purpose of this experiment is to establish the relapsing model of EAE in our laboratory.

Because we simply want to determine if we can recapitulate this established model, in this experiment we will only be using 5 mice per group. If the initial study does not demonstrate substantial phenotype during relapse, the experiment will be repeated using a higher dose of PLP. The primary outcome is clinical score during relapse (approximately weeks 6-8). Given that there are 3 groups of mice in this experiment (EAE, PLP/CFA, and PLP/CFA + pertussis toxin), this experiment will require 15-30 mice.

Experiment 2B: The purpose of this experiment is to assess whether aCeAg injections are protective in a relapsing/remitting model of multiple sclerosis. Here we will test whether repeated administration of aCeAg started before induction of EAE prevents clinical disease.

Four groups of 10 SJL/j female mice each will be used. Four weeks prior to EAE induction, 100 μg of aCeAg$^{-/+GFP}$ will be given twice weekly by intra-peritoneal injection, and treatment will continue to experiment endpoint. EAE will be induced, in 8-week old mice, via subcutaneous injections of PLP/CFA into the upper trunk, ~2cm from the base of each fore- and hind-limb. We will score these animals for clinical manifestation of disease (a peripheral neuropathy ranging from limp tail or weak hind limb to complete hind limb paralysis; see details below and in section V.4.1.2.1), as well as disease remission and relapse. Additionally, we will analyze plasma levels of IgG and IgE antibodies. We will

examine brain and spinal cord sections for inflammation, and splenocytes and draining lymph node cells for proliferation and cytokine production. As this experiment requires 10 animals per group, and as there are 4 groups, this experiment requires 40 mice.

Experiment 2C: The purpose of this experiment is to assess whether aCeAg injections are suppressive in a relapsing/remitting model of multiple sclerosis. In part 2C we will test whether aCeAg injections can ameliorate relapse in animals that have already been sensitized to develop EAE.

Four groups of 20 SJL/j female mice each will be used. EAE will be induced, in 8-week mice, via subcutaneous injections of PLP and complete Freund's adjuvant behind each shoulder and on either side of the tail. One week following sensitization, we will score these animals for clinical manifestation of disease (a peripheral neuropathy ranging from limp tail or weak hind limb to complete hind limb paralysis; see details below and in section V.4.1.2.1), as well as disease remission and relapse. Additionally, we will analyze plasma levels of IgG and IgE antibodies. We will examine brain and spinal cord sections for inflammation, and splenocytes and draining lymph node cells for proliferation and cytokine production. Given the variability in occurrence of relapsing EAE, this experiment will require 20 animals per treatment group for a total of 4 groups x 20 mice/group = 80 mice.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: Alternative methods for studying autoimmune disease outside of *in vivo* approaches do not currently exist. The studies supported by this protocol specifically examine *in vivo* autoimmune pathogenesis, a phenomena without an *in vitro* equivalent. Further, our studies will be determining potential protective and/or suppressive immunotherapies.

V.3.2. Animal Model and Species Justification: Mice were chosen based on the similarities between the models utilized and human autoimmune disease. In addition, mice are a very well characterized model system, with a multitude of immunologically relevant mouse specific tools available. No smaller, less sentient mammalian species with appropriate genetic components and inflammatory responses are readily available for studying these aspects of autoimmunity. Examination in non-mammalian species is problematic, as the autoimmune disease model is only known to occur in higher vertebrate species. Current *in vitro* systems do not recapitulate the complex interactions between the cells and extracellular components necessary to model the interactions of T-cells with the target organs of interest. *In silico* models are insufficient for dissection of pathogenic T-cell interactions with target tissues due to the vast number of still unknown players that play a role in the examined processes.

SJL/j mice were chosen for the EAE studies due to the similarities between the disease model and autoimmune mediated CNS disease in humans. This model provides a well-described system that has been used in many published studies for examination of mechanisms involved in autoimmune mediated CNS diseases such as MS. Additionally, reagents necessary for these studies are readily available.

V.3.3. Laboratory Animals

		Species #1	Species #2
V.3.3.1.	Genus & Species:	*Mus musculus*	
V.3.3.2.	Strain/Stock:	SJL/j	
V.3.3.3.	Source/Vendor:	(b)(4)	
V.3.3.4.	Age:	4-12 weeks	
V.3.3.5.	Weight:	10-25g	
V.3.3.6.	Sex:	Female	
V.3.3.7.	Special Considerations:	None	
V.3.4.	Number of Animals Required (by Species):	183	

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: All animals with EAE are observed daily for advanced disease. If advanced disease is detected food and water sources on the floor of the cage will be provided. In addition animals will be euthanized if they meet any of the alternate endpoint criteria outlined in section V4.5. Analgesics were considered but cannot be used to relieve pain due to their effects on the immune and neuronal cells being tested.

V.3.5.2. Reduction: Statistical analyses were utilized to determine the minimum number of animals required to test individual hypotheses, thus allowing us to determine with a fair degree of power the likelihood of whether our hypotheses are correct. The number of animals requested represent the minimum number we consider necessary for completion of our studies. We are using 5-20 mice per group, and every effort is made to ensure the scientific utility of data derived from each animal used in this study by monitoring, evaluating and modifying group size as needed to obtain clear and consistent data. Every effort will be made to minimize the numbers of mice used without compromising research results.

V.3.5.3. Replacement: All of our studies require animals with an intact immune system, and therefore cannot be replicated *in vitro*.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	103	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	80	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

Inhalation anesthesia for immobilization for intra-nasal administrations: We will follow IACUC policy for our procedures. Mice undergoing intranasal inoculation will first be

anesthetized with isoflurane at 1-4.5% in a chamber using a precision vaporizer. Once they are immobilized, they will be removed from the induction chamber and depth of anesthesia will be monitored to ensure both adequate anesthesia (lack of movement with toe pinch) and adequate respiration.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:
Monitoring of EAE: Experimental Allergic Encephalomyelitis (EAE) is an autoimmune disorder characterized by a chronic relapsing, and remitting paralysis secondary to central nervous system (CNS) demyelination caused by T cells, and mice can go into remission even after development of stage III disease. Ordinarily, animals will be monitored for clinical symptoms daily and weighed every other day to determine weight loss associated with autoimmune disease. Animals with score of 4 will be monitored twice daily until the symptoms have resolved to a lower score. If resolution does not occur within 3 days the animal will be euthanized. A standardized system has been devised for classification of disease severity in both atypical and classical EAE. (Abromson-Leeman, Bronson et al. 2004; Lees, Iwakura et al. 2008). Clinical symptoms will be graded and recorded daily using the following scales:

Classical EAE symptoms
1- Flaccid Tail
2- Delay in righting reflex, hind limb weakness
3- Flaccid paralysis in 1 hind limb
4- Flaccid paralysis in both hind limbs
5- Moribund

Atypical EAE symptoms
1- Tail Rigor
2- Wide stance, leaning, or directed locomotion to a single side, mild ataxia
3- Continual turning to a single side, rigor in a single limb, moderate ataxia
4- Rolling, rigor in multiple limbs, severe ataxia
5- Moribund

Ataxia symptoms
1- Mild ataxia (stumbling)
2- Moderate ataxia (weaving)
3- Severe ataxia (moribund)

Mice will be considered moribund if they are found to be incapable of directed mobility at will. Specifically, mice with paralysis, rigor, or weakness in three or more limbs will be considered moribund. Additionally any mouse that is found to be incapable of directed continuous (lasting longer than 1s) movement due to vertigo or ataxia will be defined as moribund. Any mice with scores higher than a 2 on any EAE disease score scale will be provided with moistened food and transport gel on the cage floor to insure access to food and water. Hydration will be monitored by physical examination. If animals show signs of injury or difficulty ambulating they will be relocated to a separate cage to protect them from potential negative interactions with cage-mates. Mice with distended bladders that do not urinate upon handling will be considered incapable of self-elimination and the bladder will be expressed twice daily until symptoms subside or the mouse is euthanized. Skin lesions and ulcers are expected in many of the mice after complete CFA administration. Lesions will be monitored for development of abscesses or draining lesions. If such a discharge occurs mice will be euthanized.

V.4.1.2.3. **Paralytics**: N/A

V.4.1.3. **Literature Search for Alternatives to Painful or Distressful Procedures**:

V.4.1.3.1. **Sources Searched**: AGRICOLA, PubMed

V.4.1.3.2. **Date of Search**: 7/28/15

V.4.1.3.3. **Period of Search**: All available.

V.4.1.3.4. **Key Words of Search**:

Search 1: Alternatives to CFA in EAE AND mice
Search 2: Alternatives to complete Freund's adjuvant in EAE AND mice
Search 3: Alternatives to complete Freund's adjuvant in encephalomyelitis AND mice
Search 4: Pain EAE AND mice
Search 5: Pain encephalomyelitis AND mice
Search 6: cfa AND Pain AND eae AND mice
Search 7: complete Freund's adjuvant AND Pain AND encephalomyelitis AND mice

V.4.1.3.5. **Results of Search**:

AGRICOLA: search 1-3 No results
AGRICOLA: search 4 2 unique studies, None relevant
AGRICOLA: search 5 2 unique studies; None relevant
PubMed: search 1-3 No results
PubMed: search 4 - 29 unique studies; None relevant
PubMed: search 5 - 46 unique studies; None relevant
PubMed: search 6 - 1 unique studies; None relevant
PubMed: search 7 - 7 unique studies; None relevant

V.4.1.4. **Unalleviated Painful or Distressful Procedure Justification**:

(1) Some mice used under this proposal will develop EAE following treatment. The neurological symptoms associated with EAE will be experienced without relief for the remainder of the time described in individual experiment descriptions. The induction and development of EAE is vital to all major scientific questions addressed in this proposal. As analgesics work by modulation of neuronal and inflammatory processes treatment with analgesics could compromise our neuro-immunological findings. It is my belief that the utilization of the EAE system will allow us to address unique questions relating to neuro-inflammation that may in turn provide significant human benefit, thus justifying the moderate and temporary pain and distress the animals may experience.

(2) The use of Complete Freund's Adjuvant (CFA) is needed in the EAE model to generate an inflammatory response, thus breaking the animal's immunological tolerance to the antigenic Proteolipid protein peptide ($PLP_{139-151}$). This leads to the formation of multiple small, disseminated lesions of demyelination, micro-necrosis in the brain and spinal cord and the onset of clinical symptoms. Although multiple adjuvants are sufficient to induce EAE in rats, CFA is thought to be uniquely capable of reliably generating autoimmunity in mice (Stromnes and Goverman 2006). Researchers have tried muramyldipeptide, purified lipid A, and saponins in various water-in-oil emulsions without much success. One research group claims to have achieved EAE in C57BL/6 mice using Quil A, however these results have not been duplicated, and Quil A has not been proven effective with the SJL/j mouse model of MS. As such for these studies, no adequate substitute is available. In spite of using the minimally effective amount of CFA

(CFA suspended 1:1 (v/v) with the antigen preparation; subcutaneous injection of 0.05 ml per site), the adjuvant may cause skin lesions or ulcers at the site of injection. Mice will be monitored for development of abscesses or draining lesions. If discharge is observed the veterinary staff will be consulted and/or the mice will be euthanized.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: N/A

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

SQ injection- Subcutaneous injections of aCeAg (for pharmacokinetic analysis) will be administered between the shoulder blades using a 25-27G needle with less than or equal to 100 µl total volume. Subcutaneous injections of CFA/PBS emulsion containing $PLP_{139-151}$ peptide (for induction of EAE) will be administered into the upper trunk, ~2cm from the base of each fore- and hind-limb, using a 25-27G needle with 50 µl total volume per site.

IP injection – Intraperitoneal injections of aCeAg (for pharmacokinetic analysis), aCeAg and/or PBS (for EAE therapy/control), and/or pertussis toxin in PBS (for induction of EAE) will be done using a 23-25 gauge needle into the caudal ventral abdomen of recipient mice. All IP injections will be done with no more than 200 µl of volume.

IV injection- Intravenous injections of aCeAg (for pharmacokinetic analysis), in a volume no greater than 200µl, will be done in the lateral tail vein using a 25-30 gauge needle. Mice will first be restrained in a commercial Pexiglas chamber. The chamber will be covered with a piece of paper and a heat lamp will be placed over the tail to dilate the veins. Care will be taken not to overheat the animal. After locating the tail vein, the site will be disinfected with an alcohol swab. The needle will be placed parallel to the tail pointing toward the head. Following the injection, the needle will be removed and gentle pressure will be applied to the site until bleeding stops (~30 seconds).

V.4.4.2. Biosamples:

Ante-mortem biosampling

A. Blood draws: Up to 75µl of blood will be drawn every two weeks until time of euthanasia by tail bleed. Mice will be placed in a Broome restrainer while awake. A heat lamp is then placed over the tail of the mice for one to two minutes. Then, a shallow incision is made on the ventral surface of the tail just lateral to midline across one of the two lateral tail veins with a #10 scalpel blade. Drops of blood are then

directly collected into a small test tube. At the end of collection, mice are removed from the heat lamp and direct pressure is applied to the incision site with clean gauze.

Post-mortem biosampling

A. Terminal bleed: We only conduct terminal bleeds after animals have been euthanized by carbon dioxide as described in section V.4.6. The terminal bleed is a supplemental method to ensure death. The maximum possible amount of blood obtainable will be drawn by withdrawing blood directly from the heart post-mortem using a 20-23 gauge needle inserted into the left ventricle of the heart. Collected blood will be transferred into microtube for plasma processing.

B. Recovery of lymph nodes and organs of interest: Once euthanasia and terminal bleed is complete, brachial lymph nodes, pancreatic lymph nodes thoracic cavity lymph nodes (parathymic, post-mediastinal, and paravertebral lymph nodes), spleen, etc. will be removed for further study using aseptic techniques via lymph node dissection, midline thoracotcomy, and midline laparotomy. Histology may be performed on the tissues obtained.

V.4.4.3. Adjuvants: Complete Freund's adjuvant has been commonly used for decades in immunologic studies in mice and while multiple adjuvants are sufficient to induce EAE in rats, CFA is thought to be uniquely capable of reliably generating autoimmunity in mice (Stromnes and Goverman 2006). Researchers have tried muramyldipeptide, purified lipid A, and saponins in various water-in-oil emulsions without much success. One research group claims to have achieved EAE in C57BL/6 mice using Quil A, however these results have not been duplicated, and Quil A has not been proven effective with the SJL/j mouse model of MS. As such for these studies, no adequate substitute is available. Mice will be treated with a CFA emulsion containing the PLP immuno-dominant peptide or PBS. Four subcutaneous injections will be administered into the upper trunk, ~2cm from the base of each fore- and hind-limb, using a 25-27G needle with 50 µl total volume per site. Mice will be watched for development of adverse reactions and subsequent euthanasia as described in section V.4.5.

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Cage cards will be used. Animals may be identified by 2mm ear punch.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures:

Oral gavage inoculation – Oral inoculation of aCeAg (for pharmacokinetic analysis), in a volume no greater than 200µl will be performed. Mice will be manually restrained at the neck and back scruff, with the neck vertically extended. A 22 gauge ball tip needle will be gently passed through the mouth and pharynx into the esophagus. Contents of the syringe will be slowly administered.

Intranasal inoculation – Intranasal inoculation of aCeAg (for pharmacokinetic analysis), in a volume no greater than 30µl will be performed on anesthetized mice. Animals will be anesthetized with isoflurane at 1-4.5% in a chamber using a precision vaporizer. Once they are immobilized, they will be removed from the induction chamber and depth

of anesthesia will be monitored to ensure both adequate anesthesia (lack of movement) and adequate respiration. The mice will be manually restrained with the tail anchored between the small finger and the palm (Simmons and Brick, 1970). While held in a supine position with the head elevated. The end of a micropipette will be placed at or in the external nares, and the solution will be slowly injected.

V.4.4.8. Tissue Sharing: Unused tissues will be available after termination of experimental procedures with the understanding that mice have been manipulated and may have disease-associated tissue inflammation.

V.4.5. Study Endpoint: Euthanasia at pre-determined time. Alternatively mice will be euthanized if they are judged to be moribund or display any of the following criteria associated with EAE development:

i. Total weight loss exceeding 20% of body weight at start of experiment.
ii. Inability to rest.
iii. Hunched posture, head tucked into abdomen, lethargy lasting for >24 hours.
iv. Any condition that interferes with fecal or urinary elimination.

or development of other issues sometimes found in the mouse population:

v. Formation of multiple sores on tail.
vi. Diarrhea, if debilitating or prolonged over 3 days.
vii. Spreading alopecia that covers ~25% or more body surface area.
viii. Excessive scratching with associated scabbing
ix. Persistent coughing, labored breathing, or nasal discharge.
x. Jaundice.
xi. Bleeding from any orifice and/or prolapse.
xii. Pus-like discharge from sores.

While we do not anticipate any complications as a result of aCeAg inoculations, any animal that shows signs of distress, pain, illness, or anaphylactic shock (respiratory distress, edema, wheezing) will be evaluated by the veterinary staff and the P.I. and then euthanized.

V.4.6. Euthanasia: Carbon dioxide. Animals will be euthanized by Center for LAM or research personnel using cylinderized CO_2 in compliance with the most current version of the AVMA Guidelines. Euthanasia will be performed in (b)(6) or in room (b)(6) If possible, animals will be euthanized in their home cage. If not, a clean rodent cage will be used. Animals will be placed in a chamber that is not overcrowded (each animal can place all four feet on the bottom of the cage). With animals in the chamber, the flow rate will displace 10-30% of the chamber volume per minute. Gas flow will be maintained for at least one minute after apparent clinical death. Animals will be verified as dead (lack of heart beat and lack of respirations, blanched retinas, cyanotic mucous membranes) before removal of the chamber.

Note: For all euthanized animals, a supplemental method to ensure death (cervical dislocation or bilateral thoracotomy or terminal bleed) will be used in addition to carbon dioxide.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) as assigned by LAM. (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No __X__

Fluid Restriction: Yes _____ No __X__

V.5.1.3. Exceptions: None

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be cared for by LAM personnel according to LAM SOPs.

We will also monitor daily and weigh every other day following development of EAE clinical symptoms. Arrangements will be made with the Laboratory of Animal Management (LAM) staff for placement of food and water sources on the floor of cages as needed for animals that may have difficulty accessing the normal feed and water sources.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. LAM personnel, once daily, conduct essential husbandry procedures and health rounds during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions: None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Injections	(b)(6)	9 years experience	IACUC investigator training course April 2006
Tail bleeds		9 years experience	
Euthanasia (C02, cardiac puncture)		9 years experience	

Injections	(b)(6)	4 years experience	IACUC investigator training course March 2011
Euthanasia (C02, cardiac puncture)		4 years experience	
Oral gavage		To be learned; training provided by (b)(6)	
Tail bleed		To be learned; training provided by (b)(6)	
Intranasal administration		To be learned; training provided by (b)(6)	
EAE scoring		To be learned; training provided by (b)(6)	
Colony management, genotyping		15 years experience	Univ. of Iowa IACUC Training 1999; Washington Univ. IACUC Training 2004; Univ. of MD School of Medicine IACUC rodent handling and investigator training, 2008; USUHS IACUC training 4/4/13
Injections, oral gavage, intranasal administration		15 years experience	
Euthanasia		15 years experience	
EAE animal observation and scoring		15 years experience	
Injections		7 years experience	IACUC investigator training course August 2012
Oral gavage		7 years experience	
Tail bleeds		7 years experience	
Euthanasia (C02, cardiac puncture)		7 years experience	
Mouse colony management		7 years experience	
EAE scoring		To be learned; training provided by (b)(6)	
Injections		4 years experience	IACUC investigator training course July 2013
Oral gavage		4 years experience	
Tail bleeds		4 years experience	

Euthanasia (C02, cardiac puncture)	(b)(6)	4 years experience	
Mouse colony management and observation		4 years experience	
EAE scoring		To be learned; training provided by (b)(6)	

VII. BIOHAZARDS/SAFETY: Lab personnel will wear personal protective equipment (gloves, masks, lab coats or scrubs) when handling animals. Lab personnel will be briefed on safety measures to prevent accidental needle sticks with CFA (e.g. do not recap needles, proper disposal of needles) and the potential consequences of accidental injection of CFA into human tissues (e.g. localized inflammation, possible conversion to a positive TB test). If used for tissue fixation, paraformaldehyde will be used in a hood and personnel will wear personal protective equipment.

VIII. ENCLOSURES:

(1) EAE Induction by Active Immunization in SJL Mice
- http://hookelabs.com/protocols/eaeAI_SJL.html

(2) Hooke Kits™ for EAE Induction in SJL Mice
- http://hookelabs.com/products/EK-2120/

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. **Training:** I verify that I have attended the USUHS Investigator/Animal User Training [redacted]

Principal Investigator Signature — Date: 7/30/2015

G. **Training:** The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. **Responsibility:** I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Respons[redacted] use alternatives where feasible and conducting humane a[redacted]

Principal Investigator Signature — Date: 7/30/2015

I. **Painful Procedure(s):**

I am conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have cons[redacted]es; however, using the methods and sources d[redacted]rmined that alternative procedures are not availa[redacted]his proposed experiment.

Principal Investigator Signature — Date: 7/30/2015

X. PROTOCOL ABSTRACT:

A. **Animal Protocol Number:**

B. **Animal Protocol Title:** Efficacy of *Caenorhabditis elegans* antigen as a protective treatment of experimental autoimmune encephalomyelitis (an animal model of multiple sclerosis) in mice

C. **Principal Investigators:** [redacted] M.D.

D. **Performing Organization:** USUHS

Over the last decade, studies have shown an inverse relationship between endemic helminth infections and the incidence of allergy and/or autoimmune disease in developing regions. Further, the potential use of helminth-derived immuno-regulatory therapy as a protective measure against inflammatory disease has been validated in both animal and clinical studies [1,2]. However, several obstacles impede the overall usefulness of helminic treatments including difficulties in obtaining large quantities (due to complex lifecycles), variability in purification and inter-batch efficacy, and the disease causing potential of live infections [3]. Here we propose a strategy for utilizing the beneficial effects of helminth immuno-suppression while avoiding the complicating issues associated with helminth isolation/treatment.

We have shown in a proof-of-concept study that treatment of Non-obese Diabetic (NOD) mice with soluble antigens from axenically grown *C. elegans* (aCeAg) nematodes is protective for the onset of Type I Diabetes. Mechanistically, we have shown that these antigen treatments increase the levels of basophils, eosinophils, and polyclonal and antigen-specific IgE immunoglobulins, in a similar manner to that seen with *L. sigmodontis* treatments [4]. Further, we observe increased production of the suppressive cytokine IL-10, but not of the pro-inflammatory cytokine IFNγ, from splenocytes of aCeAg-treated animals [4].

Our current study aims to analyze the potential for protective and/or suppressive effects of aCeAg treatments in the relapsing/remitting Multiple Sclerosis model of SJL/j mice. Thus, we will determine the potential therapeutic reach of this non-parasitic nematode-derived treatment, and gain a better understanding of the immuno-modulatory responses to such treatments.

References

1. H.J. McSorley and R.M. Maizels, Helminth Infections and Host Immune Regulation, Clin. Microbiol. Rev. 2012, 25(4):585
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G. Indexing Terms (Descriptors): Multiple sclerosis, autoimmunity, helminth therapy, IgE, basophil, mast cell, regulatory T-cell, immune tolerance

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January 5, 2016

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF MICROBIOLOGY

SUBJECT: IACUC Approval of Protocol –Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on January 5, 2016:

Animal Protocol Title: (b)(4)

USUHS Protocol Number: (b)(6)

Expiration Date: January 4, 2019

Supporting Grant(s) Number: (b)(6),(b)(4)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: (b)(4)

(b)(4)

GRANT TITLE (if different from above): (b)(4)

(b)(4)

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: (b)(4)

EARLIEST ANTICIPATED FUNDING START DATE: November 20, 2015

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6) 12/8/15

Principal Investigator Signature | Department | Office/Lab Telephone | Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) 12-8-15

Research Unit Chief / Dept. Head Signature | Title | Telephone | Date

Typed Name (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) 12/8/15

Statistician Signature | Department | Telephone | Date

Typed Name

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics. see next page

(b)(6) (b)(6)

Attending/Consulting Veterinarian Signature | Department | Telephone | Date

Typed Name (b)(6) VMD

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(5)

PROTOCOL TITLE: (b)(4) (b)(4)

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER:

FUNDING AGENCY: (b)(4)

EARLIEST ANTICIPATED FUNDING START DATE: November 20, 2015

PRINCIPAL INVESTIGATOR: (b)(6)

Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

Research Unit Chief / Dept. Head Signature Typed Name:	Title	Telephone	Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

Statistician Signature Typed Name:	Department	Telephone	Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)	(b)(6)	(b)(6)	12/4/15
Typed Name (b)(6) VMD	Department	Telephone	Date

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6)

ANIMAL PROTOCOL TITLE: (b)(4) (b)(4)

GRANT TITLE (if different from above): (b)(4) (b)(4)

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6) Ph.D. (b)(6) Ph.D.

TECHNICIANS(S): To be added

I. NON-TECHNICAL SYNOPSIS:

Malaria is a tropical parasitological disease that remains a global health problem, causing more than half a million deaths and 250 million cases annually. Expanded control and treatment programs in the past decade have reduced the incidence of the disease and led to the call for efforts to eliminate, possibly even eradicate, malaria. To do this new strategies are needed that target the sexual stages of the parasites, called gametocytes that are responsible for disease transmission. The current recommended chemotherapy for malaria does not effectively kill mature gametocytes, allowing malaria to be transmitted for more than a week after the clearance of asexual parasites. Previous drug screens used assays that only detected asexual replication and therefore did not monitor activity against gametocytes. (b)(4) (b)(4) (b)(4) The goal of this research is three fold 1) to use a rodent malaria model to analyze the *in vivo* safety of selected compounds with *in vitro* gametocytocidal activity 2) to use a rodent malaria model to analyze the *in vivo* malaria transmission blocking efficacy of selected compounds with *in vitro* gametocytocidal activity at doses found safe in mice 3) define the stage specificity and timing of compound inhibition from intraerythrocytic development to oocyst formation. Together these approaches should identify classes of compounds that can be targeted for further drug development, as well as advance our understanding of gametocyte metabolism and facilitate the design of effective control strategies. The mouse studies described in this work will be performed in full accordance with the Institutional Animal Care and Use Committee at USUHS

II. BACKGROUND:

II.1. Background:

Malaria transmission requires the production of male and female gametocytes in the human host. These gametocytes are then taken up in a blood meal by a mosquito and begin a multistep developmental process that leads to the formation of human-infectious sporozoites. In contrast to the 48 hr asexual replication cycle of *Plasmodium falciparum*, the development of mature gametocytes takes 10-12 days [8]. Mature gametocytes then circulate in the peripheral blood for several days and are resistant to almost all known antimalarial drugs. Consequently, transmission can continue for over a week or longer after the clearance of asexual stages [9]. Primaquine, an 8-aminoquinoline, effectively eliminates late stage gametocytes and has recently been included in mass drug administration efforts to reduce malaria transmission [10-

12], but it has a short half-life and does not affect early stage gametocytes, allowing continued production of infectious mature gametocytes. Primaquine can also have serious side effects in individuals with genetic deficiencies in glucose-6-phosphate dehydrogenase (G6PD) or NADPH-methemoglobin reductase [13]. Unfortunately, people living in malaria endemic areas have a high prevalence of these genetic mutations, and this fact has raised concerns about the widespread use of primaquine to block transmission [13]. The active metabolite of artemisinin, dihydroartemisinin, also has gametocytocidal activity, but its gametocyte IC50 is ~100 times higher than that required to kill asexual stages. Mosquito feeding assays have demonstrated that malaria patients remain infectious to mosquitoes for over a week after treatment [9]. This time course is consistent with artemisinin's preferential effect on immature gametocytes. Even this partial effect on transmission has been implicated in the decreased transmission observed over the past several years [14]. Unfortunately, the mechanism of action of neither of these compounds is known, so their efficacy does not provide clues to gametocyte vulnerabilities.

New, safe, potent gametocytocidal compounds are needed to effectively block malaria transmission and prevent the spread of malaria [3]. The biology of gametocytes and asexual parasites differ significantly, resulting in differential sensitivity to common antimalarials. One of the primary differences is the lack of DNA replication during gametocyte development, and consequently gametocytes are resistant to drugs that target de novo pyrimidine synthesis, such as sulfadoxine–pyrimethamine, atovaquone and dihydroorotate dehydrogenase inhibitors [15]. After developing into a stage II gametocyte, *P. falciparum* is no longer affected by compounds that block hemoglobin digestion or erythrocyte invasion, such as the 4-aminoquinolines and protease inhibitors [15]. Gametocytes also are not lysed by sorbitol, suggesting a reduction in permeability pathways such as the plasmodial surface anion channel (PSAC) [16-18]. Likewise, gametocytes are not cleared by antibacterial agents such as clindamycin and tetracycline analogs that target the apicoplast, a relict plastid carried by Plasmodium [19]. Additional apicoplast-specific enzyme systems have not yet been evaluated in gametocytes.

There are several reasons for the lack of drug studies on gametocytes and mosquito infectivity. First, although gametocytes can be detected in 95% of malaria patients by analyzing RNA samples, they are difficult to detect by Giemsa-stained thick smear and consequently they are often not analyzed in field studies [20, 21]. Additionally, few laboratories have experience growing gametocytes in culture and many lab-adapted lines have lost the ability to produce gametocytes. Even in lines that do make gametocytes, maturation takes 12 days and the yield is generally very low in comparison to asexual stages, because only a small subpopulation of asexual parasites differentiates into gametocytes. Consequently, ~50 fold fewer gametocytes than asexual parasites are produced from the same amount of stock culture over the same time period. The lack of DNA replication during gametocytogenesis also rules out the possibility of measuring increases in DNA content to estimate gametocyte viability, which is frequently used to estimate the growth of asexual stages. Consequently, (b)(4) (b)(4) (b)(4) does not require genetically engineered parasites that often produce few gametocytes and allows screening of field isolates to study resistance patterns. However, the production of late stage gametocytes is inherently time and resource intensive, therefore (b)(4) (b)(4) (b)(4) In contrast (b)(4) tested are inactive against mature

gametocytes. Consistent with the ineffectiveness of most approved anti-malarials against gametocytes, (b)(4)
(b)(4)
These data demonstrate the distinct biology of the transmission stages and emphasize the importance of screening additional compounds for gametocytocidal activity. The additional screening of (b)(4) provided an efficient overview of pathways that are essential for gametocyte viability and allowed comparison with previous data obtained using asexual parasites. (b)(4) and 3 had not previously been reported to have anti-malaria activity and will continue to be developed as drug candidates in (b)(4)
(b)(4) will produce and screen new derivatives *in vitro* gametocytocidal activity. Compound with potent *in vitro* gametocytocidal activity will be selected and advanced for *in vivo* testing in mice.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: Pubmed, NIH RePORTER, DTIC

II.2.2. Date of Search: Nov 10, 2015

II.2.3. Period of Search: Pubmed (English filter, 1948-present); NIH RePORTER (active awards only, 2000-2015)

II.2.4. Key Words and Search Strategy:
NIH RePORTER: (malaria transmission-blocking drug) NOT vaccine, only identified our current R01. The more general term "malaria" was used because this includes studies done in all species, including mice.

Pubmed: "(malaria transmission-blocking drug) NOT Vaccine"

DTIC: "malaria transmission blocking drug".

II.2.5. Results of Search:
Pubmed: "(malaria transmission-blocking drug) NOT Vaccine" = 97 hits, The number of articles on transmission-blocking drug discovery has increased in the last five years with 70 of these article being published from 2012-present. This increase reflects the realization that none of the current anti-malarials used for first line treatment block transmission. Consequently patients remain infectious for at least a week after symptoms resolve allowing the parasite to continue to move through the population. Three hits were from our group, including a recent Science Reports article (2014) describing a large screen of > 7000 bioactive compounds that has led to this proposal. The compounds being screened in this proposal are not being developed by other groups, many will be novel derivatives synthesized by our collaborators.

NIH RePORTER: "(malaria transmission-blocking drug) NOT vaccine", only identified our current R01 that supports this project.

DTIC: "malaria transmission blocking drug". There were 3 hits, but only one involved malaria drug development, the other two were screening for malaria immunity or vector control. The one drug development project is focused on optimizing a different chemical compound, ELQ-300, for oral administration. We will not be testing this compound unless it is in collaboration with the DOD PI. We are collaborators with that DOD PI on another grant to test some of his other compounds for transmission-blocking activity.

III. OBJECTIVE\HYPOTHESIS: The objectives of these proposed studies are:

1) Determine the *in vivo* safety of selected gametocytocidal compounds using the rodent malaria model, *Plasmodium berghei* in mice.
2) Determine the *in vivo* malaria transmission blocking efficacy of selected gametocytocidal compounds using the rodent malaria model *Plasmodium berghei* in mice at doses found to be safe.
3) Define the stage specificity and timing of compound inhibition from intraerythrocytic development to oocyst formation

IV. MILITARY RELEVANCE:

40% of the world's population lives in malaria endemic areas and, therefore, it continues to be a threat to military personnel deployed to these areas. The potential for infection was most recently demonstrated when 5 US soldiers working in Liberia to control the Ebola epidemic were diagnosed with the disease. Since 2005, 800 U.S Troops have been infected with malaria (Air Forces Health Surveillance Center). Of most concern is that resistance has been reported for all the current anti-malarials. This finding has increased the push for the identification of novel drugs that target clinical symptoms and can effectively block transmission to reduce the spread of malaria through the population.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

Compounds that are found to be 10 times more potent against *P. falciparum* gametocytes than mammalian cells *in vitro* will be selected for further *in vivo* testing using the rodent malaria *P. berghei*/mouse model. Both commercially available and newly synthesized compounds may be used during the course of this work. In accord with IACUC Policy 27, if available pharmaceutical grade compounds will be purchased. If pharmaceutical grade is not available, compounds approved for cell culture use will be obtained. For the remaining compounds the purest preparation available will be used and each lot will be tested in vitro for cytotoxicity against mammalian cells prior to use in animals. A dose escalation model will be used to evaluate *in vivo* safety (Experiment 1, V.1.1) and compound concentrations found to be safe will be tested for the ability to block parasite transmission via a mosquito (Experiment 2, V1.2). To better define the transmission-blocking kinetics and stage specificity of the selected compounds, RBCs infected with mature gametocytes will be harvested and stimulated *in vitro* to fertilize and differentiate over the next 24 hours into ookinetes as they do in the mosquito midgut. Selected compounds will be added at specific times during this process and tested for an effect on ookinete production. The results will be used to determine stage sensitivity and the time needed to block development. The goal of this work is to identify safe compounds with potent, multi-stage, transmission-blocking activity for further preclinical development as a novel

anti-malarial.

V.1.1. Experiment 1:

V.1.1.1) Safety testing: Compounds found to have potent gametocytocidal activity *in vitro*, will first be screened for cytotoxicity in the mammalian cell line, such as HepG2 cells. The compound concentrations needed to reduce viability of HepG2 cells and gametocytes will be compared. Those compounds with 10 times more activity against gametocytes will be candidates for *in vivo* studies in mice. If the compound has not been previously tested in mice, the initial safety studies described here will be completed prior to (Exp 2) transmission-blocking efficacy testing using the rodent malaria, *P. berghei* in mice (CD-1 outbred mice, (b)(4)

For the safety studies a dose escalation model will be followed starting with a dose of 1 mg/kg administered by intravenous (IV) or intraperitoneal (IP) injection (50-200 µl) to two outbred CD1 mice (b)(4) (16). For the next 4 days the mice will be monitored daily for signs of distress, including, decreased/impaired motility, hunched posture, ruffled fur, difficulty breathing, diarrhea, and skin lesions and will be euthanized if they appear unhealthy. The compound formulations will vary depending on the solubility of the compounds and could include a range of carriers. Carrier selection will be based on the properties of the compound and previous literature and could include, up to 2% methyl cellulose, 50% Polyethylene glycol (PEG), 25% n-Methylpyrrolidone, 5% Dimethyl sulfoxide (DMSO), 7.5% ethanol or 18% PEG-40 Hydrogenated Castor Oil (cremophor RH40 or kolliphor RH40). All these carriers have been used previously in animals (http://www.pharma-ingredients.basf.com/Solubilizer /Kolliphor_range/Kolliphor_RH40.aspx), http://www.bu.edu/orccommittees/iacuc/policies-and-guidelines/administration-of-drugs-and-experimental-compounds-in-mice-and-rats/).

If there are no adverse reactions two days after the injection another two mice will be treated with 2 mg/kg. In the absence of adverse reactions from the higher dose, the experiment will be repeated using doses of 4 mg/kg and then 8 mg/kg. If there continues to be no adverse effects, the compound will then be tested for transmission-blocking efficacy at 4 and 8 mg/kg as described below in Exp 2. If the compound completely blocks transmission, higher doses will not be tested. If the compound does not block transmission at these doses additional safety testing will be done at 16 mg/kg. If there continues to be no adverse effects, the dose will be increased in 2 fold increments until a maximum dose of 128 mg/kg. This upper level was selected, because this is a drug development project and it is unlikely that a compound that requires a 128 mg/kg dose for activity will be advanced without additional modification.

Compounds that are found to be safe and effective by IV/IP administration will then be tested for safety and efficacy when administered by gastric gavage first at 50 mg/kg then if safe the dose will be increased 2 fold to a maximum of 400 mg/kg.

V.1.1.2) Over the course of the 3 year project we anticipate testing up to 100 compounds for safety. At a maximum the carrier control and 8 different doses of compound will be tested by IV or IP injection and 4 doses by gavage in two mice. 100 x (1+8+4) x 2 =2,600

V.1.2. Experiment 2:

V.1.2.1) Transmission-blocking Efficacy Testing: To extend the study to the evaluation of efficacy against gametocytes, 2 mice will be restrained by hand and inoculated IP with *P. berghei* and then returned to the cage. They will be monitored for parasitemia by Giemsa-stained smear of a drop of blood from a tail nick every other day until parasites are observed. To obtain blood for the smear, the mice will be restrained briefly for a tail nick to collect a drop of blood on a microscope slide. Once parasites are observed the mice will be observed daily to monitor well-being and assess parasitemia. When the parasitemia is >5%, the mouse will be

anesthetized with inhaled isoflurane and the blood will be collected by cardiac puncture and transferred to 2 mice that will be restrained by hand and the parasites will be transferred by IP injection. The donor mouse will be euthanized after the cardiac puncture by an overdose of isoflurane and exsanguination. The gametocytemia of the newly infected mice will be followed daily by Giemsa-stained smear until it is >1% and then some of the blood from the tail nick will be diluted into sterile phosphate buffered saline (PBS) with heparin to test the ability of male gametocytes to exflagellate when cooled to room temperature (rt). Exflagellation, the production and release of motile flagella that can fertilize female gametes can be observed under the microscope at 400x magnification and is used as a marker of gametocyte sexual maturity. Once exflagellation is observed in the blood sample the highest well-tolerated dose of compound from Exp. 1 or carrier alone will be administered by IV,IP or gavage to the mice. One to two hours after drug administration the mice will be anesthetized with an IP injection of 3 mg/kg acepromazine maleate and 60 mg/kg ketamine and laid on the top of a sealed container containing *Anopheles stephensi* mosquitoes provided by the Entomology Section of Preventative Medicine and Biostatistics Department. After the blood meal the mouse will be returned to the cage to recuperate so that the course of parasitemia can continue to be followed to monitor parasitemia after drug treatment or the mouse will euthanized while still anesthetized by cervical dislocation. The blood fed mosquitoes will be maintained to assess parasite transmission. Transmission can be evaluated by harvesting mosquitoes on days 7-12 post blood meal to count the number of *Plasmodium* oocysts that have developed in the midgut. Alternatively, to determine if the mosquito can transmit malaria to a naive mouse, 21±2 days after the initial blood feed, a naive mouse will be anesthetized and placed on top of the sealed cage containing the *P. berghei*-infected blood fed mosquitoes. After the *P. berghei*-exposed mosquitoes have taken a blood meal the mouse will be allowed to recuperate and after 5 days the parasitemia will be followed by a tail bleed as described above to evaluate whether they were infected.

V.1.2.2) If all hundred compounds are found to be safe they will be tested for transmission blocking activity in triplicate in up to 6 drug concentrations by IV or IP and 4 drug concentrations by oral gavage. The concentrations will be selected based on the safety profile of the compound. Initially, a single mouse will be tested for each treatment, 1) a selected safe high dose, 2) half the high dose and a carrier control. One donor mouse will be needed to provide parasites 2 experimental mouse. 100 compounds x 15 [3 mice (2 concentrations and carrier*) x 5 (for a total of 6 IV/IP concentrations and 4 oral gavage concentrations)] x 3 (triplicates)] = 4500 experimental and 4500/2 =2250 donor mice = 6750 mice total.

*Only 4 or fewer mice can be tested at once due the time needed for setting up the mosquito feed. Since this is an initial "dose finding" screen only 2 concentrations and a carrier control will be tested at a time to inform the selection of additional doses for further experiments. This strategy will be explained in more detail in the Refine and Reduce Section.

V.1.3. Experiment 3:

V.1.3.1) Define the stage specificity and timing of compound inhibition from intraerythrocytic development to oocyst formation. *P. berghei* gametocytes can be harvested from the mouse, stimulated to fertilize and over the next 24 hours develop into motile ookinetes *in vitro* (Rodriguez et al. *Exp Parasitol. 2002 May;101(1):73-6. In vitro* development allows direct evaluation of the effects of the compound throughout this period. Understanding the kinetics of compound activity and determining the parasites stages that are susceptible will facilitate in the design of effective dosing strategies. For these experiments, the production of sexual stage parasite will be augmented by pre-treating the mouse with one IP injection of phenylhydrazine

(1.2 mg in PBS) 3 days before the inoculation of 10^8 *P. berghei*-infected RBCs from a donor mouse as described above. Three days after the IP injection of parasitized RBC the mice will be treated with pyrimethamine to eliminate any remaining asexual parasites. One to two days after the pyrimethamine treatment the mouse will be anesthetized with isoflurane and the sexual stage parasites will be harvested by heart puncture. After the cardiac puncture the donor mouse will be euthanized by an overdose of isoflurane and exsanguination. The harvested blood will be centrifuged and the cell pellet will be incubated at 18°C in ookinete medium (RPMI media (KD Biomedical) + 10% Fetal bovine serum (FBS) pH 8.0) for 15 minutes and exflagellation centers will be quantified at 400x magnification. After 1 hour, parasites can be further analyzed for fertilization and zygote formation by measuring DNA content [37] using DNA dye SYTO 59 (Life technologies) and flow cytometry. Ookinetes can be observed up to 24 hours post culture and quantified by Giemsa stained smear. To evaluate the time course and the drug sensitivity of parasites after exflagellation, compound will be administered to the mice by gavage if that is effective or by IV or IP 0-2 h prior to the cardiac puncture or the compound will be added directly to the *in vitro* culture at 20 min, 2 hr, 6 hr and 12 hr after fertilization. If there are differences between stages, additional time points and exposure times could be investigated. The *in vitro* results will be compared to the effect of 1.5 hr *in vivo* compound treatment. Together, the data from this aim will allow us to determine the timing and stage specificity of the selected compounds gametocytocidal activity in *P. berghei*, *in vivo* and *in vitro*. Having a more refined time line for the selected compound's activity in the midgut will identify which stages are susceptible and facilitate the modeling of the effects of compound administration and characterization of the target molecule.

V.1.3.2) It is anticipated that the stage specificity and kinetics of 30 compounds will be analyzed at a maximum of 5 different time points in replicate over the course of the 3 year project. For each compound a carrier control will be needed and for each phenylhydrazine treated mouse a donor mouse is need to provide the initial parasite inoculum. (30 compounds + 30 carrier controls)=60 x 5 time points x 3 (triplicate) = 900 experimental and 900 donor mice= 1,800 mice total

V.2. Data Analysis:

Exp 1: Safety will be assessed qualitatively by screening daily for signs of distress, including , decreased/impaired motility, hunched posture, ruffled fur, difficulty breathing, diarrhea, and skin lesions. If any 2 or these are observed the mouse will be euthanized immediately and no higher doses of compound will be administered. This is a conservative endpoint. It is designed to minimize the exposure of mice to untested compound, not for a statistical analysis

Exp 2: Transmission-blocking activity will be defined as a significant difference in oocyst numbers per mosquito midguts using a Mann-Whitney U Test. The mosquito feed is an established assay for transmission-blocking activity and it has been found that the numbers of oocysts in at least 20 mosquitoes need to be counted to have high confidence about the infectivity of a single mouse. We will then repeat the assay 3 more times to confirm that the effect is due to the treatment. If there is no significant difference between the treated and untreated groups in three mice at the maximum safe dose we will conclude that the compound was not effective.

Exp 3: The production of ookinetes will be counted and used to assess the kinetics and stage specificity using an ANOVA and Bonferroni posttest. The experiment will be repeated 3 times to confirm the results. Enough gametocytes are needed to generate > 30 ookinetes in the control group to have a statistical significance.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: All the compounds will first be tested in *in vitro* against parasites and the mammalian cell line, HepG2 and found to have a 10 fold higher potency against parasites than mammalian cells. These initial tests will screen out cytotoxic compounds, however *in vivo* testing is still required to evaluate compound safety and efficacy in a complex living system that contains many cell types that interact with each other and the compound. This complexity cannot yet be reproduced *in vitro* and requires testing in an intact animal.

V.3.2. Animal Model and Species Justification:
The *Plasmodium berghei*/mouse model is well established for testing malaria transmission. It has been used for successfully for years to evaluate anti-malaria drugs and vaccine strategies and found to predict compounds that will also work against human malarias (Ramkaran AE. (1969) Trans R Soc Trop Med Hyg 63(1):8.The sensitivity of *P. berghei* in *A. stephensi* to chemotherapy) and is still the gold standard used today (Baragaña et al. (2015) Nature. 522:315-20).

V.3.3. Laboratory Animals

		Species #1	Species #2
V.3.3.1.	Genus & Species:	*Mus musculus*	
V.3.3.2.	Strain/Stock:	CD-1	
V.3.3.3.	Source/Vendor:	(b)(4)	
V.3.3.4.	Age:	5-10 weeks	
V.3.3.5.	Weight:	20-35 gm	
V.3.3.6.	Sex:	Female so that they can be housed together	
V.3.3.7.	Special Considerations:	NA	

V.3.4. Number of Animals Required (by Species):

Mice 8450 over 3 years

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: The mice will be group housed with environmental enrichment and anesthesia will be used prior to potentially painful or distressful procedures, including the IV

injection, cardiac puncture and Mice will also be monitored closely once they are inoculated with compound or parasites and euthanized if found to exhibit at least 2 of the following signs of distress, decreased/impaired motility, hunched posture, ruffled fur, difficulty breathing, diarrhea, and skin lesions.

V.3.5.2. Reduction:

To minimize the number of compounds that will be tested, initially the compounds pre-screened *in vitro* for a 10 fold great effect on parasites than mammalian cells. Any compounds that have already been tested for safety in mice will be tested for transmission activity at safe concentrations. Only if the compound does not block transmission and there is no published data indicating that higher doses are toxic, will additional higher doses be tested for safety. Additionally, to further reduce the number of mice used only a maximum of 4 compound doses (1, 2, 4, & 8 mg/kg) will be tested for safety before efficacy is tested. These are drug levels that have been found to effectively block transmission with other compounds and it is likely that the safety of higher doses will not have to be tested. Only if the compound is not effective, will higher IV/IP doses up to 128 mg/kg will be tested for safety. 128 mg/kg chosen as the maximal level, since if the compound is not effective at that IV/IP dose it is unlikely to be advanced as an antimalarial.

V.3.5.3. Replacement: All the compounds will be tested for safety and efficacy *in vitro* first and only compounds that are 10 times more effective against parasites than mammalian cells will be advanced for *in vivo* safety testing.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1 CD-1	Species #2
V.4.1.1.1.1. Column C:	2200*	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	8950*	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

	Substance/Agent	DEA	Amount Given	Delivery Route
1	isoflurane	No	3-5%	inhalation
2	Acepromazine maleate	No	3 mg/kg	IP

3	ketamine	Yes	60 mg/kg	IP

Description and Adverse Effects

Ketamine and Acepromazine maleate are used together as a pharmacological restraint during the 20 min mosquito feed. They are mixed prior to administration by a single IP injection. The correct dose per animal size is needed to avoid an overdose. The loss of eye blink or reflex movement just as a pinch between the toes will be used to confirm anesthesia. For isoflurane breathing rate will also be used to determine anesthesia level

Isoflurane is used as a short acting pharmacological restraint during the IV injection and the cardiac puncture. It is administered by inhalation in a fume hood for the safety of the experimenter. A small piece of gauze soaked in isoflurane is placed at the base of a 50 ml conical tube and then a piece of filter paper or sintered glass filter will be inserted to prevent the mouse from coming into contact with liquid isoflurane. The tube will be held over the mouse's nose. The loss of eye blink or reflex movement just as a pinch between the toes will be used to confirm anesthesia and breathing rate will be used to determine anesthesia level. After cardiac puncture the mouse will be euthanized, while after the IV injection the mouse will be isolated from other mice until it regains consciousness.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: N/A

V.4.1.2.3. Paralytics: None

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: Altweb, PubMed

V.4.1.3.2. Date of Search: Nov 10, 2015

V.4.1.3.3. Period of Search: 1947-present

V.4.1.3.4. Key Words of Search:
Altweb: Berghei, Transmission-blocking, drug development
Pubmed: "Berghei AND Transmission-blocking"

V.4.1.3.5. Results of Search:
Altweb: 0 results were found for berghei as well as transmission-blocking. An article was found for drug development that concluded that following *in vitro* cytotoxicity assays, animal testing was still needed assess the effect of potential drug candidates in intact organisms Rovida, et al. Altex 32(3), 2015

Pubmed: "Berghei AND Transmission-blocking" had 99 articles, including 11 in the past year that used the *in vivo P. bergheil*mouse model to assess malaria transmission.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification

The long term goal of this work is to identify a compound that can be developed as an anti-malaria drug that could be used in humans to eliminate malaria, which currently is responsible for >200 million clinical cases and > half a million deaths/ annually. We are using the well-established rodent malaria, P. berghei/mouse model to evaluate both safety and efficacy as a first step to determine which compounds should be advanced for further development of a drug that could be used in humans. No alternative in vitro models have yet been developed that predict the complex interactions of chemical compounds in the context of an intact organism.

Selected compounds that in vitro are 10 time more active against malaria parasites will be tested for safety in healthy mice using the dose escalation protocol described in Exp 1. The mice cannot be anesthesized during this test, because we need to know the maximum safe dose for the efficacy experiments (Exp 2) and the presence or absence of physical signs of distress are how we are determining safety. Once 2 signs of distress are observed the animal will be euthanized and no higher doses will be tested.

All the mice that are infected with P. berghei including the donors will also be in category E. The parasites required for the experiments proposed in Exp 2 and 3 need to be generated in the mice because Exp 2 is evaluating in vivo efficacy and for Exp 3 there is no other in vitro assay to produce ookinetes. After the initial parasite inoculation it can take a week or longer for the parasitema to reach a high enough level (>10 % parasitemia) for in vivo testing or gametocyte isolation. Generally physical symptoms are not observed until the parastema is >30%, but the course of the infection cannot be precisely predicted. It is not possible to keep the mouse anesthetized during the entire infection therefore, although we monitor the mice daily, we cannot guarantee that the mice will not experience distress and they have been included in category E.

V.4.2. Prolonged Restraint: NA

V.4.3. Surgery: NA

V.4.3.1. Pre-surgical Provisions: NA

V.4.3.2. Procedure: NA

V.4.3.3. Post-surgical Provisions: NA

V.4.3.4. Location: NA

V.4.3.5. Surgeon: NA

V.4.3.6. Multiple Major Survival Operative Procedures: NA

V.4.3.6.1. Procedures: NA

V.4.3.6.2. Scientific Justification: NA

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

-IV injection:the mouse will be pharmacologically restrained using isoflurane, and no more than 200 ul fluid will be injected into the retro orbital venous sinus using a ≤27.5 gauge needle or tail vein using a ≤25 gauge needle.

-IP injection: the mouse will be restrained by hand and less than 410 ul of liquid will be introduced into the peritoneum via a 23 gauge syringe needle. A 23 gauge needle is used to decrease shear stress to the *P. berghei* infected RBCs. Enough shear stress can be generated during rapid flow through a 25 gauge needle to lyse infected and uninfected RBCs.

V.4.4.2. Biosamples:

Samples that will be collected from the mice

Species	Fluid	Amount	Frequency	Site/method	Postmortem
Mus musculus	blood	20 microliters	daily	tail bleed	No
Mus musculus	blood	200 microliters	once	mosquito bite	No
Mus musculus	blood	1 milliliter	once	heart puncture	No

The mice will also be infected with *P. berghei* parasites, which can be lethal if untreated, therefore after infection the mice will be monitored daily for signs of lethargy or distress and will be euthanized if they appear unhealthy.

V.4.4.3. Adjuvants:

In addition to the test compounds the following substances may administered to the mice during the course of the study.

	Substance/Agent	DEA	Amount Given	Delivery Route
1	phenylhydrazine	No	200 ul of 6 mg/ml PBS	IP
2	pyrimethamine	No	200 ul 1 mg/ml PBS-2%Tween20	IP IV or PO
3	RPMI	No	400 ul	IP IV or PO
4	methylcellulose	No	200 ul of a 2% solution	IP IV or PO
5	Polyethylene glycol (PEG)	No	200 ul of a 40% solution	IP IV or PO
6	n-Methylpyrrolidone	No	200 ul of a 25% solution	IP IV or PO
7	DMSO Dimethyl sulfoxide	No	200 ul of a 5% solution	IP IV or PO
8	kolliphor RH40 /cremophor RH40	No	200 ul of a 18% solution	IP IV or PO
9	Ethanol	No	200ul of a 7.5% solution	IP IV or PO

1) Phenylhydrazine induces the parasites to undergo sexual differentiation. The phenylhydrazine protocol is routinely used to enhance the production of sexual-stage *P. berghei* parasites (Rodriquez et al (2002) Exp Parasitol 101:73-76.

2) Pyrimethamine kills asexual parasites and the Tween20 is a nontoxic detergent that helps dissolve the pyrimethamine. Pyrimethamine is actually used therapeutically in human to treat human malaria.
3) RPMI is a type of tissue culture media that can be used in place of PBS to resuspend the parasites to provide more nutrient rich environment which enhances viability. RPMI is used to grow eukaryotic cells *in vitro* and will have no adverse efect on the mice.
4) Methylcellulose is a nontoxic compounds that is used as a thickener and emulsifier in food. It decreases clearance of the compound. Methyl cellulose is not toxic and even approved for use in food.
5) Polyethylene glycol (PEG) is used to enhance the solubility of hydrophobic compounds in aqueous solutions.
6) n-Methylpyrrolidone, like DMSO, it contains both polar and nonpolar regions allowing it to dissolve in water and oil and therefore is used to enhance the solubility of hydrophobic compounds in aqueous solutions.
7) DMSO Dimethyl sulfoxide, Contains both polar and nonpolar regions allowing it to dissolve in water and oil and therefore is used to enhance the solubility of hydrophobic compounds in aqueous solutions.
8) kolliphor RH40 or cremophor RH40 (PEG-40 Hydrogenated Castor Oil) is used to enhance the solubility of hydrophobic compounds in aqueous solutions. It is approved for use in human drug formulations.
9) Ethanol is used to enhance the solubility of hydrophobic compounds in aqueous solutions.

V.4.4.4. **Monoclonal Antibody (MAbs) Production**: NA

V.4.4.5. **Animal Identification**:

-Ear tag: the mouse will be restrained by hand and a small hole will be made in its ear with an ear punch to identify the mouse. There is usually little or no bleeding.

V.4.4.6. **Behavioral Studies**: NA

V.4.4.7. **Other Procedures**:

- PO administration by oral gavage: the mouse will be restrained by hand and a bulb tipped 18 and 20 gauge gastric gavage needle will be used to deliver defined amounts of compound directly into the stomach of mice $\geq$ 25 gms and 20-25 gms. respectively.

-Tail bleed: the mouse will be restrained by hand and a small nick will be made at the tip of the tail. A drop of blood will be collected on a microscope slide, then the nick will be allowed to clot.

-Monitoring for Distress. Following the administration of a compound or the appearance of circulating *P. berghei* parasites, mice will be evaluated daily for distress. Since the compounds we are using have not been administered to mice before the possible symptoms are not known and will require a general screen for decreased or impaired motility, hunched posture, ruffled fur, difficulty breathing, diarrhea, and skin lesions. If any 2 of these are observed the mouse will be euthanized. In the case of *P. berghei*, the parasitemia usually increases to ~10% over the first 5-7 days with few associated symptoms. Depending on the age and genetic background of mouse the parasitemia can plateau at ~30% with few symptoms for another week before the parasites are cleared or the mouse can become anemic. Alternatively, some mice at ~30% parasitemia develop symptoms of cerebral malaria and can die if not treated. The physical

manifestations of cerebral malaria are quite obvious and include difficulty moving (ataxia or paralysis), hunched posture, hyperventilation and convulsions. If a mouse exhibits any two of these after *P. berghei* infection or just one of these signs in combination with a parasitemia of >15% the mouse will be euthanized.
-Mosquito feed: The mouse will be anesthetized with ketamine and acepromazine and laid on the top of a sealed cage containing no more than 90 mosquitoes for 20 min. After the blood meal the mouse will be euthanized by exsanguination under anesthesia or in some cases returned to the cage to recuperate and the mosquitoes will be maintained for 21 days. At that time a naïve mouse will be anesthetized and placed on top of a sealed cage containing the mosquitoes that had previously taken a blood meal from a *P. berghei* infected mouse. After the Plasmodium-exposed mosquitoes have taken a blood meal the mouse is allowed to recuperate and after 5 days the parasitemia will be followed by tail bleed as described above to evaluate whether they were infected.

-Exsanguination by cardiac puncture. The mouse will be anesthetized with isoflurane and a 25 gauge needle will be used to withdraw blood from the heart.

V.4.4.8. Tissue Sharing: A portion of the tissue obtained in the course of the study may be provided to collaborators to assess compound levels for pharmacokinetic evaluation to determine drug absorption, bioavailability, distribution and metabolism.

V.4.5. Study Endpoint:

Exp 1: Mice will be monitored daily for signs of distress for 4 days after compound administration. If no distress is observed they could be transferred to another protocol or euthanized by CO_2 narcosis or anesthetized and exsanguinated by cardiac puncture. If 2 signs of distress are observed including, decreased/impaired motility, hunched posture, ruffled fur, difficulty breathing, diarrhea, and skin lesions and will be euthanized by CO_2 narcosis or anesthetized and exsanguinated by cardiac puncture.

Exp 2: *P. berghei* donor mice will be anesthetized and exsanguinated by cardiac puncture to harvest *P. beghei* infected RBCs for transfer to another mouse.
After exposure of *P. beghei* infected experimental mice to mosquitoes for 20 minutes they will either be euthanized by exsanguination under anesthesia immediately or allowed to revive and continue to be monitored for a week. At this time they could be transferred to another protocol or euthanized by exsanguination under anesthesia or CO_2 narcosis.

Exp 3: *P. berghei* donor and experimental mice will be anesthetized and exsanguinated by cardiac puncture to harvest *P. beghei* infected RBCs for transfer to another mouse or to test in vitro ookinetes development.

If any time a mouse is observed to have 2 signs of distress including, decreased/impaired motility, hunched posture, ruffled fur, difficulty breathing, diarrhea, and skin lesions and will be euthanized by CO_2 narcosis or anesthetized and exsanguinated by cardiac puncture.

V.4.6. Euthanasia:

The mice will be euthanized after being anesthetized and exsanguinated by cardiac puncture to harvest blood. This procedure will be done in a fume hood to protect personnel from inhaling

the anesthetic. Once the mouse has lost consciousness and reflex movement, a 25 gauge syringe needle will be inserted into the heart and the blood will be withdrawn into a 1 ml syringe.

Following the procedure the lack vital signs (breathing and heartbeat) will be observed for 5 minutes to confirm death will be done. The mouse carcasses will be disposed of by incineration.

If a blood sample is not needed a mouse could also be euthanized by CO_2 narcosis followed by cervical dislocation or bilateral thoracotomy.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:
Building(s) (b)(6) Room Number(s) TBD

V.5.1.2. Special Husbandry Provisions: N/A

Food Restriction:	Yes	_____	No	X
Fluid Restriction:	Yes		No	X

V.5.1.3. Exceptions: N/A _____ _____

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be observed and cared for by the Center for LAM personnel according to LAM SOPs.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein	Name of person performing activity	Qualifications of person performing activity (e.g.,	Specific training in this activity or procedure

injections, euthanasia)		research technician, 2 yrs experience)	(e.g., rodent handling class, 1999)
(handling or care of animals, euthanizing animals, or non-surgical procedures (e.g., administration of drugs or other experimental procedures).	(b)(6)	PI, >20 years of experience work with mice and Plasmodium parasites	Citi and USUHS training courses
(handling or care of animals, euthanizing animals, or non-surgical procedures (e.g., administration of drugs or other experimental procedures).)		Post-doc, > 4 years experience working with the P. berghei/mouse model of malaria	Citi and USUHS training courses
(handling or care of animals, euthanizing animals, or non-surgical procedures (e.g., administration of drugs or other experimental procedures).)		Post-doc, > 4 years' experience working with parasite infected mice.	Citi and USUHS training courses

VII. BIOHAZARDS/SAFETY:

P. berghei is not infectious to humans and there is no mouse to mouse transmission.

Good laboratory practices will be followed. A lab coat and gloves will be worn when working with the compounds, the mice and in the LAM facility. Face mask are not required but will be provided if requested by a staff member.

Isoflurane will be used in a fume hood and all needles will be disposed of in sharps containers.

VIII. ENCLOSURES:

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) 12/8/15

Principal Investigator Signature Date

have attended

G. Training: The following personnel ~~will attend~~ the next USUHS Investigator/Animal User Training Course:

(b)(6) (b)(6)

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) 12/8/15

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature

12·8·15

Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: (b)(4) (b)(4)

C. Principal Investigator: (b)(6)

D. Performing Organization: Uniformed Services University of the Health Sciences

E. Funding: (b)(4)

F. Objective and Approach: Malaria is a tropical parasitological disease that remains a global health problem, causing more than half a million deaths and 250 million cases annually. Expanded control and treatment programs in the past decade have reduced the incidence of the disease and led to the call for efforts to eliminate, possibly even eradicate, malaria. To do this new strategies are needed that target the sexual stages of the parasites, called gametocytes that are responsible for disease transmission. The current recommended chemotherapy for malaria does not effectively kill mature gametocytes, allowing malaria to be transmitted for more than a week after the clearance of asexual parasites. Previous drug screens used assays that only detected asexual replication and therefore did not monitor activity against gametocytes. (b)(4) (b)(4) (b)(4) The goal of this research is three fold 1) to use a rodent malaria model to analyze the *in vivo* safety of selected compounds with *in vitro* gametocytocidal activity 2) to use a rodent malaria model to analyze the *in vivo* malaria transmission blocking efficacy of selected compounds with *in vitro* gametocytocidal activity at doses found safe in mice 3) define the stage specificity and timing of compound inhibition from intraerythrocytic development to oocyst formation. Together these approaches should identify classes of compounds that can be targeted for further drug development, as well as advance our understanding of gametocyte metabolism and facilitate the design of effective control strategies. The mouse studies described in this work will be performed in full accordance with the Institutional Animal Care and Use Committee at USUHS. The sequential dose escalation and low dose efficacy testing are designed to minimize the animal usage.

G. Indexing Terms (Descriptors): animals, mice, malaria, antimalarials, Plasmodium berghei, drug development

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

April 2, 2014

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF MEDICAL AND CLINICAL PSYCHOLOGY

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on April 2, 2014:

Title of Application: "Behavioral Investigations of Nicotine and Caffeine in rats (Rattus Norvegicus)"

USUHS Protocol Number: (b)(6)

Expiration Date: April 1, 2017

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc: Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER:

PROTOCOL TITLE: Behavioral investigations of nicotine and caffeine in rats (Rattus norvegicus)

GRANT TITLE (if different from above): Behavioral effects of stress, nicotine, and caffeine in male and female rats (Rattus norvegicus)

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: USUHS

EARLIEST ANTICIPATED FUNDING START DATE: 10 January 2014

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6) MPS (b)(6) 27 Feb '14
P Department Office/Lab Telephone Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) Chair, MPS (b)(6) 27 Feb'14
Research Unit Chief / Dept. Head Signature Title Telephone Date
Typed Name: (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) MEM (b)(6) 25 FEB 14
D. Department Telephone Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) LAM (b)(6) 28 Feb 2014
Attending/Consulting Veterinarian Signature Department Telephone Date
Typed Name: (b)(6) D.V.M.

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

ANIMAL PROTOCOL TITLE: Behavioral investigations of nicotine and caffeine in rats (Rattus norvegicus)

GRANT TITLE (if different from above): Behavioral effects of stress, nicotine, and caffeine in male and female rats (Rattus norvegicus)

USUHS PROJECT NUMBER (b)(6)

CO-INVESTIGATOR(S): (b)(6) (b)(6)

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

Stress is a pervasive experience that affects behaviors, cognitions, and biological responses. Insufficient sleep is a particularly disruptive stressor that is experienced by civilians and Warriors. Threat of attack also is a stressor that is commonly experienced by Warriors. Nicotine and caffeine are legal drugs that are used by many civilians and service members. These drugs are often consumed during stressful situations. Each of these variables (i.e., insufficient sleep, threat of attack, nicotine, and caffeine) alone can have psychological effects (on behavior and cognition). It is important to examine if nicotine or caffeine use alters psychological effects of stress – either potentiating or attenuating effects of stressors. The experiments described in this application use a rodent model of Warrior Stress and sleep disruption to determine how psychological responses to stress are altered by nicotine or caffeine administration in male and female rats. The behaviors assessed include motor responses (to index anxiety, depression, . and learning), sensorimotor (including reflexes and nociception), and cognitive responses (including attention, memory, learning). Blood samples will be assessed for stress hormones (corticosterone). It is hypothesized that: the stressors will deleteriously affect the measured behaviors; nicotine will attenuate these effects; caffeine will potentiate these effects; and females will be more sensitive than males to the effects of stress, nicotine, and caffeine.

II. BACKGROUND:

II.1. Background:

Stress is a psychobiological process that can involve physical, psychological (i.e., behavioral, cognitive, motivational/emotional), social demands, or stressors (negative [distress] and positive [eustress]). Responses to acute stressors usually are adaptive, whereas responses to chronic stress often are disruptive to health (McEwen, 2007). Stress is experienced by everyone (civilians and service members) and can affect behaviors and cognitions (Baum, Gatchel, & Krantz, 1997). Nicotine (in tobacco products and in other delivery systems) and caffeine (in beverages and food stuffs) are licit substances that are consumed by many people and are commonly consumed while under stress (Grunberg, Berger, & Hamilton, 2011). Research has examined effects of

stress on drug self-administration (including nicotine and caffeine), but there has been little study of whether consumption of each of these drugs potentiates or attenuates effects of stress. Because these drugs are legal and use is so prevalent, actions of these drugs can be mistakenly underestimated. Whether use of these drugs potentiates or attenuates effects of stress is important to determine whether consumption of nicotine and caffeine while stressed, makes performance and mental health better or worse. This information is needed to advise individuals and decision-makers responsible for the health and performance of individuals who consume these substances while under stress.

The proposed experiments will use an animal model to investigate psychological effects of stressors (sleep disruption, a rat model of "Warrior Stress," or both stressors together). This experiment also will examine whether nicotine and caffeine alter psychological effects of stress. Additionally, we will examine if males and females respond differently to the combination of stress and these licit drugs. Animals (rats) will be used as subjects to allow for true experiments that: manipulate all relevant independent variables; maintain controlled environments and housing conditions; measure all behaviors in comparable ways; and include genetically similar subjects. This type of preclinical investigation has proved to be valuable to gather information about stress, stress effects, and effects of drugs (including nicotine and caffeine; Faraday, 2005; Grunberg, 1992; Grunberg, Berger, et al., 2011; Jacob, Wilson, & Benowitz, 1981). Findings from this investigation will provide information that could later be tested in humans. The following sections provide relevant information about effects of stress and the drugs nicotine and caffeine on behavior and provide the rationale for conducting the proposed experiments.

Stress is pervasive and has short and long term mental and physical health effects (Baum et al., 1997; McEwen, 1998). Additionally, stress affects health behaviors, cognition, and performance. Stressed individuals increase health-harming behaviors (e.g., cigarette smoking; Grunberg, Berger, et al., 2011) and decrease health-promoting behaviors (e.g., exercise; American Psychological Association, 2012). The experience of stress has been associated with decreased attention (Liston et al., 2006) and impairments in learning and memory (Diamond, Fleshner, Ingersoll, & Rose, 1996). Chronic stress and extreme stress have been linked to psychiatric problems such as anxiety, depression, and PTSD (Ford & Kidd, 1998; Goenjian et al., 2000). Stress also negatively affects performance (Beilock, 2008; Motowidlo, Packard, & Manning, 1986; Westman, 1990).

A particularly powerful stressor is **insufficient sleep**. Disrupted and insufficient sleep is a commonly reported condition among civilians and service members (see Military Relevance section below). According to the Center for Disease Control (2011), 37% of Americans report that they sleep less than the recommended 7-8 hours per night. Disturbed and insufficient sleep has been associated with increased use of tobacco and alcohol (McKnight-Eily et al., 2011), poor cardiovascular health (Franzen et al., 2011), and increased suicide attempts (Fitzgerald, Messias, & Buysse, 2011). Poor sleep adversely affects cognitive functioning, including deficits in alertness, attention, and psychomotor vigilance (Wesensten, Killgore, & Balkin, 2005b) as well as results in slow, unstable mental processes filled with errors (Doran, Van Dongen, & Dinges, 2001). Sleep disruption also has serious negative effects on executive (cognitive) function (Killgore, Kahn-Greene, Grugle, Killgore, & Balkin, 2009). This proposal seeks

to incorporate sleep interruption as a stressor of interest to examine effects of insufficient sleep on behavior and potential interactions with nicotine and caffeine.

It is noteworthy that stress is directly associated with drug use, including nicotine and caffeine (Grunberg & Baum, 1985; Grunberg, Berger, & Starosciak, 2010; Kassel, Stroud, & Paronis, 2003; Ratliff-Crain, 1991; Shaham, Klein, Alvares, & Grunberg, 1993; Shiffman, 1993). Several explanations have been offered to explain this association: drug use decreases stress ("self-medication hypotheses"); drugs are used to relieve withdrawal-like symptoms that resemble or are caused by or exacerbated by stress ("withdrawal hypotheses"); stress alters drug actions ("pharmacodynamic hypotheses"); and drug use increases stress which then increases drug use ("positive feedback loop hypotheses"; Grunberg et al., 2010). Despite these speculative mechanisms (b)(6) (b)(6) there has been little direct, empirical examination of psychological effects of stress and nicotine or stress and caffeine, even though these drugs are commonly self-administered. The proposed work is designed to begin to fill this gap.

Nicotine and caffeine are two drugs that can be used legally and are easily accessible. In the U.S. 19% of adults smoke cigarettes (Center for Disease Control and Prevention, 2012) that contain nicotine and 90% of individuals consume caffeine (Iversen, Iversen, Bloom, & Roth, 2009). These numbers rise when other nicotine-containing substances (e.g., chew, dip, cigars, nicotine replacement products) are included. These numbers rise even more for military personnel for nicotine (see below).

Nicotine. Nicotine, the primary pharmacologically-active drug in tobacco, has many effects that reinforce its self-administration. Nicotine's reinforcing effects include: physical dependence, decreased appetite, improved attention, perceived stress relief, and pain reduction. Our laboratory has contributed to these findings using rat paradigms to conduct true experiments which have produced results that have been later confirmed in human samples (Acri, Morse, Popke, & Grunberg, 1994; Faraday & Grunberg, 1999; Grunberg, 1982, 1989, 1991, 1997; Grunberg & Phillips, 2004; Winders & Grunberg, 1989). The question remains, however, whether using nicotine while under stress makes stress responses better or worse – a question that has not yet been examined.

Nicotine is reported to improve cognitive functioning (Amitai & Markou, 2009; Potter & Newhouse, 2008). Nicotine also is associated with improved cognitive and motor performance (Rose, Ross, Kurup, & Stein, 2010). This drug is unusual in that it has been classified as a sympathomimetic drug (*Goodman & Filman's The Pharmacological Basis of Therapeutics*, 2006) but it also has a paradoxical dual action to either increase or decrease sympathetic nervous system activity depending on the physiological and psychological state of the individual (Grunberg & Starosciak, 2010; USDHHS, 1988). As mentioned above, cigarette smoking increases during stress. Some investigators have suggested that this increase results because nicotine (acting via *positive reinforcement*) reduces stress and that smokers are self-medicating to relieve the symptoms of stress (e.g., irritability; Grunberg, Berger, et al., 2011). However, this suggestion has not been the focus of careful empirical investigations in animals or in human subjects. Because nicotine in tobacco products are addictive (USDHHS, 1988), studies of nicotine over the past 20 years have focused on mechanisms underlying the addiction process and development of tobacco cessation products, including new nicotine delivery systems and medications that mimic effects of nicotine. Investigators have not examined effects of nicotine to potentially reduce stress.

Considering that stress often disrupts cognitive processing and other behaviors along with reported effects of nicotine, we hypothesize that nicotine will attenuate disruptive effects of stress on cognitive function and other behaviors.

Caffeine. Caffeine is another licit substance that is associated with improved cognitive functioning and performance (Seidl, Peyrl, Nicham, & Hauser, 2000). Many studies have reported that caffeine can be used safely and effectively to improve performance and stave off fatigue (Baranski et al., 2001; IOM-CMNR Institute of Medicine Committee on Military Nutrition Research, 2001). Yet, few studies have examined effects of caffeine under stressful situations (Sunram-Lea, Owen-Lynch, Robinson, Jones, & Hu, 2012). The research that is available reports that caffeine is associated with increases in glucocorticoids (e.g., cortisol, ACTH; Dhingra et al., 2007; Gavrieli et al., 2011; Hino et al., 2007; Lin, Uhde, Slate, & McCann, 1997) – these previous studies have not focused on behavioral or cognitive effects of caffeine and stress. Because caffeine indirectly activates some of the same pathways that stress activates (e.g., sympathetic nervous system), the combination of caffeine and stress may affect behavior and health in undesirable ways (James, 2004, 2011). In addition, caffeine may interfere with sleep and, thereby, increase stress. Sleep hygiene guidelines, for example, suggest avoiding caffeine consumption 6 hours before going to sleep (Alabama Sleep Clinic, 2012). Most of the reported research studying caffeine and sleep has investigated how caffeine can be used to offset deleterious effects of sleep disruption and to delay sleep onset. These investigations, however, have not considered caffeine's effects when other stressors (e.g., threat of attack – see below under Independent Variables "Warrior Stress Paradigm") are present.

The relationship between caffeine, stress, and poor sleep has not been examined. It is important to understand how these variables interact to affect behavior because caffeine is used by most civilians and almost all service members and these individuals experience stress. It also is important to examine how these variables interact because the use of these drugs increases during times of stress and potentially (especially in the case of caffeine) during times of limited sleep. We hypothesize that caffeine will potentiate effects of stress.

Males and females. The male psychological and biological stress responses have been extensively studied in animals and humans and can be summarized as the "fight-or-flight response" (Cannon, 1935; Faraday, 2005; Mason, 1975; Selye, 1946, 1973). In response to stress, males generally become aggressive (fight) or escape the threatening situation (flight). The female stress response involves similar biological systems (i.e., the sympathetic branch of the autonomic nervous system and the hypothalamic-pituitary-adrenal axis) but seems to involve different behavioral responses that have been called the "tend-and-befriend" response (Taylor et al., 2000). When a female experiences stress, the fight-or-flight response still occurs but she also increases care for her young and/or seeks social interaction. There has been relatively little experimental examination of sex differences in stress responses; animal subjects allow for true experiments to be conducted ethically and logistically. For example, we recently discovered that female rats display more anxiety-related behavior and more depression-related behavior after exposure to a "Warrior Stress" (see below) than do male rats (Yarnell, 2012a). In addition, we have found that female rats are more sensitive to stress than male rats with regard to peripheral stress hormones (i.e., corticosterone).

The proposed experiments include male and female rats as subjects to investigate sex differences in effects of nicotine and caffeine during stress. We

hypothesize that females will be particularly sensitive to effects of stress and to effects of these drugs on stress.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:

Medline (PubMed), PsycInfo, F1000Biology (and electronic journal review service), BRD, and RePORT

II.2.2. Date of Search:

February, 2014

II.2.3. Period of Search:

All years available on each database (ranging from past year to past 30 years)

II.2.4. Key Words and Search Strategy:

Nicotine, chronic nicotine administration, acute nicotine administration, caffeine, stress, rats, sex differences, age differences, locomotion, activity, neurobehavioral functioning, hot plate, nociception, acoustic startle reflex, pre-pulse inhibition, passive avoidance, memory, anxiety, depression, cognition (words are entered separately and in combination; rat is entered with each term [i.e., rat+nicotine+stress+locomotion/activity, rats+nicotine+stress+neurobehavioral functioning, rat+nicotine+stress+hotplace/nociception, rat nicotine+stress+acoustic startle response/prepulse inhibition, rats+nicotine+stress+passive avoidance/learning, rats+nicotine+stress+anxiety/depression, rats+nicotine+stress+cognition, etc)) have been searched in data base searches. We also read electronic and paper journals regularly and search for phrases, titles, and abstracts relevant to our work.

II.2.5. Results of Search:

There has been extensive work on stress, nicotine, and the various behavioral measures included in our work (i.e., nicotine + various behavioral measures, stress + behavioral measures). There has been some work on effects of caffeine in rats and the various behavioral measures. However, there has been little work combining these variables in the ways that we do (e.g., effects stress and nicotine on behavioral responses). The Warrior Stress Paradigm (b)(6) methods/results have not been published. Sleep disruption has been studied in rats (>60,000 hits), but not looking as extensively at the different behavioral measures or in combination with nicotine, caffeine, or the Warrior Stress Paradigm. Also, research has focused more on male rats. Overall, literature searches on the topics to be covered in our experiments indicate the value of our approaches and have not revealed existing literature or data that would supercede our experiments.

III. OBJECTIVE\HYPOTHESIS:

The current proposal focuses on psychological (especially behavioral and cognitive) effects of stress, nicotine, and caffeine using animal models (male and female rats) to allow for carefully controlled experimental examination of behavioral measures related to anxiety, depression, sensorimotor reflexes, attention, learning, and memory. The overarching aim of this project is to enhance the health of Warriors under stressful conditions by investigating whether nicotine (which Warriors self-administer via smokeless and smoked tobacco products) and caffeine (which Warriors self-administer via caffeinated beverages and caffeine pills) alter behavioral responses to stress. We hypothesize that nicotine will attenuate effects of stress, whereas caffeine will potentiate

effects of stress, and that effects of stress and of these drugs will be greater in females than in males.

Specific Aim 1: To determine effects of military-relevant stressors (threat of attack; sleep disruption) on behaviors (motor, sensorimotor, cognition) in male and female rats.

Hypothesis 1: Stress will deleteriously affect the measured behaviors, including: motor (increased indices of anxiety and depression based on particular locomotor activity parameters); sensorimotor (impaired neurobehavioral functioning); and cognition (impaired attention, learning, and memory).

Specific Aim 2: To determine whether nicotine alters the effects of stress.

Hypothesis 2: Nicotine will attenuate deleterious effects of stress on motor (i.e., decrease behavioral measures related to anxiety and depression), sensorimotor (i.e., improve sensorimotor responses), and cognitive measures (i.e., attenuate deleterious effects of stress on attention, learning, and memory).

Specific Aim 3: To determine whether caffeine alters the effects of stress.

Hypothesis 3: Caffeine will potentiate deleterious effects of stress on motor (i.e., increase behavioral measures related to anxiety and depression), sensorimotor (i.e., worsen sensorimotor responses), and cognitive measures (i.e., worsen deleterious effects of stress on attention, learning, and memory).

Specific Aim 4: To determine if males and females are affected differently by the combination of stress and licit drugs (nicotine or caffeine).

Hypothesis 4: Females will be particularly sensitive to effects of stress and to effects of these drugs on stress (i.e., nicotine will be more beneficial for female than for male rats under stress; caffeine will be more deleterious for female than for male rats under stress).

IV. MILITARY RELEVANCE:

Warriors experience unique **stressors** associated with deployment and combat exposure, and recent reports indicate a dramatic increase in combat exposure and greater rates of anxiety, depression, and acute stress disorder (Joint Mental Health Advisory Team, 2011). In addition, psychological stress is highly correlated with drug use and abuse (Grunberg, Berger, et al., 2011). Deployment stressors place Warriors at significant risk of physical and mental health consequences that disrupt day-to-day life as well as performance under pressure (Vasterling et al., 2006). These consequences include neurological compromise, anxiety, depression, and posttraumatic stress disorder (PTSD). Hoge and colleagues (Hoge et al., 2004) reported that a significant percentage of service members met criteria for major depression, PTSD, and drug use after deployment. In another sample, 35% of Veterans who served in Iraq sought mental health services in the year following their return and 12% were diagnosed with a mental health problem (Hoge, Auchterlonie, & Milliken, 2006). Additionally, multiple deployments have been associated with more psychological problems, decreased morale, and increased use of psychopharmaceuticals (Joint Mental Health Advisory Team, 2011).

Many of the stressors experienced by service members are comparable to stressors experienced by civilians (e.g., work demands, family pressures, and insufficient sleep). Even the threat of death (that is experienced by service members in combat) may be experienced by police officers, firefighters, and civilians who are in dangerous situations (e.g., domestic violence, living or traveling in high crime rate

areas). What is unique about the Warriors experience is that the stressors are often extreme versions of stressors and are often experienced simultaneously and for extended periods of time. For example, it is typical for a Warrior to have interrupted sleep, go on a life threatening mission, and be required to perform (behaviorally and cognitively) under environmentally unpleasant conditions (e.g., loud noises) day after day. A majority (70-91%) of Warriors returning from combat report sleep-related disturbances (Ulmer, Edinger, & Calhoun, 2011). Service members who have disturbed and insufficient sleep are at risk for poor cardiovascular health (Franzen et al., 2011) and increased suicide attempts (Fitzgerald et al., 2011). Additionally, they may increase the use of licit drugs (e.g., nicotine and alcohol) because of poor sleep (McKnight-Eily et al., 2011).

The proposed experiments will evaluate behavioral and cognitive effects of **military-relevant stressors using rat paradigms** developed in our laboratory to model sleep disruption, threat of attack, and exposure to environmental mild stressors (Grunberg, 2011; Kwon et al.; Perry, 2009; Yarnell, 2012a; Yarnell, Chwa, Hamilton, & Grunberg, 2011; Yarnell & Grunberg, 2012). These experiments also will examine whether nicotine and caffeine (legal drugs that are consumed by many service members while in stressful situations) alter behavioral, cognitive, and biological effects of stress.

Although about 1/5 American civilians smoke cigarettes, almost 1/3 military personnel currently smoke cigarettes and even more use smokeless **nicotine**-containing tobacco products (Bray & Hourani, 2007). Many young never-smokers and occasional smokers become regular smokers after military basic training (Haddock, Klesges, Talcott, Lando, & Stein, 1998), suggesting that the military environment (e.g., high stress) may be a factor in smoking initiation. Perhaps, intake of nicotine is a way to help attenuate the stress of military life.

Caffeine consumption is not monitored in the military and there are no statistics available for the use or abuse of caffeine in this organization. Nonetheless, any member of the military will report that Warriors consume vast quantities of caffeine (James, 2011; Moosey, 2012; Yarnell, 2012b). Popular energy drinks are sold in the exchange and others are free in the dining hall. Coffee is also a staple for most Troops. Standard rations include caffeine supplements in the form of caffeinated gums and powdered drinks (Armed with Science, 2011). The use of caffeine is encouraged as a way to enhance performance and counter effects of sleep disruption (IOM-CMNR Institute of Medicine Committee on Military Nutrition Research, 2001). This recommendation was made based on extensive scientific investigation (IOM-CMNR Institute of Medicine Committee on Military Nutrition Research, 2001). However, effects of caffeine while under stress (e.g., in combat situations where service members may be exposed to stressful and traumatic events) have not been examined. We hypothesize that caffeine intake while under stress may exacerbate effects of stressors.

The experiments that we propose focus on behavioral, cognitive, and biological effects of nicotine, caffeine, and stress in rat paradigms to better understand how these variables are interacting. This work is intended and designed to be relevant to the health and well-being of military personnel living and working in various environments.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

(b)(6) research program has produced a robust characterization of effects of **nicotine**, including effects on: reward and addictive processes, food consumption and body weight, attention, anxiety-like behavior, nociception, and heart morphology (Elliott, Faraday, & Grunberg, 2003; Grunberg & Phillips, 2004; Winders & Grunberg, 1989). (b)(6) discovered that there are sex and age differences in responses to nicotine in adolescent and adult rats (Faraday, Elliott, & Grunberg, 2001; Grunberg, Winders, & Wewers, 1991). (b)(6) reported effects of **caffeine** to alter cognitive processes in rats (Faraday & Grunberg, 1999) and humans (Stine, O'Connor, Yatko, Grunberg, & Klein, 2002).

(b)(6) have examined how **stress** changes the actions of nicotine and related self-administration and withdrawal behaviors. (b)(6) discovered that stress alters pharmacokinetic and pharmacodynamic effects of nicotine consistent with increased nicotine self-administration during stress (Grunberg, Morse, & Barrett, 1983; Winders, Grunberg, Benowitz, & Alvares, 1998). (b)(6) discovered that: stress exacerbates behavioral sensitization to nicotine (an index of the development of drug dependence; Hamilton, Starosciak, Chwa, & Grunberg, 2012); nicotine withdrawal occurs in male and female rats and is affected by environment relevant to stress (Hamilton, Berger, Perry, & Grunberg, 2009); and withdrawal effects of nicotine differ based on rat strains relevant to stress-sensitivity (Perry, 2009). We have yet to examine how nicotine or caffeine alter psychological effects of stress. In other words, does nicotine or caffeine change responses to stress?

(b)(6)

For example, one military-relevant project (b)(6) focused on effects of **predator stress** and **sleep disruption** (SD) on the behavior of rats. (b)(6) developed an effective sleep disruption paradigm for rats. Results indicated that rats in the sleep disruption condition displayed greater anxiety-like behavior, increased alcohol consumption, and higher corticosterone blood levels (a biomarker of stress; Perry, 2009). Moreover, sleep disrupted female rats were more anxious than males.

(b)(6) "Warrior Stress Paradigm" (WSP) that uses rats to model impending threat of attack in novel environments (Kwon et al.; Yarnell, 2012a; Yarnell et al., 2011; Yarnell & Grunberg, 2012) based on previous studies (Berger, 2009; Hamilton, 2010; Hayley, Borowski, Merali, & Anisman, 2001; Long, 2010; Meyerhoff, 2006; Perry, 2009; Starosciak, 2010; Willner, Towell, Sampson, Sophokleous, & Muscat, 1987) . The WSP attempts to model the anticipatory stress that a Warrior experiences prior to conducting a life threatening mission and the disruptive stimuli that are found in a deployed setting. Rats are exposed to a combination of the threat of death (predator scent) and specific, disruptive environmental stimuli (e.g., flashing lights, loud noise) for 20 minutes per day for 7 days. This combination produces a mild stress response in the rat.

The proposed experiments build on this work by investigating effects of two powerful, military-relevant stressors (sleep disruption and the Warrior Stress Paradigm) individually and in combination. Additionally, these experiments will examine whether nicotine and caffeine alter the behavioral effects of stress.

(b)(6) (b)(6) The Revised Neurobehavioral Severity Scale (NSS-R) is a series of 10 sensory and motor tests (Grunberg, Yarnell, Chwa, Hutchison, & Barry, 2011; Yarnell, 2012a; Yarnell, Barry, & Grunberg, in preparation; Yarnell & Grunberg, 2012). This test battery is based on similar versions found in the literature (Hamm, 2001; Mahmood et al., 2001; Shohami, Novikov, & Bass, 1995), but offers a more reliable and sensitive way to conduct the assessment for changes in neurobehavioral functioning (b)(6) (b)(6) to investigate five different forms of brain injury in rats and mice (Cole et al., 2011; A. Sharma et al., in preparation; P. Sharma, Su, Barry, Grunberg, & Lei, 2012). (b)(6) found changes in response to stress using this test battery (Yarnell, 2012a; Yarnell & Grunberg, 2012). In the proposed experiment the NSS-R will be used to test how sleep disruption and "Warrior stress" affect **sensory and motor function**. Rats' performance as a result of these stressors and under the influence of nicotine and caffeine will be tested.

The experiments that are included in this research application are designed to build upon our recent findings. We have the relevant conceptual knowledge and technical experience to perform the proposed experiments. We have developed all of the procedures to manipulate non-painful stress and to measure behavioral indices of sensory and motor performance, memory, attention, anxiety, and depression. These measures will be used in the proposed project to determine if nicotine or caffeine alters effects of stress on behavior.

V.1.1. Experiment 1: Biobehavioral effects of nicotine and stress in a rat model

This experiment is designed to determine the behavioral and biological effects of stress and nicotine in rats. It is hypothesized that: (a) stress will deleteriously affect the measured behaviors, including motor (increased indices of anxiety and depression based on particular locomotor activity parameters); sensorimotor (impaired neurobehavioral functioning; and cognition (impaired attention, learning, and memory); (b) nicotine will attenuate deleterious effects of stress on motor (i.e., decrease behavioral measures related to anxiety and depression), sensorimotor (i.e., improve sensorimotor responses), and cognitive measures (i.e., attenuate deleterious effects of stress on attention, learning, and memory); (c) effects of stress, nicotine, and the combination will be more pronounced in female than in male rats (i.e., nicotine will be more beneficial for female than for male rats under stress).

Design: This experiment is a 3 (control, 3 mg/kg, 6 mg/kg) x 4 (no stress, WSP, SD, WSP+SD) x 2 (male, female) full factorial mixed design = 24 treatment groups. Each animal will be evaluated before and after injury. There will be 10 animals per each of the 24 treatment groups for a total of 240 animals (plus 20 animals for pilot work). **Grand total of 260 rats are requested.** This experiment will be run in cohorts of 24 – 48 animals per cohort. The "pilot" animals are needed to train personnel in the techniques used in this experiment.

Subjects: Subjects will be young adult male and female Sprague-Dawley rats (2 - 3 months old). This strain and age is comparable to subjects used in many of our previous experiments with rats.

Induction of "Stress": For animals in the WSP group, non-painful stress will be manipulated by exposure for 20 min per day for 7 days to fox urine and lights or sounds as developed and used in our laboratory (Acri et al., 1994; Berger, 2009; Faraday,

2000; Hamilton, 2010; Kant, Leu, Anderson, & Mougey, 1987; Kant, Mougey, & Meyerhoff, 1986; Long, 2010; Perry, 2009; Raygada, Shaham, Nespor, Kant, & Grunberg, 1992; Shaham, Klein, Alvares, & Grunberg, 1993; Starosciak, 2010; Yarnell et al., 2011). Environmental stimuli to manipulate stress include lights, noises (e.g., whistle, laboratory timer), and tilting the cage based on Willner et al. (1987). A combination of these stimuli avoids habituation of the stress response. Details are provided below (See section V.4.4.7). For animals in the SD group, their sleep will be disrupted for 9 out of 12 of the animals' 12 hour sleep period for 7 days as developed and used in out laboratory (Perry, 2009). Details are provided below (See section V.4.4.7).

Drug administration. Nicotine will be administered chronically via subcutaneous minipump implanted in a brief surgical procedure followed procedures develop by Grunberg (1982) to model exposure to nicotine comparable to levels achieved by human cigarette smoking and to be comparable to dosages that can be achieved by medicinal nicotine patches (Winders et al., 1998). Details are provided below (See section V.4.3.2).

Dependent measures. Measures include body weight, food and liquid consumption, activity, attention, memory, anxiety, depression, sensory responses, and blood chemistry (corticosterone [to index stress], nicotine, cotinine [primary metabolite of nicotine]). Details are provided below (See section V.4.4.6).

Procedure. Animals will acclimate to the facility for 2 days. Each animal then will be handled 3 - 5 min/day for 3 days to minimize handling stress. Animals will be assigned to treatment groups such that same-sex groups have similar body weights. Minipumps will be implanted to deliver nicotine or saline. Stress will be started after minipump implantation and will continue for 7 days, then behavior will be measured, then continued for another 7 days with behavior measured at the end before the end of the study. See Timeline below for details (See section V.4.5). Animals will be euthanized by exposure to carbon dioxide followed by decapitation following LAM procedures. Blood will be collected, prepared, and stored for later analyses. Brains will be removed and stored for later tissue sharing analyses.

V.1.2. <u>Experiment 2: Biobehavioral effects of of caffeine and stress in a rat model</u>

This experiment is designed to determine the behavioral and biological effects of stress and caffeine in rats. It is hypothesized that: (a) stress will deleteriously affect the measured behaviors, including motor (increased indices of anxiety and depression based on particular locomotor activity parameters); sensorimotor (impaired neurobehavioral functioning; and cognition (impaired attention, learning, and memory); (b) caffeine will potentiate deleterious effects of stress on motor (i.e., increase behavioral measures related to anxiety and depression), sensorimotor (i.e., worsen sensorimotor responses), and cognitive measures (i.e., worsen deleterious effects of stress on attention, learning, and memory); (c) effects of stress, caffeine, and the combination will be more pronounced in female than in male rats (i.e., caffeine will be more deleterious for female than for male rats under stress).

Design: This experiment is a 3 (control, 1.5 mg/ml, 3 mg/ml) x 4 (no stress, WSP, SD, WSP+SD) x 2 (male, female) full factorial mixed design = 24 treatment groups. Each animal will be evaluated before and after injury. There will be 10 animals per each of the 24 treatment groups for a total of 240 animals (plus 20 animals for pilot work). **Grand total of 260 rats are requested.** This experiment will be run in cohorts of 24 – 48

animals per cohort. The "pilot" animals are needed to train personnel in the techniques used in this experiment.
Subjects: identical to Experiment 1
Induction of stress: identical to Experiment 1
Exposure to caffeine: Caffeine will be administered through the drinking water based on the procedures of Holtzman (1983) and Goldberg and colleagues (Gasior, Shoaib, Yasar, Jaszyna, & Goldberg, 1999; Jaszyna, Gasior, Shoaib, Yasar, & Goldberg, 1998; Shoaib, Swanner, Yasar, & Goldberg, 1999) to model exposure to caffeine comparable to levels achieved by human consumption. In order to reduce the amount of additional stress (since stress is an independent variable), we decided not to use a gavage to administer caffeine. This oral self-administration method has been reported to be reliable. Water bottles are weighed daily to measure consumption. In addition, biological samples are taken at the end of the study to validate caffeine exposure. Details are provided below (See section V.4.1.2.2).
Dependent variables: Identical to Experiment 1
Procedure: Identical to Experiment 1 except that caffeine will be manipulated instead of nicotine.

V.2. Data Analysis:

Samples sizes (n=10 per experimental "cell") for each experiment were based on previous experiments in our laboratory and in the published literature that have yielded statistically significant findings with behavioral and biological dependent variables comparable to those to be used in the proposed experiments. Specifically, moderate to large effect sizes (η^2= 0.06-0.14) are expected for behavioral measures in response to stress and drug manipulations. Power analyses (G*Power) are based on previous experiments in our laboratory which yielded these effect sizes in samples of ten per "cell." The data will be analyzed by ANOVA or MANOVA (with and without covariates) with repeated measures as appropriate. Tukey HSD *post hoc* tests or Dunnett's t will be used to compare differences between conditions. All analyses will be two-tailed with alpha = 0.05.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

This research project relies on behavioral responses in living organisms. Rats are subjects because their responses are similar to and predictive of human responses. Non-animal alternatives such as computer modeling and cell cultures were considered but are not suitable to assess behaviors in organisms because there currently are no non-animal alternatives that provide information about effects of stress, nicotine, and caffeine in living organisms.

V.3.2. Animal Model and Species Justification:

Rats are the subjects because they allow for true experiments to be conducted and because biobehavioral findings with rats have been excellent predictors of information relevant to the human condition. There is an abundance of research literature using rats in animal models of a variety of human conditions including drug abuse, stress, and mental and physical health disorders. With regard to the present research, for example, rat models have been used extensively to investigate stress, nicotine, and caffeine (e.g., Acri et al., 1994; al'Absi, 2012; Balfour et al., 1986; Barron et al., 2005; Benwell & Balfour, 1985; Collins et al., 1986; Corrigall & Coen, 1989, 1991;

Gasior et al., 1999; Glick et al., 1970; Goldberg, 2012; Goldberg et al., 1981; Grunberg, 1982, 1992; Hansen, 1979; Holtzman, 1983; Jaszyma et al., 1998; Larson et al., 1961; Marks et al., 1986; Shoaib et al., 1999; Slotkin et al., 1986). Our laboratory has used rats, mice, other rodents, and primates in our research. Our findings with rats have been reliable (in our laboratory and in other laboratories) and have proven to be valid in predicting effects in human subjects and human populations with regard to the topics that we study.

Subjects. Subjects are male and female Sprague-Dawley rats, the most commonly used lab rats in various studies, including investigations of stress and licit drugs. Sprague-Dawley albino rats are considered to be the standard test rat for nicotine and stress experiments. Adult animals are approximately 55 days old upon arrival (Spear, 2000) and in consultation with the breeder.

V.3.3. Laboratory Animals		**Species #1**	**Species #2**
V.3.3.1.	**Genus & Species:**	Rattus norvegicus (Rat)	
V.3.3.2.	**Strain/Stock:**	Sprague-Dawley	
V.3.3.3.	**Source/Vendor:**	(b)(4)	
V.3.3.4.	**Age:**	Adult (about 55 days)	
V.3.3.5.	**Weight:**	Adult males (about 200 grams); adult females (about 150 grams)	
V.3.3.6.	**Sex:**	Males and females	
V.3.3.7.	**Special Considerations:**	N/A	

V.3.4. Number of Animals Required (by Species): 520 rats

Please note: Each experiment will be run as a series of smaller sub-experiments for logistical reasons:
Experiment 1 = 240 rats (plus 20 additional pilot rats) = 260 rats
Experiment 2 = 240 rats (plus 20 additional pilot rats) = 260 rats

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: Animals are carefully monitored to limit or avoid pain and distress. Animals are observed daily for any behavioral signs of distress, including vocalizations, weight loss, changes in food consumption, changes in reactivity, and any signs of injury. If there are any indications of pain or distress, then the LAM staff is consulted. With regard to logistical and technical refinements, many of the behavioral assessments use automated equipment interfaced with computers to allow for the collection of dozens of parameters simultaneously. The use of sophisticated software and computerized technologies allows us to collect fine-grained and complete data sets for

every animal included in the experiment, eliminating the need for additional studies that might otherwise have been necessary. Our many years of behavioral investigations regarding stress and various drugs also have allowed us to minimize the sample sizes as we have refined testing to be sensitive and reliable.

V.3.5.2. Reduction: The sample sizes for the proposed work were derived from power analyses of pilot data and past work in our laboratory and in other laboratories. These samples sizes, therefore, constitute the minimum number of animals necessary to obtain meaningful results and represent a useful application of knowledge of statistical effect size and the concept of power. In addition, in each experiment multiple dependent variables are measured. Measurement of many variables in the same animals markedly reduces the total number of experiments conducted.

V.3.5.3. Replacement: There are no appropriate replacement methods (in-vitro, computer models) for the proposed work.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

Animals will be anesthetized during implantation of minipumps (in Experiment 1). Animals will be observed daily by investigators and by LAM staff to check health and well-being and to insure that there is no undue pain or distress based on behaviors and vocalizations. Any indications of pain or distress will be dealt with by alleviating the cause of the problem and/or by contacting LAM personnel for assistance and guidance

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	80	
V.4.1.1.1.2. Column D:	40	
V.4.1.1.1.3. Column E:	400	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

In Experiment 1 (to implant minipumps), subjects are first anesthetized by inhalation anesthetic to prevent pain. Isoflurane is mixed with oxygen (i.e., 2 – 4% Isoflurane; with an oxygen flow rate of 0.5 - 1.0 liters/minute). Subjects are placed inside an induction chamber saturated with Isoflurane vapor. Subjects are removed from the chamber when tail pinch produces no reflex movement and anesthesia is maintained using a mask/nose cone attached to the anesthetic vaporizer (with Isoflurane concentration ranging from 0.25 – 3% to maintain appropriate depth of anesthesia)

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

The non-surgical procedure that may induce momentary discomfort (e.g., hot plate test of nociceptive responses) is included for scientific purposes and is short in duration (< 60 sec). Animals are observed daily by investigators and by LAM staff to check on their health and well-being and to assure that there is no undue pain or distress. Animals also are carefully monitored during behavioral testing procedures. In addition, animals are closely monitored during all procedures that involve any pain or potential distress (e.g., during and after minor surgery; during and after injections). Pain/distress is assessed based on behaviors and vocalizations. Any indications of

pain or distress are dealt with by alleviating the cause of the problem or by contacting LAM personnel for assistance and guidance.

Caffeine (obtained from Sigma) will be administered based on the procedures of Holtzman (1983) and Goldberg and colleagues (Gasior, Shoaib, Yasar, Jaszyna, & Goldberg, 1999; 1981; Jaszyna, Gasior, Shoaib, Yasar, & Goldberg, 1998; Shoaib, Swanner, Yasar, & Goldberg, 1999). This paradigm is considered to be the optimal strategy to model caffeine consumption in humans (al'Absi, 2012; Gasior et al., 1999; Goldberg, 2012; Jaszyna et al., 1998; Shoaib et al., 1999). Rats in the lower caffeine condition will receive drinking water with caffeine increased from 0.25 mg/ml to 1.5 mg/ml over the course of 10 days. After the initial 10 days, rats in this condition will receive drinking water with 1.5 mg/ml for the remaining 10 days of the experiment. Rats in the higher caffeine condition will receive drinking water with caffeine base increased from 0.25 mg/ml to 3 mg/ml over the course of 15 days. After the initial 15 days, rats in the higher caffeine condition will receive drinking water with 3 mg/ml caffeine for the remaining 5 days of the experiment. It is noteworthy that the route of administration (oral ingestion) at the particular concentrations that will be used produced behavioral effects in rats and are considered to be (based on the published literature and communication with the investigators cited) the optimal approach to parallel human consumption of caffeine of servicemen. Currently in the military, consumption of approximately 200-300 mg of caffeine is encouraged to enhance performance and delay sleep onset during continuous operations (IOM-CMNR, 2001). This recommendation is based on extensive research (Killgore et al., 2009; Lieberman, Tharion, Shukitt-Hale, Speckman, & Tulley, 2002; McLellan, Bell, & Kamimori, 2004; McLellan et al., 2005; McLellan, Kamimori, Voss, Tate, & Smith, 2007; Wesensten et al., 2002; Wesensten, Killgore, & Balkin, 2005a) that examined effects of caffeine on performance in rested and sleep-deprived service members. Warriors report consuming greater than 300 mg per day (Lieberman, 2012) and those who use energy drinks may be consuming 80 mg - 500 mg per serving ("Caffeine content of drinks," 2012). Therefore, it is likely that service members are consuming more than moderate doses of 200-300 mg.

We also will use two mild stress paradigms ("Warrior Stress Paradigm" and "Sleep Disruption" described in Section V.4.4.7. below). WSP involves 10 – 20 min exposure to fox urine on a cotton ball and some environmental mild stressors detailed below (e.g., overhead lights turned on and off). This procedure is repeated once/day for 14 days (with a 3 day break for behavior after the first 7 days) for animals in the WSP and WSP+SD groups. Animals are free to move around a cage and are not restrained in any way. For animals in the SD and WSP+SD groups, it will involve their natural sleep being disrupted by noises (e.g., loud banging, bells, voices) during 9 total hours of the animals' 12 hour sleep period for 14 days (with a 3 day break for behavior after the first 7 days). Sounds will vary from 65 - 80 dB. Total duration of the sounds range from 6 seconds to approximately 1 minute. Total hourly sound exposure will not exceed 6 minutes at any time during this study. Animals are free to move around a cage and are not restrained in any way.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched:

Medline (PubMed), PsycInfo, F1000Biology, AGRICOLA

V.4.1.3.2. Date of Search:
February, 2014
V.4.1.3.3. Period of Search:
All years available on each database are searched (from recent work up to 30 years in the past).
V.4.1.3.4. Key Words of Search:
osmotic minipumps, minipump implantation, chronic administration paradigms, pain, alternatives, analgesia, anesthesia, surgery, acute drug administration, stress, rat (these words are searched in various combinations[i.e., rat+osmotic minipump+pain, rat+osmotic minipump+alternatives, rat+osmotic minipump+analgesia, rat+osmotic minipump+anesthesia, rat+osmotic minipump+surgery, etc)
V.4.1.3.5. Results of Search:
Searches looking at literature from the last 30 years, focusing more on the past 5 years, did not discuss the rats being in pain from the minipump implantations (>9500 hits). Besides daily injections, which cause discomfort each time, no other alternatives to the minipump implantation procedure have been found (>9000 hits). No alternatives to the nociception procedures have been found (>19,000 hits). There are many stressors used in rodent literature (immobilization, tail shock, feline exposure), but no milder alternative for the stress manipulations have been found (>80,000 hits). No alternatives are available to avoid using live animals for the assessment of effects of stress, caffeine, and nicotine in intact rats and the procedures that are followed.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: Animals receiving the Warrior Stress Paradigm receive the stress paradigm in a cage separate from their home cage and are returned to their home cages once the paradigm is complete (for details, see section V.4.4.7). On the days that the animals receive stress, they receive no behavioral measures (i.e., during this time, they only receive the stress paradigm and are left to rest after). This paradigm's stressors are mild and brief (exposure to fox urine, lights, and sound). It is used to model the impending threat of attack in a novel environment that Warriors face while in deployed settings.

Animals receiving the Sleep disruption paradigm receive the stress paradigm in their home cage. The animals are exposed to sounds periodically during their sleep cycle. This paradigm is used to model interrupted sleep that many Warriors face.

V.4.2. Prolonged Restraint: No prolonged restraint will be used.

V.4.3. Surgery:
V.4.3.1. Pre-surgical Provisions:

All minipump implantation surgeries will be performed in the LAM facility by the Grunberg research team and in consultation with LAM staff to insure proper preparation and safeguards. Implantation of osmotic minipumps uses aseptic technique. Subjects will be anesthetized by inhalation anesthetic LAM equipment (vaporizer) and Isoflurane mixed with oxygen. Subjects will be placed inside an induction chamber saturated (at 4-5%) with Isoflurane vapor. Subjects will be removed from the chamber when tail pinch produces no reflex movement and anesthesia will be maintained (at 1-1.5%) using a mask/nose cone attached to the anesthetic vaporizer.

V.4.3.2. **Procedure:**

Subjects are anesthetized by inhalation anesthetic using LAM anesthetic equipment (vaporizer) and Isoflurane and oxygen. Subjects are placed inside an induction chamber saturated with Isoflurane vapor. Subjects are removed from the chamber when tail pinch produces no reflex movement and anesthesia is maintained using a mask/nose cone attached to the anesthetic vaporizer. A 3 x 5 cm area between the withers is shaved and cleaned with the antiseptic Betadine. A 2 cm transverse incision within the shaved region approximately 1 cm below the scapulae is made with blunt-nosed, curved-tipped Mayo surgical scissors, a pocket is created by gently spreading the subcutaneous tissues with the scissor tips, and the minipump is inserted. Incisions are closed with two-three 9 mm stainless steel wound clips. This procedure takes approx. 4 min per subject. The surgical area is thoroughly cleaned before and after procedures. Investigators wear surgical masks, gloves, and laboratory coats or surgical scrubs. The procedures are performed under aseptic conditions. All surgical material will be sterilized between animals using the glass bead sterilizers.

Minipump anchoring. The minipump is not anchored. For the majority of animals, it remains in the subcutaneous pocket created between the withers (shoulder blades) and can be palpated easily. On rare occasions, it migrates under the skin to other subcutaneous locations. When migration occurs, animals are monitored daily for minipump position to be sure that it does not interfere with normal behaviors (i.e., eating, drinking, grooming) or animal comfort.

Instruments. Surgical instruments are cleaned and sterilized by the (b)(6) lab before each procedure and in between animals.

V.4.3.3. **Post-surgical Provisions:**

One dose of buprenorphine (0.05-0.1mg/kg) given subcutaneously after surgery will be given for pain relief. Subjects will be returned to their cage and observed post-surgery until they are able to demonstrate recovery such as return of righting reflex, or coordinated voluntary movement. They also will be monitored three times a day until they have completely recovered from the minor surgical procedures.

Surgical incision monitoring. The incision site is closely examined daily for signs of infection or lack of healing. If signs of infection or lack of healing are observed, then veterinary consult will be requested for antibiotic administration or other appropriate care. Over 30 years of our experience and the implantation of several thousand rats with these devices, the incidence of infection has been less than 0.1%. In addition, we have never observed any problems with the surgical incisions in animals exposed to water (as in behavioral tests described below).

Post-op antibiotics. Post-operative antibiotics are not administered unless there are signs of infection (i.e., reddening or swelling of the incision site). A veterinarian will be consulted for appropriate course of action, antibiotic choice and route of administration in cases of infections.

Surgery-Experimental procedure time lag. Animals are allowed 16 hours to recover from surgery before the commencement of any behavioral testing. In our experience, by 15 minutes post-surgery animals are moving and by 2 hrs post-surgery animals are eating, drinking, and grooming normally. By 16 hrs post-surgery, the incision site exhibits signs of healing.

Animal disposition if surgical complications occur. In our experience, the only surgical complication that might occur is infection of the incision site, and this complication is rare. Should this complication develop, treatment with antibiotics will be

requested from the veterinary staff. If the infection is mild and resolves quickly with treatment, then the animal will remain in the experiment. If the infection is debilitating (i.e., the animal's behaviors indicate that it is in pain), or any other surgical complications occur, then a request will be placed for the animal to be euthanized by LAM personnel (carbon dioxide inhalation followed by decapitation).

V.4.3.4. Location:

Surgeries to implant the minipumps will be conducted in the LAM facility. Behavioral measures will be conducted in the laboratories assigned to Dr. (b)(6) in LAM at USUHS.

V.4.3.5. Surgeon: PI and all co-investigators and technicians

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: N/A

V.4.4.2. Biosamples:

At the end of each experiment, animals will be euthanized as described below. Blood samples and some tissue samples will be collected and stored for later analysis.

Biological and Physiological Measures

1) Corticosterone assessment. Trunk blood samples are obtained during sacrifice in some experiments. Total serum corticosterone is measured by ELISA/EIA. All samples and standards are run in duplicate.

2) Nicotine and cotinine levels. Blood samples are assayed for nicotine and cotinine by HPLC in some experiments (Jacob et al., 1981). Structural analogs of both nicotine and cotinine are used as internal standards. Calibration curves are linear from 1-100 ng/ml for nicotine and 10-1000 ng/ml for cotinine. The limit of quantification is 1 ng/ml for nicotine and 10 ng/ml for cotinine. The detection limits are 0.5 ng/ml for nicotine and 5 ng/ml for cotinine. Inter-day and intra-day variability have been determined from the repeated analysis of spiked plasma and brain tissue samples. The mean coefficient of variation ranges from 1.1 to 7.8% for nicotine concentrations ranging from 1 to 100 ng/ml. The mean coefficient of variation ranges from 0.8 to 9.5% for cotinine concentrations ranging from 10-1000 ng/ml. No interference is found from various nicotine metabolites. Assay specificity is verified by comparing concentrations of nicotine and cotinine in pooled plasma and in pooled brain samples.

3) Brain assays. Brains are harvested following decapitation and rapidly frozen with dry ice or liquid nitrogen. Brains will be stored for possible, later analysis (please see Tissue Sharing section V.4.4.8).

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification:

Animals cages will be identified with a cage card generated by LAM according to SOP upon arrival to USU. In addition, cages are numbered corresponding to the animals in that cage and rat tails are coded with a marker using a stripe system that corresponds to units of ten or ones, depending on the location of the mark on the tail.

V.4.4.6. Behavioral Studies:

This project focuses on behavioral evaluations in rats. Each measure is described below. The duration of behavioral testing on each day depends on the

specific measure used and is listed below. The specific behavioral measures used in each experiment is listed under the dependent variables section of each experiment. No more than three types of behavioral measure are made of each animal on a given day and will take less than 1½ hours per day.

1) Body weight, and food and liquid consumption (minimal handling of animals)
2) Open field activity: 1 hr
3) Neurobehavioral Severity Scale-Revised: 5 min
4) Hot Plate: < 60 sec
5) Acoustic startle and pre-pulse inhibition: 20 min
6) Passive Avoidance: 5 min

Open field locomotion. Locomotor activity is a collection of sensitive unconditioned behaviors that occur when an animal moves in its environment. Various parameters of open field locomotion provide information about gross motor movement, general health, anxiety, depressive-like behavior, and learning (i.e., habituation). These measures have been used in rat studies (b)(6) for many years and have provided reliable and valuable data (e.g., Bowen, Eury, & Grunberg, 1986; Elliott, Faraday, Phillips, & Grunberg, 2004; Elliott & Grunberg, 2005; Faraday, Elliott, Phillips, & Grunberg, 2003; Faraday, O'Donoghue, & Grunberg, 2003; Faraday, Scheufele, Rahman, & Grunberg, 1999; Grunberg & Bowen, 1985; Morse et al., 1997). Horizontal activity provides information about gross motor performance and general health; center time provides an index of anxiety (with an inverse relationship between center time and anxiety); vertical activity provides an index of depression (with escape behaviors inversely related to depression) (Berger, 2009; Faraday, 2005; Grippo, Beltz, & Johnson, 2003; Hamilton, 2010; Long, 2010; Perry, 2009; Sarkisova, Kulikov, Midzyanovskaya, & Folomkina, 2008; Shafer, 2006; Starosciak, 2010; Zhuang, Xu, & Chun-Zhi, 2007). Locomotor activity is measured using an Accuscan Electronics infrared photocell system (Accuscan Electronics, Columbus, OH), located in a dedicated room within the animal facility. This room is constructed of cinderblock walls, acoustic tile ceiling and steel doors so that sound is kept to a minimum. One hour activity measurements will be obtained during animals' active or dark cycle. Dark cycle measurement is done because animals' baseline level of activity during this part of the circadian cycle is sufficiently high to allow measurement of activity decrements and also low enough that activity increases can be reliably measured. Animals will be placed singly in a 40 x 40 x 30 cm clear Plexiglas arena and a Plexiglas lid with multiple holes to allow air flow. The lid ensures that subjects have adequate ventilation but cannot escape during data collection. A photocell array measures horizontal locomotor activity and vertical activity. Data will be automatically gathered and transmitted to a computer equipped with "Fusion" software (from Accuscan Electronics). Once subjects are placed in the test areas, the experimenter turns off the lights and leaves the room. Chambers are cleaned between subjects with a clidox-s solution. The duration of locomotor activity testing is a maximum of 60 min per day.

Neurobehavioral observation. Observations will be made and recorded using the Neurobehavioral Severity Scale-Revised (NSS-R) (b)(6) (b)(6) (Grunberg, Yarnell, Chwa, Hutchison, & Barry, 2011) and based on neurological severity scales in the literature (Hamm, 2001; Shohami, Novikov, & Bass, 1995) using a Likert-format scoring system. These measures include observation of a righting response, leg flexion, balance, and sensory responses to mild stimuli (e.g.,

touching ear with cotton-tipped stick, response to light). The measures are similar to those described in CNRM SOP #2 (on USUHS LAM web page).

Hot plate nociception. Hot plate response measures supraspinal nociceptive responses (Berry et al., 2007; Ding, Shum, Ko, & Zhuo, 2005). The hot plate task will be performed with an Omnitech/Accuscan Electronics Analgesia Monitor (Model HPrx). Subjects are placed on a 26 x 26 cm square hot plate platform, enclosed on all sides by Plexiglas walls and covered with a removable Plexiglas top. The metal plate heated to 51°C (a temperature that will elicit a nociceptive response without tissue damage). Hotplate latencies are measured as the time from placement on the heated surface until the animal raised and licked a rear paw. To prevent tissue damage, a maximum latency of 60 sec is allowed. Animals that do not lick their paws at all during the 60-sec period are removed from the heated surface. The platform is cleaned between subjects.

Acoustic startle response with and without pre-pulse inhibition. Acoustic startle reflex (ASR) amplitudes and pre-pulse inhibition (PPI) are measured in a Med Associates Acoustic Response Test System based on procedures of Davis and Sollberger (1971) and Faraday and Grunberg (2000). ASR and PPI provide information about information processing and attention. ASR and PPI are measured in a Med Associates Acoustic Response Test System consisting of weight-sensitive platforms inside individual sound-attenuated chambers. Subjects' movements in response to stimuli are measured as a voltage change by a strain gauge inside each platform. Each rat is placed individually in a ventilated holding cage. Startle stimuli range from 110 to 120 dB and are white noise bursts of 20 ms duration sometimes preceded 100 ms by 68 or 82 dB 1kHz pure tones (pre-pulses). Each stimulus combination is presented 8 times. Total testing period is about 20 min.

Passive Avoidance. Passive avoidance is an index of learning and general cognition. An animal is placed into an apparatus with two chambers separated by a sliding door. After being placed in one of the chambers, a light turns on and the door opens. The other chamber is dark, which rodents prefer. During the training period, crossing over into the dark chamber causes the animal to receive an electrical shock. The animal is tested the next day with no electrical shock. Remaining in the lit chamber, against the animal's natural instincts, indicates that learning has occurred. The Gemini model (San Diego Instruments, San Diego, CA) consists of two chambers (21 cm x 25 cm x 17 cm). A 50 watt light bulb is present in both compartments. The shocks (0.8 mA) are delivered through a grid floor. The training and testing operations are controlled by computer software ("PA", San Diego Instruments, San Diego, CA). During the training period, rats will be placed in a chamber. After 60s, the light will turn on and the door to the second, ark chamber will open. Upon crossing into the dark chamber, a 0.8 mA shock will be delivered for a period of one second. The latency to cross will be recorded by the computer. Rats will be removed after 300 seconds. The testing period occurs 24 hours after the training period. The procedure is identical, except that the rats will not be shocked upon crossing into the darkened chamber.

V.4.4.7. Other Procedures:

Nicotine chronic administration and surgical procedure. Nicotine or physiologic saline (also used as vehicle for the nicotine solution) is administered via Alzet osmotic mini-pumps for one to three weeks. Nicotine solution is made from nicotine dihydrochloride or nicotine bitartrate (obtained from Sigma); concentrations are expressed as nicotine base. Dosages are calculated based on body weight.

Stress manipulation. [(b)(6)] a non-painful "**Warrior Stress Paradigm**" to model impending threat of attack in novel environments (Yarnell et al., 2011) based on previous studies, [(b)(6)] e.g., Berger, 2009; Hamilton, 2010; Long, 2010; Perry, 2009; Starosciak, 2010 [(b)(6)] [(b)(6)] Hayley, Borowski, Merali, & Anisman, 2001; Meyerhoff, 2006; Willner et al., 1987). Predator stress is manipulated by introducing a cotton ball with commercially purchased synthetic fox urine into a test cage with the rat. Unpredictable, non-painful stressors include noise, flashing light, and cage shaking. The animal is transferred from its home cage to the stress cage with lid. The fox urine (15mL) is absorbed by a large cotton ball and placed in varying spots in the stress cage. The procedure is conducted in a room separate from the housing room and the behavioral rooms. A bright florescent overhead light remains on during the procedure. This procedure lasts 20 minutes. The stimuli are presented for 14 days (7 consecutive days, the 3 days of behavioral measurements, then 7 additional consecutive days; see table below) in a manner designed to minimize habituation of the response. Each manipulation occurs no more than once per day. A combination of these stimuli avoids habituation of the response.

Timeline of Stress Days		
Stress Day	**Predator Stress**	**Unpredictable Event**
1	Fox Urine (20 min)	None
2	Fox Urine (10 min)	Whistle at 12, 15 & 19 min
3	Fox Urine (10 min)	Coin Shake at 11, 14, & 17 min
4	Fox Urine (10 min)	Flashing Lights at 13, 16, & 19 min
5	Fox Urine (10 min)	Cage Shake at 12, 15, & 18 min
6	Fox Urine (10 min)	Flashing Lights at 12, 16, & 19 min
7	Fox Urine (10 min)	Whistle at 11, 13, 16 & 18 min
8	Fox Urine (10 min)	Coin Shake at 12, 16, & 19 min
9	Fox Urine (10 min)	Flashing Lights at 11, 15, 19 min
10	Fox Urine (10 min)	Cage Shake at 11, 14, & 17 min
11	Fox Urine (10 min)	Coin Shake at 13, 16, & 19 min
12	Fox Urine (10 min)	Whistle at 12, 14, 17 min
13	Fox Urine (10 min)	Flashing Lights at 11, 14, 18 min
14	Fox Urine (10 min)	Cage Shake at 12, 15, & 18 min

Sleep Disruption. Sleep disruption will be manipulated based on methods previously used [(b)(6)] (Perry, 2009). The natural sleep of the rats will be intermittently disrupted by varying high and low frequency environmental sounds (to prevent habituation) for a period of 14 days. These noises (loud banging, bells, voices, shattering glass, vehicular traffic, etc.) are pre-recorded onto a CD and will be programmed to play on an hourly loop for 9 total hours of the animals' 12 hour sleep period. Sounds will vary from 65 - 80 dB. Total duration of the sounds range from 6 seconds to approximately 1 minute. Total hourly sound exposure will not exceed 6 minutes at any time during this study.

Body weight, food and liquid consumption. Body weight is measured using electronic balances programmed to take multiple weighings within a short period of time to account for movement artifacts. Food and liquid consumption are calculated by weighing food and water on subsequent days. Foods includes standard rodent laboratory chow (available in LAM) and sugar-containing foods and solutions to assess taste preferences in response to the independent variables.

V.4.4.8. Tissue Sharing:

Some brain samples may be shared with other investigators to perform additional assays.

V.4.5. Study Endpoint:

The study endpoint is euthanasia in each experiment. Each experiment will be run in five separate counter-balanced cohorts. The timeline below summarizes daily activities for each cohort within each experiment. Considering pilot work, time between cohorts, and time to run each cohort, each experiment will require about 6 months to collect behavioral data and blood samples. Biochemical assays and data analyses will require several additional months per experiment.

Timeline for Experiment 1

	Mon	Tues	Wed	Thurs	Fri	Sat	Sun
Week 1		Arrive	Gentle	Gentle ASR acclimation 1	Gentle OFA Acclimation ASR Acclimation 2		
Week 2	BL OFA BL NSS-R BL HP	PA Training	BL PA BL ASR	Minipump	Nic/Sal Stress/NS	Nic/Sal Stress/NS	Nic/Sal Stress/NS
Week 3	Nic/Sal Stress/NS	Nic/Sal Stress/NS	Nic/Sal Stress/NS	Nic/Sal Stress/NS	Nic/Sal T1 OFA T1 NSS-R T1 HP	Nic/Sal T1 PA	Nic/Sal T1 ASR
Week 4	Nic/Sal Stress/NS	Nic/Sal Stress/NS	Nic/Sal Stress/NS	Nic/Sal Stress/NS	Nic/Sal Stress/NS	Nic/Sal Stress/NS	Nic/Sal Stress/NS
Week 5	Nic/Sal T2 OFA T2 NSS-R T2 HP	Nic/Sal T2 PA	Nic/Sal T2 ASR	Euthanasia			

Timeline for Experiment 2

	Mon	Tues	Wed	Thurs	Fri	Sat	Sun
Week 1		Arrive	Gentle	Gentle	Gentle		
				ASR acclimation 1	OFA Acclimation		
					ASR Acclimation 2		
Week 2	BL OFA BL NSS-R BL HP	PA Training	BL PA		Caf/Sal	Caf/Sal	Caf/Sal
			BL ASR		Stress/NS	Stress/NS	Stress/NS
Week 3	Caf/Sal	Caf/Sal	Caf/Sal	Caf/Sal	Caf/Sal	Caf/Sal	Caf/Sal
	Stress/NS	Stress/NS	Stress/NS	Stress/NS	T1 OFA T1 NSS-R T1 HP	T1 PA	T1 ASR
Week 4	Caf/Sal	Caf/Sal	Caf/Sal	Caf/Sal	Caf/Sal	Caf/Sal	Caf/Sal
	Stress/NS	Stress/NS	Stress/NS	Stress/NS	Stress/NS	Stress/NS	Stress/NS
Week 5	Caf/Sal	Caf/Sal	Caf/Sal	Euthanasia			
	T2 OFA T2 NSS-R T2 HP	T2 PA	T2 ASR				

Gentling involves 2 - 3 minutes of gentle handling
ASR/PPI = acoustic startle reflex with and without pre-pulse inhibition
ASR acclimation entails putting rat in ASR chamber for 15 min without taking measurements.
OFA acclimation entails putting rat in open field activity (OFA) chamber for 60 min without taking measurements.
BL = baseline (i.e., pre-nicotine/caffeine) measurements
NSS-R = Neurobehavioral Severity Scale-Revised
HP = hot plate nociception measure
PA Training entails putting rat in the passive avoidance (PA) box for up to 5 minutes or until they cross to the other side of the chamber
Nicotine admin = minipump implantation to deliver nicotine or saline continuously throughout Exp. 1
Nic = Nicotine
Sal = Saline
Caf = Caffeine
Stress = either 20 min/day of Warrior Stress Paradigm to half of the subjects in each experiment and/or sounds playing for 9 out of 12 of animals' sleep period to half of the subjects in each experiment

V.4.6. Euthanasia:

Rats will be euthanized using carbon dioxide inhalation from a compressed gas cylinder. Euthanasia will be done in a LAM designated procedure room followed by

rapid decapitation by guillotine. This euthanasia procedure is in compliance with the 2013 AVMA Guidelines on Euthanasia.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations:

Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6) (b)(6)

V.5.1.2. Special Husbandry Provisions: Animals will be single housed

Food Restriction: Yes _____ No __X__

Fluid Restriction: Yes _____ No __X__

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:

Animals will be observed and cared for by the Center for LAM veterinary technicians and veterinarians according to USUHS Center for LAM SOPs. Animals will be monitored for health, human treatment and husbandry considerations twice daily by LAM personnel during routine weekday rounds and at least once daily on weekends/holidays. This is in addition to, and not in place of, the monitoring that is done by the principal investigator and their staff. In the event of a debilitating illness or adverse reaction, the decision to treat or euthanize an animal will be made by either the veterinarian and/or the principal investigator.

V.5.2.2. Emergency Veterinary Medical Care:

All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays. In the case of an emergency health problem, if the responsible person (e.g. investigator) is not available or if the investigator and veterinary staff cannot reach consensus on treatment, the veterinarian has the authority to treat the animal, remove it from the experiment, institute appropriate measures to relieve severe pain or distress, or perform euthanasia as necessary.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy:

Animals will be single housed without toys because these experiments are designed to determine effects of several interventions. Enrichment itself attenuates effects of injury as reported by (b)(6) (Elliott & Grunberg, 2005).

V.5.3.2. Enrichment Restrictions:

Animals will be single housed without toys to assess effects of stress, nicotine, and caffeine. Enrichment would interfere with the specific aims of this research project.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Personnel	Procedure	Qualifications/Training
(b)(6)	Animal Handling	USUHS Investigator Training and Animal Handling courses (several sessions, including sessions in 1980 and in early 1990s); NIH/NIMH training courses (several in mid 1980s); > 30 years experience with rats and other rodents
	Drug Administration	USUHS Investigator Training and Animal Handling courses; NIH/NIMH training courses; > 30 years experience with rats and other rodents
	Surgical Implant of Osmotic Mini-pumps	NIH/NIMH training courses; > 30 years experience with rats and other rodents; 1200+ surgeries performed
	Behavioral Measures	NIH/NIMH training courses; > 30 years experience with rats and other rodents
	Biological sample collection	NIH/NIMH training courses; > 30 years experience with rats and other rodents
	Anesthetized Decapitation	NIH/NIMH training courses; 25+ years experience with rats; 1000+ decapitations performed
(b)(6)	Animal Handling	USUHS Investigator Training (Summer, 2009) and Animal Handling course (July, 2009); 5 years experience with rats; training by Dr (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel
	Surgical Implant of Osmotic Mini-pumps	Training by Dr (b)(6) and lab personnel; 2 years experience with rats
	Behavioral Measures	Training by Dr (b)(6) and lab personnel; 5 years experience with rats
	Biological sample collection	Training by Dr (b)(6) and lab personnel; 5 years experience with rats
	Anesthetized Decapitation	Training by Dr (b)(6) and lab personnel
(b)(6)	Animal Handling	USUHS Investigator Training and Animal Handling courses (Fall, 2010); 4 years experience with rats; training by Dr (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel
	Surgical Implant of Osmotic Mini-pumps	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Behavioral Measures	Training by Dr (b)(6) and lab personnel; 4 years experience with rats
	Biological sample collection	Training by Dr (b)(6) and lab personnel; 4 years experience with rats
	Anesthetized Decapitation	Training by Dr (b)(6) and lab personnel
(b)(6)	Animal Handling	USUHS Investigator Training (Fall, 2010) and Animal Handling course (Fall, 2010); 4 years experience with rats; training by Dr (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel
	Surgical Implant of Osmotic Mini-pumps	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Behavioral Measures	Training by Dr (b)(6) and lab personnel; 4 years experience with rats

	Biological sample collection	Training by Dr. (b)(6) and lab personnel; 4 years experience with rats
	Anesthetized Decapitation	Training by Dr (b)(6) and lab personnel
(b)(6)	Animal Handling	USUHS Investigator Training (Fall, 2011) and Animal Handling course (Fall, 2011); 3 years experience with rats; training by Dr (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr. (b)(6) and lab personnel
	Surgical Implant of Osmotic Mini-pumps	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Behavioral Measures	Training by Dr (b)(6) and lab personnel; 3 years experience with rats
	Biological sample collection	Training by Dr. (b)(6) and lab personnel; 3 years experience with rats
	Anesthetized Decapitation	Training by Dr. (b)(6) and lab personnel
(b)(6)	Animal Handling	USUHS Investigator Training (Fall, 2012) and Animal Handling course (Fall, 2012); 2 years experience with rats; training by Dr (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel
	Surgical Implant of Osmotic Mini-pumps	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Behavioral Measures	Training by Dr (b)(6) and lab personnel; 2 years experience with rats
	Biological sample collection	Training by Dr (b)(6) and lab personnel; 2 years experience with rats
	Anesthetized Decapitation	Training by Dr (b)(6) and lab personnel
(b)(6)	Animal Handling	USUHS Investigator Training (Fall, 2013) and Animal Handling course (Fall, 2013); ½ year experience with rats; training by Dr (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr. (b)(6) and lab personnel
	Surgical Implant of Osmotic Mini-pumps	Training by Dr (b)(6) and lab personnel
	Behavioral Measures	Training by Dr. (b)(6) and lab personnel; ½ year experience with rats
	Biological sample collection	Training by Dr. (b)(6) and lab personnel; ½ year experience with rats
	Anesthetized Decapitation	Training by Dr (b)(6) and lab personnel
(b)(6)	Animal Handling	USUHS Investigator Training (Fall, 2013) and Animal Handling course (Fall, 2013); ½ year experience with rats; training by Dr. (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel
	Surgical Implant of Osmotic Mini-pumps	Training by Dr (b)(6) and lab personnel
	Behavioral Measures	Training by Dr (b)(6) and lab personnel; ½ year experience with rats
	Biological sample collection	Training by Dr (b)(6) and lab personnel; ½ year experience with rats
	Anesthetized Decapitation	Training by Dr (b)(6) and lab personnel

(b)(6)	Animal Handling	USUHS Investigator Training (Fall, 2013) and Animal Handling course (Fall, 2013); ½ year experience with rats; training by Dr. (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr. (b)(6) and lab personnel
	Surgical Implant of Osmotic Mini-pumps	Training by Dr. (b)(6) and lab personnel
	Behavioral Measures	Training by Dr. (b)(6) and lab personnel; ½ year experience with rats
	Biological sample collection	Training by Dr. (b)(6) and lab personnel; ½ year experience with rats
	Anesthetized Decapitation	Training by Dr. (b)(6) and lab personnel
(b)(6)	Animal Handling	USUHS Investigator Training (Spring, 2010) and Animal Handling (Spring, 2010) courses; 4 years experience with rats; training by Dr. (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr. (b)(6) and lab personnel
	Surgical Implant of Osmotic Mini-pumps	Training by Dr. Grunberg and lab personnel
	Behavioral Measures	Training by Dr. (b)(6) and lab personnel; 4 years experience with rats
	Biological sample collection	Training by Dr. (b)(6) and lab personnel; 4 years experience with rats
	Anesthetized Decapitation	Training by Dr. (b)(6) and lab personnel
(b)(6)	Animal Handling	USUHS Investigator Training (Spring, 2011) and Animal Handling (Spring, 2011) courses; training by Dr. (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr. (b)(6) and lab personnel
	Surgical Implant of Osmotic Mini-pumps	Training by Dr. Grunberg and lab personnel
	Behavioral Measures	Training by Dr. (b)(6) and lab personnel; 3 years experience with rats
	Biological sample collection	Training by Dr. (b)(6) and lab personnel; 3 years experience with rats
	Anesthetized Decapitation	Training by Dr. (b)(6) and lab personnel

VII. BIOHAZARDS/SAFETY:

When lab personnel work with nicotine, personnel wear lab coats, two layers of gloves, a mask, and protective eye wear. When personnel work with anesthetic vapors, personnel wear protective masks. All laboratory personnel wear laboratory coats, gloves, and masks when working with animals.

VIII. ENCLOSURES: N/A

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) [redacted] 2/27/14
Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

N/A

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) [redacted] 2/27/14

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

2/27/14
Date

X. PROTOCOL ABSTRACT:

A. **Animal Protocol Number:** (b)(6)

B. **Animal Protocol Title:** Behavioral investigations of nicotine and caffeine in rats (Rattus norvegicus)

C. **Principal Investigator:** (b)(6) Ph.D.

D. **Performing Organization:** Uniformed Services University of the Health Sciences

E. **Funding:** USUHS

F. **Objective and Approach:**

Stress is a pervasive experience that affects behaviors, cognitions, and biological responses. Insufficient sleep is a particularly disruptive stressor that is experienced by civilians and Warriors. Threat of attack also is a stressor that is commonly experienced by Warriors. Nicotine and caffeine are legal drugs that are used by many civilians and service members. These drugs are often consumed during stressful situations. Each of these variables (i.e., insufficient sleep, threat of attack, nicotine, and caffeine) alone can have psychological effects (on behavior and cognition). It is important to examine if nicotine or caffeine use alters psychological effects of stress – either potentiating or attenuating effects of stressors. The experiments described in this application use a rodent model of Warrior Stress and sleep disruption to determine how psychological responses to stress are altered by nicotine or caffeine administration in male and female rats. The behaviors assessed include motor responses (to index anxiety, depression, and learning), sensorimotor (including reflexes and nociception), and cognitive responses (including attention, memory, learning). Blood samples will be assessed for stress hormones (corticosterone). It is hypothesized that: the stressors will deleteriously affect the measured behaviors; nicotine will attenuate these effects; caffeine will potentiate these effects; and females will be more sensitive than males to the effects of stress, nicotine, and caffeine.

G. **Indexing Terms (Descriptors):**

Stress, nicotine, caffeine, behavioral responses of rats to stress, nicotine, caffeine, behavioral responses in rats, tobacco,

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March 14, 2013

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF NEUROLOGY

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on March 14, 2013:

Animal Protocol Title: "Phenserine, an Acetylcholinesterase Inhibitor with Possible Neuroprotective Efficacy Against soman-Induced Neuropathology in Male Sprague-Dawley Rats"

USUHS Protocol Number: (b)(6)

Expiration Date: March 13, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

Revised

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Phenserine, an acetylcholinesterase inhibitor with possible neuroprotective efficacy against soman-induced neuropathology in male Sprague-Dawley rats

GRANT TITLE (if different from above): Phenserine, an acetylcholinesterase inhibitor with possible neuroprotective efficacy against soman-induced neuropathology

USUHS PROJECT NUMBER:

FUNDING AGENCY: DTRA (b)(6)

EARLIEST ANTICIPATED FUNDING START DATE: January 19, 2013

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D., M.D.

(b)(6) _____ Neurology (b)(6) 1/8/13
Principal Investigator Signature Department Office/Lab Telephone Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) _____ Acting Chair (b)(6) 1/07/13
Research Unit Chief / Dept. Head Signature Title Neurology Telephone Date
Typed Name: COL (b)(6)
CAPT

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) _____ PMB (b)(6) 1/4/13
Statistician Signature Department Telephone Date
Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress if relieved by anesthetics or analgesics.

(b)(6)	LAM	(b)(6)	14 JAN 13
Attending/Consulting Veterinarian Signature	Department	Telephone	Date

Typed Name: (b)(6) DVM, MPH

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6)

ANIMAL PROTOCOL TITLE: Phenserine, an acetylcholinesterase inhibitor with possible neuroprotective efficacy against soman-induced neuropathology in male Sprague-Dawley rats

GRANT TITLE (if different from above): Phenserine, an acetylcholinesterase inhibitor with possible neuroprotective efficacy against soman-induced neuropathology

USUHS PROJECT NUMBER:

CO-INVESTIGATOR(S): (b)(6) Ph.D. (b)(6) Ph.D., (b)(6) (b)(6) Ph.D. (b)(6) Ph.D., (b)(6) (b)(6) (graduate student in MCB program)

TECHNICIANS(S): N/A

I. **NON-TECHNICAL SYNOPSIS:** Nerve agents cause mass casualties and are a threat to military and civilian populations. Nerve agents inactivate acetylcholinesterase, an enzyme that breaks down acetylcholine. Inactivation of acetylcholinesterase leads to the accumulation of acetylcholine in synapses and results in the overactivation of muscarinic and nicotinic acetylcholine receptors in the brain and periphery. Acetylcholine is an excitatory neurotransmitter in the brain. Accumulation of acetylcholine in synapses causes status epilepticus via the overactivation of muscarinic type acetylcholine receptors. Status epilepticus is a dangerous complication of nerve agent exposure that can lead to brain damage. The muscarinic antagonist, atropine, is effective in controlling the seizures but only if given within several minutes after onset of status epilepticus. Diazepam, an anti-convulsant, is efficacious if given within minutes after onset of status epilepticus. Exposure to nerve agents produces rapid inactivation and "aging" of acetylcholinesterase. Aging results in an irreversible bond between agent and the enzyme and results in irreversible inhibition of acetylcholinesterase. Because of the rapid onset and nature of the nerve agent-enzyme reaction, neuroprotective agents are needed to reduce brain damage and improve outcome.

II. BACKGROUND:

II.1. Background:

The organophosphorus nerve agents, sarin, soman, VX, and tabun are among the most lethal chemical weapons ever developed by man. Some of these agents were used in wars and more recently in terrorist attacks in Japan in the early 1990s. The possibility that the warfighter will be exposed to nerve agents and the possibility of another terrorist attack underscore the required need to develop effective and safe countermeasures against nerve agents.

The major mechanism of acute nerve agent toxicity is the irreversible inhibition of acetylcholinesterase (AChE). This enzyme degrades the neurotransmitter

acetylcholine. When AchE is inhibited, excessive levels of acetylcholine (Ach) accumulate in the synaptic cleft leading to progression of toxic signs including a muscarinic receptor activation syndrome consisting of hypersecretions, miosis, profuse sweating, diarrhea, bradycardia, hypotension and bronchoconstriction. Central nervous system-related effects include: anxiety, confusion, ataxia, seizures, cardiorespiratory paralysis, and coma.

The current therapeutic strategies to reduce nerve agent toxicity include atropine, an anti-cholinergic drug that reduces the muscarinic receptor activation syndrome, an oxime, compounds that reactivate nerve agent inhibited AChE and a benzodiazepine, drugs that effectively control nerve agent-induced convulsions when given soon after seizure initiation. Because of the limitations of these treatments, alternative therapies that combine with current therapies have been sought to provide greater survival, protection of the brain against nerve agents and improved functional outcome.

Phenserine is a reversible AChE inhibitor that was synthesized on the physostigmine backbone. By virtue of its phenyl group, phenserine is more lipophilic compared to physostigmine. Phenserine readily and preferentially enters the brain following its systemic administration and achieves levels that are 10-fold higher than concomitant levels in plasma. Phenserine selectively inhibits AChE in plasma and brain and has a minimal effect on butyrylcholinesterase (BChE). Phenserine is dramatically less acutely toxic than is physostigmine and is safe. An additional feature of phenserine, that is not available for huperzine A, galantamine or similar anticholinesterases, is that phenserine has a chiral center and naturally exists in the active form as (-)-phenserine. Phenserine entered Phase III trials for the treatment of Alzheimer's Disease and is well tolerated (1-5). Recently, phenserine has been formulated for transdermal application and has been tested in animals (6).

Phenserine has been used in over 1,000 Alzheimer patients and the drug is well-tolerated. Memory testing showed significant improvement in a test for short-term memory. A four week trial of phenserine in Alzheimer patient showed improvement on Alzheimer's Disease Assessment Scale-cognitive Subscale and on Clinically Interview-based Impression of Change but the results never did reach statistical significance. The reasons for pointing out these studies is to demonstrate that this compound is ready for translational neuroscience in that it can be readily and rapidly brought to the warfighter if neuroprotective efficacy is established.

Huperzine A, a reversible AChE inhibitor that crosses the blood brain barrier, protects against soman-induced convulsions and the neuropathological changes in the hippocampus. The efficacy of huperzine A appears to be primarily related to the selectivity of huperzine A for peripheral AChE which preserves scavenger capacity of plasma butyrylcholinesterases (BChE) for nerve agents and to the protection conferred by huperzine A on cerebral AChE. Thus, efficacy of huperzine A seems to be related to a protection of both peripheral and central stores of AChE. In a similar fashion, phenserine has a 10:1 brain to plasma ratio and selectively binds to acetylcholinesterase. By selectively inhibiting acetylcholinesterase, phenserine protects acetylcholinesterase and butyrylcholinesterase (BChE) is free to bind nerve agent. In so doing, BChE acts as a chemical sponge (scavenger) to reduce the amount of

nerve agent available for binding to AChE. Thus, efficacy by phenserine may relate to protection of both peripheral and central stores of AChE.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: PubMed, DTIC, Crisp, Toxline, BRD

II.2.2. Date of Search: November 27, 2012

II.2.3. Period of Search: 1980-2012

II.2.4. Key Words and Search Strategy: phenserine, soman, rat, hippocampus, nerve agent,

II.2.5. Results of Search:

November 27, 2012

Toxline: 500 references on soman and effects in vivo. No references on the possible neuroprotective effect of phenserine against soman.
Crisp: 17 grants but none deal with phenserine and soman.
BRD: no hits
PubMed: No hits
No duplication of this proposal.

III. OBJECTIVE\HYPOTHESIS: The long-term objective is to develop an effective combinatorial drug regimen to maximize neuronal survival and functional outcome against soman-induced neuropathology. We believe there is a high likelihood of achieving a higher level of neuroprotection and improved functional outcome by targeting different pathways that are involved in soman-induced brain damage.

(-)-Phenserine has properties similar to huperzine A and these properties make it an attractive candidate drug to protect against soman-induced seizures and brain damage. An additional feature of phenserine, that is not available for Hyperzine A, galantamine and similar anticholinesterases, is that phenserine has a chiral center and naturally exists as the (-)-enantiomeric form as (-)-phenserine. This is the chirally pure form that has anticholinesterase activity that moved into phase III clinical assessment in Alzheimer's disease and is the basis for the proposed work.

Only one study exists in the literature which shows neuroprotective efficacy by Huperzine A in the hippocampus of guinea pigs following soman injection. Whether Huperzine A protects against soman-induced damage in other vulnerable brain regions is unknown. In this proposal, we will test the neuroprotective efficacy of (-)-phenserine and compare the results to (+)-phenserine which lacks anticholinesterase activity and is utilized as a pharmacological tool to determine whether actions by (-)-phenserine to protect the brain against soman are due to anticholinesterase inhibition or some other

mechanism.

IV. MILITARY RELEVANCE: The neurological effects of chemical weapons on the performance and operations of our military would be devastating. This is, in fact, a major goal of the terrorists. They want to cripple the military at a sufficient level and long enough so that when the levels of the chemical weapons are no longer toxic the terrorists can penetrate into and destroy military facilities, equipment and soldiers.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1: To test a dose and time course of neuroprotective efficacy of (-)-phenserine as a pretreatment strategy (-)-Phenserine selectively inhibits AChE and has a minimal effect on BChE in plasma and brain. (-)-Phenserine achieves a 10-fold higher level in brain compared to plasma, and (-)-phenserine is safe and has fewer side effects than physostigmine.

We have data suggesting that pretreatment of (-)-phenserine at a dose of 1 mg/kg iv is neuroprotective against soman-induced brain damage. Percent neuroprotection for each of the four vulnerable brain regions are: amygdala (50), piriform cortex (48), hippocampus (85) and prefrontal cortex (45). The results are significant but we don't know where the 1 mg/kg dose is on the neuroprotection curve. It is possible that a lower dose may achieve similar levels of neuroprotection. Since dose selection is of utmost importance to maximize brain protection and minimize side effects in humans exposed to nerve agents, the proposed experiments set out to determine levels of neuroprotection using two additional pretreatment doses of (-)-phenserine (0.3 and 3 mg/kg iv) using the same two time points (30 min and 4 hours) proposed in the grant proposal (30 min and 4 hours). All three doses (0.3, 1, 3 mg/kg, iv) will be considered as part of the original protocol.

Phenserine-preconditioned rats (male, Sprague-Dawley 250-300 gm) will be intravenously injected with the active stereoisomer of phenserine [(-)-phenserine] or the inactive isomer of phenserine [(+)-phenserine] (0.3 and 3 mg/kg) **4 hours or 30 min** before nerve agent. Rats receiving nerve agent will be injected with the oxime HI-6 (125 mg/kg, ip) and 30 min later will receive soman (180 ug/kg, sc) and post-exposure treatment with atropine (2 mg/kg, im, 1 min after soman im). These treatments, HI-6 (125 mg/kg, ip) and 30 min later soman (180 ug/kg, sc) and post-exposure treatment with atropine (2 mg/kg, im) 1 min after soman, have been shown to reliably produce electrographic seizure activity within approximately10 min. Rats are allowed to seize for 40 min and then treated with 10 mg/kg, im, diazepam to stop/attenuate the seizures. There will be 10 rats/group in an attempt to get at least 6 survivors/soman-exposure group (Table 1-2)

Table 1: Phenserine Pretreatment Groups and Group Sizes for Experiment 1

Treatment Groups	HI-6 30 min Soman 9 min Atropine 1 min Diazepam 40 min	Treatment (Time before seizure onset)	Group Size	Pain Category
Group 1 Adult Male Rats	HI-6→Soman→atropine→seizure →diazepam	(-)-phenserine (30 min)	20 Male Rats (10 for each dose)	E

Table 2: Control groups - No Soman Exposure and Group Sizes for Experiment 1

Treatment Group	HI-6 30 min Saline 0 min, Atropine 1 min Diazepam 40 min	Treatment (Time before Saline injection)	Group Size	Pain Category
Group 5 Adult Male Rats	HI-6→Saline→atropine →Diazepam	(-)-phenserine (30 min)	12 Male Rats (6 for each dose)	C
Group 6 Adult Male Rats	HI-6→Saline →atropine →diazepam	(+)-phenserine (30 min)	12 Male Rats (6for each dose)	C
Group 7 Adult Male Rats	HI-6→Saline →atropine →diazepam	(-)-phenserine (4 h)	12 Male Rats (6 for each dose)	C
Group 8 Adult Male Rats	HI-6→Saline →atropine →diazepam	(+)-phenserine (4 h)	12 Male Rats (6 for each dose)	C
TOTAL			**48 Male Rats**	**48 C**

Table 3: Control groups - Soman Exposure No Treatment and Group Sizes for Experiment 1

Treatment Group	HI-6 30 min Soman 0 min, Atropine 1 min Diazepam 40 min	Saline (Time before seizure onset)	Group Size	Pain Category
Group 9 Adult Male Rats	HI-6→Soman→atropine→ seizure→diazepam	Saline (30 min)	20 Male Rats (10 for each dose)	E
Group 10 Adult Male Rats	HI-6→Soman→atropine→ seizure→diazepam	Saline (4 h)	20 Male Rats (10 for each dose)	E
TOTAL			**40 Male Rats**	**40 E**

We will weigh all animals and determine the survival rate of all animals just before perfusion.

Rats will be perfused 24 hours after soman injection, perfused and brains fixed. Brain sections will be made on a cryostat and placed on slides. Alternate slides will be stained with cresyl violet and fluoroJade C. Animal survival in all groups will be recorded just prior to the animals being perfused.

V.1.2. Experiment 2: To test the neuroprotective efficacy of (-)-phenserine as a treatment strategy. (-)-Phenserine-treatment rats (male, Sprague-Dawley 250-300 gm) will be intravenously injected with the active stereoisomer of phenserine [(-)-phenserine] or the inactive stereoisomer of phenserine [(+)-phenserine] (0.3 or 3 mg/kg) either 5 min or 30 min after soman injection. Rats receiving nerve agent will be injected with the oxime HI-6 (125 mg/kg, ip) and 30 min later will receive soman (180 ug/kg, sc) and post-exposure treatment with atropine (AMN, 2 mg/kg, im) 1 min after soman. Rats are allowed to seize for 40 min and then treated with diazepam (10 mg/kg, im) to stop/ attenuate the seizures.

Treatment Group	HI-6 30 min Soman 0 min, Atropine 1 min Diazepam 40 min	Treatment (Time after seizure onset)	Group Size	Pain Category
Group 11 Adult Male Rats	HI-6→Soman→atropine→seizure →diazepam	(-)-phenserine (5 min)	20 Male Rats (10 for each dose)	E

Table 4. Phenserine Treatment Groups and Group Sizes for Experiment 2.

Table 5. Control Groups No Soman Exposure and Group Sizes for Experiment 2

Treatment Group	HI-6 30 min Saline 0 min, Atropine 1 min Diazepam 40 min	Treatment (Time after Saline injection)	Group Size	Pain Category
Group 15 Adult Male Rats	HI-6→Saline→atropine →Diazepam	(-)-phenserine (5 min)	12 Male Rats (6 for each dose)	C
Group 16 Adult Male Rats	HI-6→Saline →atropine →diazepam	(+)-phenserine (5 min)	12 Male Rats (6 for each dose)	C
Group 17 Adult Male Rats	HI-6→Saline →atropine →diazepam	(-)-phenserine (30 min)	12 Male Rats (6 for each dose)	C
Group 18 Adult Male Rats	HI-6→Saline →atropine →diazepam	(+)-phenserine (30 min)	12 Male Rats (6 for each dose)	C
TOTAL			**48 Male Rats**	**48 C**

Control groups - Soman Exposure No Treatment and Group Sizes for Experiment 1

Treatment Group	HI-6 30 min Soman 0 min, Atropine 1 min Diazepam 40 min	Saline (Time after seizure onset)	Group Size	Pain Category
Group 19 Adult Male Rats	HI-6→Soman→atropine→ seizure→diazepam	Saline (5 min)	20 Male Rats (10 for each dose)	E
Group 20 Adult Male Rats	HI-6→Soman→atropine→ seizure→diazepam	Saline (30 min)	20 Male Rats (10 for each dose)	E
TOTAL			**40 Male Rats**	**40 E**

METHODS

Chemicals. (-)-Phenserine and (+)-phenserine are provided by Dr. (b)(6) All other reagents except soman will be purchased from commercial sources or as indicated. Soman, atropine, HI-6, and diazepam will be supplied by Dr. (b)(6) (b)(6) at the Institute of Chemical Defense.

Drug treatments. (-)-Phenserine and (+)-phenserine will be freshly prepared on the day of experimentation. Phenserine readily dissolves in physiological saline and an appropriate working stock concentration will be used for injection purposes for a final doses of 0.3 and 3 mg/kg IV.

Treatment and pretreatment procedure (Experiments 1-2). Phenserine-treated rats will be intravenously injected into the jugular vein by an indwelling catheter as a bolus with (-)-phenserine or (+)-phenserine prior to (Specific Aim 1) or after (Specific Aim 2) soman challenge. Saline-injected animals will be treated in an identical manner and will

be used as controls for the treatment and pretreatment group.

Interval from catheterization to soman injection. The interval from catherization to injection of soman is standardized.

Rats will be euthanized at 24 hours after injection of soman (Specific Aims 1, 2).

V.2. Data Analysis: Values from experimental and control groups are averaged and compared using analysis of variance ($p<0.05$ to reject the null hypothesis that the group means were equal). Post hoc analyses will use the Tukey test for multiple group comparison. A sample size of ten animals per group will have 80% power to detect differences between groups of 1.8 standard deviations, based on one-way ANOVA with Tukey's post-hoc multiple comparisons among 10 group means with a 5%, two-sided significance level. Even with death of four animals per group, a sample size of six animals per group will have 80% power to detect differences between groups of 2.4 standard deviations, based on one-way ANOVA with Tukey's post-hoc multiple comparisons among 10 group means with a 5%, two-sided significance level. For the categorical outcome of mortality, the Fisher exact test has 80% power to detect a significant difference between groups when the mortality is at least 85% in one group compared with no more than 15% in the second group, based on a 5% two-sided significance level with ten animals per group.

V.3. Laboratory Animals Required and Justification: Chemical warfare agents or nerve agents are some of the most lethal compounds known to mankind. Nerve agents induce severe seizures, brain damage and death. Anticholinergic drugs are only effective if given within 5 minutes after the initiation of seizures. Thus, these agents have limited value in protecting the brain against nerve agent-induced seizures. Therefore, efficacious drugs are needed to reduce brain damage and improve outcome. Phenserine is a reversible acetylcholinesterase inhibitor that has a high brain to periphery penetration. Our goal is to determine whether phenserine protects vulnerable neurons against soman-induced brain damage. Because we are studying cellular mechanisms and responses, there are currently no non-animal alternatives that can meet our needs for these studies.

V.3.1. Non-animal Alternatives Considered: N/A

V.3.2. Animal Model and Species Justification: The male Sprague-Dawley rats for the in vivo studies were selected because this is the common gender and species of rat used in the literature. This allows us to compare our results directly with the many published results from other laboratories using chemical warfare agents or other acetylcholinesterase inhibitors such as paraoxon.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	Rattus Norvegicus	

V.3.3.2. **Strain/Stock**: Sprague-Dawley

V.3.3.3. **Source/Vendor**: (b)(4)

V.3.3.4. **Age**: adult

V.3.3.5. **Weight**: 250-275 grm

V.3.3.6. **Sex**: male

V.3.3.7. **Special Considerations**: N/A

V.3.4. **Number of Animals Required (by Species)**: 336 (240 Category E and 96 Category C)

V.3.5. **Refinement, Reduction, Replacement (3 Rs)**:

V.3.5.1. **Refinement**: All surgeries will be performed under appropriate anesthesia. Any rat exhibiting signs of pain or impairment beyond that from this model will be euthanized at an early endpoint. No other refinement alternatives were considered.

V.3.5.2. **Reduction**: We are using 10 rats/group in the treated group which provides a sufficient number to determine statistical significance even if 4 rats/group die (see V.2). We will use 6 rats/group in the control groups (no soman) as no deaths are expected in this group of animals. We will minimize the number of animals used in this study as much as possible but without affecting statistical significance. Using rats will allow us to study whether phenserine protects against soman-induced neuropathology without using higher species.

V.3.5.3. **Replacement**: This type of study requires a whole biological system because there is no currently available in-vitro or computer modeling systems that can be used to study the physiologic effects of treatments and exposures proposed in this protocol. No other replacements were considered.

The requirement for seizures (40 min). The protocol involving the use of soman was developed by Dr. (b)(6) my collaborator at (b)(6) Dr. (b)(6) is a senior scientist at (b)(6) and an established investigator in the nerve agent field. According to Dr (b)(6) the dose of soman used to inject the animals is the dose that elicits seizures in 100% of the animals. Animals must seize for 40 min in order for the brain to be damaged. If the animals don't seize, they have to be euthanized and cannot be used for further study. The dose of soman used in this study is 1.6 x LD_{50}. The LD_{50} is the dose that kills 50% of the animals so 1.6xLD_{50} will result in more than 50% death in soman-treated animals alone. You can see from Dr (b)(6) publications that he uses 2xLD_{50} often to determine efficacy (21-26). We are working on another project where we have convincing data indicating that alpha-linolenic acid protects against soman-induced brain damage. Our work was recently published using

the method listed in this animal protocol (27).

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	96	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	240	

V.4.1.2. Pain Relief / Prevention: There is momentary discomfort upon injection of HI-6, atropine (AMN), and soman. Because the animals are in status epilepticus which involves hyperexcitability of both hemispheres, it is unlikely that the animals feel any discomfort when they are injected with diazepam.

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:
When the rats are delivered from the supplier (b)(4) they will be allowed to acclimate to LAM for 1 week. The animals will then be subjected to anesthesia for placement of the external jugular catheters. Animals will be allowed to recover for 1 week and will then be injected via external jugular catheter (see V.4.4.1.). When the animals have stabilized at (b)(6) following soman exposure, they will be brought back to USUHS where they will be placed in quarantine, observed by laboratory personnel and injected with 5 ml lactate ringers solution sc every 6 hours. All animals will be euthanized 24 hours after soman exposure.

Anesthesia for placement of external jugular catheter:
Rats will be placed in an induction chamber then anesthesia will be induced with 3-4% isoflurane from a precision vaporizer delivered in 1-2 Liters per minute of oxygen. Once the rats are in lateral recumbancy, they will be moved to a mask and prepared for surgery. Maintenance anesthesia will be delivered at 1-3% isoflurane in 0.5 to 1 Liter per minute of oxygen. Anesthesia will be adjusted based on response to toe pinch and depth of respirations. Once animal is adequately anesthetized (no response to toe pinch) then surgical procedures will begin. At the completion of the procedure, the rats will be removed from anesthetic and allowed to awaken in a home cage. All rats except for naïve will have external jugular catheters placed for injection of either vehicle or a stereoisomer of phenserine. The procedure itself takes 15-20 min. Animals are not kept under anesthesia any longer than necessary.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Animals will have three days to acclimate to their surroundings once delivered to USUHS and before being used for any in vivo experiments. Rats will be observed by the PI or technician to ensure they appear healthy prior to undergoing surgery.

Animal monitoring post-soman. The animals will be watched throughout the 24 hour period. Monitoring will be performed in the rats' home cage, and will continue through the night until they are perfused. Any animals that show unexpected neurologic signs following soman injection or hunched posture, lethargy, anorexia, or other clinical signs potentially indicative of infection may be euthanized at an early endpoint or treated at the discretion of the veterinarian and/or PI. Research personnel will weight rats just prior to being perfused (24 hours after soman). Supplemental feeding in the form of wet mash will be provided by LAM personnel as directed by the veterinarian or requested by the PI and 5 ml Lactated Ringer's sc every 6 hours will be administered for rats over the 24 hour period. Gel squares will be placed in each cage as a fluid supplement for the animals. The survival rate will also be determined just prior to the animals being perfused (24 hours after soman injection).

Supplemental (alternative approach) feeding. LAM personnel may place wet mash/ chow on the cage floor for rats that may not be able to gnaw pellets to get food – as requested by the PI, PI staff or LAM veterinarian.

Soman-induced seizures: The animals will not receive anesthesia or analgesia during the time the animals seize following injection of soman. Rats receiving soman will be injected with the oxime HI-6 (125 mg/kg, ip) and 30 min later will receive soman (180 ug/kg, sc) and post-exposure treatment with atropine methyl nitrate (2 mg/kg, im, 1 min after soman. All soman-treated animals will receive diazepam (10 mg/kg im) 40 min after onset of status epilepticus.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures: There is no alternative for inducing brain damage in rats that mimics the damage caused by soman to determine whether the proposed research plan using phenserine protects against soman-induced brain damage or improves functional outcome. The only way to carry out the research plan is to allow the animals to seize for 40 min. This is the protocol established by Dr. (b)(6) at (b)(6) to induce brain damage followed by injection of diazepam to stop/attenuate the seizures.

V.4.1.3.1. Sources Searched: BRD, DTIC Private STINET, CRISP

V.4.1.3.2. Date of Search: November 27, 2012

V.4.1.3.3. Period of Search: 1980-2012

V.4.1.3.4. Key Words of Search: rat, pain, kainic acid, seizure, hippocampus, piriform cortex, amygdala, neuronal damage, brain damage, gluatamate receptor, glutamate receptor, animal welfare, BHLHB2, alternative, reduce, refine, prevent, control, manage,

eliminate, alleviate, decrease, diminish

V.4.1.3.5. Results of Search:

DTIC Private STINET: 520 hits. One study discussed seizures as it relates to chemical warfare agents and measuring seizures on the battlefield. These studies offered no advantage over the proposed methods.

BRD: no hits

CRISP: 17 hits. Several discussed reduce or refine but were not relevant to the proposed study.
Soman-induced convulsions: The treatment plan for inducing status epilepticus by soman in rats is a standard procedure that is routinely performed by many laboratories including our laboratory and outlined in the scientific literature. Sprague-Dawley rats have been used in the literature.

Interpretation: There presently is no alternative to employment of rat as an *in vivo* animal model to study the role of phenserine to protect against soman-induced neuropathology.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: 240 male rats will receive soman at a dose that will induce seizures, an effect that is seen in humans exposed to nerve agents. The rats will experience some level of physical stress and discomfort for some period of time during the experiments. Analgesic, sedatives and/or anesthetic agents cannot be used because they will modify the pain but also seizure threshold preventing us from fully evaluating the effect of soman and as such we will not be able to draw any conclusions on the proposed effect of phenserine on neuronal survival or functional outcome. The objective of this study is to determine the effect of a single injection of phenserine on neuronal survival 24 hours after soman treatment. The purpose of the study is to find additional therapies to protect the brain so emergency room physicians will have more than one drug to use on patients exposed to these deadly weapons. Without exposing the animals to soman, it is not possible for us to conduct these studies. These studies are justified because the use of soman is a model for nerve agent-induced seizures, an expected effect of nerve agent exposure of military personnel in international conflicts and civilian populations. Identification of new neuroprotective agents is highly relevant to enhance treatment response capabilities. Thus, the knowledge obtained from the proposed investigations may benefit many active duty personnel as well as civilians.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: N/A

V.4.3.1. Pre-surgical Provisions: Surgeons will wear sterile gloves, mask, lab coat or scrub top. A general assessment of physical and behavioral health will be carried out on each rat before surgery. LAM veterinarians will be consulted should any sign of

abnormality in appearance and/or behavior be observed.

V.4.3.2. Procedure:
Rats will be anesthetized and maintained with isoflurane in oxygen by nosecone (see **V.4.1.2.1.**). After the induction of anesthesia, the rats (with the nosecone) will be placed in lateral recumbancy and prepared for surgery. Maintenance anesthesia will be delivered at 1-3% isoflurane in 0.5 to 1 Liter per minute of oxygen. Anesthesia will be adjusted based on response to toe pinch and depth of respirations. Once animal is adequately anesthetized (no response to toe pinch) then surgical procedures will begin. Only sterile instruments will be used. A supplemental heat source, such as an electric heating pad or circulating warm water blanket, will be set at 37°C throughout the duration of anesthesia. Rats will be monitored for dermal burns.

Placement of external jugular catheter. Anesthetized rats will be placed in dorsal recumbancy. Fur will be clipped from the ventral neck and interscapular region. Skin will be prepared for aseptic surgery using either betadine or alcohol or chlorhexidine scrub. The animal will be draped using sterile towels. A 1.5 to 2 cm long incision by a scalpel will be made in the ventrolateral aspect of the neck, parallel and approximately 0.5 cm lateral to midline. The external jugular vein will be dissected free of surrounding tissue and stabilized with a proximally placed tie of 4-0 suture. A catheter introducer will be used to guide the placement of the jugular catheter. A 4-0 ligature will be placed at the distal end of the catheter to secure it in place. Tissue adhesive may also be used to help stabilize the catheter in the jugular vein. Catheter will be tested for patency by flushing with sterile saline. The catheter will then be tunneled subcutaneously to exit via an approximately 2.5 cm incision in the interscapular region. Wound clips or suture material will be used to close the interscapular area. Tissue glue or suture material (3-0-5-0 nylon) will be used to close the ventral neck incision. If placement in one side of the jugular area is problematic, the procedure will be repeated on the opposite jugular vein. The procedure takes 15-20 min from start to finish. All animals will be allowed to be awakened and they are kept on a 37 degree C warmer until awake. When animals are awake, they are placed in a home cage.

All animals are awake when they are injected with soman.

Tail vein injections. I have much experience with tail vein injections. If you master the technique successfully, this route is superior over surgical placement of indwelling catheters. However, the problem with tail vein injections is failure to deliver the entire amount of drug into the vein. If some of the drug escapes into the extravascular space, we may not be able to show neuroprotective efficacy. Determining drug blood levels are beyond the scope of this grant proposal.

V.4.3.3. Post-surgical Provisions: Immediate post-operative care will be provided by the investigators and rats will be monitored continuously until they are ambulatory. Animals are expected to ambulate. Animals will be returned to their cages post-surgery only after they have recovered sufficiently from anesthesia. Food and water will be provided *ad libitum*. Room maintenance and climate control is as per LAM SOP. Investigators will weigh the animals just prior to perfusion (24 hours after injection of soman). Following surgery, rats will be monitored continuously until they are

ambulatory. The incision site will be monitored daily for signs of infection (erythema, edema, presence of an exudate, induration etc). Because the catheter will be inserted into the external jugular vein (indwelling catheter), we will also examine the area for signs of bleeding. Catheters will remain in place for about one week. Each catheter will be flushed once daily with heparinized saline to prevent clotting of the tip.

All animals undergoing catherization will receive buprenorphine, 0.01-0 05 mg/kg, subcutaneous (SC), every 8-12 hrs for 2 days. The alternative drug is acetaminophen, 6 mg/ml in drinking water for 2 days.

In addition to Center for LAM SOP's, study personnel will monitor animals using the checklist for animals exhibiting pain/distress beyond expectations of this study. If LAM personnel notice any of the following: persistent convulsions or tremors, self mutilation, continuous circling, or persistent labored breathing, and study personnel are unavailable, the LAM veterinarian may make the decision to euthanize the animal.

V.4.3.4. Location: LAM procedure room

V.4.3.5. Surgeon: To be determined. Dr. (b)(6) will instruct new personnel on how to insert external jugular catheters. Dr. (b)(6) is currently inserting jugular vein catheters in rats successfully. Dr. (b)(6) has six years of experience in placing these catheters in rats.

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures:

V.4.3.6.2. Scientific Justification:

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

Drug	Concentration	Dose	Route	Site	Frequency
Phenserine or saline	1 mg/ml	1-3 mg/kg	Iv Needle : 22-25 gauge Vol : 250-270 µl	Into indwelling catheter between shoulders	once
Posiphen	1 mg/ml	1-3 mg/kg	Iv Needle : 22-25 gauge Vol : 250-270 µl	Into indwelling catether between shoulders	once
HI-6	250 mg/ml	125 mg/kg	Ip (150 µl), 25 gauge needle	Abdomen	once
soman	360 ug/ml	180 ug/kg	Sc (150 µl) ; 25 gauge	Above thigh posteriorly	once

			needle		
Atropine	4 mg/ml	2 mg/kg	Im (120 µl) ; 23-25 gauge needle	thigh	once
Diazepam	5 mg/ml	10 mg/kg	Im (600 µl) ; 23-25 gauge needle	thigh	once

Soman treatment (Specific Aims 1-3). Male Sprague-Dawley rats (250-300 gm) receiving nerve agent will be injected with the oxime HI-6 (125 mg/kg, ip) and 30 min later will receive soman (180 ug/kg, sc) and post-exposure treatment with AMN (2 mg/kg, im, 1 min after soman. These treatments, HI-6 (125 mg/kg, ip) and 30 min later soman (180 ug/kg, sc) and post-exposure treatment with ANM (2 mg/kg, im) 1 min after soman, have been shown to reliably produce electrographic seizure activity within approximately10 min. Rats are allowed to seize for 40 min and then treated with 10 mg/kg, im, diazepam to stop/attenuate the seizures (as per Dr. (b)(6) protocol at (b)(6)

Phenserine will be injected via catheter in either a pretreatment or post-treatment paradigm followed by nerve agent + oxime injection. We will use a needle size ranging from 22 to 25 gauge.

IP Injections: For animals requiring fluid replacement, 5 ml of sterile lactate ringers will be injected sc. The rat will be manually restrained and tilted so that the head is facing downward and its abdomen is exposed. The injection site will be disinfected and the needle (23-25 gauge) attached to the syringe containing the sterile solution inserted cranially into the abdomen at a 30-45 degree angle caudal to the umbilicus and lateral to the midline. Once the needle is inserted, we will aspirate. If nothing is aspirated, the lactated ringer solution is injected.

V.4.4.2. Biosamples:

In vivo experiments. The brains from the male Sprague-Dawley rats will be collected at the time of euthanize. Brain will be preserved for future studies. Brain tissue may be removed to determine phenserine levels to ensure proper injection.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification:

A patch will be shaved on each animal's back and then marked with non-toxic permanent ink with an individual ID. Sometimes, the ink fades in which case the ID will

be re-inked on the patch. All cages have cage cards per LAM. Rats that receive nerve agent injection will be checked for their ink mark labels prior to injection of nerve agent to make sure they are clearly labeled.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures:

Transcardial perfusion. When rats are euthanized, they will be placed under deep pentobarbital (50-75 mg/kg) anesthesia. Once adequate anesthesia is achieved (lack of response to toe pinch, slow even respirations), a midsternal thoracotomy will be performed to expose the heart and the rats will undergo transcardial perfusion with cold PBS and 10% formalin. Generally, about 200 cc of each solution is sufficient *per animal*, but, more will be used if required. Transcardial perfusion will be performed in (b)(5)

General histological procedures. For each brain studied, we will place two sections per slide and prepare 60 slides per rat. Every other slide will be stained with cresyl violet and every other slide will be stained with FluoroJade.C. Nissl-positive undamaged neurons and fluorescent-positive degenerating neurons are counted in the four brain regions [piriform cortex, hippocampus (CA1, CA3), cerebral cortex, amygdala]. Counts will be performed without knowledge of the treatment history of the rats.

Cresyl Violet staining (Experiments 1,2). Dried slides will be stained with cresyl violet in the following manner:

Treatment	Time
95% ETOH	15 min.
70% ETOH	1 min.
50% ETOH	1 min.
DH2O	2 min.
DH2O	1 min
Cresyl Violet Stain*	2 min.
DH2O	1 min.
50% ETOH	1 min.
70% acid ETOH**	2 min.
95% ETOH	2 min
95% ETOH	a few dips
100% ETOH	1 min
Histoclear	mounting media

*Cresyl violet preparation: add 1.25g cresyl violet acetate and 0.75 ml glacial acetic acid to 250 ml warm DH20, cool and filter.
**2 ml glacial acetic acid in 200 ml 70% ETOH.
Note: Some people find histoclear vapor to be irritating and may require a mask.

Histofluorescent labeling of degenerating neurons with Fluoro-Jade B (Experiments 1 and 2). Formalin-fixed brains will be prepared as outlined above (Transcardial perfusion of rats for brain fixation) and brain sections will be cut through

the designated brain regions on a cryostat.
The sections will be collected in 0.1M neutral phosphate buffer and mounted on 2% gelatin coated slides and then air dried on a slide warmer at 50 degrees C for at least 30 min. The slides will be transferred to a solution of 0.06% potassium permanganate for 10 minutes on a shaker table to insure consistent background suppression between sections. The slides will be rinsed in distilled water for 2 minutes. From this point forward, the protocol is performed as much as possible away from the light. The staining solution will be prepared from a 0.01% stock solution for Fluoro-Jade C (10 mg of Fluoro-Jade C in 100 mL of distilled water). To make up 200 mL of staining solution, 20 mL of stock solution will be added to180 mL 0.1% acetic acid (180 µL Acetic Acid in 180 mL distilled water). This results in a final dye concentration of 0.001%. The stock solution is stable for 2 months when stored at 4 °C but the freshly made staining solution is prepared within 10 minutes of use and will not be reused. After 30 minutes in the staining solution in the dark, the slides will be rinsed for one minute in each of three distilled water washes. Excess water will be removed by briefly (about 15 seconds) draining the slides vertically on a paper towel. The slides will be rapidly placed on a slide warmer, set at approximately 50 °C, until they are fully dry (about 5-10 minutes). The dry slides will be cleared by immersion in xylene for at least one minute before coverslipping with DPX (Sigma-Aldrich, St. Louis, MO), a non-aqueous non-fluorescent plastic mounting media. Sections spaced 125 µm apart will be viewed under fluorescein (FITC) optics in an Axiovert Zeiss microscope equipped for epifluorescence and photographed (10x magnification) using Axiovision software. A grid (170 x 170 µm) will be placed over a representative field in each of the four brain regions and the number of fluorescent cells will be counted, providing an estimate of the mean number of degenerating neurons for each animal. The mean number of Fluoro-Jade B-positive neurons ± SD will be calculated and graphed as a histogram.

Quantification of viable neurons (Experiments 1,2). The neuronal density of the four brain regions will be quantified. For example, analysis of neuronal density in the hippocampus will be performed on coronal sections of the dorsal hippocampus stained with cresyl violet and corresponding to brain sections located between 3.14 and 4.16 mm posterior to bregma. Neurons will be scored as undamaged if they are Nissl-positive with a round to oval shaped cell body that exhibits no evidence of cell shrinkage. Degenerating neurons characterized by shrunken cell bodies will be excluded. Neuronal density values will be expressed as mean ± SD. Counts are performed by an investigator without knowledge of the treatment history of the rats. Slides are placed on a Zeiss standard microscope with fluorescence capability. This microscope is connected to a PC for imaging and photography. Three representative fields are photographed from each of the four brain regions (cortex, hippocampus, amygdala, piriform cortex) from cresyl violet- and FluoroJade C-stained slides. The viable (cresyl violet) and degenerating (fluoroJade C) neurons are quantified; graphed and standard statistical methods are used to determine significance.

Animal transportation: The USUHS cargo van will be used to transport animals and the anticipated drive time one-way is 80 min and the number of miles one-way is 63. The maximum time the animals will be in the cargo van is 160 min. This van will be wiped down with disinfectant prior to and after the transport. The cargo van is heated and has air conditioning; LAM personnel usually place a small device that monitors the

temperature in the van. Only laboratory personnel that work on this project will drive the van to and from [(b)(6)] All animals will be transported to [(b)(6)] on the morning of soman injection. Animals will be pre- or post-injected with phenserine or saline at USUHS or at [(b)(6)] as per the experimental design. **An animal protocol will also be submitted to [(b)(6)] for approval.**

After the rats are stabilized following the injection of diazepam (40-60 min), the rats will be brought back from [(b)(6)] by lab personnel on the day of nerve agent injection and placed in a room designated by LAM. All animals will be in their home cage (1 rat/cage). See **V.4.1.2.2 for animal monitoring.**

All soman injections will be performed by Dr. [(b)(6)] at [(b)(6)]

V.4.4.8. Tissue Sharing: The brains from Specific Aim 1-2 will be collected at the time of euthanasia and remain available for future studies. All other tissues (organs etc) will be made available to other investigator upon request.

V.4.5. Study Endpoint: The study endpoint for rats used for Specific Aim 1-2 is survival until the pre-determined post-operative time point, after which they will be euthanized.

V.4.6. Euthanasia:

Experiment 1-2: Research personnel will perform all euthanasias except in cases where a LAM veterinarian make the decision that a particular animal needs to be euthanized. At the indicated endpoint, each rat will be deeply anesthetized with Pentobarbital (50-75 mg/kg ip). Animals should be deeply anethetized within 30 minutes. A midsternal thoracotomy will be performed to expose the heart and the rats will undergo transcardial perfusion with PBS and the 10% formalin in PBS. This procedure results in euthanasia from exsanguination under deep nembutal anesthesia.

Rats that reach an early endpoint (see **V.4.5**) will be euthanized via cylinderized CO_2. The research technician or the PI will perform this early euthanasia. The apparatus for euthanasia by CO_2 is located in [(b)(6)] Alternately, if requested by the PI, animals will be euthanized by Center for LAM personnel) using cylinderized CO_2 in compliance with the 2007 AVMA guidelines on Euthanasia. Euthanasia will he performed in the LAM CAF. If possible, animals will be euthanized in their home cage. If not, a clean rodent cage will be used. Animals will be placed in the cage so that it is not overcrowded (each animal can place all four feet on the bottom of the cage.) With animals in the chamber, the flow rate of CO_2 will displace at least 20% of the chamber volume per minute. Gas flow will be maintained for at least one minute after respiration has stopped before removing animals from the cage. A supplemental method to ensure death (cervical dislocation or bilateral thoracotomy) may be used.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:
Building(s) (b)(6) Room Number(s) _____

Surgery Room: External jugular catheter placement will occur in a (b)(6) (see V.4.3.2).

Soman Injection: All animals will be transported in their home cages to (b)(6) from the USUHS loading dock on the day of soman injection. Dr. (b)(6) will inject HI-6, atropine, soman and valium. Once the animals are stabilized, the animals will returned to USUHS on the same day. LAM personnel will assist in loading and off loading the animals in their home cages. The animals will be placed in a room assigned by LAM when they return to USUHS.

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No x

Fluid Restriction: Yes No x

V.5.1.3. Exceptions: _____ _____

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions:

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Animal handling,	(b)(6)	3 years experience	Rodent handling

External jugular vein catherization, euthanasia by transcardial perfusion	(b)(6)	insering jugular vein catheter,15 year experience in rodent handling, surgery and tissue dissection, 5 years experience in transcardial perfusion	course June 1, 2007 at USUHS.
External jugular vein catherization, animal handling, drug injections, euthanasia via transcardial perfusion		6+ yr experience rodent handling, surgery and tissue dissection; 6 month experience in euthanasia by transcardial perfusion. External jugular vein catherization – 2 years of experience	Animal investigator training course (May 2008). Rodent Handling Course (30 May 2008).
Animal handling, agent injections, drug injections		39+ years rodent handling, surgery and tissue dissection; 15 years in EEG electrode emplacement in primates; 39 years EEG electrode emplacement in rodents	Occ Health/zoonotic disease (01/05/04), Rodent Handling (02/05/04), aseptic techniques and rodent anesthesia (November 2006), Anesthesia (March 2006) and XCSM (nerve agent) custodian
Animal handling, drug injections		B.S. Environmental Scinece; 2+ yr rodent handling (MRICD), 2+ yr rodent surgery (MRICD)	Occ Health/zoonotic disease (21 June 2005), Rodent Handling (21 Jun 2005) and XCSM (nerve agent) User
Animal handling, drug injections		M.S. Biology, 3 yr experience assistant small animal veterinary practice; 7 yr experience ORISE research assistant	Occ Health/zoonotic disease (21 Jun 2005), Rodent Handling (21 Jun 2005), Anesthesia (03 Feb 2006) and XCSM (nerve agent) user
Euthanasia via transcardial perfusion		30+ yr rodent handling, surgery, tissue dissection,	30 + years experience and on-the-job training

	(b)(6)	euthanasia	in handling small laboratory animals, anesthesia, guillotine usage, perfusion. Rodent handling course and investigator training course at NIH
Animal handling, tissue dissection, drug injections, transcardial perfusion		30+ yr rodent handling, surgery and tissue dissection; 3 yr experience on transcardial perfusion	Investigator training course at USUHS (Nov 1993).
External jugular vein catherization, animal handling, drug injections, euthanasia via transcardial perfusion		3 years of rodent handling, and experience in animal behavior and transcardial perfusion	Investigator training course and rodent handling at USUHS (July 2012)
Supply of phenserine		Over 30 yr experience as medicinal chemist	

VII. BIOHAZARDS/SAFETY:

The first grant proposal in my laboratory that used soman was initiated in 2006. None of my laboratory personnel developed any side effects of acetylcholinesterase inhibition from working on this project. There is no risk to my laboratory personnel as a result of handling animals exposed to Soman and their bedding materials and excreta.

All soman injections will be performed by Dr. (b)(6) at (b)(6)

VIII. ENCLOSURES:

REFERENCES:

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IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 1/4/13

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 1/4/13

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL** or **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) _____ 1/4/13

Principal Investigator Signature Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: Phenserine, an acetylcholinesterase inhibitor with possible neuroprotective efficacy against soman-induced neuropathology in male Sprague-Dawley rats

C. Principal Investigator: (b)(6)

D. Performing Organization: USUHS and (b)(6)

E. Funding: DTRA

F. Objective and Approach: The objective is to find treatment modalities to reduce brain damage caused by nerve agents and improve outcome. Phenserine is a compound developed by Dr (b)(6) at the (b)(6) NIH, Baltimore, MD. This drug was tested in a phase 3 trial as a therapy for Alzheimer's Disease. The drug was very well tolerated with no reported side effects. Phenserine is more lipophilic compared to physostigmine. Phenserine readily and preferentially enters the brain following its systemic administration and achieves levels that are 10-fold higher than concomitant levels in plasma. Phenserine selectively inhibits AChE in plasma and brain and has a minimal effect on butyrylcholinesterase (BChE). Phenserine is dramatically less acutely toxic than is physostigmine and is safe. An additional feature of phenserine, that is not available for huperzine A, galantamine or similar anticholinesterases, is that phenserine has a chiral center and naturally exists in the active form as (-)-phenserine. Phenserine entered Phase III trials for the treatment of Alzheimer's Disease and is well tolerated (1-5). Recently, phenserine has been formulated for transdermal application and has been tested in animals (6). The approach is to pretreat or post-treat animals with phenserine and determine neuroprotective efficacy in soman-injected animals. Neuroprotective efficacy will be determined quantitatively by counting the number of surviving and degenerating neurons in brain sections stained with cresyl violet and FluoroJade C respectively.

G. Indexing Terms (Descriptors): phenserine, rat, soman, neuroprotection, acetylcholinesterase, neuropathology.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
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March 15, 2013

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF NEUROLOGY

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on March 15, 2013:

Animal Protocol Title: "Linolenic Acid: An Omega 3 Fatty Acid That Protects Against Soman-Induced Neuropathology in Sprague-Dawley Rats"

USUHS Protocol Number: (b)(6)

Expiration Date: March 14, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

Revised

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Linolenic Acid: An omega 3 fatty acid that protects against soman-induced neuropathology in Sprague-Dawley rats

GRANT TITLE (if different from above): Linolenic Acid: An omega 3 fatty acid that protects against soman-induced neuropathology

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: DTRA

EARLIEST ANTICIPATED FUNDING START DATE: October 1, 2009

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D., M.D.

(b)(6) _____ NEY (b)(6) 12/26/12
Principal Investigator Signature — Department — Office/Lab Telephone — Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) _____ Chair (acting) (b)(6) 01/02/13
Research Unit Chief / Dept. Head Signature — Title — Telephone — Date
Typed Name: COL (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) _____ PMB (b)(6) 12/14/12
Statistician Signature — Department — Telephone — Date
Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress even relieved by anesthetics or analgesics.

(b)(6)

Attending/Consulting Veterinarian Signature

LAM
Department

Telephone (b)(6)

1/4/13
Date

Typed Nar (b)(6)

(b)(6)

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6)

ANIMAL PROTOCOL TITLE: Linolenic Acid: An omega 3 fatty acid that protects against soman-induced neuropathology in Sprague-Dawley rats

GRANT TITLE (if different from above): Linolenic Acid: An omega 3 fatty acid that protects against soman-induced neuropathology

USUHS PROJECT NUMBER:

CO-INVESTIGATOR(S): Dr. (b)(6) Dr. (b)(6) Dr. (b)(6) (b)(6)

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS: Nerve agents represent a key threat to the United States military and civilian populations. These agents block a crucial brain enzyme, acetylcholinesterase, which in turn leads to brain hyperactivity. Brain hyperactivity results in seizures. A severe type of seizure is one lasting more than thirty minutes and is called status epilepticus and is commonly observed following exposure to chemical warfare agents. Status epilepticus is a medical emergency because it can lead to severe brain damage and/or death. Certain drugs are effective but must be given within five minutes after onset of seizures. Thus, compounds that protect the brain against nerve agents are required to attenuate or prevent damage and improve outcome.

Linolenic acid is an omega-3 fatty acid and found in canola oil. Linolenic acid is a dietary supplement that can be purchased over the counter. Linolenic acid has been shown to reduce brain damage induced by kainic acid, a compound that is found in a particular type of seaweed and induces seizures. Therefore, we tested whether linolenic acid may protect against soman-induced brain damage. Our data suggest that injecting linolenic acid either before or after soman significantly protects vulnerable brain cells against soman-induced brain damage twenty four hours after nerve agent injection. Our next goal is to determine whether the protection by linolenic acid is long lasting. Brain networks will be examined through behavioral testing.

Catheters will be placed into a medium sized vein and patency of the catheters will be maintained by a heparin-saline solution. Drugs to improve the survival of the animals will be administered before and after injection of the nerve agent, soman and a well-established agent that stops the seizures will be injected in all animals that receive soman. The neuroprotective agent, α-linolenic acid, will be injected into the animals three times over a 7 day period via the catheters either prior to or after nerve agent.

Our recent data show that three injections of linolenic acid further improve brain cell survival and exert an anti-depressant effect. We will test the hypothesis that chronic administration of α-linolenic acid either prior to or after soman improves functional outcome

II. BACKGROUND:

II.1. Background: Nerve agents and neurodegeneration. Nerve agents irreversibly inhibit the cholinesterase enzyme. Inhibition of acetylcholinesterase (AchE) results in elevated acetylcholine (Ach) levels in synapses in the peripheral and CNS leading to the progression of toxic signs including hypersecretions, tremors, seizures, respiratory distress and ultimately death.

It is established that prolonged seizures are responsible for the neuropathology caused by nerve agents. Termination of seizures within 10 min results in no neuronal cell death whereas 20 minutes of seizure activity results in mild neuronal cell death; further delay in controlling seizures dramatically increases the neuropathology in animal models. Moreover, the longer seizures continue the higher the probability for the development of status epilepticus, a serious complication of seizure activity defined as a prolonged seizure or continuous seizures lasting more than thirty minutes without regaining consciousness. Neurodegeneration occurs most frequently in the piriform cortex, followed by the amygdala, hippocampus, thalamus, cerebral cortex and caudate/putamen. The brain region that shows the most severe damage is the piriform cortex followed by the hippocampus, cortex, thalamus, amygdala and caudate/putamen. Anticonvulsants such as benzodiazepines decrease seizure activity and improve outcome but their efficacy to attenuate or abolish nerve agent-induced seizures depends upon the administration time post-exposure of the nerve agent. For example, the mean latency for seizure termination by midazolam (the most effective anticonvulsant) given five minutes after the induction of seizure activity induced by either soman or sarin was twenty minutes. Thus, even under the most ideal conditions, it still requires twenty minutes to terminate seizure activity induced by these agents (2). In addition, benzodiazepines such as midazolam can cause respiratory depression and exacerbate nerve agent-induced respiratory depression leading to increased brain injury and/or death.

Glutamate and excitotoxicity. Neurodegeneration caused by prolonged seizure activity is an example of hypoxic-ischemic neuronal injury. The primary event in nerve agent-induced seizures is the excessive accumulation of Ach in synapses which results in the release of the excitatory amino acid glutamate. Glutamate, the endogenous neurotransmitter required for normal physiological excitation, is involved in the pathophysiology of hypoxic-ischemic neuronal injury. Although this neuropathological process can be mediated by any of the excitatory amino acid receptors, the N-methyl-D-aspartate (NMDA) glutamate receptor subtype plays a crucial role. Overactivation of postsynaptic NMDA receptors is thought to result in a massive influx of calcium leading to biochemical and metabolic events that result in neurodegeneration. This process is referred to as excitotoxicity. The NMDA glutamate receptor subtype plays a crucial role in neuronal cell death mediated by nerve agents. These results confirm the role of NMDA receptors in nerve agent-induced neurodegeneration and validate the mechanism of neuronal cell death.

The vulnerable brain regions that are involved in nerve agent-induced neuropathology would be expected to impair learning and memory (hippocampus, dorsal medial nucleus of the thalamus), emotional memory (amygdala), cognition (cortex) and movement (caudate/putamen). Because of the long latency required to terminate seizures,

soldiers are at high risk for developing the neuropathology. Thus, it is essential to develop effective neuroprotective treatments and preventative therapies.

Linolenic acid is an essential fatty acid and the only omega-3 fatty acid found in vegetable products. It is most abundant in canola oil. It is commercially available and can be purchased over the counter as a nutritional supplement. Beneficial effects of PUFAs have been shown in the cardiovascular field and in preventing fatal ventricular arrhythmias by reducing cardiac excitability. PUFA's may also have beneficial effects on brain function such as depression. Also, there is some evidence that PUFA's may reduce seizure activity by decreasing neuronal excitability perhaps by opening potassium channels.
Injection of linolenic acid (500 nmol/kg iv) thirty minutes after an epileptic insult (kainic acid 10 mg/kg ip) is neuroprotective. Linolenic acid also exhibits delayed neuroprotective properties. Thus, injection of linolenic acid (500 nmol/kg, iv) three days prior to an epileptic insult (kainic acid 7.5 mg/kg, ip) almost completely inhibited kainic acid-induced neuronal damage in the CA1 and CA3 subfields of the rat hippocampus. The severity of seizure activity was attenuated but was not thought to contribute to delayed neuroprotection. Finally, linolenic acid (10 uM, i.c.v.) thirty minutes prior to 10-30 minutes global ischemia almost completely blocked ischemic-induced neuronal cell loss in the hippocampus whereas injection of palmitic acid, a saturated fatty acid, exerted no neuroprotection. Linolenic acid is also neuroprotective when injected intravenously thirty minutes after a ten minute global ischemic insult. These results show that linolenic acid protects against an epileptic insult when given as a treatment or pretreatment paradigm. The fact that linolenic acid also protects against ischemia provides further confirmation of its neuroprotective effect since ischemic and epileptic-induced neurodegeneration are examples of hypoxic-ischemic neurodegeneration that involve NMDA receptor-mediated excitotoxicity.

Linolenic acid increases activated NF-κB in the hippocampus. Thus, NF-κB is a biomarker. We first showed and confirmed that injection of linolenic acid intravenously at a dose of either 100 nmol/kg or 500 nmol/kg increased activated NF-kappaB in hippocampal nuclear extracts. Injection of linolenic acid three days prior to soman injection protects vulnerable neurons against soman-induced neuropathology in four critical brain regions: piriform cortex, amygdala, hippocampus and cortex. Our results show that pretreatment with linolenic acid (500 nmo/kg IV) protects vulnerable neurons in the cortex [35.6%], in the hippocampus [81.02%], in the amygdala [63.34%], and in the piriform cortex [45.14%] using the FluoroJade C staining method. Moreover, linolenic acid reduces the degree of swelling in the piriform cortex in soman-treated animals. Injection of linolenic acid thirty minutes after soman injection is also neuroprotective in the four brain regions but there is no difference in the degree of swelling in the piriform cortex from linolenic acid-treated and soman-treated animals.

This is the first study to show that linolenic acid, an over the counter dietary supplement provides significant protection against the neuroprotection by a potent nerve agent. We now extend our findings by: 1) determining whether protection extends out to a 7 day time frame; 2) determining whether the observed neuroprotection by linolenic acid improves functional outcome; and 3) determine whether multiple injections of linolenic acid improve neuronal survival over and above a single injection.

II.2. **Literature Search for Duplication:**

II.2.1. **Literature Source(s) Searched**: BRD, PubMed, DTIC, ToxNet

II.2.2. **Date of Search**: December 1, 2012

II.2.3. **Period of Search**: 1980-2012

II.2.4. **Key Words and Search Strategy**: linolenic acid, soman, rat, nerve agent, nuclear factor kappaB, neuroprotection.

II.2.5. **Results of Search:**

BRD: no hits
DTIC: no hits
PubMed: 3 hit. One paper (b)(6) described the neuroprotective effect of linolenic acid against kainic acid-induced status epilepticus and the second paper described that the protective effect against ischemic stroke requires activated NF-κB. The third paper (b)(6) which was published in the journal *Neurotoxicology* in 2012 (33(5):1219-29). The current project is an extension of the first project and seeks to determine the long-term efficacy and the efficacy of three injections of α-linolenic acid on delayed neurodegeneration and behavior outcome.

TOXNET: No hits
No duplication of the proposed study.

III. **OBJECTIVE\HYPOTHESIS:** Linolenic acid protects against soman-induced neuropathology. If iv linolenic acid is neuroprotective, we will develop subcutaneous neuroprotective doses of linolenic acid for future studies.

IV. **MILITARY RELEVANCE**: The neurological effects of chemical weapons on the performance and operations of our military would be devastating. This is, in fact, a major goal of the terrorists. They want to cripple the military at a sufficient level and long enough so that when the levels of the chemical weapons are no longer toxic the terrorists can penetrate into and destroy military facilities, equipment and soldiers.

V. **MATERIALS AND METHODS:**

V.1. **Experimental Design and General Procedures:**

V.1.3. Specific Aim 3. To determine behavior measures in LIN500-treated rats prior to or after soman injection. Status epilepticus induced by soman damages the following brain regions: piriform cortex, amygdala, hippocampus, thalamus, cerebral cortex and caudate/putamen. Since linolenic acid provides significant protection against soman-induced neuropathology, we wish to determine whether the increased neuronal survival improves functional outcome by performing behavior tests designed to test

specific areas of the brain known to be damaged by soman. The behavior measures performed in this specific aim are: locomotor activity and rotarod test, open field center time, forced swim test, shuttle box performance test, and social interactions. The software also detects seizure activity including onset, duration and frequency. One group of rats will be **pretreated** (500 nmol/kg, iv; 3 doses) with linolenic acid (500 nmol/kg iv, LIN500; ≤300 µL) prior to soman injection (day 1, 3, 7 prior to injection of soman) and a second group will be **post-treated** with 3 doses of linolenic acid (500 nmol/kg, LIN500 iv) on day 1, 3, 7 after injection of soman.

For the pretreatment paradigm: groups of male Sprague-Dawley rats:

Table 1: α-Linolenic acid Pretreatment Groups and Group Sizes for Experiment 1

Treatment Group	**HI-6 30 min Soman 0 min, Atropine 1 min Diazepam 40 min**	**Treatment (Time before seizure onset)**	**Group Size**	**Pain Category**
Group 1 Adult Male Rats	HI-6→Soman→atropine→seizure →diazepam	α-linolenic acid (1 day, 3 days, 7 days)	17 Male Rats	E
TOTAL			**17 Male Rats**	**17 E**

Table 2: α-Linolenic acid Pretreatment Groups and Group Sizes for Experiment 1

Treatment Group	**HI-6 30 min Saline 0 min, Atropine 1 min Diazepam 40 min**	**Treatment (Time before Saline injection)**	**Group Size**	**Pain Category**
Group 2 Adult Male Rats	HI-6→Saline→atropine →Diazepam	α-linolenic acid (1 day, 3 days, 7 days)	9 Male Rats	C
TOTAL			**9 Male Rats**	**9 C**

Table 3: Control Groups and Group Sizes for Experiment 1

Treatment Group	**HI-6 30 min Soman 0 min, Atropine 1 min Diazepam 40 min**	**Saline (Time before seizure onset)**	**Group Size**	**Pain Category**
Group 3 Adult Male Rats	HI-6→Soman→atropine→ seizure→diazepam	Vehicle (1 day, 3 days, 7 days)	17 Male Rats	E
TOTAL			**17 Male Rats**	**17 E**

Because we are performing behavioral measures on the animals injected with α-linolenic acid, all groups of animals, including controls, need to be included in this study.

For the treatment paradigm: groups of male Sprague-Dawley rats:

Table 4: α-Linolenic acid Treatment Groups and Group Sizes for Experiment 1

Treatment Group	HI-6 30 min Soman 0 min, Atropine 1 min Diazepam 40 min	Treatment (Time after seizure onset)	Group Size	Pain Category
Group 4 Adult Male Rats	HI-6→Soman→atropine→seizure →diazepam	α-linolenic acid (1 day, 3 days, 7 days)	17 Male Rats	E
TOTAL			**17 Male Rats**	**17 E**

Table 5: Control groups - No Soman Exposure and Group Sizes for Experiment 1

Treatment Group	HI-6 30 min Saline 0 min, Atropine 1 min Diazepam 40 min	Treatment (Time after Saline injection)	Group Size	Pain Category
Group 5 Adult Male Rats	HI-6→Saline→atropine →Diazepam	α-linolenic acid (1 day, 3 days, 7 days)	9 Male Rats	C
TOTAL			**9 Male Rats**	**9 C**

Table 6: Control groups - Soman Exposure No Treatment and Group Sizes for Experiment 1

Treatment Group	HI-6 30 min Soman 0 min, Atropine 1 min Diazepam 40 min	Saline (Time after seizure onset)	Group Size	Pain Category
Group 6 Adult Male Rats	HI-6→Soman→atropine→ seizure→diazepam	Saline (5 min)	17 Male Rats (17 for each dose)	E
TOTAL			**17 Male Rats**	**17 E**

When the behavioral testing of the rats is completed, all rats will be euthanized, brains fixed, sectioned and stained to quantify neuronal survival and degenerating neurons. **All animals will be weighed daily. During the first 24 hours after soman injection, laboratory personnel will stay with animals in LAM. After the first 24 hours, laboratory personnel will check animals every 8 hours. All animals will receive Lactate Ringer's (5 ml, sc) or more if the rats are not drinking up to 25 ml/day, wet mash, apple slices and gel squares. We will determine the survival rate of all animals and quantify the results just prior to perfusion.**

Methods

Chemicals. Linolenic acid will be purchased from Nu-Chek Prep, Elysian, Minnesota. All other reagents except soman will be purchased from commercial sources or as

indicated. Soman will be supplied by Dr. (b)(6) at the (b)(6) (b)(6)

Drug treatments. Linolenic acid will be freshly prepared on the day of experimentation. Linolenic acid will be first dissolved in ethanol at a molar concentration and then diluted in NaCl 0.9% solution to reach a final concentration of 500 µM. The pH of the solution will be adjusted to 7.0 for bolus intravenous injection. Vehicle (0.05% ethanol) will be prepared in an identical fashion but in the absence of linolenic acid.

Soman injections: Soman (180 ug/kg, sc in Sprague Dawley male rats, ~300g weight) will be injected in conjunction with pretreatment with the oxime HI-6 (125 mg/kg, ip, 30 min before soman) and post-exposure treatment with methyl atropine (2mg/kg, im, 1 min after soman). Under these conditions, seizures are produced with a high probability (>95%). Animals are allowed to seize for 40 min and then treated with 10 mg/kg, im, diazepam (valium) to stop the seizures.

Linolenic acid treatment procedure (Specific Aim 1). Linolenic acid-treated rats will be intravenously injected into the jugular vein as a bolus (500 nmol/kg body weight) 30 min after soman challenge. Vehicle-injected animals will be treated in an identical manner and will be used as controls for the treatment group.

Quantification of viable neurons. The neuronal density of the six brain regions will be quantified. For example, analysis of neuronal density in the hippocampus will be performed on coronal sections of the dorsal hippocampus stained with cresyl violet and corresponding to brain sections located between 3.14 and 4.16 mm posterior to bregma. Neurons will be scored as undamaged if they are Nissl-positive with a round to oval shaped cell body that exhibits no evidence of cell shrinkage. Degenerating neurons characterized by shrunken cell bodies will be excluded. Neuronal density values will be expressed as mean ± SD. Counts are performed by an investigator without knowledge of the treatment history of the rats.

V.2. Data Analysis: Values from experimental and control groups are averaged and compared using analysis of variance ($p<0.05$ to reject the null hypothesis that the group means were equal). Post hoc analyses will use the Tukey test for multiple group comparison. A sample size of 17 animals per group will have 80% power to detect differences between groups of 1.2 standard deviations, based on one-way ANOVA with Tukey's post-hoc multiple comparisons among 3 group means with a 5%, two-sided significance level. Even with death of 8 animals per group, a sample size of 9 animals per group will have 80% power to detect differences between groups of 1.6 standard deviations, based on one-way ANOVA with Tukey's post-hoc multiple comparisons among 3 group means with a 5%, two-sided significance level.

V.3. Laboratory Animals Required and Justification: Chemical warfare agents or nerve agents are some of the most lethal compounds known to mankind. Nerve agents induce severe seizures, brain damage and death. Anticholinergic drugs are only effective if given within 5 minutes after the initiation of seizures. Thus, these agents have limited value in protecting the brain against nerve agent-induced seizures. We have convincing data indicating that linolenic acid, a dietary supplement that can be

purchased over the counter, is an efficacious neuroprotective compound against soman-induced neuropathology.

Mortality Rate:
The protocol involving the use of soman was developed by Dr. (b)(6) my collaborator at (b)(6) Dr. (b)(6) is a senior scientist at (b)(6) and an established investigator in the nerve agent field. According to Dr (b)(6) the dose of soman used to inject the animals is the dose that elicits seizures in 100% of the animals. Animals must seize for 40 min in order for the brain to be damaged. If the animals don't seize, they have to be euthanized and cannot be used for further study. The dose of soman used in this study is 1.6 x LD_{50}. The LD_{50} is the dose that kills 50% of the animals so 1.6xLD_{50} will result in more than 50% death in soman-treated animals alone. You can see from Dr (b)(6) publications that he uses 2xLD_{50} often to determine efficacy (21-26). Also, our work showing that alpha-linolenic acid is protective against soman-induced neuropathology was recently published as well using the method listed in this animal protocol (27).

V.3.1. **Non-animal Alternatives Considered**:
Because we are studying cellular mechanisms and responses, there are currently no non-animal alternatives that can meet our needs for these studies.

V.3.2. **Animal Model and Species Justification**: The male Sprague-Dawley rats for the *in vivo* studies were selected because this is the common gender and species of rat used in the literature. This allows us to compare our results directly with the many published results from other laboratories using chemical warfare agents or other acetylcholinesterase inhibitors such as paraoxon.

V.3.3. **Laboratory Animals**

		Species #1	Species #2
V.3.3.1.	**Genus & Species**:	Rattus norvegicus	
V.3.3.2.	**Strain/Stock**:	Sprague-Dawley	
V.3.3.3.	**Source/Vendor**:	(b)(4)	
V.3.3.4.	**Age**:	adult	
V.3.3.5.	**Weight**:	250-275 gm	
V.3.3.6.	**Sex**:	male	
V.3.3.7.	**Special Considerations**:	N/A	

V.3.4. **Number of Animals Required (by Species)**: 86

V.3.5. **Refinement, Reduction, Replacement (3 Rs)**:

V.3.5.1. Refinement: All surgeries will be performed under appropriate anesthesia. Any rat exhibiting signs of pain or impairment beyond that from this model will be euthanized at an early endpoint. No other refinement alternatives were considered. All animals will be weighed daily. During the first 24 hours after soman injection, laboratory personnel will stay with animals in LAM. After the first 24 hours, laboratory personnel will check animals every 8 hours. All animals will receive Lactate Ringer's (5 ml, sc) as needed (up to 25 ml/day), wet mash, apple slices and gel squares. We will determine the survival rate of all animals and quantify the results just prior to perfusion (the day after the behavioral measures are completed).

V.3.5.2. Reduction: We are using 17 rats/group which provides a sufficient number to determine statistical significance.

V.3.5.3. Replacement: Using rats will allow us to study whether linolenic acid protects against soman-induced neuropathology without using higher species. No other replacements were considered.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	18	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	68	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

Anesthesia for placement of external jugular catheter:
Rats will be placed in an induction chamber then anesthesia will be induced with 3-4% isoflurane from a precision vaporizer delivered in 1-2 Liters per minute of oxygen. Once the rats are in lateral recumbancy, they will be moved to a mask and prepared for surgery. Maintenance anesthesia will be delivered at 1-3% isoflurane in 0.5 to 1 Liter per minute of oxygen. Anesthesia will be adjusted based on response to toe pinch and depth of respirations. Once animal is adequately anesthetized (no response to toe pinch) then surgical procedures will begin. All rats except for naïve will have external jugular catheters placed for injection of either vehicle or linolenic acid.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Animals will have 7 days to acclimate to their surroundings once delivered to USUHS and before being

used for any *in vivo* experiments. Rats will be observed by the PI or technician to ensure they appear healthy prior to undergoing surgery.

Animal monitoring. The animals will be watched continuously for 1h and then checked every 30 min until the animal is able to move. Researcher personnel will monitor the rats twice a day beginning on the following day after soman. Monitoring will be performed in the rat's home cage, and will continue every day up to the point they areeuthanized Any animals that show unexpected neurologic signs following soman injection or hunched posture, lethargy, anorexia, or other clinical signs potentially indicative of infection may be euthanized at an early endpoint or treated at the discretion of the veterinarian and/or PI. Research personnel will also weigh rats once a day to ensure they have not lost >20% of their body weight compared to baseline weights. Supplemental feeding in the form of wet mash will be provided by LAM personnel at the request of the PI or as directed by the LAM Veterinarians and fluids (5 ml Lactated Ringer's sc 2 to 3 times a day) will be administered to rats injected with soman by research personnel. Gel squares will also be placed on the floor of each cage and replaced every day by laboratory personnel.

Supplemental (alternative method) feeding. LAM personnel may place wet mash/chow on the cage floor for rats that may not be able to gnaw pellets to get food.

Soman-induced seizures: The animals will not receive anesthesia or analgesia during the time the animals seize following injection of soman. Rats receiving soman will be injected with the oxime HI-6 (125 mg/kg, ip) and 30 min later will receive soman (180 ug/kg, sc) and post-exposure treatment with AMN (2 mg/kg, im, 1 min after soman. All soman-treated animals will receive diazepam (10 mg/kg im) 40 min after onset of status epilepticus.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures: There is no alternative for inducing brain damage in rats that mimics the damage caused by soman to determine whether the proposed research plan using linolenic acid protects against soman-induced brain damage or improves functional outcome. The only way to carry out the research plan is to allow the animals to seize for 40 min as recommended by Dr. (b)(6) at (b)(6) followed by injection of diazepam to stop the seizures.

V.4.1.3.1. Sources Searched: BRD, DTIC Private STINET, NIH Reporter

V.4.1.3.2. Date of Search: December 1, 2012

V.4.1.3.3. Period of Search: 1980-2012

V.4.1.3.4. Key Words of Search: rat, pain, kainic acid, seizure, hippocampus, piriform cortex, amygdala, neuronal damage, brain damage, gluatamate receptor, glutamate receptor, animal welfare, BHLHB2, alternative, reduce, refine, prevent, control, manage, eliminate, alleviate, decrease, diminish.

V.4.1.3.5. Results of Search:

DTIC Private STINET: 520 hits. One study discussed seizures as it relates to chemical warfare agents and measuring seizures on the battlefield. These studies offered no advantage over the proposed methods.

BRD: no hits

NIH Reporter: 17 hits. Several discussed reduce or refine but were not relevant to the proposed study.
Soman-induced convulsions: The treatment plan for inducing status epilepticus by soman in rats is a standard procedure that is routinely performed by many laboratories including our laboratory and outlined in the scientific literature. Sprague-Dawley rats have been used in the literature.

Interpretation: There presently is no alternative to employment of rat as an *in vivo* animal model to study the role of multiple doses of linolenic acid to improve neuroprotection against soman-induced neuropathology or to determine whether improved neuronal survival in linolenic acid-treated rats improves overall functional outcome by behavioral testing.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: Sixty eight male Sprague-Dawley rats will receive soman at a dose that will induce seizures, an effect that is seen in humans exposed to nerve agents. The rats will experience some level of seizure or physical stress and discomfort for some period of time during the experiments. Analgesic, sedatives and/or anesthetic agents cannot be used because they will modify the pain and seizure threshold preventing us from fully evaluating the effect of soman and as such we will not be able to draw any conclusions on the proposed effect of linolenic acid on neuronal survival or functional outcome. The objective of this study is to determine whether the increase in neuronal survival in linolenic acid-treated rats improves functional outcome. Without exposing the animals to soman, it is not possible for us to conduct these studies. These studies are justified because the use of soman is a model for nerve agent-induced seizures, a expected effect of nerve agent exposure of military personnel in international conflicts and civilian populations. Identification of new neuroprotective agents is highly relevant to enhance treatment response capabilities. Thus, the knowledge obtained from the proposed investigations may benefit many active duty personnel as well as civilians.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: N/A

V.4.3.1. Pre-surgical Provisions:
Surgeons will wear sterile gloves, mask, lab coat or scrub top. A general assessment of physical and behavioral health will be carried out on each rat before surgery. LAM veterinarians will be consulted should any sign of abnormality in appearance and/or behavior be observed.

V.4.3.2. Procedure: Rats will be anesthetized and maintained with isoflurane in oxygen by nosecone (see **V.4.1.2.1.**). After the induction of anesthesia, the rats (with the nosecone) will be placed in lateral recumbancy and prepared for surgery. Maintenance anesthesia will be delivered at 1-3% isoflurane in 0.5 to 1 Liter per minute of oxygen. Anesthesia will be adjusted based on response to toe pinch and depth of respirations. Once animal is adequately anesthetized (no response to toe pinch) then surgical procedures will begin. Only sterile instruments will be used. A supplemental heat source, such as an electric heating pad or circulating warm water blanket, will be used to keep the rats warm throughout the duration of anesthesia. Rats will be monitored for dermal burns.

Placement of external IV catheter. Anesthetized rats will be placed in dorsal recumbancy. Fur will be clipped from the ventral neck and interscapular region. Skin will be prepared for aseptic surgery using either betadine or alcohol or chlorhexidine scrub. The animal will be draped using sterile towels. A 1.5 to 2 cm long incision will be made in the ventrolateral aspect of the neck using a scalpel, parallel and approximately 0.5 cm lateral to midline. The external jugular vein will be dissected free of surrounding tissue and stabilized with a proximally placed tie of 4-0 suture. A catheter introducer will be used to guide the placement of the IV catheter. A 4-0 ligature will be placed at the distal end of the catheter to secure it in place. Tissue adhesive may also be used to help stabilize the catheter in the jugular vein. Catheter will be tested for patency by flushing with sterile saline. The catheter will then be tunneled subcutaneously to exit via an approximately 2.5 cm incision in the interscapular region. Wound clips or suture material will be used to close the interscapular area. Tissue glue or sterile 4 O silk will be used to close the ventral neck incision.
If placement in one side of the jugular area is problematic, the procedure will be repeated on the opposite jugular vein.

V.4.3.3. Post-surgical Provisions:
Immediate post-operative care will be provided by the investigators and rats will be monitored continuously until they are ambulatory. Animals are expected to ambulate. Animals will be returned to their cages post-surgery only after they have recovered sufficiently from anesthesia. Food and water will be provided ad libitum. Room maintenance and climate control is as per LAM SOP. Investigators will perform daily weights, provide supplemental calories (as appropriate) via wet mash and sc lactate ringers (5 ml) if weight loss is greater than 20% until study completion. Following surgery, rats will be monitored continuously until they are ambulatory. The incision site will be monitored daily for signs of infection (erythema, edema, presence of an exudate, induration etc). Because the catheter will be inserted into the external jugular vein, we will also examine the area for signs of bleeding. Catheters will remain in place for about 3 weeks (Specific Aim 3). Each catheter will be flushed once daily with heparinized saline to prevent clotting of the tip.

In addition to Center for LAM SOP's, study personnel will monitor animals using the checklist for animals exhibiting pain/distress beyond expectations of this study. If LAM personnel notice any of the following: constant convulsions or tremors, self mutilation, continuous circling, or persistent labored breathing, and study personnel are

unavailable, the LAM veterinarian may make the decision to euthanize the animal. If animals continue to lose weight despite supplements (wet mash etc) or remain comatose for more than 24 hours, these animals will be euthanized by laboratory personnel or LAM.

V.4.3.4. Location: (b)(6)

V.4.3.5. Surgeon: Dr. (b)(6) (competent in placement of jugular vein catheter)
Ms. (b)(6) (graduate student)

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures:

V.4.3.6.2. Scientific Justification:

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

Soman treatment (Specific Aims 3). Male Sprague-Dawley rats (250-300 gm) receiving nerve agent will be injected with the oxime HI-6 (125 mg/kg, ip) and 30 min later will receive soman (180 ug/kg, sc) or saline and post-exposure treatment with atropine methyl nitrate (AMN, 2 mg/kg, im, 0.16-0.19 mL), 1 min after soman. These treatments, HI-6 (125 mg/kg, ip) and 30 min later soman (180 ug/kg, sc) and post-exposure treatment with ANM (2 mg/kg, im) 1 min after soman, have been shown to reliably produce electrographic seizure activity within approximately 10 min. Rats are allowed to seize for 40 min and then treated with 10 mg/kg, im, diazepam (25 gauge needle, 0.67-.74 ml) to stop/attenuate the seizures (as per Dr. (b)(6) protocol at (b)(6)

Linolenic acid will be injected via catheter. We will use a needle size ranging from 22 to 25 gauge and the injected volume is 0.3-35 mL.

Lactate Ringer's solution. After the first 24 hours, laboratory personnel will check animals every 8 hours. All animals will receive Lactate Ringer's (5 ml, sc) or more if the rats are not drinking up to 25 ml/day, wet mash, apple slices and gel squares.

Transcardial perfusion. When rats are euthanized, they will be placed under deep pentobarbital (50-75 mg/kg, i.p.) anesthesia. Once adequate anesthesia is achieved (lack of response to toe pinch, slow even respirations), a midsternal thoracotomy will be performed to expose the heart and the rats will undergo transcardial perfusion with cold PBS and 10% formalin. Generally, about 200 cc of each solution is sufficient per animal, but, more will be used if required.

V.4.4.2. Biosamples:

In vivo experiments. The brains from the male Sprague-Dawley rats will be collected at the time of euthanasia Brain will be preserved for future studies. Hippocampi may need to be removed to determine activated NF-κB levels, a biomarker of linolenic acid.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification:

A patch will be shaved on each animal's back and then marked with non-toxic permanent ink with an individual ID. Sometimes, the ink fades in which case the ID will be re-inked on the patch. All cages have cage cards per LAM. Rats that receive nerve agent injection will be checked for their ink mark labels prior to injection of nerve agent to make sure they are clearly labeled.

V.4.4.6. Behavioral Studies:

Activity and Movement: General activity, movement, and coordination can be used as indices of overall health and well-being of rodents. Activity and movement serve as negative controls (brain areas relevant to movement should not be disrupted in the current study), and as a covariates for the other behavioral tasks that rely on animal locomotion for task completion. The locomotor and rotarod tasks have been used reliably for these purposes in Dr. (b)(6) lab for many years.

Open field locomotor activity. Locomotor activity is measured using an Omnitech Electronics Digiscan infrared photocell system. One-hour activity measurements are obtained (preferably during animals' active or dark cycle). Animals are placed singly in a 40 X 40 X 30 cm clear Plexiglas arena covered by a perforated Plexiglas lid. Data are automatically gathered and transmitted to a computer via an Omnitech Model DCM-I-BBU analyzer. The interfaced software measures 21 activity variables, including total distance and horizontal and vertical activity. Chambers are cleaned between subjects with 35% isopropyl alcohol solution.
The duration of locomotor activity testing is a maximum of 60 min per day and can be performed on multiple days throughout the duration of the experiment.

Rotarod: The rotarod test measures an animal's ability to maintain balance by coordinating movement and making postural adjustments. It also measures the ability to improve these skills with practice. Each rat rotarod treadmill (Med Associates, Inc, St. Albans, VT) consists of a motor-driven drum with constant speed or accelerating speed modes of operation. The drum (7.0 cm diameter) allows each animal to maintain a suitable grip. It is divided into four test zones (8.9 cm wide) so that up to four animals may be tested at the same time. The device consists of a smooth hard plastic cylinder with concentric circular plastic sides (39.7 cm diameter) to prevent the rat from laterally climbing off the cylinder.
Rats are placed on the device facing the wall with the rod rotating in the direction opposite from the animal (so that the rat uses its paws to pace forwards and avoid

falling off the rod). When a rat falls off the rotating drum (height of 26.7 cm), it breaks a photobeam, stopping the timer associated with that zone. In each trial, the speed of rotation is increased from 0 revolutions per minute (rpm) to 35 rpm for a maximum of 3 or 5 min. The mean duration on the device (based on 3 consecutive trials) is recorded. The procedures are based on reports in the literature (6-9). Chambers are cleaned between subjects with 35% isopropyl alcohol solution. The duration of rotarod measurement is 9 or 15 min per day can be performed on multiple days throughout the duration of the experiment.

Activation of the amygdala, a brain region vulnerable to soman-induced neuronal cell death, is involved in anxiety and stress-related behaviors. This can be measured using the total time spent in the center of an open field arena or number of entries into the center of the field.

Anxiety

Open field center time. The interfaced software measures time spent in the center vs. the margin of the open field. More time spent in the center of the apparatus is indicative of lower anxiety because the center of an open field may be more dangerous than the sides and corners. Chambers are cleaned between subjects with 35% isopropyl alcohol solution. The duration of open field center time measurement is 60 minutes per day, and data is gathered at the same time as general activity described above under "Open field locomotor activity" and can be performed on multiple days throughout the duration of the experiment.

Research in humans and animals has clearly established that the hippocampus is involved in learning and memory (2). Rats lesioned in the hippocampus have a difficult time acquiring active avoidance responses (3,4) and retaining passive avoidance responses over a period of time (3-5).

Learning and Memory

Passive avoidance: Animals are trained and tested using an automated avoidance training system (Med Associates, St. Albans, VT) consisting of two 21 x 25 x 17 cm chambers separated by a vertically-sliding door. This task consists of a training day and a consecutive testing day. During training, the animal is placed in one chamber of the darkened apparatus. After a delay of 60 seconds, a light comes on and the door to the other, still darkened chamber, opens. Naive rats instinctively move quickly from the lit chamber into the dark chamber. When the rat crosses completely into the darkened chamber, the door closes, latency to cross is recorded by the interfaced computer, and a 0.8 mA (mild, non-damaging) shock is delivered through the grid floor for 1 sec. The rat is left in the darkened chamber in which the shock has been delivered for 30 sec to facilitate the formation of memory for the aversive event, and then removed. The procedure on testing day is identical except that the shock is not delivered if/when the animal crosses into the darkened chamber. Memory is presumed to have occurred if the animal does not cross into the chamber in which it previously was shocked, or if latency to cross is significantly longer during the testing trial than during the training trial. The duration of passive avoidance testing is 5 min on each of two consecutive days,

and is only performed once during an experiment. Chambers are cleaned with 35% isopropyl alcohol between animals.

Active Avoidance: The active avoidance task is a fear-motivated associative avoidance test based on electric current as a source of punishment. In this task, a rodent learns to predict an aversive event (0.8 mA shock for 1 sec; **1 shock on day of training and 1 shock on testing day if they don't remember the cue that they will be shocked**) based on the presentation of a specific stimulus (lit chamber becomes darkened), and avoid the aversive event by actively moving into a lit compartment (10-11). **The total number of shocks each animal receives on the training day is one. If the animal remembers the cue that he will be shocked on the testing day, the rat does not get shocked. If he doesn't remember, the rat gets shocked only once. Maximum number of shock sessions is 2 (one training day and one testing day).**
The measures recorded are: number of avoidances (rodent crossing to other compartment during stimulus signal), number of non-responses (rodent failing to cross during stimulus signal), and response latency (latency to avoid aversive stimulus). These measures serve as an index of learning and allow memory to be assessed. This task takes place in the shuttlebox apparatus described above for passive avoidance. It is performed on 2 consecutive days once during an experiment. Chambers are cleaned with 35% isopropyl alcohol between animals.

The prefrontal cortex is involved in complex executive function, including planning, decision making, and controlling social behavior in humans. The social interaction paradigm has been used in rats to assess functioning of the prefrontal cortex in a rat model of schizophrenia (12). Further, rodent social interaction is correlated with neuronal morphology in the prefrontal cortex (13). The piriform cortex, also called the olfactory cortex, is related to processing of olfactory cues. Because rodent-rodent interaction involves sniffing and smelling one another, the social interaction paradigm will also be used to assess piriform cortex function.

Interaction with Conspecifics
Social interaction observation: Social interaction is videotaped in a 40 X 40 X 30 cm clear Plexiglas arena. A shield made of cardboard measuring 45 cm in height is placed around three sides of the test arena at a distance of 10 cm to minimize subject distraction. The camera is placed 30 cm away from the unshielded side of the arena. Video cables connect cameras to taping equipment nearby. Following each social interaction, arenas are cleaned with 35% isopropyl alcohol solution. Animals are paired for testing with another animal of the same sex and in the same experimental condition. Pairs are determined so that body weights are comparable. Prior to taping of the interaction, animals are placed in opposite corners of the arena and a slotted Plexiglas lid is placed on top of the arena. Social interactions are observed or videotaped for 10 min for later scoring and analyses. We have not observed fighting to occur during the social interaction test. Animals typically engage in a range of social behaviors, including grooming, following and sniffing one another, and wrestling behavior (similar to wrestling behaviors exhibited by cage mates) but we have never seen an instance of biting. Animals are monitored continuously during the test both on video monitor and by listening for vocalizations (because fighting generally is accompanied by vocalizations). Should fighting occur, animals will be separated immediately and the test will be

terminated. The duration of the social interaction test is 10 min and can be performed multiple times throughout the experiment.
Live scoring and/or videotapes made during the testing sessions will be quantified by a sampling procedure in which the behavior of each animal is scored by a trained rater once every three seconds for the entire 10 min period. Behaviors are separated into three categories for analysis: Exploratory, Social, and Other. These procedures are based on classic protocols used to score and analyze behavior of children interacting in pairs (57, 58) and we have applied these same scoring categories to analyses of social interactions by rats (14, 15).

Human and animal studies have shown that the amygdala may be implicated in depressive states and expression of depressive behaviors. In humans, increased amygdale volumes positively correlated with depressive symptoms in temporal lobe epilepsy (67). Rodent models have found that greater depressive-like behavior on the forced swim test is correlated with amygdala activation (16).

Depression
Swim test: The Porsolt swim test is widely used as an index of learned helplessness or depression (17-20). The procedure involves a repeated procedure in which rats are placed into a cylinder (approximately 65 cm tall and 25 cm diameter cylinder filled to a height of 48 cm with water that is between 28-32 °C on the first day for 15 minutes. After 15 minutes of swimming, the rats are removed from the water and are dried and warmed with towels and heat lamps. On subsequent days, rats are retested for 5 minutes under identical conditions to determine the latency to become immobile (i.e., the rat initiates a stationary posture that does not reflect attempts to escape from the water), amount of time spent immobile, and number of times the animal becomes immobile, as determined by video analysis software (AnyMaze, Stoelting, Wood Dale, IL). After 5 minutes, the animals are removed and then dried and warmed with towels and heat lamps. On the second day of testing, shorter latencies to assume an immobile, floating position, more instances of immobility, and longer durations of immobility are interpreted as increased helplessness or depression. All rats are monitored the entire time and are removed from the water if they cannot swim or show signs of distress. Feces will be removed from the water between animals, and water will be replaced if it becomes too murky. The test lasts 15 minutes on the training day, and 5 minutes the consecutive testing day. This procedure can be performed several times throughout the experiment.
Seizure Observation
Animals will be placed into a cage separate from their homecage for observation and video analysis (AnyMaze, Stoelting, Wood Dale, IL) of seizure activity. Seizure activity is manually keyed for the duration of the seizure. AnyMaze software can analyze the video for latency to seize, seizure duration, and number of seizure occurrences.

Seizure Observation
Animals will be placed into a cage separate from their homecage for observation and video analysis (AnyMaze, Stoelting, Wood Dale, IL) of seizure activity. Seizure activity is manually keyed for the duration of the seizure. AnyMaze software can analyze the video for latency to seize, seizure duration, and number of seizure occurrences.

Timing of behavioral Tests

Day 1	Day 2	Day 3	Day 4
Open field activity (training) Rotarod	**Open field activity testing Passive Avoidance training**	**Passive avoidance testing Forced Swim Test training**	**Forced swim testing**

Behavior tests are performed 14 days after soman exposure. We are performing four behavior tests: rotarod, open field activity, passive/active avoidance and the forced swim test. On any given day, animals are subjected to 1 training and testing/day or a training combined with the rotarod because rotarod does not require training. Animals that undergo testing for the forced swim test are not subjected to any other training or testing during the remainder of the day. All behavior tests are completed in four days/group of animals.

V.4.4.7. Other Procedures:

Transcardial perfusion. When rats are euthanized they will be placed under deep pentobarbital (50-75 mg/kg) anesthesia as described above. Once adequate anesthesia is achieved (lack of response to toe pinch, slow even respirations), a midsternal thoracotomy will be performed to expose the heart and the rats will undergo transcardial perfusion with cold PBS and 10% formalin. Generally, about 200 cc of each solution is sufficient per animal, but, more will be used if required. Transcardial perfusion will be performed in (b)(6)

Animal transportation: All animals will be transported to (b)(6) on the morning soman is to be injected with soman in accordance with the LAM transportation SOP. Animals will be pre- or post-injected with linolenic acid or saline. **An animal protocol will also be submitted to** (b)(6) **for approval.** The rats will be brought back from (b)(6) to LAM by lab personnel on the day of nerve agent injection where they will be placed in home cages (1 rat/cage).

General histological procedures. For each brain studied, we will place two sections per slide and prepare 60 slides per rat. Every other slide will be stained with cresyl violet and every other slide will be stained with FluoroJade. Nissl-positive undamaged neurons and fluorescent-positive degenerating neurons are counted in the six brain regions [piriform cortex, hippocampus (CA1, CA3), cerebral cortex, amygdala, thalamus, caudate/putamen]. Counts will be performed without knowledge of the treatment history of the rats.

Cresyl Violet staining. Dried slides will be stained with cresyl violet in the following manner:

Treatment	Time
95% ETOH	15 min.
70% ETOH	1 min.

50% ETOH	1 min.
DH2O	2 min.
DH2O	1 min
Cresyl Violet Stain*	2 min.
DH2O	1 min.
50% ETOH	1 min.
70% acid ETOH**	2 min.
95% ETOH	2 min
95% ETOH	a few dips
100% ETOH	1 min
Histoclear	mounting media

*Cresyl violet preparation: add 1.25g cresyl violet acetate and 0.75 ml glacial acetic acid to 250 ml warm DH20, cool and filter. **2 ml glacial acetic acid in 200 ml 70% ETOH. Note: Some people find histoclear vapor to be irritating and may require a mask.

Histofluorescent labeling of degenerating neurons with Fluoro-Jade B (Specific Aim 1 and 2). Formalin-fixed brains will be prepared as outlined above (Perfusion of rats for brain fixation) and brain sections will be cut through the designated brain regions on a cryostat.
The sections will be collected in 0.1M neutral phosphate buffer and mounted on 2% gelatin coated slides and then air dried on a slide warmer at 50 □C for at least 30 min. The slides will be transferred to a solution of 0.06% potassium permanganate for 10 minutes on a shaker table to insure consistent background suppression between sections. The slides will be rinsed in distilled water for 2 minutes. From this point forward, the protocol is performed as much as possible away from the light. The staining solution will be prepared from a 0.01% stock solution for Fluoro-Jade B (10 mg of Fluoro-Jade B in 100 mL of distilled water). To make up 200 mL of staining solution, 20 mL of stock solution will be added to180 mL 0.1% acetic acid (180 µL Acetic Acid in 180 mL distilled water). This results in a final dye concentration of 0.001%. The stock solution is stable for 2 months when stored at 4 □C but the freshly made staining solution is prepared within 10 minutes of use and will not be reused. After 30 minutes in the staining solution in the dark, the slides will be rinsed for one minute in each of three distilled water washes. Excess water will be removed by briefly (about 15 seconds) draining the slides vertically on a paper towel. The slides will be rapidly placed on a slide warmer, set at approximately 50 °C, until they are fully dry (about 5-10 minutes). The dry slides will be cleared by immersion in xylene for at least one minute before coverslipping with DPX (Sigma-Aldrich, St. Louis, MO), a non-aqueous non-fluorescent plastic mounting media. Sections spaced 125 µm apart will be viewed under fluorescein (FITC) optics in an Axiovert Zeiss microscope equipped for epifluorescence and photographed (10x magnification) using Axiovision software. A grid (170 x 170 µm) will be placed over a representative field in each of the six brain regions and the number of fluorescent cells will be counted, providing an estimate of the mean number of degenerating neurons for each animal. The mean number of Fluoro-Jade B-positive neurons ± SD will be calculated and graphed as a histogram.

V.4.4.8. Tissue Sharing: The brains from Specific Aim 1-3 will be collected at the time of euthanasia and remain available for future studies. All other tissues (organs etc) will be made available to other investigator upon request.

V.4.5. Study Endpoint: The study endpoint for rats used for Specific Aim 1-3 is survival until the pre-determined post-operative time point, after which they will be euthanized. Prior to euthanization rats will be anesthetized then euthanized for tissue collection as indicated above.

V.4.6. Euthanasia:

Experiment 1-3: Research personnel will perform all euthanasias except in cases where a LAM veterinarian makes the decision that a particular animal needs to be euthanized. At the indicated endpoint, each mouse will be deeply anesthetized with Pentobarbital (60-75 mg/kg ip). Animals should be deeply anesthetized within 30 minutes. A midsternal thoracotomy will be performed to expose the heart and the mice will undergo transcardial perfusion with PBS and the 10% formalin in PBS. This procedure results in euthanasia from exsanguination under deep nembutal anesthesia.

Rats that reach an early endpoint (see **V.4.5**) will be euthanized via cylinderized CO_2. Research personnel or the PI will perform this early euthanasia. The apparatus for euthanasia by CO_2 is located in (b)(6) Alternately, if requested by the PI, animals will be euthanized by Center for LAM personnel) using cylinderized CO_2 in compliance with the 2008 AVMA Guidelines on Euthanasia. Euthanasia will be performed in the LAM CAF. If possible, animals will be euthanized in their home cage. If not, a clean rodent cage will be used. Animals will be placed in the cage so that it is not overcrowded (each animal can place all four feet on the bottom of the cage.) With animals in the chamber, the flow rate of CO2 will displace at least 20% of the chamber volume per minute. Gas flow will be maintained for at least one minute after respiration has stopped before removing animals from the cage. A supplemental method to ensure death (cervical dislocation or bilateral thoracotomy) may be used.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes	_____	No	x
Fluid Restriction:	Yes		No	x

V.5.1.3. Exceptions: All animals will be housed individually after insertion of IV catheter. Animals injected with soman will be given apple slices, wet mash and gel squares as food supplements on the floor of each cage.

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions:

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
small animal surgery, Drug injections, tissue dissection, euthanasia, transcardial perfusion	(b)(6)	1 year experience with animal surgery; 3 + years rodent handling and 3 years experience with tissue dissection and transcardial perfusion	Investigator course and rodent handling course (July, 2012)
Catheter placement, small animal surgery, drug injections, tissue dissection, euthanasia, transcardial perfusion, brain section cutting, brain section staining and counting		15 year experience in rodent handling, surgery and tissue dissection, 5 years experience in transcardial perfusion	Investigator course and rodent handling course (1 June 2007)

Animal handling, agent injections, drug injections	(b)(6)	39+ years rodent handling, surgery and tissue dissection; 15 years in EEG electrode emplacement in primates; 39 years EEG electrode emplacement in rodents	Occ Health/zoonotic disease (01/05/04), Rodent Handling (02/05/04), aseptic techniques and rodent anesthesia (November 2006), Anesthesia (March 2006) and XCSM (nerve agent) custodian
Euthanasia via transcardial perfusion		30+ yr rodent handling, surgery, tissue dissection, euthanasia	30 + years experience and on-the-job training in handling small laboratory animals, anesthesia, guillotine usage, perfusion. Rodent handling course and investigator training course at NIH
Animal handling, tissue dissection, drug injections, transcardial perfusion		30+ yr rodent handling, surgery and tissue dissection; 3 yr experience on transcardial perfusion	Investigator training course at USUHS (Nov 1993).
Collaborator for behavioral measures		Animal handling 25+ yrs experience in: injections, drug administration, surgical implant/expaint of osmotic mini-pumps, behavioral measures, biological sample collection anesthesia, decapitation	USUHS investigator Training and Animal Handling courses (several sessions, including sessions in 1980 and in early 1990s); NIH/NIMH training courses (several in mid 1980s); 25+ years experience with rats and other rodents.
Animal Handling, drug injections		Animal handling 3+ years,	USUHS investigator Training and Animal Handling courses; 2010; 5 + years experience with rats.

VII. BIOHAZARDS/SAFETY:

VIII. ENCLOSURES:

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IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 01 | 08 | 13

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 01 | 08 | 13

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) _____ 01/08/13

Principal Investigator Signature Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number: (b)(6)

B. Animal Protocol Title: Linolenic Acid: An omega 3 fatty acid that protects against soman-induced neuropathology in Sprague-Dawley rats

C. Principal Investigator: (b)(6)

D. Performing Organization: USUHS

E. Funding: DTRA

F. Objective and Approach: The objective is to develop innovative and safe strategies to protect the brain against soman-induced neuropathology. The approach is to inject multiple doses of linolenic acid as a post-treatment strategy and determine neuronal survival. We will determine whether the neuroprotective effect of linolenic acid translates in an improvement of functional outcome through behavioral testing.

G. Indexing Terms (Descriptors): linolenic acid, soman, rat, neuroprotection, neurodegeneration, nerve agents, rotorod, open field, depression,

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

April 1, 2014

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF NEUROLOGY

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on April 1, 2014:

Animal Protocol Title: "Possible involvement of Alpha-liolenic acid-induced neuroplastic effects against soman-induced brain damage"

USUHS Protocol Number: (b)(6)

Expiration Date: March 31, 2017

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6). The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6). This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

Revised

IACUC Date Stamp

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Possible involvement of Alpha-linolenic acid-induced neuroplastic effects against soman-induced brain damage

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: approved conveyance to (b)(6)

FUNDING AGENCY: NIH

EARLIEST ANTICIPATED FUNDING START DATE: January 2014

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6)		(b)(6)	
Principal Investigator Signature	INDY Department	Office/Lab Telephone	1/7/14 Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	Chair, Neurology	(b)(6)	8JAN14
Research Unit Chief / Dept. Head Signature Typed Name:	Title	Telephone	Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

Statistician Signature Typed Name:	Department	Telephone	Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)	LAM		1/28/14
Attending/Consulting Veterinarian Signature Typed Name:	Department	Telephone	Date

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6)

ANIMAL PROTOCOL TITLE: Possible involvement of Alpha-linolenic acid (LIN)-induced neuroplastic effects against soman-induced brain damage in male Sprague-Dawley rats

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER:

CO-INVESTIGATOR(S): (b)(6) Ph.D., (b)(6) Ph.D. (b)(6)

TECHNICIANS(S):

I. NON-TECHNICAL SYNOPSIS:

Nerve agents represent a key threat to the United States military and civilian populations. These agents block a crucial brain enzyme, acetylcholinesterase, which in turn leads to brain hyperactivity. Brain hyperactivity results in seizures. A severe type of seizure is status epilepticus, seizures lasting more than 5 minutes, and is commonly observed following exposure to chemical warfare agents. Status epilepticus is a medical emergency because it can lead to severe brain damage and/or death. The current therapy for nerve agent-induced status epilepticus is ineffectual in preventing the brain damage. Thus, compounds that protect the brain against nerve agents are required to attenuate or prevent damage and improve outcome.

α-Linolenic acid (LIN) is an omega-3 fatty acid found in vegetable products such as flax and pumpkin seeds. Linolenic acid is a dietary supplement that can be purchased over the counter. α-Linolenic acid has been shown to significantly protect against the brain damage caused by status epilepticus induced by kainic acid, a compound that is found in a particular type of seaweed. Therefore, we tested whether linolenic acid may protect against soman-induced brain damage caused by status epilepticus. Our data show that administration of a single dose of α-linolenic acid injected intravenously either before or after soman significantly protects vulnerable brain cells against soman-induced brain damage twenty four hours after nerve agent injection. Our most recent results provide convincing evidence that three doses of α-linolenic acid administered by intravenous injection after soman significantly protects against soman-induced brain damage. Brain networks were examined through behavioral testing. Administration of 3 doses of α-linolenic acid injected after soman exerts an anti-depressant-like activity as measured by the Porsolt swim test, significantly improved performance on the rotarod as well as memory consolidation and memory processes (see below).

Catheters will be placed into a medium sized vein and patency of the catheters will be maintained by a heparin-saline solution. The oxime, HI-6, ill be administered before and atropine and diazepam will be administered after injection of the nerve agent, soman. HI-6, atropine and diazepam improve survival of animals exposed to soman. The

neuroprotective agent, α-linolenic acid, will be injected into the animals three times over a 7 day period via the catheters after nerve agent.

We now wish to test the hypothesis that the well-established *in vivo* α-linolenic acid-induced neuroplastic effects contribute at least in part to the observed increase in animal survival, neuronal survival and improvement in functional outcome by activating the mammalian target of rapamycin in the hippocampus.

II. BACKGROUND:

II.1. Background:

Nerve agents and neurodegeneration. Nerve agents irreversibly inhibit the cholinesterase enzyme. Inhibition of acetylcholinesterase (AchE) results in elevated acetylcholine (Ach) levels in synapses in the peripheral and central nervous system (CNS) leading to the progression of toxic signs including hypersecretions, tremors, seizures, respiratory distress and ultimately death.

It is established that prolonged seizures are responsible for the neuropathology caused by nerve agents. Termination of seizures within 10 min results in no neuronal cell death whereas 20 minutes of seizure activity results in mild neuronal cell death; further delay in controlling seizures dramatically increases the neuropathology in animal models. Moreover, the longer seizures continue the higher the probability for the development of status epilepticus, a serious complication of seizure activity defined as a prolonged seizure or continuous seizures lasting more than five minutes without regaining consciousness. The brain region that shows the most severe damage is the amygdala followed by the piriform cortex, hippocampus, cortex, thalamus, and caudate/putamen. Anticonvulsants such as benzodiazepines decrease seizure activity and improve outcome but their efficacy to attenuate or abolish nerve agent-induced seizures depends upon the administration time post-exposure of the nerve agent. For example, the mean latency for seizure termination by midazolam (the most effective anticonvulsant) given five minutes after the induction of seizure activity induced by either soman or sarin was twenty minutes. Thus, even under the most ideal conditions, there is attendant brain damage (McDonough et al., 1997). In addition, benzodiazepines such as midazolam can cause respiratory depression and exacerbate nerve agent-induced respiratory depression leading to increased brain injury and/or death.

Glutamate and excitotoxicity. Neurodegeneration caused by prolonged seizure activity is an example of hypoxic-ischemic neuronal injury. The primary event in nerve agent-induced seizures is the excessive accumulation of Ach in synapses which results in the release of the excitatory amino acid glutamate. Glutamate, the endogenous neurotransmitter required for normal physiological excitation, is involved in the pathophysiology of hypoxic-ischemic neuronal injury. Although this neuropathological process can be mediated by any of the excitatory amino acid receptors, the N-methyl-D-aspartate (NMDA) glutamate receptor subtype plays a crucial role. Overactivation of postsynaptic NMDA receptors is thought to result in a massive influx of calcium leading to biochemical and metabolic events that result in neurodegeneration. This process is

referred to as excitotoxicity. The NMDA glutamate receptor subtype plays a crucial role in neuronal cell death mediated by nerve agents. These results confirm the role of NMDA receptors in nerve agent-induced neurodegeneration and validate the mechanism of neuronal cell death.
The vulnerable brain regions that are involved in nerve agent-induced neuropathology would be expected to impair learning and memory (hippocampus, dorsal medial nucleus of the thalamus), emotional memory (amygdala), cognition (cortex) and movement (caudate/putamen). Because of the long latency required to terminate seizures, soldiers are at high risk for developing the neuropathology. Thus, it is essential to develop effective neuroprotective treatments and preventative therapies.

Linolenic acid is an essential polyunsaturated fatty acid (PUFA) and the only omega-3 fatty acid found in vegetable products. It is most abundant in flaxseed oil. It is commercially available and can be purchased over the counter as a nutritional supplement. Protective effects of α-linolenic acid have been convincingly demonstrated in models of stroke, spinal cord injury and brain damage due to status epilepticus induced by kainic acid. PUFAs also have beneficial effects on mood as alpha-linolenic acid has been shown to exert an anti-depressant effect in women. Our most recent work demonstrated that three doses of α-linolenic acid increases neurogenesis, synaptogenesis, synaptic function and brain-derived neurotrophic factor (BDNF) levels in brain.

We have demonstrated previously that administration of a single dose of alpha-linolenic acid injected intravenously into adult male Sprague-Dawley rats either 3 days before or 30 minutes after soman protects significantly against soman-induced brain damage (Pan et al., 2012). We now have convincing evidence that administration of three doses of alpha-linolenic acid injected 30 min, 3 days and 7 days after soman increases animal survival, neuronal survival and improves functional outcome.

Alpha-linolenic acid (LIN) exerts an anti-depressant-like activity. Administration of 3 doses of LIN exerts an anti-depressant activity in soman-treated animals. Groups of animals were administered soman (1.6xLD50 s.c.) followed by α-linolenic acid (500 nmol/kg, iv; LIN500) at 30 min, 3 days and 7 days after soman.
Control groups consisted of saline for soman and vehicle (0.05% ethanol) for LIN500; vehicle (veh) was injected intravenously.
Two weeks after soman/veh exposure, all groups of animals were subjected to the Porsolt forced swim test (Porsolt et al., 1977). The forced swim test (FST) is a behavioral despair test and is the most widely used test to screen antidepressant drugs. Exposure to soman significantly increased immobility time; post-treatment with three doses of LIN500 following soman elicited a significant antidepressant-like effect, producing a 3.8-fold decrease in the FST immobility time compared with soman/vehicle (Figure 1). The locomotor activity in LIN500-injected rats

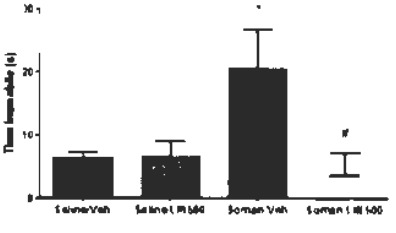


Figure 1. Three doses of α-linolenic acid exert an anti-depressant effect in soman-treated animals. Performance in the Porsolt forced swimming test was measured as time spent being immobile in seconds (s). FST was conducted two weeks after administration of soman/veh. *p < 0.05 vs saline/veh (vehicle) or saline/LIN (α-linolenic acid) group. #p <0.05 vs soman/veh (n = 8-10 animals/group).

was comparable with that observed in vehicle-treated animals ensuring that the increased active behaviors in FST were not secondary to a nonspecific increase in motor activity. These results demonstrate for the first time that 3 doses of α-linolenic acid exert an anti-depressant effect on animals damaged by soman.

A critical mechanism in the modulation of antidepressant treatment response occurs through increased brain-derived neurotrophic factor (BDNF) synthesis and its signaling pathways in brain. Converging lines of evidence have shown that the mechanisms of antidepressants are related to several mechanisms of neuroplasticity, ranging from changes in gene expression (including neurotrophic mechanisms) to synaptic transmission and plasticity, and neurogenesis (Lemaire et al., 2000; Racagni et al., 2008; Shors et al., 2001). Our previous results demonstrated that three doses of LIN500 administered intravenously over a seven day period increased BDNF levels, neurogenesis synaptogenesis and synaptic function in specific brain regions (Blondeau et al., 2009), properties well known for the efficiency of antidepressant drugs (Castren et al., 2007).

Research activities focusing on neural stem cells have shown that promoting their proliferation or grafting/infusing them by different routes into the brain leads to neurological improvement in different brain disease models (Capone et al., 2007; Maysami et al., 2008; Nomura et al., 2005; Pallini et al., 2005; Bramham, 2008; Cohen and Greenberg, 2008). This benefit does not come only by their capacity to replace lost neurons. They can also trigger several other mechanisms, such as the induction of survival-promoting neurotrophic factors or promote the restoration of synaptic transmitter release function by integrating into existing synaptic networks, and thus improve the functional circuitry. Three doses of α-linolenic acid promote neural stem cell proliferation, synaptogenesis and synaptic function (Blondeau et al., 2009). It is worth noting that neural stem cells can induce the transcription of protective factors, such as BDNF, which would be expected to modify the ischemic environment and promote neuroprotection. The in vitro and in vivo studies show that the expression of BDNF levels was increased after α-linolenic acid treatment in neural stem cells, hippocampal mature neurons, and in cortical/hippocampal tissue (5). BDNF is a potent enhancer of neurogenesis in intact (Lee et al., 2002) and ischemic brains (Liu et al., 1998; Zhu et al., 2011). Soman-induced status epilepticus would be expected to create an ischemic environment and it has been demonstrated that neurogenesis is impaired in this model (Joosen et al., 2009). The increase of mature BDNF, enhanced neurogenesis, synaptogenesis and synaptic function (see below) induced by α-linolenic acid may lead to increased brain protection and an environmental change favorable toward recovery of functional network circuitry.

In a mouse model of stroke, a single injection of α-linolenic acid, after the onset of stroke, significantly reduced infarct size and improved the neurological score 24-h post-ischemia. Although the single injection of α-linolenic acid did not improve long-term survival rate, repeated α-linolenic acid injections significantly improved survival, suggesting that subchronic α-linolenic acid treatment triggers other protective pathways (Heurteaux et al., 2006). Mature BDNF protein levels increased in the cortex and hippocampus following the injection of three doses of α-linolenic acid given iv (day 1, day 3, day 7) in control mice (Blondeau et al., 2009). BDNF is widely expressed in brain

where it carries out diverse functions including neuronal maintenance, learning and memory, enhanced neuronal survival in vivo and in vitro (Acheson et al., 1995; Fawcett et al., 1998; Ghosh et al., 1994; Gottschalk et al., 1998; Jiang et al., 2005; Korte et al., 1995; Lu and Figurov, 1997; Marini et al., 1998; Mattson et al., 2006; Miller and Kaplan, 2001; Suen et al., 1997; Tong et al., 1998; Yan et al., 1992); BDNF mediates these effects through its high affinity receptor, TrkB (Aloyz et al., 1999; Friedman and Greene, 1999; Kafitz et al., 1999; Kaplan and Miller, 2000; Klein et al., 1991; Soppet et al., 1991). It has been previously shown that BDNF increases neurogenesis, synapse formation and synaptic transmission (Katoh-Semba et al., 2002; Lee et al., 2002a; Lee et al., 2002b; Pencea et al., 2001).

Administration of three doses α-linolenic acid given intravenously on day 1, day 3 and day 7 in control mice also enhanced neurogenesis over baseline levels on day 10 in the hippocampus. Western blot experiments showed a 1.7 and 3.2-fold increase in synaptophysin in the cortex and hippocampus respectively. Increases in the expression of synaptobrevin and soluble synaptosomal-associated protein of 25kD (SNAP-25) by approximately 2-fold were also found in the cortex and hippocampus. Both proteins play a key role in exocytosis by participating in the fusion process. The protein expression of vesicular glutamate transporters V-GLUT1 and V-GLUT2 increased in the cortex and hippocampus; both are expressed in adult brain (Blondeau et al., 2009). Neuronal injury itself can lead to neural proliferation as a compensatory mechanism for cell death in the hippocampus (Gould and Tanapat, 1997; Liu et al., 1998) and growth factors such as BDNF play a prominent role in promoting neuronal differentiation and survival (Lowenstein and Arsenault, 1996). α-Linolenic acid may further promote the restoration of synaptic transmitter release function thus improving the function of damaged circuits. We hypothesize that chronic treatment with α-linolenic acid restores network circuitry damaged by soman via enhanced neurogenesis, synaptic function and synaptogenesis in the hippocampus and cortex, two major brain regions involved in plasticity and heavily damaged after nerve agent exposure.

We now show that three doses of α-linolenic acid administered 30 min, 3 days and 7 days after soman exposure increases animal survival.

The neuroprotective effect of 3 doses of α-linolenic acid administered intravenously (i.v.) 30 min, 3 days and 7 days after soman (soman/LIN500) is accompanied by a 2-fold increase in survival rate 21 days after soman (soman/Veh) exposure (Figure 2). We observed no increase in survival when α-linolenic acid (LIN500) was injected once, 30 min after soman exposure. There were no deaths in control groups (saline/veh; saline/LIN500). These results are comparable to our previous results where a single injection of LIN500 failed to improve animal survival following middle cerebral artery occlusion model

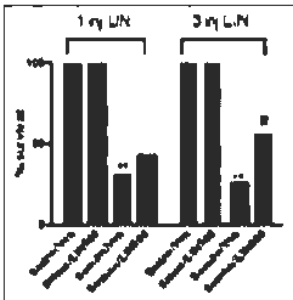


Figure 2. Administration of three injections of α-linolenic acid (LIN) significantly improves survival rate. Young male Sprague-Dawley rats were injected with α-linolenic acid intravenously (500 nmol/kg; LIN500) or vehicle (0.05% ETOH; veh) 30 min (1 injection; 1 inj), or 30 min, 3 days and 7 days (3 injections; 3 inj) after soman exposure. Controls consisting of saline in place of soman were also incorporated into the experiment. Survival rate was measured on day 21. **p < 0.001 *vs* saline/veh or saline/LIN500 group and #p < 0.05 *vs* soman/veh group by Fisher exact test. n=13-15/group.

(MCAO) of stroke but where three injections (30 min, 3 days, 7 days) after MCAO increased animal survival 3-fold (Blondeau et al., 2009).

We hypothesize that enhanced neurogenesis, synaptogenesis, synaptic neurotransmission and BDNF levels in cortex and hippocampus, play a critical role in the observed increase in animal survival and anti-depressant-like activity in animals that received three doses of α-linolenic acid after soman. We further hypothesize that one of the known downstream targets of BDNF via its high affinity receptor, TrkB, is the mammalian target of rapamycin (mtor).

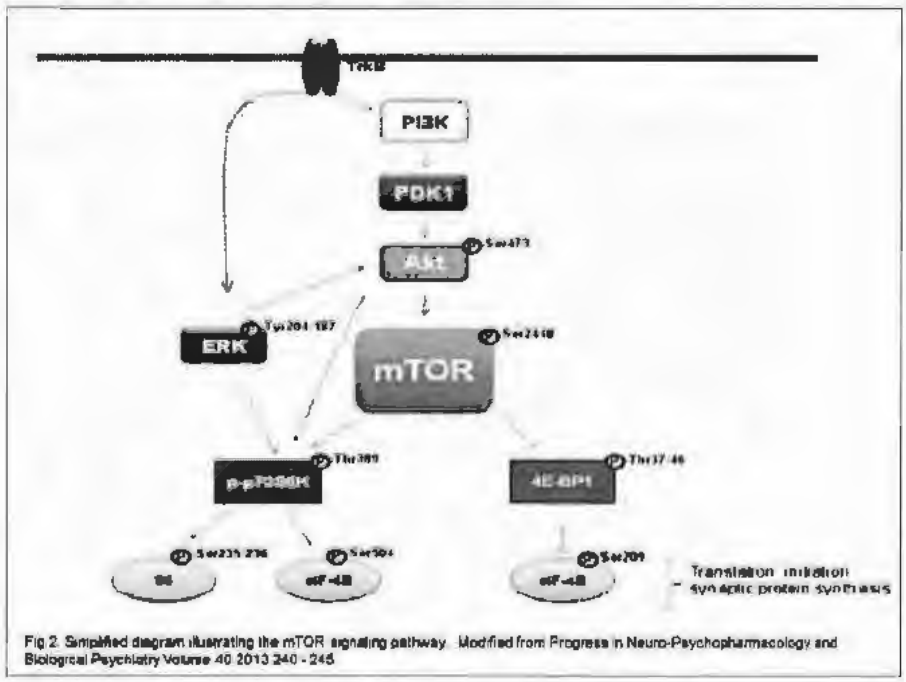


Fig 2. Simplified diagram illustrating the mTOR signaling pathway. Modified from Progress in Neuro-Psychopharmacology and Biological Psychiatry Volume 40 2013 240 - 245

BDNF-induced activation of TrkB lead to the activation of three main intracellular signaling pathways: 1) the phospholipase Cγ (PLCγ) pathway, which leads to activation of protein kinase C; 2) the mitogen-activated protein kinase [MAPK, or extracellular signal related kinase (ERK)] pathway, which activates several downstream effectors; and 3) the phosphatidylinositol 3-kinase (PI3K) pathway that activates the serine/threonine kinase Akt. MAPK and PI3K play crucial roles in neuronal survival, protein-synthesis dependent plasticity and neurogenesis.

The MAPKs are a specific class of serine/threonine kinases which respond to extracellular signals such as growth factors, mitogens, cellular stress and mediate proliferation, differentiation, and cell survival in mammalian cells. One of the MAPKs within mammalian cells is the extracellular signal-related kinases (ERKs). Several investigations have indicated that ERK promotes cell survival and proliferation. ERK is a threonine-glutamic acid-tyrosine (Thr-Glu-Tyr) motif divided into two subtypes: ERK1 and ERK2. Activation of ERK by neurotrophins stimulates ras, which activates MAPKKK, MAPKK and ERK [Lu and Chow, 1999; Mcallister et al., 1999; Tyler et al., 2002; West et al., 2001]. The PI3Ks are enzymes that initiate signaling cascades. Akt is a serine/threonine protein kinase and a major downstream target of PI3K. This pathway is known for regulating metabolism, cell growth and survival and recently has been associated with synaptic plasticity and antidepressant behavior.

Low expression of Akt has been related to neurodegeneration, whereas the increased activation of Akt is neuroprotective [Lee et al., 2006]. The phosphorylation of Thr308 and Ser473 of Akt fully activates Akt, which then phosphorylates a diverse number of

protein substrates containing the consensus sequence of RXRXXS/T [LaPiccolo et al., 2008]. Akt phosphorylation at ser473 is associated with neuroprotection after seizure-induced neuronal death [Piermartiri et al., 2009]. The substrates activated by Akt include Bcl-2-associated death promoter (BAD), Caspase 9, mammalian target of rapamycin (mTOR), IκB kinase (IKK), NF-Kappa B, glycogen synthase kinase 3β (GSK-3β), cAMP response element-binding (CREB) among others. The phosphorylation of these substrates eventually elicits multiple biological responses.

The PI3K–Akt and ERK are the two pathways involved in the activation of the mammalian target of rapamycin (mTOR) signaling (Fig 2) [Chandran et al., 2013). The mTOR is a large serine-threonine kinase that exists in two distinct heteromeric protein complexes referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTORC1 is sensitive to the selective inhibitor rapamycin and it is activated by phosphorylation of serine 2448 in the canonical PI3K- Akt pathway after growth factor stimulation. However, additional signaling cascades also activate mTOR [Chong et al., 2012]. Rapamycin does not directly inhibit mTOR kinase activity; instead, it binds to FKBP12 and disrupts the mTOR-RAPTOR interaction, e.g. the complex of mTORC1. By contrast, mTORC2 is resistant to rapamycin, and recent studies have suggested that mTORC2 may phosphorylate Akt at S473 [Sarbassov et al., 2005; Sarbassov et al., 2004] but its mechanisms in brain plasticity and neurogenesis are not well understood.

The role of mTORC1 in neurological disorders. Treatments that activate Akt and mTOR mediate neuroprotective effects against ischemic brain injury [Koh, 2008], and contribute to recover motor function after spinal cord injury [Hu et al., 2010]. Activation of AKT/ERK/mTOR signaling pathway in the brain mediates protein-dependent synaptic plasticity [Slipczuk et al., 2009], neural differentiation and proliferation during neurogenesis [Han et al., 2008; Otsegi et al., 2006; Paliouras et al., 2012], and has been proposed to be part of the mechanism underlying antidepressant effects (Marsden, 2012). The antidepressant effect in rats is abolished when animals are treated with either rapamycin or with the PI3K inhibitor (LY294002) or the ERK inhibitor (U0126), an effect possibly related to concomitant inhibition of synaptogenesis [Li et al., 2010].
However, hyperactive mTOR leads to epilepsy, and inhibition of mTOR has antiepileptogenic properties in some model of epilepsy [Zeng et al., 2008; McMahon et al., 2012; Buckmaster et al., 2011]. But chronic inhibition of mTOR has been reported to induce prodepressant effects in a rat model of absence epilepsy [Russo et al., 2013]. In a recent extensive review of mTOR and epilepsy, Russo et al. [Russo et al., 2012] concluded that the activation status of mTOR is "complex and variable" and "might be limited to certain types of epilepsy and epileptogenic process", suggesting that the nature of the brain insult may contribute to the degree of mTOR activation.
The activation of mTOR signaling by neurotrophins increases the translation of synaptic proteins, which is essential for synaptic plasticity, by two mechanisms. First, mTORC1 phosphorylates and inactivates the eIF4E-binding protein (4E-BPs), which facilitates translation initiation by releasing the inhibition of eukaryotic initiation factor 4E (eIF4E), a crucial initiation factor in cap-dependent translation. The association of 4E-BPs with eIF-4E inhibits the ability of eIF-4E to associate with eIF-4G and initiate translation. Second, it activates p70S6 kinase, an enzyme that controls translation at a number of

levels, including synthesis of the S6 ribosomal subunit, phosphorylation of RNA helicase cofactor eIF4A and inhibition of eukaryotic elongation factor 2 (eEF2) kinase [Kuhn et al., 1996]. The p70S6 kinase and 4E-BP are also regulated by MAPK/ERK to control protein-synthesis dependent plasticity [Hartman et al., 2012], and p70S6 can further phosphorylate mTOR setting up an autoregulatory mechanism. Protein-dependent synaptic plasticity strengthens the neuronal connection and helps to regulate memory storage in the brain. On the other hand, excessive protein synthesis results in behavioral deficits, instead of improving neuroplasticity, suggesting a temporal window to where mTOR should be carefully modulated (Troca-Marin et al., 2012). Taken together, the LIN-induced increase in BDNF to enhance neuronal survival, synaptic plasticity and neurogenesis may play a central role in ameliorating the soman-induced neuronal damage and cognitive impairment in rodents.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: PubMed, DTIC, BRD, ToxNet, NIH Reporter

II.2.2. Date of Search: January 27, 2014

II.2.3. Period of Search: 1980-2013

II.2.4. Key Words and Search Strategy: alpha-linolenic acid, soman, rat, neuropathology, brain damage, excitotoxicity

II.2.5. Results of Search:

BRD: no hits
DTIC: no hits
PubMed: 3 hit. One paper (Lauritzen et al., 2000) described the neuroprotective effect of linolenic acid against kainic acid-induced status epilepticus and another paper described that the protective effect against ischemic stroke requires activated NF-κB (Blondeau et al., 2001). The third paper (b)(6) which was published in the journal Neurotoxicology in 2012 (33(5):1219-29). The current project is an extension of our work where we have convincing data indicating that three doses of alpha-linolenic acid increase animal survival, neuronal survival in four major brain regions known to be vulnerable to soman and an improvement in functional outcome in animals exposed to a highly toxic dose of soman.

III. **OBJECTIVE\HYPOTHESIS:** The objective of this project is to determine the role of the known alpha-linolenic acid-induced neuroplastic effects in the observed increase in animal survival, neuronal survival and the improvement in functional outcome in soman-induced brain damage.

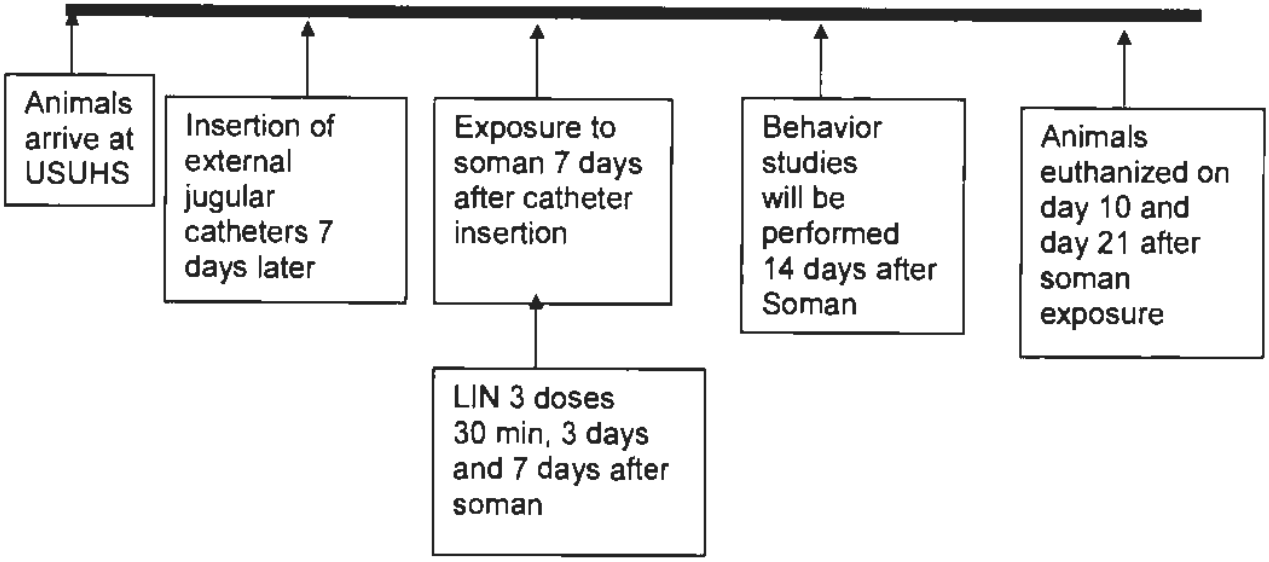
IV. **MILITARY RELEVANCE:**
These studies are justified because the use of soman is a model for nerve agent-induced seizures, an expected effect of nerve agent exposure of military personnel in international conflicts and civilian populations. Identification of new neuroprotective

agents is highly relevant to enhance treatment response capabilities. Thus, the knowledge obtained from the proposed investigations may benefit many active duty personnel as well as civilians.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

Experimental Design:



Experimental Design: On either day 10 or day 21 after soman exposure, rats will be euthanized, brains fixed and sectioned for immunohistochemistry. A subset of rats will be euthanized 10 days after soman exposure, the brains harvested and stored at -80°C for western blot analysis. The brain regions investigated will be the hippocampus and prefrontal cortex. Another group of rats will be subjected to behavior studies and will be euthanized on day 21.

Experiment 1) Investigation of BDNF/TrkB and signaling pathways: western blot will be performed to determine changes in expression of BDNF, the presynaptic proteins synaptophysin-1, SNAP25 and postsynaptic density protein-95 (PSD-95).

Experiment 2) Correlation of mTOR signaling pathway with neurogenesis, synaptic plasticity and behavior: We will further test the role of the activation of mTOR in the well-known neuroplastic effects of LIN and correlate mTOR signaling to LIN-induced neuroprotection by blocking mTOR pathway with rapamycin (an mTOR antagonist),

Drug treatment

All of the procedures for exposing the animals to soman and post-treatment with LIN are described below. LIN will be administered 30 min, 3 days and 7 days after soman exposure by intravenous injection via an external jugular catheter.

Soman-induced seizures: The animals will not receive anesthesia or analgesia during the time the animals seize following injection of soman. Rats receiving soman will be injected with the oxime HI-6 (125 mg/kg, ip) and 30 min later will receive soman (180 ug/kg, sc) followed by post-exposure treatment with atropine methyl nitrate [AMN] (2 mg/kg, im, 1 min after soman. All soman-treated animals will receive diazepam (10 mg/kg im) 40 min after onset of status epilepticus.

Additional drug treatments are alpha-linolenic acid, BrdU, rapamycin and paraformaldehyde for perfusion (see V.4.4.1. Injections)

Histological procedures include cresyl violet staining, immunohistochemistry, FluroJade C staining.

V.1.1. Experiment 1: Determine neurogenesis in the subgranular zone of the dentate gyrus induced by Linolenic acid after soman exposure.

V.1.2. Experiment 2: To determine the role of the mTOR pathway in LIN-induced neuroprotection and improved functional outcome following soman exposure.

V.2. Data Analysis: Immunohistochemistry, Western blot and behavioral outcomes will be compared between groups using analysis of variance (ANOVA) followed by post-hoc t tests for specific comparisons of interest. Data will be examined for normality and constant variance and will be transformed if necessary, and nonparametric tests, e.g. Kruskal-Wallis and Mann-Whitney will be used if transformation is not sufficient. A sample size of 6 per group will have 80% power to detect a difference of 1.8 standard deviations between groups based on a two-group t test with a 5%, two-sided significance level. Differences of this magnitude have been observed in previous studies in this laboratory.

Experiment 1. Determine neurogenesis induced by Linolenic acid after soman exposure.

BrdU administration and Measurement of Neurogenesis: This experiment is divided in two sections:
1) In the first part the animals will receive two BrdU injections (i.p.). BrdU is a nucleotide analog, after injection BrdU is permanently incorporated into replicating DNA in place of thymidine, in the S-phase of cell cycle. BrdU-labeled cells can undergo apparently normal division, differentiation, migration and process integration in studies of adult neurogenesis [Bondeau et al., 2009).

2) The second part is the BrdU staining after the animals are euthanized. Neurogenesis will be quantified in the subgranular zone (SGZ) of the dentate gyrus (DG) of the

hippocampus. Fluorescent BrdU labeling can be imaged on a standard fluorescent microscope after immunohistochemistry to positively identify the number of BrdU-positive (anti-BrdU) cells. The phenotype of the BrdU-positive cells will be determined using fluorescent double-labeling with double cortin (DCX), an immature neuronal marker, NeuN (Feminizing Locus on X-3, Fox-3, or Hexaribonucleotide Binding Protein-3), a marker that is expressed in mature neurons, and GFAP (glial fibrillary acidic protein), an astrocyte marker.

Experiment 2. To determine the role of the mTOR pathway in LIN-induced neuroprotection and improved functional outcome following soman exposure. Rationale: Soman exposure results in profound cognitive deficits in humans and rodents. The mammalian target of rapamycin (mTOR) is a key mediator of protein-dependent synaptic plasticity (e.g. synaptogenesis), an important mechanism for memory storage in the brain. The mTOR inhibitor, rapamycin has been shown to impair cognitive function, disrupting memory consolidation and processing in the hippocampus. Moreover, mTOR has been reported to promote neuronal differentiation during neurogenesis. Increased synaptic plasticity and neurogenesis in the adult brain are necessary for positive behavioral effects of antidepressant drugs in rodent models of depression. The mTOR pathway can be activated by growth factors such as BDNF through the activation of TrkB receptors, and downstream activation of Akt and ERK signaling. Previous results from our group have demonstrated that repeated LIN treatment per se increases BDNF levels, synaptogenesis, neurogenesis and antidepressant effect, but the intracellular signaling mechanism(s) remain unclear. Hence I want to investigate whether soman–induced neurodegeneration is associated with changes in BDNF, TrkB and the downstream pathways i.e. PI3K, MAPK and mTOR in the hippocampus and propose to address whether 3 doses of LIN treatment (500 nmol/kg, iv) modulates these pathways to exert, in part, its neuroprotective effect to improve neuronal survival and functional outcome after soman exposure.

Treatment Groups and Group Sizes for Experiments 1 and 2

Table 1 LIN Treatment Group and Group Sizes for Experiment 1 (neurogenesis)

Treatment Group	Soman 0 min, Atropine min	Treatment (time after seizure onset)	Group Size	Pain Category
Group 1 Adult Male rats	HI-6→Soman→atropine →seizure→diazepam	LIN (30 min, 3 da 7 days), BrdU on day 9 and day 10 and 2 time points (10 days and 21 days)	12 Male Rats (6 for each time point)	**E**
Total			12 male rats	**12E**

Table 2 LIN Treatment Group and Group Sizes for Experiment 1 (neurogenesis)

Treatment Group	Soman 0 min, Atropine 1 min	Treatment (time After seizure onset)	Group Size	Pain Category
Group 2 Adult Male rats	HI-6→Soman →atropine →seizure →diazepam	LIN (30 min, 3 days, 7 days) saline on day 9 and day 10 and 2 time points (10 days and 21 days)	12 Male Rats (6 for each time point)	**E**
Total			12 male rats	**12E**

Table 3 Control groups - No Soman Exposure and Group Sizes for Experiment 1 (neurogenesis)

Treatment Group	HI-6 -30 min→Saline 0 min →Atropine 1 min→ diazepam 40 min	Treatment (Time after Saline injection)	Group Size	Pain Category
Group 3 Adult male rats	HI-6→Saline →atropine→diazepam	LIN (30 min, 3 days, 7 days), BrdU (day 9 and day 10) and 2 time points (10 and 21 days)	12 Male Rats (6 for each time point)	D
TOTAL			**12 Male Rats**	**12 D**

Table 4 Control groups - No Soman Exposure and Group Sizes for Experiment 1 (neurogenesis)

Treatment Group	HI-6 -30 min→Saline 0 min →Atropine 1 min→ diazepam 40 min	Treatment (Time after Saline injection)	Group Size	Pain Category
Group 4 Adult male rats	HI-6→Saline →atropine→diazepam	LIN (30 min, 3 days, 7 days), saline (day 9 and day 10) and 2 time points (10 and 21 days)	12 Male Rats (6 for each time point)	D
TOTAL			**12 Male Rats**	**12 D**

Table 5: Control groups - Soman Exposure and no LIN and Group Sizes for Experiment 1 (neurogenesis)

Treatment Group	HI-6 -30 min→Soman 0 min→atropine 1 min →diazepam 40 min	vehicle (Time after seizure onset)	Group Size	Pain Category
Group 5 Adult male rats	HI-6→Soman→ atropine →seizure→diazepam	vehicle (30 min, 3 days, 7 days), BrdU (day 9 and day 10) and 2 time points (10 and 21 days)	12 Male Rats (6/group)	E
TOTAL			**12 Male Rats**	**12 E**

Table 6: Control groups - Soman Exposure and no LIN and Group Sizes for Experiment 1 (neurogenesis)

Treatment Group	HI-6 -30 min→Soman 0 min→atropine 1 min →diazepam 40 min	vehicle (Time after seizure onset)	Group Size	Pain Category
Group 6 Adult male rats	HI-6→Soman→ atropine →seizure→diazepam	vehicle (30 min, 3 days, 7 days), saline (day 9 and day 10) and 2 time points (10 and 21 days)	12 Male Rats (6/group)	E
TOTAL			**12 Male Rats**	**12 E**

Table 7: Control groups – No soman Exposure and no LIN and group sizes for Experiment 1 (neurogenesis)

Treatment Group	HI-6 -30 min→saline 0 min→atropine 1 min →diazepam 40 min	vehicle (Time after seizure onset)	Group Size	Pain Category
Group 7 Adult male rats	HI-6→saline→ atropine →vehicle→diazepam	vehicle (30 min, 3 days and 7 days), BrdU (day 9 and day 10) and 2 time points (10d, and 21 days)	12 Male Rats (6/group)	D
TOTAL			**12 Male Rats**	**12 D**

Table 8: Control groups – No soman exposure and no LIN and group sizes for Experiment 1 (neurogenesis)

Treatment Group	HI-6 -30 min→saline 0 min→atropine 1 min →diazepam 40 min	vehicle (Time after seizure onset)	Group Size	Pain Category
Group 8 Adult male rats	HI-6→saline→ atropine →vehicle→diazepam	vehicle (30 min, 3 days and 7 days), saline (day 9 and day 10) and 2 time points (10 days and 21 days)	12 Male Rats (6/group)	D
TOTAL			**12 Male Rats**	**12 D**

Table 9: Soman and LIN Treatment Groups and Group Sizes for Experiment 2 (rapamycin/neurogenesis)

Treatment Group	Soman 0 min, Atropine 1 min	Treatment	Group Size	Pain Category
Group 9 Adult male rats	HI-6→Soman→ atropine→seizure→diazepam	LIN (500 nmol/kg, 30 min, 3 days, 7 days after seizure onset), rapamycin (3 h prior to first LIN injection and day 2-7, BrdU (day 9 and day 10) x 2 time points (10 and 21 days)	6 Male Rats (6/group) x 2 time points	E
TOTAL			**12 Male Rats**	**12 E**

Table 10: Soman and LIN Treatment Group and Group Sizes for Experiment 2 (rapamycin/neurogenesis)

Treatment Group	Soman 0 min, Atropine 1 min	Treatment	Group Size	Pain Category
Group 10 Adult male rats	HI-6→Soman→ atropine→seizure→diazepam	LIN (500 nmol/kg, 30 min, 3 days, 7 days after seizure onset), vehicle (-3 hours prior to and day 1-7 after first LIN injection), BrdU (day 9 and day 10) x 2 time points (10 and 21 days)	6 Male Rats (6/group) x 2 time points	E
TOTAL			**12 Male Rats**	**12 E**

Table 11: Control groups – Soman exposure and no LIN and group sizes for Experiment 2 (rapamycin/neurogenesis)

Treatment Group	HI-6 -30 min→Soman 0 min→atropine 1 min →diazepam 40 min	vehicle (Time after seizure onset)	Group Size	Pain Category
Group 11 Adult male rats	HI-6→Soman→ atropine →seizure→diazepam	vehicle (30 min, 3 days, 7 days), rapamycin (3h, prior to first vehicle injection and day 2-7), BrdU (day 9 and day 10) and 2 time points (10 and 21 days)	12 Male Rats (6/group)	E
TOTAL			**12 Male Rats**	**12 E**

Table 12: Control groups – Soman exposure and no LIN and group sizes for Experiment 2 (rapamycin/neurogenesis)

Treatment Group	HI-6 -30 min→Soman 0 min→atropine 1 min →diazepam 40 min	vehicle (Time after seizure onset)	Group Size	Pain Category
Group 12 Adult male rats	HI-6→Soman→ atropine →seizure→diazepam	vehicle (30 min, 3 days, 7 days), vehicle (3h, prior to first vehicle injection and day 2-7), BrdU (day 9 and day 10) and 2 time points (10 and 21 days)	12 Male Rats (6/group)	E
TOTAL			**12 Male Rats**	**12 E**

Table 13: Control group – No Soman Exposure and LIN and Group Sizes for Experiment 2 (rapamycin/neurogenesis)

Treatment Group	HI-6 -30 min→Saline 0 min →Atropine 1 min→ diazepam 40 min	Treatment (Time after Saline injection)	Group Size	Pain Category
Group 13 Adult male rats	HI-6→Saline →atropine→diazepam	LIN (500 nmol/kg iv, (30 min, 3 days, 7 days), rapamycin (3 h prior to first LIN injection and day 2-7), BrdU (day 9,10) x 2 time points (10 days, 21 days)	6 Male Rats (6/group) x 2 time points	D
TOTAL			**12 Male Rats**	**12 D**

Table 14: Control group – No Soman Exposure and LIN and Group Sizes for Experiment 2 (rapamycin/neurogenesis)

Treatment Group	HI-6 -30 min→Saline 0 min →Atropine 1 min→ diazepam 40 min	Treatment (Time after Saline injection)	Group Size	Pain Category
Group 14 Adult male rats	HI-6→Saline →atropine→diazepam	LIN (500 nmol/kg iv, (30 min,3 days, 7 days), vehicle (3 h prior to first LIN injection and day 2-7), BrdU (day 9, 10) x 2 time points (10 days, 21 days)	6 Male Rats (6/group) x 2 time points	D
TOTAL			**12 Male Rats**	**12 D**

Table 15: Control group –No Soman and No LIN and Group Sizes for Experiment 2 (rapamycin/neurogenesis)

Treatment Group	HI-6 -30 min→Saline 0 min →Atropine 1 min→ diazepam 40 min	Treatment (Time after Saline injection)	Group Size	Pain Category
Group 15 Adult male rats	HI-6→Saline →atropine→diazepam	vehicle (30 min,3 days, 7 days), rapamycin (3 h prior to first vehicle injection and day 2-7), BrdU (day 9, 10) x 2 time points (10 days, 21 days)	6 Male Rats (6/group) x 2 time points	D
TOTAL			**12 Male Rats**	**12 D**

Table 16: Control group –No Soman and No LIN and Group Sizes for Experiment 2 (rapamycin/neurogenesis)

Treatment Group	HI-6 -30 min→Saline 0 min →Atropine 1 min→ diazepam 40 min	Treatment (Time after Saline Injection)	Group Size	Pain Category
Group 16 Adult male rats	HI-6→Saline →atropine→diazepam	vehicle (30 min,3 days, 7 days), vehicle (3 h prior to first vehicle injection and day 2-7), BrdU (day 9, 10) x 2 time points (10 days, 21 days)	6 Male Rats (6/group) x 2 time points	D
TOTAL			**12 Male Rats**	**12 D**

Table 17: Soman and LIN/rapamycin Treatment Group and Group Sizes for Experiment 2 (western blot)

Treatment Group	Soman 0 min, Atropine 1 min	Treatment	Group Size	Pain Category
Group 17 Adult male rats	HI-6→Soman→ atropine→seizure→diazepam	LIN (500 nmol/kg, 30 min, 3 days, 7 days), rapamycin (3 h prior to first LIN injection and day 2-7, x 2 time points (10 days)	6 Male Rats (6/group) x 1 time point	E
TOTAL			**6 Male Rats**	**6 E**

Table 18: Soman and LIN but no rapamycin Treatment Group and group sizes for Experiment 2 (western blot)

Treatment Group	Soman 0 min, Atropine 1 min	Treatment	Group Size	Pain Category
Group 18 Adult male rats	HI-6→Soman→ atropine→seizure→diazepam	LIN (500 nmol/kg, 30 min, 3 days, 7 days after seizure onset), vehicle (-3 hours prior to and day 1-7 after first LIN injection) x 1 time point (10 days)	6 Male Rats (6/group) x 2 time points	E
TOTAL			**6 Male Rats**	**6 E**

Table 19: Control groups—Soman exposure and no LIN but with rapamycin and group sizes for Experiment 2 (western blot)

Treatment Group	Soman 0 min, Atropine 1 min	Treatment	Group Size	Pain Category
Group 19 Adult male rats	HI-6→Soman→ atropine→seizure→diazepam	vehicle (30 min, 3 days, 7 days after seizure onset), rapamycin (-3 hours prior to and day 1-7 after first vehicle injection) x 2 time points (10 days)	6 Male Rats (6/group) x 1 time point	E
TOTAL			**6 Male Rats**	**6 E**

Table 20: Control groups—Soman exposure and no LIN and no rapamycin and group sizes for Experiment 2 (western blot)

Treatment Group	Soman 0 min, Atropine 1 min	Treatment	Group Size	Pain Category
Group 20 Adult male rats	HI-6→Soman→ atropine→seizure→diazepam	vehicle (30 min, 3 days, 7 days after seizure onset), vehicle (-3 hours prior to and day 1-7 after first vehicle injection) x 1 time points (10 days)	6 Male Rats (6/group) x 1 time point	E
TOTAL			**6 Male Rats**	**6 E**

Table 21: Control groups –No Soman plus LIN and with rapamycin and group sizes for Experiment 2 (western blot)

Treatment Group	HI-6 -30 min→Saline 0 min →Atropine 1 min→ diazepam 40 min	Treatment (Time after Saline injection)	Group Size	Pain Category
Group 21 Adult male rats	HI-6→Saline →atropine→diazepam	LIN (500 nmol/kg iv, (30 min,3 days, 7 days), rapamycin (3 h prior to first LIN injection and day 2-7), x 1 time points (10 days)	6 Male Rats (6/group) x 1 time point	D
TOTAL			**6 Male Rats**	**6 D**

Table 22: Control groups –No Soman and LIN but no rapamycin and group sizes for Experiment 2 (western blot)

Treatment Group	HI-6 -30 min→Saline 0 min →Atropine 1 min→ diazepam 40 min	Treatment (Time after Saline injection)	Group Size	Pain Category
Group 22 Adult male rats	HI-6→Saline →atropine→diazepam	LIN (500 nmol/kg iv, (30 min,3 days, 7 days), vehicle (3 h prior to first LIN injection and day 2-7), x 1 time point (10 days)	6 Male Rats (6/group) x 1 time point	D
TOTAL			**6Male Rats**	**6 D**

Table 23: Control Group – No Soman and no LIN but with rapamycin exposure and group sizes for Experiment 2 (western blot)

Treatment Group	HI-6 -30 min→Soman 0 min→atropine 1 min →diazepam 40 min	vehicle (Time after seizure onset)	Group Size	Pain Category
Group 23 Adult male rats	HI-6→saline→ atropine →vehicle→diazepam	vehicle (30 min, 3 days, 7 days), rapamycin (3h prior to first vehicle injection and days 2-7), x 1 time point (10 days)	6 Male Rats (6/group) x 1 time point	D
TOTAL			**6Male Rats**	**6 D**

Table 24: Control Group – No Soman and no LIN and no rapamycin exposure and group sizes for Experiment 2 (western blot)

Treatment Group	HI-6 -30 min→Soman 0 min→atropine 1 min →diazepam 40 min	vehicle (Time after seizure onset)	Group Size	Pain Category
Group 22 Adult male rats	HI-6→saline→ atropine →vehicle→diazepam	vehicle (30 min, 3 days, 7 days), vehicle (3h prior to first vehicle injection and days 2-7), x 1 time point (10 days)	6 Male Rats (6/group) x 1 time point	D
TOTAL			**6 Male Rats**	**6 D**

V.3. Laboratory Animals Required and Justification: Because we are studying cellular mechanisms and responses, there are currently no non-animal alternatives that can meet our needs for these studies.

V.3.1. Non-animal Alternatives Considered: Because we are studying cellular mechanisms and responses, there are currently no non-animal alternatives that can meet our needs for these studies.

V.3.2. Animal Model and Species Justification: The male Sprague-Dawley rats for the in vivo studies were selected because this is the common gender and species of rat used in the literature. This allows us to compare our results directly with the many published results from other laboratories using chemical warfare agents or other acetylcholinesterase inhibitors such as paraoxon.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	Rattus norvegicus	
V.3.3.2. Strain/Stock:	Sprague-Dawley	
V.3.3.3. Source/Vendor:	(b)(4)	
V.3.3.4. Age:	Young adult	
V.3.3.5. Weight:	250-275 gm	
V.3.3.6. Sex:	Male	
V.3.3.7. Special Considerations:	N/A	

V.3.4. Number of Animals Required (by Species): 288

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: All surgeries will be performed under appropriate anesthesia as stated in section V.4.1.2.1. Any rat exhibiting signs of pain or impairment beyond that from this model will be euthanized at an early endpoint. No other refinement alternatives were considered.
All animals will be weighed daily. During the first 24 hours after soman injection, laboratory personnel will stay with animals in LAM. After the first 24 hours, laboratory personnel will check animals every 8 hours. All animals will receive Lactate Ringer's (5 ml, sc) by research staff as needed (up to 25 ml/day), wet mash, apple slices and gel

squares. We will determine the survival rate of all animals and quantify the results just prior to perfusion.

V.3.5.2. Reduction: All animals that are injected with soman will receive drugs to improve their survival (HI-6, atropine and diazepam). By using these drugs, survival is improved thereby reducing the overall number of animals required for this work.

V.3.5.3. Replacement: Computer modeling or lower animals such as worms cannot be used for these studies because we are quantifying cellular and molecular changes that can only be measured in living brain cells. The rat has been used extensively for cellular and molecular studies involving nerve agents and we will be able to compare our results with the existing literature.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment: In addition to Center for LAM SOP's, research personnel will monitor animals using the checklist (in section VIII, Enclosures) for animals exhibiting pain/distress beyond expectations of this study. If LAM personnel notice any of the following: constant convulsions or tremors, self-mutilation, continuous circling, or persistent labored breathing, and study personnel are unavailable, the LAM veterinarian may make the decision to euthanize the animal. If animals continue to lose weight despite supplements (wet mash etc) or remain comatose for more than 24 hours, these animals will be euthanized by laboratory personnel or LAM.

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:		
V.4.1.1.1.2. Column D:	144	
V.4.1.1.1.3. Column E:	192	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

Anesthesia for placement of external jugular catheter: Rats will be placed in an induction chamber then anesthesia will be induced with 3-4% isoflurane from a precision vaporizer delivered in 1-2 Liters per minute of oxygen. Once the rats are in lateral recumbancy, they will be moved to a mask and prepared for surgery. Maintenance anesthesia will be delivered at 1-3% isoflurane in 0.5 to 1 Liter per minute of oxygen. Anesthesia will be adjusted based on response to toe pinch and depth of respirations. Once animal is adequately anesthetized (no response to toe pinch) then

surgical procedures will begin. All rats except for naïve will have external jugular catheters placed for injection of either vehicle or linolenic acid.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Animals will have 7 days to acclimate to their surroundings once delivered to USUHS and before being used for any in vivo experiments. Rats will be observed by the PI or technician to ensure they appear healthy prior to undergoing surgery.

Animal monitoring. The animals will be watched continuously for 1h and then checked every 30 min until the animal ambulatory Research personnel will monitor the rats three times a day beginning on the following day after soman. Monitoring will be performed in the rat's home cage, and will continue every day up to the point they are euthanized. Any animals that show unexpected neurologic signs following soman injection or hunched posture, lethargy, anorexia, or other clinical signs potentially indicative of infection may be euthanized at an early endpoint or treated at the discretion of the veterinarian and/or PI. Research personnel will also weigh rats once a day to ensure they have not lost >20% of their body weight compared to baseline weights. Supplemental feeding in the form of wet mash will be provided by LAM personnel at the request of the PI or as directed by the LAM Veterinarians and fluids (5 ml Lactated Ringer's sc 2 to 3 times a day) will be administered to rats injected with soman by research personnel. Gel squares will also be placed on the floor of each cage and replaced every day by laboratory personnel.

Supplemental (alternative method) feeding. LAM personnel may place wet mash/chow on the cage floor for rats that may not be able to gnaw pellets to get food. They may also provide apple slices once animals are awake and moving around their home cage.

Soman-induced seizures: The animals will not receive anesthesia or analgesia during the time the animals seize following injection of soman. Rats receiving soman will be injected with the oxime HI-6 (125 mg/kg, ip) and 30 min later will receive soman (180 ug/kg, sc) followed by post-exposure treatment with atropine methyl nitrate [AMN] (2 mg/kg, im, 1 min after soman. All soman-treated animals will receive diazepam (10 mg/kg im) 40 min after onset of status epilepticus.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: BRD, DTIC Private STINET, NIH Reporter

V.4.1.3.2. Date of Search: December 31, 2013

V.4.1.3.3. Period of Search: 1980-2013

V.4.1.3.4. Key Words of Search: rat, pain, kainic acid, seizure, hippocampus, piriform cortex, amygdala, neuronal damage, brain damage, gluatamate receptor, glutamate

receptor, animal welfare, alternative, reduce, refine, prevent, control, manage, eliminate, alleviate, decrease, diminish

V.4.1.3.5. Results of Search:

There is no alternative for inducing brain damage in rats that mimics the damage caused by soman to determine whether the proposed research plan using linolenic acid protects against soman-induced brain damage or improves functional outcome. The only way to carry out the research plan is to allow the animals to seize for 40 min as recommended by Dr. (b)(6) at (b)(6) followed by injection of diazepam to stop the seizures.

DTIC Private STINET: 520 hits. One study discussed seizures as it relates to chemical warfare agents and measuring seizures on the battlefield. These studies offered no advantage over the proposed methods.

BRD: no hits

NIH Reporter: 17 hits. Several discussed reduce or refine but were not relevant to the proposed study.
Soman-induced convulsions: The treatment plan for inducing status epilepticus by soman in rats is a standard procedure that is routinely performed by many laboratories including our laboratory and outlined in the scientific literature. Sprague-Dawley rats have been used in the literature.

Interpretation: There presently is no alternative to employment of rat as an in vivo animal model to study the role of multiple doses of linolenic acid to improve neuroprotection against soman-induced neuropathology or to determine whether improved neuronal survival in linolenic acid-treated rats improves overall functional outcome by behavioral testing.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

One hundred and forty-four male Sprague-Dawley rats will receive soman at a dose that will induce seizures, an effect that is seen in humans exposed to nerve agents. The rats will experience some level of seizure or physical stress and discomfort for some period of time during the experiments. Analgesic, sedatives and/or anesthetic agents cannot be used because they will modify the pain and seizure threshold preventing us from fully evaluating the effect of soman and as such we will not be able to draw any conclusions on the proposed effect of linolenic acid on the neuroplastic effects or functional outcome. The objective of this study is to determine the role of LIN-induced neuroplastic effects in the observed increase in neuronal survival, animal survival, and improvement in functional outcome. Without exposing the animals to soman, it is not possible for us to conduct these studies.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions:
Surgeons will wear sterile gloves, mask, lab coat or scrub top and use aseptic technique. A general assessment of physical and behavioral health will be carried out on each rat before surgery. LAM veterinarians will be consulted should any sign of abnormality in appearance and/or behavior be observed.

V.4.3.2. Procedure: Rats will be anesthetized and maintained with isoflurane in oxygen by nosecone (see V.4.1.2.1.). After the induction of anesthesia, the rats (with the nosecone) will be placed in lateral recumbancy and prepared for surgery. Maintenance anesthesia will be delivered at 1-3% isoflurane in 0.5 to 1 Liter per minute of oxygen. Anesthesia will be adjusted based on response to toe pinch and depth of respirations. Once animal is adequately anesthetized (no response to toe pinch) then surgical procedures will begin. Only sterile instruments will be used. A supplemental heat source, such as an electric heating pad or circulating warm water blanket, will be used to keep the rats warm throughout the duration of anesthesia. Rats will be monitored for dermal bums.

Placement of external IV catheter. Anesthetized rats will be placed in dorsal recumbancy. Fur will be clipped from the ventral neck and interscapular region. Skin will be prepared for aseptic surgery using either betadine or alcohol or chlorhexidine scrub. The animal will be draped using sterile towels. A 1.5 to 2 cm long incision will be made in the ventrolateral aspect of the neck using a scalpel, parallel and approximately 0.5 cm lateral to midline. The external jugular vein will be dissected free of surrounding tissue and stabilized with a proximally placed tie of 4-0 suture. A catheter introducer will be used to guide the placement of the IV catheter. A 4-0 ligature will be placed at the distal end of the catheter to secure it in place. Tissue adhesive may also be used to help stabilize the catheter in the jugular vein. Catheter will be tested for patency by flushing with sterile saline. The catheter will then be tunneled subcutaneously to exit via an approximately 2.5 cm incision in the interscapular region. Wound clips or suture material will be used to close the interscapular area. Tissue glue or suture material (sterile 4 O silk) will be used to close the ventral neck incision. If placement in one side of the jugular area is problematic, the procedure will be repeated on the opposite jugular vein.

V.4.3.3. Post-surgical Provisions:

Immediate post-operative care will be provided by the investigators and rats will be monitored continuously until they are ambulatory. Animals are expected to ambulate. Animals will be returned to their cages post-surgery only after they have recovered sufficiently from anesthesia. Food and water will be provided ad libitum. Room maintenance and climate control is as per LAM SOP. Following surgery, rats will be monitored continuously until they are ambulatory. The incision site will be monitored daily for signs of infection (erythema, edema, presence of an exudate, induration etc). Because the catheter will be inserted into the external jugular vein, we will also examine the area for signs of bleeding. Each catheter will be flushed once daily with heparinized saline to prevent clotting of the tip by research personnel or PI.

Soman-injected animals: Food and water will be provided ad libitum. Room maintenance and climate control is as per LAM SOP. Investigators will perform daily weights, provide supplemental calories (as appropriate) via wet mash and sc lactate ringers (5 ml) four times a day while comatose after soman. Once the animal is awake, research personnel will watch animals for their ability to drink from their water bottle. Those animals that have difficulty drinking due to poor ambulation will be injected with lactate ringers (5 ml) s.c. 2-4 times per day until animals are able to drink on their own.. Early Endpoints: In addition to Center for LAM SOP's, research personnel will monitor animals using the checklist for animals exhibiting pain/distress beyond expectations of this study. If LAM personnel notice any of the following: constant convulsions or tremors, an animal that is comatose for more than 2 hours, self-mutilation, continuous circling, persistent labored breathing, or behavior that LAM personnel feels is causing the animal to suffer, and research personnel are unavailable, the LAM veterinarian may make the decision to euthanize the animal. If animals continue to lose weight despite supplements (wet mash etc) or remain comatose for more than 24 hours, these animals will be euthanized by laboratory personnel or LAM.

V.4.3.4. Location: (b)(6)

V.4.3.5. Surgeon: Dr (b)(6) (competent in placement of jugular vein catheter)
Ms. (b)(6) (graduate student)

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures:

V.4.3.6.2. Scientific Justification:

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

Soman treatment. Male Sprague-Dawley rats (250-300 gm) receiving nerve agent will be injected with the oxime HI-6 (125 mg/kg, 250 mg/ml (0.15 ml for 300 gram rat/1 ml syringe and 25 gauge 1/2 inch needle) ip) and 30 min later will receive soman (180 ug/kg, 360 ug/ml (0.15 ml for 300 gram rat/1 ml syringe and 25 gauge1/2 inch needle; sc) or saline and post-exposure treatment with atropine methyl nitrate (AMN, 2 mg/kg, 4 mg/ml (0.15 ml for 300 gram rat/1 ml syringe 25 gauge 1/2 inch needle) im into right thigh muscle,), 1 min after soman. These treatments, HI-6 (125 mg/kg, ip) and 30 min later soman (180 ug/kg, sc) and post-exposure treatment with ANM (2 mg/kg, im) 1 min after soman, have been shown to reliably produce electrographic seizure activity within approximately 10 min. Rats are allowed to seize for 40 min and then treated with 10 mg/kg, im, diazepam (0.6 ml for 300 gram rat/1 ml syringe 25 gauge 1/2 inch needle,injected into left thigh muscle) to stop/attenuate the seizures.

Alpha-Linolenic acid (500 nmol/kg) or vehicle will be injected via catheter 30 min, 3 days and 7 days after soman exposure. We will use a needle size ranging from 22 to 25 gauge and the injected volume is 0.3-0.35 mL.

Rapamycin: Rapamycin (LC Labs, Woburn, MA, USA) is initially dissolved in 100% ethanol to prepare a stock solution of 20mg/ml and stored at −20°C. As previously described [(Hartman et al., 2012), immediately prior to use, rapamycin will be diluted to a final concentration of 4% ethanol and 1 mg/ml rapamycin in a vehicle solution containing 5% Tween 80 (sigma), 5% PEG 400 (low-molecular-weight grade of polyethylene glycol) (Sigma). Rats will receive daily administration of rapamycin by intraperitoneal injection in the right lower quadrant (25-26 gauge needle, a total volume of 1.12 ml) x 7 days. The rapamycin dose of 4.5 mg/kg proposed in this major modification has been demonstrated to inhibit mTOR activity in the hippocampus [Hartman et al., 2012]. The dose which inhibits mTOR without blocking soman-induced seizures and neuronal degeneration will be used in this proposal. Control groups will receive an equal volume of vehicle. After blocking this signaling pathway, a subset of the rats will be euthanized on day 10 to identify changes in synaptic proteins and neurogenesis (n= 6 rats/per group). Another group will be submitted to behavioral tests and euthanized on day 21 (n= 6 rats/per group).

Bromodeoxyuridine (BrdU): A stock solution of BrdU will be diluted in saline to a final concentration of 20 mg/ml and the pH adjusted to 7.4. The solution will be filtered sterilized prior to injection. BrdU will be injected at a dose of 300 mg/kg on day 9 and day 10 after soman exposure. To maintain BrdU availability during a full cell cycle, which has 24.7 hours, BrdU or vehicle will be administered to rats by intraperitoneal injection in the right lower quadrant by restraint (25-26 gauge needle, a total volume of 4.5 ml/300 gram rat) prior to euthanasia (Blondeau et al., 2009). This high dosage has been demonstrated to maximize BrdU incorporation into proliferating cells in the subgranular zone of the dentate gyrus in rats allowing specific and quantitative assessment [Cameron and McKay, 2001].

Transcardial perfusion. When rats are euthanized, they will be placed under deep Fatal Plus (60-75 mg/kg) anesthesia. Once adequate anesthesia is achieved (lack of response to toe pinch, slow even respirations), a midsternal thoracotomy will be performed to expose the heart and the rats will undergo transcardial perfusion with cold PBS and 10% formalin. Generally, about 200 cc of each solution is sufficient per animal, but, more will be used if required.

Euthanasia for western blot experiments: Rats will be placed under deep Fatal Plus (60-75 mg/kg, i.p.) anesthesia. Once adequate anesthesia is achieved (lack of response to toe pinch, slow even respirations), the animal will be decapitated using a sharp blade to avoid tissue-altering effects of gases and anesthetics. Many euthanization methods have been shown to influence protein expression levels and confound interpretation of protein expression results. For example, the use of carbon dioxide or ether has been shown to distress animals and deoxygenate blood. Thus, the physiological effects associated with hypoxia and hypercapnia would alter protein expression profiles.

V.4.4.2. Biosamples:

In vivo experiments. The brains from the male Sprague-Dawley rats will be collected at the time of euthanasia. Brain will be preserved for future studies. Hippocampi may need to be removed to determine activated NF-κB levels, a biomarker of linolenic acid.

V.4.4.3. **Adjuvants**: N/A

V.4.4.4. **Monoclonal Antibody (MAbs) Production**: N/A

V.4.4.5. **Animal Identification**:

A patch will be shaved on each animal's back and then marked with non-toxic permanent ink with an individual ID. Sometimes, the ink fades in which case the ID will be re-inked on the patch. All cages have cage cards per LAM. Rats that receive nerve agent injection will be checked for their ink mark labels prior to injection of nerve agent to make sure they are clearly labeled.

V.4.4.6. **Behavioral Studies**: Behavior studies will be conducted 14 days after soman exposure.

Behavior Outline:
One group of 48 animals

Day 14	Day 18	Day 19
Rotarod test	Forced swim pretest 15 min in water	Forced Swim test 5 min in water

Second group of 48 animals

Day 14	Day 15	Day 19	Day 20
Open field (accommodation)	Open field testing	passive avoidance training day	Passive avoidance testing day

Each group of treated (6 animals/group) and control animals (6 animals/group) will undergo only two behavior tests. There are 8 groups (treated and control) of animals (see table 17 through 24). Therefore, 48 animals will be subjected to the open field behavior test and the passive avoidance task. Another group of 48 animals (treated and controls) will be subjected to the rotarod task and forced swim task. A period of four days will separate the two behavior measurements to reduce any influence of one test on another. Interactions with co-specifics will not be performed in this study and this section was deleted.

Activity and Movement: General activity, movement, and coordination can be used as indices of overall health and well-being of rodents. Activity and movement serve as negative controls (brain areas relevant to movement should not be disrupted in the current study), and as a covariates for the other behavioral tasks that rely on animal

locomotion for task completion. The locomotor and rotarod tasks have been used reliably for these purposes in Dr. (b)(6) lab for many years.

Open field locomotor activity. Locomotor activity is measured using an Omnitech Electronics Digiscan infrared photocell system. One-hour activity measurements are obtained (preferably during animals' active or dark cycle). Animals are placed singly in a 40 X 40 X 30 cm clear Plexiglas arena covered by a perforated Plexiglas lid. Data are automatically gathered and transmitted to a computer via an Omnitech Model DCM-I-BBU analyzer. The interfaced software measures 21 activity variables, including total distance and horizontal and vertical activity. Chambers are cleaned between subjects with 35% isopropyl alcohol solution.
The duration of locomotor activity testing is a maximum of 60 min per day and can be performed on multiple days throughout the duration of the experiment.

Rotarod: The rotarod test measures an animal's ability to maintain balance by coordinating movement and making postural adjustments. It also measures the ability to improve these skills with practice. Each rat rotarod treadmill (Med Associates, Inc, St. Albans, VT) consists of a motor-driven drum with constant speed or accelerating speed modes of operation. The drum (7.0 cm diameter) allows each animal to maintain a suitable grip. It is divided into four test zones (8.9 cm wide) so that up to four animals may be tested at the same time. The device consists of a smooth hard plastic cylinder with concentric circular plastic sides (39.7 cm diameter) to prevent the rat from laterally climbing off the cylinder.
Rats are placed on the device facing the wall with the rod rotating in the direction opposite from the animal (so that the rat uses its paws to pace forwards and avoid falling off the rod). When a rat falls off the rotating drum (height of 26.7 cm), it breaks a photobeam, stopping the timer associated with that zone. In each trial, the speed of rotation is increased from 0 revolutions per minute (rpm) to 35 rpm for a maximum of 3 or 5 min. The mean duration on the device (based on 3 consecutive trials) is recorded. The procedures are based on reports in the literature (6-9). Chambers are cleaned between subjects with 35% isopropyl alcohol solution. The duration of rotarod measurement is 9 or 15 min per day can be performed on multiple days throughout the duration of the experiment.

Activation of the amygdala, a brain region vulnerable to soman-induced neuronal cell death, is involved in anxiety and stress-related behaviors. This can be measured using the total time spent in the center of an open field arena or number of entries into the center of the field.

Anxiety

In our recent study, we did not find evidence of anxiety in rats exposed to soman. Therefore, we will not determine open field center time in this protocol.

Research in humans and animals has clearly established that the hippocampus is involved in learning and memory (2). Rats lesioned in the hippocampus have a difficult

time acquiring active avoidance responses (3,4) and retaining passive avoidance responses over a period of time (3-5).

Learning and Memory

Passive avoidance: Animals are trained and tested using an automated avoidance training system (Med Associates, St. Albans, VT) consisting of two 21 x 25 x 17 cm chambers separated by a vertically-sliding door. This task consists of a training day and a consecutive testing day. During training, the animal is placed in one chamber of the darkened apparatus. After a delay of 60 seconds, a light comes on and the door to the other, still darkened chamber, opens. Naive rats instinctively move quickly from the lit chamber into the dark chamber. When the rat crosses completely into the darkened chamber, the door closes, latency to cross is recorded by the interfaced computer, and a single 0.8 mA (mild, non-damaging) shock is delivered through the grid floor for 1 sec. The rat is left in the darkened chamber in which the shock has been delivered for 30 sec to facilitate the formation of memory for the aversive event, and then removed. The procedure on testing day is identical except that the shock is not delivered if/when the animal crosses into the darkened chamber. Memory is presumed to have occurred if the animal does not cross into the chamber in which it previously was shocked, or if latency to cross is significantly longer during the testing trial than during the training trial. The duration of passive avoidance testing is 5 min on each of two consecutive days, and is only performed once during an experiment. Chambers are cleaned with 35% isopropyl alcohol between animals.

The prefrontal cortex is involved in complex executive function, including planning, decision making, and controlling social behavior in humans. The social interaction paradigm has been used in rats to assess functioning of the prefrontal cortex in a rat model of schizophrenia (12). Further, rodent social interaction is correlated with neuronal morphology in the prefrontal cortex (13). The piriform cortex, also called the olfactory cortex, is related to processing of olfactory cues. Because rodent-rodent interaction involves sniffing and smelling one another, the social interaction paradigm will also be used to assess piriform cortex function.

Depression

Swim test: The Porsolt swim test is widely used as an index of learned helplessness or depression (17-20). The procedure involves a repeated procedure in which rats are placed into a cylinder (approximately 65 cm tall and 25 cm diameter cylinder filled to a height of 48 cm with water that is between 28-32 °C on the first day for 15 minutes. The rationale for the 15 min pretest is to induce a state of behavioral despair since the animals become more immobile as the test progresses during the 15 min pretest (Porsolt et al., 1978). As defined, immobility is floating in the water using only movements necessary to keep the animal's nose above the surface. Immobility time and latency to the initial immobility period are the primary dependent measures in this task (Castagne et al., 2009).

After 15 minutes of swimming, the rats are removed from the water and are dried and warmed with towels and heat lamps. On subsequent days, rats are retested for 5 minutes under identical conditions to determine the latency to become immobile (i.e.,

the rat initiates a stationary posture that does not reflect attempts to escape from the water), amount of time spent immobile, and number of times the animal becomes immobile, as determined by video analysis software (AnyMaze, Stoelting, Wood Dale, IL). After 5 minutes, the animals are removed and then dried and warmed with towels and heat lamps. On the second day of testing, shorter latencies to assume an immobile, floating position, more instances of immobility, and longer durations of immobility are interpreted as increased helplessness or depression. All rats are monitored the entire time and are removed from the water if they cannot swim or show signs of distress. Feces will be removed from the water between animals, and water will be replaced if it is difficult to observe the animals in the water. The test lasts 15 minutes on the training day, and 5 minutes on the consecutive testing day.

Seizure Observation
Animals will be placed into a cage separate from their homecage for observation and video analysis (AnyMaze, Stoelting, Wood Dale, IL) of seizure activity. Seizure activity is manually keyed for the duration of the seizure. AnyMaze software can analyze the video for latency to seize, seizure duration, and number of seizure occurrences.

V.4.4.7. Other Procedures:

Transcardial perfusion. When rats are euthanized they will be placed under deep Fatal Plus (50-75 mg/kg) anesthesia as described above. Once adequate anesthesia is achieved (lack of response to toe pinch, slow even respirations), a midsternal thoracotomy will be performed to expose the heart and the rats will undergo transcardial perfusion with cold PBS and 10% formalin. Generally, about 200 cc of each solution is sufficient per animal, but, more will be used if required. Transcardial perfusion will be performed in (b)(6)

Animal transportation: All animals will be transported to (b)(6) on the morning soman is to be injected in accordance with the LAM transportation SOP. Animals will be post-injected with linolenic acid or saline. Laboratory personnel who will transport animals to and from (b)(6) include: (b)(6) (b)(6) USUHS laboratory personnel will not be "working" on the animals at (b)(6) as only (b)(6) employees can handle nerve agents. Animals will be transported to and from (b)(6) using the LAM van. The rats will be brought back from (b)(6) to LAM by lab personnel on the day of nerve agent injection where they will be placed in home cages (1 rat/cage).

It is important to note that this study cannot be conducted without the use of the nerve agent, soman. Dr. (b)(6) will be submitting an IACUC protocol to (b)(6) The experiments using animals that will receive soman will commence when we receive approval of the animal protocol at (b)(6)

V.4.4.8. Tissue Sharing: The brains from Experiments 1-2 will be collected at the time of euthanasia and remain available for future studies. All other tissues (organs etc) will be made available to other investigator upon request.

V.4.5. Study Endpoint: The study endpoint for rats used for Experiments 1-2 is survival until the pre-determined post-operative time point, after which they will be euthanized. Prior to euthanization rats will be anesthetized then euthanized for tissue collection (V.4.4.7).

Early endpoints: Any of the following endpoints will represent a reason for early euthanasia: persistent seizures, persistent tremors, comatose for more than 2 hours after exposure to soman, self mutilation, continuous circling, persistent labored respiration. These signs, if observed by LAM personnel and no study personnel can be reached, may be taken by the LAM veterinarian as cause for euthanasia.

V.4.6. Euthanasia:

Experiment 1-2: Research personnel will perform all euthanasias except in cases where a LAM veterinarian makes the decision that a particular animal needs to be euthanized. At the indicated endpoint, each rat will be deeply anesthetized with Fatal Plus (60-75 mg/kg ip). Animals should be deeply anesthetized within 30 minutes. A midsternal thoracotomy will be performed to expose the heart and the mice will undergo transcardial perfusion with PBS and the 10% formalin in PBS. This procedure results in euthanasia from exsanguination under deep nembutal anesthesia.

Rats that reach an early endpoint will be euthanized via cylinderized CO_2. Research personnel, PI or a LAM veterinarian will perform this early euthanasia. The apparatus for euthanasia by CO_2 is located in (b)(6) Only one animal will be placed in the apparatus located in (b)(6) for euthanasia. With one animal in the apparatus, the flow rate of CO_2 will displace at least 20% of the apparatus volume per minute. Gas flow will be maintained for at least one minute after respiration has stopped before removing animals from the cage. A supplemental method to ensure death (cervical dislocation or bilateral thoracotomy) may be used. Alternately, if requested by the PI, animals will be euthanized by Center for LAM personnel) using cylinderized CO_2 in compliance with the 2013 AVMA Guidelines on Euthanasia. Euthanasia will be performed in the LAM CAF.

Euthanasia for western blot. Rats will be placed under deep Fatal Plus (60-75 mg/kg, i.p.) anesthesia. Once adequate anesthesia is achieved (lack of response to toe pinch, slow even respirations), the animal will be decapitated using a sharp blade to avoid tissue-altering effects of gases and anesthetics.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No __x__

Fluid Restriction: Yes No x

V.5.1.3. Exceptions: All animals will be housed individually after insertion of IV catheter. Animals injected with soman will be given apple slices, wet mash and gel squares as food supplements on the floor of each cage.

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be monitored for health, humane treatment, and husbandry considerations, twice daily by LAM staff during routine weekday rounds and at least once on weekends/holidays. This is in addition to, and not in place of, the monitoring that is done by the PI and their staff. In the event of a debilitating illness or adverse reaction, the decision to treat or euthanize an animal will be made by either the veterinarian and/or the PI.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions:

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Insertion of external jugular venous catheters, i.p. injection, euthanasia	(b)(6)	More than 15 years experience in in vivo rodent procedures including perfusions. 7 years experience inserting jugular catheters	Investigator course and rodent handling (1 June 2007)
small animal surgery, Drug injections, tissue		2 years experience with animal surgery; 3 + years rodent	Investigator course and rodent handling course (July, 2012)

dissection, euthanasia, transcardial perfusion	(b)(6)	handling and 3 years experience with tissue dissection and transcardial perfusion	
Animal handling, agent injections, drug injections		39+ years rodent handling, surgery and tissue dissection; 15 years in EEG electrode emplacement in primates; 39 years EEG electrode emplacement in rodents	Occ Health/zoonotic disease (01/05/04), Rodent Handling (02/05/04), aseptic techniques and rodent anesthesia (November 2006), Anesthesia (March 2006) and XCSM (nerve agent) custodian
Animal handling, tissue dissection, drug injections, transcardial perfusion		30+ yr rodent handling, surgery and tissue dissection; 4 yr experience on transcardial perfusion	Investigator training course at USUHS (Nov 1993).

VII. BIOHAZARDS/SAFETY: All personal protective equipment (PPE) will be properly disposed of in LAM or the laboratory. Waste gas is scavenged by appropriate filters in LAM-designated surgery rooms. All sharps will be placed in Sharps containers and disposed of properly. There are no precautions necessary for animals exposed to soman.

VIII. ENCLOSURES:

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Protocol for pain/ distress checklist (based on Kirsch 2002)

1. Appearance score:

Score of 0: Normal. Haircoat normal: smooth, lies flat, normal sheen. Eyes clear, open, no discharge/staining. Normal posture/movements

Score of 1: Lack of grooming apparent (i.e., animal does not clean off eye lubricant). No other marked changes

Score of 2: Haircoat roughened. Eyes and nose discharges or porphyrin (red) staining

Score of 3: Haircoat very roughened. External orifices ungroomed. Abnormal posture not explainable by brain damage. Eyes pale, sunken, glazed. Pupils enlarged.

2. Respiratory score

Score of 0: Respiration even and frequency unchanged. Limbs and feet warm. Mucous membranes (gums and anus pink), and extremities normal (ears and feet pink)

Score of 1/2: Respiration uneven. Shallow breathing, more abdominal breathing evident.

Score of 3: Respiration visibly labored. Limbs and feet cold. Mucous/membranes and/or extremities abnormal.

3. Unprovoked behavior score

Score of 0: Normal behavior pattern (i.e., exploring cage, grooming, feeding). Animal bright, alert, responsive.

Score of 1/2: Abnormal behavior. Less mobile, alert, responsive than normal. Inactive when hyperactivity expected (nighttime, feeding time). Guarding potentially painful area (i.e., leg, head, neck). Twitching, lameness.

Score of 3: Unsolicited vocalization, self-mutilation, expiratory grunts, grinding teeth, teeth chattering, salivating, very restless or does not move. Exaggeration of score 2 signs. Unresponsive, unaware of extraneous activity.

4. Reactive behavior score

Score of 0: Behavioral responses normal for expected conditions (i.e., reaching into cage, handling animal, response to tapping on cage, response to holding food pellet in grommet opening, taking rectal temperature).

Score of 1: Shows minor depression or minor exaggeration of responses.
Score of 2: Shows moderate signs of abnormal responses. There may be behavioral changes (i.e., animal more aggressive or more docile).
Score of 3: Animal overreacts to external stimuli, has weak responses as in pre-comatose state, or is non-responsive.

5. Body weight score (only at ≥24 hours): monitor body weight, skin turgor

Score of 0: body weight (BW) maintained or increased with respect to baseline. Skin hydration unchanged (pinch test – skin should "snap" back)
Score of 1: loss of BW < 10%
Score of 2: loss of BW 10-20%
Score of 3: loss of BW >20%

Body weight score and skin turgor will be checked daily until animals are euthanized.

Whenever a subscore of 3 or a total score of >10 is reached, the PI and/or veterinarian will be contacted and the rat will be considered for euthanasia.

5. Body weight score (only at ≥24 hours): monitor body weight, skin turgor

Score of 0: body weight (BW) maintained or increased with respect to baseline. Skin hydration unchanged (pinch test – skin should "snap" back)
Score of 1: loss of BW < 10%
Score of 2: loss of BW 10-20%
Score of 3: loss of BW >20%

Body weight score and skin turgor will be checked daily until animals are euthanized.

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6)

Principal Investigator Signature

29/1/14
Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6)

Principal Investigator Signature

29 JAN 14

Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature

29 JAN 14

Date

X. PROTOCOL ABSTRACT:

Alpha-linolenic acid (ALA) is an omega-3 polyunsaturated fatty acid shown to exhibit potent neuroprotective properties against various animal models of hypoxic-ischemic neuronal cell death. This compound is found in vegetable products such as flaxseed, rapeseed and pumpkin seeds and can be purchased over-the-counter. Humans can consume large quantities (up to 6 grams) of alpha-linolenic acid without side effects. We have shown previously that administration of a single intravenous injection of ALA given either 3 days prior or 30 min after soman reduces soman-induced brain damage. We now show that three doses of ALA given over a seven day period reduces brain damage, improves functional outcome and increases animal survival. In this proposal, we wish to test whether the well-established neuroplastic effects of ALA contribute to the reduction in soman-induced brain damage, improved functional outcome and increased animal survival.

A. Animal Protocol Number:

B. Animal Protocol Title: The possible involvement of LIN-induced neuroplastic effects against soman-induced brain damage in Sprague-Dawley adult rats

C. Principal Investigator: (b)(6)

D. Performing Organization: USUHS

E. Funding: NIH

F. Objective and Approach: The objective of this project is to determine whether the known alpha-linolenic acid-induced neuroplastic effects contribute to the observed increase in animal and neuronal survival, the anti-depressant-like activity and the improved functional outcome.

Indexing Terms (Descriptors): rat, soman, neurogenesis, synaptogenesis, synaptic function, rapamycin, BrdU, Porsolt forced swim test, hippocampus, BDNF, mammalian target of rapamycin.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

February 24, 2016

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF NEUROLOGY

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on February 24, 2016:

Animal Protocol Title: "Traumatic Brain Injury Recovery with n-3 Highly Unsaturated Fatty Acids (HUFAs) in Mice: A Biomarker Driven Approach"

USUHS Protocol Number: (b)(6)

Expiration Date: February 23, 2019

Supporting Grant(s) Number: TBD

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

Revised
IACUC Date Stamp

PROTOCOL NUMBER: To be assigned (b)(6)

PROTOCOL TITLE: Traumatic brain injury recovery with n-3 highly unsaturated fatty acids (HUFAs) in mice: A biomarker-driven approach

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6) USUHS # TBD

FUNDING AGENCY: Center for Neuroscience and Regenerative Medicine (USUHS)

EARLIEST ANTICIPATED FUNDING START DATE:

PRINCIPAL INVESTIGATOR:

(b)(6)
(b)(6) MD, PhD

Neurology	(b)(6)	9-18-2015
Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)
Research Unit Chief / Dept. Head Signature
Typed Name: (b)(6) COL, MC, USA

Chair, NEU	(b)(6)	12-7-2015
Title	Telephone	Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)
Statistician Signature
Typed Name: (b)(6) MD, PhD

Neurology	(b)(6)	9-18-2015
Department	Telephone	Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)
Attending/Consulting Veterinarian Signature
Typed Name: (b)(6) DVM

LAM	(b)(6)	12/9/15
Department	Telephone	Date

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) MD, PhD

ANIMAL PROTOCOL TITLE: Traumatic brain injury recovery with n-3 highly unsaturated fatty acids (HUFAs) in mice: A biomarker-driven approach

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: To be assigned

CO-INVESTIGATOR(S):

(b)(6) MD, PhD
(b)(6) PhD
(b)(6) PhD

TECHNICIANS(S):

(b)(6)

I. NON-TECHNICAL SYNOPSIS: Traumatic brain injury (TBI) is a major cause of mortality and morbidity in civilian and military populations. In pre-clinical animal models, therapies aimed at modulating excitatory neurotransmission, inflammation, oxidative stress, and neurotrophic factors, among others, have been effective in limiting secondary neurodegeneration and improving neurological outcome. However, none of these therapies has proved effective when tested in Phase III clinical trials. A major reason for the failure to translate the preclinical findings to the bedside is that human TBI is heterogeneous, and there is a lack of appropriate biomarkers that correlate to outcome. The discovery of useful biomarkers for TBI severity will allow for better diagnosis and treatment. Development of non-invasive measures of drug efficacy in pre-clinical models will allow for easy translation into early phase clinical trials. Polyunsaturated fatty acids (PUFAs) of the omega-3 type, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are abundant in cell membranes of the brain and are released after injury. Free DHA has neuroprotective and neuroreparative effects. DHA supplementation in mice improves recovery from experimental TBI. The typical human diet is deficient in DHA while lab mouse chow is abundant in DHA. We have modified lab mouse chow to match a human DHA diet to accurately measure efficacy of DHA as a neuroprotective therapy in a mouse model of TBI. DHA is thus a potentially viable drug treatment for TBI. Additionaly, blood biomarkers and neuroimaging of the injured, DHA-deficient mice may allow for the discovery of non-invasive and accurate biomarkers that can readily be translated into clincal settings. This study aims to investigate non-invasive imaging biomarkers for DHA deficiency and TBI, as well as elucidate the neuroprotective value of DHA as a treatment for TBI.

II. BACKGROUND:

II.1. Background:

Traumatic Brain Injury (TBI):
In the United States, approximately 230,000 patients are hospitalized each year with TBI, in addition to 1.5 million cases of milder TBI that do not require hospitalization (Langlois, et al. 2004). A large number of molecular events have been postulated, primarily on the basis of experimental animal models, to influence the response of neural tissue to brain injury (Siesjo 1993). In animal models, therapeutic interventions aimed at modulating several of these molecular steps have been successful in limiting the extent of injury and improving neurologic recovery (McIntosh 1993; McIntosh, et al. 1998). These experimental observations constitute a convincing demonstration of the principle that not all neural damage occurs at the time of injury, and that a window of opportunity exists for therapeutic intervention. However, Phase III clinical trials of several of these therapies in patients with severe brain injuries have failed to demonstrate efficacy (Doppenberg and Bullock 1997). Differences between animal models of brain injury and human TBI account for part of the failure to translate these important preclinical observations to the bedside (Narayan and Michel 2002). Further, the molecular mechanisms involved in neurodegeneration resulting from focal contusions may differ from those triggered by diffuse injuries (Maxwell, et al. 1997), and past clinical trials have made no attempt to identify injury mechanism or stratify by injury type. Thus, it is likely that further advances in the therapy of TBI will require a detailed understanding of the injury subtypes involved, and targeting of therapies to specific pathologic mechanisms (Stiefel, et al. 2004). MRI holds much promise as a tool for identifying such mechanisms.

Translation of Preclinical TBI Studies to the Clinic:
The failure of multiple Phase 3 clinical trials in TBI has forced a re-thinking of the approach to early phase human clinical trials. The conclusions of several workshops convened by DoD and NIH over the past several years is that biomarker-driven approaches are needed, in order to prove that the therapy is engaging its proposed molecular target and to provide proof of principle by demonstrating efficacy on a pharmacodynamic biomarker. Since the biomarker is mechanistically closer to the biological effect of the therapy than clinically relevant outcome measures, such studies can achieve adequate power with modest sample sizes, and can be useful for fine-tuning important issues such as dose, timing, and duration of therapy before launching large and expensive Phase 3 trials. This approach, while in principle straightforward, has very rarely been attempted. This is primarily because of a low level of collaboration between preclinical scientists and clinical investigators. Most preclinical studies use outcome measures such as histopathology and behavioral function which are not appropriate for human clinical trials. Additionally, important issues such as timing, dosing, duration of therapy, pharmacokinetics and pharmacodynamics are infrequently addressed in animal models. CNRM is ideally positioned to overcome this gap between preclinical and clinical investigators. Investments in preclinical and clinical imaging, as well as in preclinical and clinical blood-based biomarkers show substantial promise for identifying pharmacodynamic biomarkers in parallel between animal and human studies. Omega-3 fatty acids are nutritional supplements which have been extensively studied for the treatment of cardiovascular disease, stroke, and neurodegenerative disorders. It is recognized to have an excellent safety profile. In animal models of experimental TBI, omega-3 fatty acids has been shown to be effective in improving

outcome after impact acceleration injury and controlled cortical impact. The therapeutic window in rats is at least 24 hours, indicating that it is a feasible agent for human therapy.

DHA as a Neuroprotective Agent for TBI:

Among polyunsaturated fatty acids (PUFAs) of the omega-3 type, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), DHA is particularly abundant in the brain where it is primarily found in membrane phospholipids (Kim 2007). Immediately after injury, tissue phospholipases are activated, leading to an increase in free fatty acids. Free DHA has several prominent effects promoting neuroprotection and neurorepair, including promoting neurite outgrowth, synaptogenesis, and activating transcriptional activity via interaction with retinoid acid X receptors (Cao, et al. 2009; de Urquiza, et al. 2000). Further, DHA is converted to anti-inflammatory, neuroprotective, synaptogenic and pro-neurogenic mediators such as synaptamide, resolvins, and protectins (Hong, et al. 2003; Bazan 2005; Kim, et al. 2011; Rashid, et al. 2013), while AA-derived eicosanoids are often pro-inflammatory, exacerbating the initial injury (Farooqui and Horrocks 2006). In rats, omega-3 FA supplementation post-injury reduced axonal injury, microglial activation, and improved behavioral function after impact acceleration TBI and fluid percussion injury (FPI) (Mills, et al. 2011; Bailes and Mills 2010). Raising brain DHA by dietary supplementation of omega-3 fatty acids improves recovery from controlled cortical impact (CCI) injury in mice (Desai, et al. 2014). Interestingly, DHA levels in mice fed standard lab chow are significantly higher than in the human brain (Hamazaki, et al. 2010), and in our optimized mouse model the diet can be modified to match DHA levels found in humans. In this proposal we will develop these measures of efficacy of DHA as a neuroprotective therapy in a mouse model of TBI. DHA has an excellent safety profile, and has been widely tested in humans with cardiovascular disease, stroke, and neurodegenerative diseases. Only a few small trials have tested DHA supplementation after TBI, with widely publicized but anecdotal results. DHA is thus a potentially viable drug treatment for patients with TBI. The next steps for the pre-clinical investigation are to develop measurements of drug efficacy through measurements of biomarkers in serum, and imaging studies, which can be used as noninvasive indications of efficacy in early phase clinical trials. Our objective is therefore to determine whether DHA supplementation alters plasma levels of bioactive lipids known to be neuroprotective, anti-inflammatory, and neuroregenerative, such as synaptamide, 17-hydroxy-DHA, resolvins and endocannabinoids, the mechanisms through which DHA supplementation is proposed to improve recovery from TBI.

MR imaging of DHA treatment after TBI:

The failure of over 30 drugs in clinical trials for TBI suggests the need for different processes to evaluate suitability of potential therapies. Identification of pharmacodynamic biomarkers in the laboratory and then translation of those biomarkers to early phase human studies has not yielded successful therapies. One suggested change is the development of non–invasive measures of drug efficacy in pre-clinical models. Non-invasive measures, such as MRI, can easily be transferred to early phase clinical trials, in order to demonstrate that the therapy is engaging the molecular target and resulting in a biologic effect.

This program proposal is centered on the evaluation of supplementing diet with omega-

3 fatty acids (FA), such as docosahexaenoic acid (DHA), as a potential therapeutic strategy for traumatic brain injury (TBI). An extensive literature suggests a neuroprotective role for omega-3 FA in stroke, neurodegenerative diseases, and traumatic spinal and brain injury (Michael-Titus and Priestley 2014; Lee, et al. 2015; Sorg, et al. 2015; Wilde, et al. 2015). There is also evidence of improved recovery of motor functions and lower anxiety in mice with adequate supplementation of DHA versus DHA depleted mice after TBI induced by controlled cortical impact (Desai, et al. 2014). Nothing is known, however, about potential Magnetic Resonance Imaging (MRI) biomarkers of structural brain changes induced by omega-3 FA. As omega-3 FA alter the chemical composition of the plasma membrane of neuronal cells it is reasonable to assume that MRI measurable brain tissue properties such as T1 and T2 relaxation times and diffusion properties may be altered. Preliminary experiments from our laboratory in mice where brain tissue omega-3 FA levels have been enriched or depleted by dietary manipulations shows significant changes in diffusivity and relaxometry. Moreover, if omega-3 FA dietary intake affects the outcome of TBI, MRI metrics should be able to document different levels of severity of the structural damage in the injured brain depending on the therapeutic regime. Finally, MRI metrics may help elucidate the mechanisms of improved recovery, revealing potential effects of omega-3 FA on edema, inflammation, gliosis, and neuroregeneration. Parallel histological assessment and ex-vivo MRI scanning will enable us to elucidate the biological substrate of the structural changes detected using the MRI techniques.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: PubMed, PowER Search through USUHS Library, DTIC database, and NIH RePorter

II.2.2. Date of Search: 11/2/2015

II.2.3. Period of Search: Pubmed: 1950-present
PowER Search: 1916-present
DTIC database: all available
NIH RePorter: 1990-2015

II.2.4. Key Words and Search Strategy: The PubMed database was searched using the following strategy:

#1: TBI AND DHA
#2: TBI and MRI and omega-3 fatty acid

The following keywords were used in PowER Search:

Traumatic brain injury
Omega-3 fatty acids
DHA
Magnetic resonance imaging

Diffusion tensor imaging
Neuroprotective
Biomarker
Mouse/ Mice

The following keywords were used for DITC database:

TBI
DHA
Mouse
MRI

The following keywords were used for the

TBI
DHA
Mouse
MRI

II.2.5. Results of Search: Search strategy 1 for Pubmed yielded several studies that investigate DHA as a neuroprotective treatment for TBI (16 publications). Only one publication utilizes MRI in combination with DHA supplementation after TBI (search strategy 2 for Pubmed, 1 publication.) This publication is a case study, as opposed to a scientific experiment, of 1 patient that utilizes MRI throughout the patient's recovery (Lewis, et al. 2013). PowER Search through the USUHS library did not yield a similar study. DTIC database yielded 0 studies when all keywords were searched together. When MRI was excluded from the search (TBI and DHA and Mouse) one study came up from our collaborator Dr. (b)(6) researching DHA metabolites after TBI. However that study did not have the same scope regarding MRI biomarkers and study time points as the current study. The NIH RePorter searched yielded 0 projects/matches. After a thorough literature search, there was no indication of duplication efforts to answer the particular questions posed in our aims and no comparable use of methods. There are currently no drug treatments or accurate biomarkers for TBI. It is thus important that we work towards studying the potential for DHA and Synaptamide as pharmacodynamic biomarkers for TBI outcome as well as a potential therapeutics.

III. OBJECTIVE\HYPOTHESIS:

Hypothesis: Our hypothesis is that DHA supplementation after TBI is neuroprotective and this neuroprotection can be detected via blood biomarkers and advanced magnetic resonance imaging strategies. We will show this with the following specific aims:

Aim 1: Innovative imaging study, designed to determine whether advanced neuroimaging strategies, including diffusion imaging and relaxometry, are sensitive to the omega-3 fatty acid content of brain membranes. In this study we propose to use quantitative MRI techniques to identify potential biomarkers of brain

changes induced by Omega-3 fatty acids in animals. We plan to investigate the effects of DHA intake on the brain in mice that are chronically depleted of Omega-3 FA vs. animals with an Omega-3 FA enriched diet, using a model based on the one developed by Desai et al. (2014) in Dr (b)(6) lab. We will acquire MRI data of mice *in vivo* prior to DHA supplementation and then longitudinally. The MRI measures we plan to apply are quantitative and informative about different tissue properties (a detailed description of the MRI techniques can be found in the research plan section). Ex-vivo, high resolution MRI scanning will be performed on the same brains studied with histological techniques. The histological analysis will enable us to elucidate the biological substrate of the structural changes detected using MRI.

Aim 2: Preclinical project designed to identify MRI biomarkers sensitive to omega-3 supplementation after TBI. Such MRI and blood biomarkers could readily be translated to clinical trials. These include measurements of bioactive lipids in plasma and RBC membranes such as synaptamide, 17-hydroxy-DHA, D-resolvins, endovanilloids, and endocannabinoids. These bioactive lipids have potent anti-inflammatory and pro-neurogenic effects that will simultaneously be tested in a parallel clinical study. Our collaborators at (b)(6) will be conducting parallel studies aimed to: (1) Reproduce neuroprotective effect of DHA in mice with humanized tissue levels of omega-3 FA. To determine which bioactive lipids are altered by DHA after TBI in mice. C57BL/6 mice will be raised in a special chow designed to produce brain tissue omega-3 FA levels comparable to what is found in human brain tissue. Eight-10 weeks old male mice will be injured by an experimental TBI model, moderate controlled cortical impact in the parietal cortex. DHA and Synpatamide supplementation (40 mg/kg) will start 6 hours after injury and continue for 30 days. Blood will be collected at sacrifice. Accessible biological tissues (plasma, serum, RBC membranes, potentially others) will be assayed using sensitive HPLC-Mass Spectrometry (MS) assays in Dr (b)(6) lab. Several bioactive lipids will be measured. The focus will be on bioactive lipids which are products of DHA and have anti-inflammatory and pro-neurogenic properties, such as synaptamide, D-resolvins, and 17-hydroxy-DHA. We expect to see an increase in tissue and plasma levels of neuroprotective bioactive lipids. Our lab will acquire MRI data of mice *in vivo* prior to injury and then longitudinally after TBI induced by controlled cortical impact to investigate the effects of DHA treatment. We will apply the same in-vivo and ex-vivo MRI techniques from aim 1, as well as histological characterization of the brains after injury.

IV. MILITARY RELEVANCE:

At present there are no effective treatments for TBI, other than stabilizing the patient, preventing excessive intracranial pressure and symptomatic relief. Military physicians and their patients are in desperate need of interventions that will lessen the initial lesion, before the process of regeneration and repair can begin. This study is focused on furthering development of treatment with a naturally occurring dietary supplement, omega-3 fatty acids. Over 30 drugs that were effective in animal TBI models have failed in clinical trials for TBI suggesting that different tactics are required for establishing efficacy of a drug both in animal models, and within the clinical trial. A recent pharmaceutical working group convened by (b)(6) supported the development of pharmacodynamic biomarkers, proteins that could be easily measured in blood, that

would demonstrate the efficacy of the drug. Non-invasive biomarkers, such as MRI abnormalities, are also promising. These non–invasive measures of drug efficacy in pre-clinical models can be transferred to early-phase clinical trials, in order to demonstrate that the therapy is engaging the molecular target and resulting in a biologic effect. This proposal seeks to establish blood biomarkers to determine the efficacy of candesartan action by non-invasive measures. It therefore will link seamlessly with early phase clinical trials to provide better measurements for assessing efficacy in patients. These experiments therefore are developing a new methodology for assessing suitable therapies in pre-clinical models, in order to allow clinical trials to have different outcome measures. This proposal will therefore have direct relevance to recovery from TBI for military service members as it seeks to develop a functional working therapy with a widely available and safe dietary supplement. The experiments will provide more complete information to decide whether to proceed to phase III clinical trials with omega-3 fatty acid supplementation. Additionally, the knowledge of blood and MRI biomarkers associated with different outcomes and responses to the therapy would be very important for the clinical management of service members suffering from TBI.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

Specific Aim 1: Innovative imaging study, designed to determine whether advanced neuroimaging strategies, including diffusion imaging and relaxometry, are sensitive to the omega-3 fatty acid content of brain membranes.

Specific Aim 2: Innovative imaging study, designed to determine whether advanced neuroimaging strategies, including diffusion imaging and relaxometry, are sensitive to the omega-3 fatty acid content of traumatically injured brain membranes and structures.

V.1.1. Experiment 1: 1) To measure quantitative MRI parameters in the brain of healthy mice that received adequate amount of dietary Omega-3 FA, versus animals that were chronically depleted.

Two-week pregnant dams will be purchased from an approved vendor and housed at USUHS. Control dams and pups will receive a diet with adequate DHA levels throughout the experiment. Experimental dams will receive a DHA-deficient feed once they arrive in the animal facility and throughout the rest of their pregnancy (see v.5.1.2). The pups will be fed the DHA-deficient diet throughout the experiment. We expect approximately 8 pups per litter (based on normative data for C57/6 mice) 50% male and 50% female. We intend to use all mouse pups for this study.

Mice will either receive DHA (40 mg/kg), Synaptamide (40 mg/kg), DHA plus Synaptamide, or vehicle injections. Animals will begin receiving supplement injections at 3 months of age. Animals will receive daily injections for a maximum of 30 days. The MRI will be performed both in vivo at 7 or 30 days. Then the brain will be fixed via cardiac perfusion and removed for ex-vivo high resolution scanning. After scanning the entire brain would be available to Dr. (b)(6) team for histological

characterization.

Total: N=140 (Class D)	**Aim 1**	**Aim 1**	**Aim 1**	**Aim 1**	**Aim 1**
Imaging Time Point	**Sufficient Dietary DHA**	**DHA-Deficient, vehicle injected**	**DHA-deficient, DHA supplemented**	**DHA-deficient, Synaptamide supplemented**	**DHA-deficient, DHA/Synaptamide supplemented**
Acute (7 days)	Male=7 Female=7	Male=7 Female=7	Male=7 Female=7	Male=7 Female=7	Male=7 Female=7
Chronic (30 days)	Male=7 Female=7	Male=7 Female=7	Male=7 Female=7	Male=7 Female=7	Male=7 Female=7

V.1.2. Experiment 2: To measure quantitative MRI parameters in the brain of mice with TBI and assess potential effects of dietary Omega-3 FA on outcome and brain structural recovery. Two groups of animals will be included, mice which received adequate amount of dietary Omega- 3 FA, versus animals which were chronically depleted. This aim includes both in vivo, and ex-vivo scanning at high resolution. The in-vivo component will investigate the effects of Omega-3 FA on the evolution of the TBI lesion.

Two-week pregnant dams will be purchased from an approved vendor and housed at USUHS. All dams will receive a DHA-deficient feed once they arrive in the animal facility and throughout the rest of their pregnancy. The pups will be fed the DHA-deficient diet throughout the experiment. Our plan is to scan *in-vivo* all animals before inducing the lesion and then at selected time-points after experimental TBI. The injury will be produced via the controlled cortical injury experimental TBI model on 8-10 week old DHA-deficient mice. One group of CCI mice will receive supplemental DHA, synaptamide, or DHA plus synaptamide injections. The other group of CCI injured mice will receive vehicle injections. Animals will be scanned at various time points, then immediately euthanized via transcardial perfusion. For transcardial perfusion, animals will be injected with Pentobarbital Na. When they are nonresponsive to toe pinch, the animnals will be perfused with phosphate buffered saline and 4% paraformaldehyde and the brain extracted. The brains will undergo high resolution ex vivo MRI and then they will be cut at 40 um thickness and undergo immunohistochemistry to reveal astrogliosis, axonal number, myelin thickness and neurons. The brains will then be scanned via high-resolution *ex-vivo* MRI. Then the brain will be histologically characterized. This will enable direct comparison of *in vivo* scanning with high resolution *ex-vivo* scanning and histology.

Fifty additional animals will come directly from our collaborator, Dr. [b)(6) redacted] to be scanned at USUHS and sacrificed immediately after scanning. These animals will not be housed at USUHS, however, will be transferred to USUHS according to LAM protocol.

N=360 (Class E)	**Aim 2**	**Aim 2**	**Aim 2**	**Aim 2**	**Aim 2**
Imaging Time Point	Sham-CCI, DHA-deficient,	CCI, DHA-deficient, vehicle	CCI, DHA-deficient, DHA	CCI, DHA-deficient, Synaptamide	CCI, DHA-deficient, DHA/Synaptamide

	vehicle injected	injected	supplemented	supplemented	supplemented
Baseline (pre-lesion)	Male=9 Female=9	Male=9 Female=9	Male=9 Female=9	Male=9 Female=9	Male=9 Female=9
1 day post CCI	Male=9 Female=9	Male=9 Female=9	Male=9 Female=9	Male=9 Female=9	Male=9 Female=9
7 day post CCI	Male=9 Female=9	Male=9 Female=9	Male=9 Female=9	Male=9 Female=9	Male=9 Female=9
60 days post CCI	Male=9 Female=9	Male=9 Female=9	Male=9 Female=9	Male=9 Female=9	Male=9 Female=9

Magnetic Resonance Imaging of CCI and DHA-deficiency: In vivo Animal MRI acquisitions will be performed in the 7 Tesla Bruker scanner at the CNRM Translational imaging facility, while high resolution ex-vivo scans of fixed brains will be performed on the NIH vertical bore scanners. We plan to focus on three quantitative MRI acquisition methods: 1) Diffusion MRI with Diffusion Tensor (DTI) dual compartment DTI assessment as proposed by Pierpaoli et al. (2004), (2) High resolution DESPOT-based T1and T2 and myelin water compartment assessment as proposed by Deoni et al., (2008), and, in ex-vivo animal brains, 3) High Angular Resolution Diffusion Image (HARDI) analysis using the MAP-MRI approach suggested by Ozarslan et al. (2013) for high quality investigations of diffusion properties.

The first task for diffusion MRI development is to improve the anatomical quality of the images, implementing optimal diffusion MRI (DWI) sequences that would allow correction for distortions in Echo planar Images. While DWI has opened up a new window on studying brain architecture, some brain regions and structures are still invisible in conventional DWI acquisitions. In particular, EPI, the most widely used sequence to acquire clinical DWI data, produces almost no measurable signal in brain areas near the sinuses and oral cavities, making white matter pathways there difficult to study. We plan to work on reducing these artifacts in EPI. The current version of our processing software TORTOISE (www.tortoise.org) corrects distortion from susceptibility artifacts by warping the EPI images to a unwarped reference image (usually a fast spin-echo echo image). The method works very well in most regions, but it is unable to correct regions of "signal pileup".

V.2. Data Analysis:

Magnetic Resonance Imaging: We plan to focus on three quantitative MRI acquisition methods: 1) Diffusion MRI with Diffusion Tensor (DTI) dual compartment DTI assessment as proposed by Pierpaoli et al. (2004), (2) High resolution DESPOT-based T1and T2 and myelin water compartment assessment as proposed by Deoni et al., (2008), and, in ex-vivo animal brains, 3) High Angular Resolution Diffusion Image (HARDI) analysis using the MAP-MRI approach suggested by Ozarslan et al. (2013) for high quality investigations of diffusion properties. If MAP-MRI will prove informative in the animal model, an appropriate clinical implementation of the MAP-MRI sequences will be also be used for the clinical studies.

The following parameters will be used for the diffusion studies in the animal experiments, both in vivo and ex-vivo are.

In-vivo diffusion acquisition. Images will be acquired using a single-shot 2D EPI pulse sequence with slice and spatial dimensions to attempt isotropic voxel sizes (approximately 250 microns isotropic). Multiple repetitions will be used with a single average and a "blip-up/blip-down" approach will be used where half of the repetitions will have a reversed phase encode direction. Diffusion parameters will be multiple non-weighted reference images (b=0s/mm2) and for DTI diffusion weighting, a b-value of 800-1000 s/mm2 will be used with 14-32 directions. For MAP-MRI an optimized diffusion-weighting paradigm will be used with multiple shells in q-space (likely 4 shells ranging from 1000-5000 s/mm2).

Ex-vivo diffusion acquisition. Fixed brain tissue will be imaged at either 7T or 14T using a 10-15mm volume coil. A segmented 3D EPI pulse sequence will be used to rapidly acquire each diffusion weighted volume (4-8 minutes per image volume) at high resolution (100 microns isotropic). A blip- up/blip-down approach will also be used for this acquisition as described in the previous section. The diffusion parametes will include a set of low b-value image volumes (100-1000s/mm2) for use in calculating the reference image and multiple shells in q-space including b(in s/mm2)/#directions=1700/32, 3800/32, 6700/56 and 10,000/87. Gaussian DTI will use only the reference images and b=1700 s/mm2 shell, while non-Gaussian DTI and MAP-MRI will use the entire sampled q-space.

Diffusion image processing. In-vivo and ex-vivo images will be processed offline using our TORTOISE software package (www.tortoise.org) including: blip-up/blip-down correction for geometric distortions, registration-based correction of motion and eddy-current artifacts, alignment with a standard template, non-linear estimation of the diffusion tensor, modeling of MAP-MRI parameters. HARDI and mcDESPOT (Deoni 2008) analysis will be performed. Each processing modality offers different information regarding the water diffusion magnitude, direction, and restriction; providing a more complete picture of the underlying microstructure of the brain.

The data acquired from this study will also be used to improve existing DWI techniques and processing protocols. Removing cerebral spinal fluid signal (CSF), distinguishing myelin from other aniostotropic regions, lack of contrast in certain grey matter regions, correcting for "signal pileup", and interpreting diffusion parameters in relation to biological mechanisms are some of the current issues with conventional DWI that need further development. Our lab has already made progress in correcting distortion from susceptibility artifacts by warping the EPI images to an unwarped reference image (usually a fast spin-echo echo image) using the current version of our processing software TORTOISE (www.tortoise.org). The method works very well in most regions, but it is unable to correct regions of "signal pileup." "Signal pileup" occurs when the amount of distortion changes in such a way that a single voxel in the reconstructed image contains signal from a region containing several voxels in the undistorted image. In this case, information is lost and no correction method can restore an undistorted image. The lost information can be recovered by acquiring a second image of the same slice with the polarity of the phase encode blips reversed. The two images together

contain all the information required to reconstruct an undistorted image. We have developed methods and written code to efficiently correct image distortion using information from blip-up and blip-down images, and we intend to include both blip-up and blip-down image acquisitions in our protocol. We expect this correction method will increase the geometrical fidelity of our images, reduce the systematic error in the DTI results, and increase our ability to correlate imaging findings to histopathology.

The dual compartment DTI method we plan to use has enhanced tissue characterization capabilities compared to conventional DTI. It will enable us to extract specific information about the parenchymal water compartment by removing the effect of CSF contamination. Diffusion measurements in brain tissue adjacent to CSF-filled cavities suffer from CSF partial volume contamination. As the diffusivity of water in CSF is 3 times larger than in normal brain parenchyma, even a small amount of CSF can severely bias diffusion measurements in the cortex and periventricular areas. As the cortical thickness is ~2mm, it is virtually impossible to obtain diffusion data in the cortex unaffected by CSF contamination at the image resolution of clinical DTI. In animal experiments image resolution is higher (in this project we expect to collect in vivo brain DTI data with isotropic voxels of 300 microns) but still significant CSF contamination at interfaces can occur. One additional interesting feature of this approach is the handling of the vascular signals that in conventional DTI contaminates the measured diffusivity of the parenchyma. With the dual compartment DTI analysis, vascular spins will affect the diffusivity and the volume fraction of the CSF water compartment, not that of the parenchymal compartment. This is particularly important for this experiment in which subtle diffusivity changes are expected due to plastic changes in the brain. We expect to be able to differentiate plasticity that may affect the vasculature, which will be reflected in the fast diffusivity water compartment from changes that will affect the brain tissue, either glial cells or neurons, which will be reflected in the low diffusivity, parenchymal water compartment.

Magnetization transfer imaging (MTI) and DTI provide information related to myelin, however, these measures are non-specific (the MTI signal is generally related to lipid content, and significant diffusion anisotropy is observed even in non-myelinated white matter). Currently, multi-component relaxometry (MCR) is the most direct means of quantifying myelin volume, with estimates having strong agreement with histopathological assessments. In conventional T2 MCR, the measured MRI signal is decomposed into contributions from two water pools, which in brain tissue, are attributed to an intra/extra-cellular pool and water trapped between the hydrophobic bilayers of the myelin sheath. The T1 and T2 MCR method we plan to use is based on rapid steady-state imaging techniques. Unlike conventional T2 MCR approaches which suffer lengthy acquisition times and poor spatial resolution (typically more than 16 minutes for a single image slice with 1 mm3 voxels), this new mcDESPOT technique permits reproducible whole-brain high-resolution myelin quantification in a clinically reasonable time-frame (less than 20 minutes), presenting the first opportunity for whole-brain quantitative evaluation of myelin in vivo in human subjects. The importance of myelin in the pathophysiology of TBI and the role of myelin products as potential biomarkers in TBI are reported in several recent studies (Schumacher 2007; Hergenroeder 2008; Ottens 2008). We hypothesize that the diffusion anisotropy changes that have been measured in white matter following learning paradigms could

be related to changes in the degree of myelination of existing axons rather then in the number of axonal projections. This view is supported by recent work by (b)(6) at the NIH who has demonstrated that electrical activity signals oligodendrocytes to trigger the initial events in formation of myelin. This mechanism would preferentially myelinate electrically active axons (Wake 2011). The use of mcDESPOT to assess myelin-specific changes in brain plasticity is a completely novel approach, and we believe that it would be more informative than previously used MRI methods.

Overall the use of these quantitative MRI metrics, in conjunction with histopathological validation, will represent a first step in achieving biological specificity in the characterization of plastic brain changes that are affected by Omega-3 FA intake. An important aspect of this project is that we aim at using the same quantitative acquisitions in both the animal and clinical portion of the project so that the results of the histological assessment performed by Dr (b)(6) lab would be valuable for the interpretation of MRI findings obtained in the clinical setting.

Histological Characterization: All brains will be sections at 40-30 microns and stained for markers of neurodegeneration and neuroinflammation. After staining, digital images of the whole section will be acquired. The digital images will be analyzed using imageJ software.

Statistical Analysis: Histology and DTI differences between treatment groups at different times will be analyzed using two-way repeated measures ANOVA. Male v. Female differences will be analyzed via one-way ANOVA both within groups and within time points. Correlation plots will then be done to compare DTI metrics with histology markers.

Statistical power analysis: Number of animals for each experiment was determined via a power-based assessment. The sample sizes (for each group) are considered for an alpha=.05 and power=.80. Experiment 1 has 5 treatment groups and 2 time points. Experiment 2 has 4 treatment groups and 4 times points. Assuming an effect size of 1.25, experiment 1 requires n=7 and experiment 2 requires an n=9. These groups were then doubled to include both male and female mice as separate groups per treatment and time point.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

We study the impact of injury on the molecular mechanisms and structural integrity of the brain which requires an accurate representation of the structure and biology of the brain. Since this study requires a specific structural and molecular biology that is not reproducible in non-animal models, we are required to use an animal model. Culture and *in vitro* techniques as well as computer models are applied to this research whenever possible.

V.3.2. Animal Model and Species Justification:

CCI is a highly reproducible and widely studied model for traumatic brain injury. There is a large literature studying the effects of CCI on the neurobiology of mice, which will assist in understanding our results later on. Additionally, the previous research studying the effect of DHA on the brain was done in CCI injured mice.

V.3.3. Laboratory Animals

		Species #1	Species #2
V.3.3.1.	**Genus & Species**:	*Mus musculus*	*Mus musculus*
V.3.3.2.	**Strain/Stock**:	C57Black/6	C57Black/6
V.3.3.3.	**Source/Vendor**:	1) (b)(4)	1) Weaned from pregnant dams at USUHS 2) (b)(6)
V.3.3.4.	**Age**:	~8 weeks	8-10 weeks
V.3.3.5.	**Weight**:	25-30g	~30g
V.3.3.6.	**Sex**:	Female, 2-weeks pregnant	Male and Female
V.3.3.7.	**Special Considerations**:	Given special chow: See v.5.1.2	Given special chow: See v.5.1.2

V.3.4. Number of Animals Required (by Species): Species 1: 71
Species 2: 500
plus 50 animals from (b)(6)

V.3.5. Refinement, Reduction, Replacement (3 Rs): also see CNRM SOP #1

V.3.5.1. Refinement:
The experiments are conducted with every effort to eliminate the possibility of pain. Animals will be anesthetized prior to exposure to CCI to minimize animal distress at the time of injury. Immediately following CCI, the animals will be monitored for at least two hours for signs of pain and/or distress i.e. changes in appearance, respiration, provoked and unprovoked behavior, and changes in body weight. Body weight will be measured once before surgery and then weekly after that. A total body weight loss of >10% from baseline weight for a sustained period of 1 week will constitute a sign of excess pain or distress. Animal handlers will adjust animals to new environments or experimental situations by allowing animal to acclimate to the new environment for at least 15 minutes prior to the experiment. New environments include the USUHS MRI facility and the surgery rooms.

V.3.5.2. Reduction:
The number of animals used in these experiments is dictated by considerations of statistical power. We will use the same animal to test multiple potential TBI biomarkers, which includes blood and MRI biomarkers.

V.3.5.3. Replacement:
No adequate replacement for live animal use was identified for this study. We have considered use of in vitro techniques and computer simulations whenever possible, however, these approaches cannot address the complex structural and molecular interactions required to study how DHA treatment effect's the brain after injury, and have therefore been rejected.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:
Depth of anesthesia will be checked prior to any potentially painful procedure by administration of a foot-pinch test. The test should not elicit a response from the animal. Assessment of pain will be accomplished by close visual monitoring both during and after potentially painful procedures by trained study personnel. Signs of excessive pain or distress will result in immediate removal from the study and euthanasia.

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	71	0
V.4.1.1.1.2. Column D:	0	212
V.4.1.1.1.3. Column E:	0	338

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: see CNRM SOP #1
Isoflurane will be the anesthetic. Mice will be anesthetized in an induction chamber with isoflurane + 100% oxygen containing (4% isoflurane.) After anesthesia is induced (indicated by non-responsiveness to toe pinch and decreased respiratory rate), the isofluorane is reduced to 1.5-2% and the animals will be maintained via nose cone.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:
Animals will be gently handled for approximately 3min daily for 3-5 days prior to the start of the experiments to reduce stress on the animal. A general assessment of physical and behavioral health will be carried out on each mouse before any procedure. A veterinarian will be consulted should any sign of abnormality in appearance and/or

behavior be observed. Animals will be housed in a static cage in a room with stable temperature and humidity and on a reversed 12-h light/dark cycle per LAM SOP. Food and water will be *ad libitum*. For at least two hours following exposure to CCI, mice will be observed for signs of pain and distress and/or gross neurologic deficits. Assessment of pain will be accomplished by close visual monitoring both before and during potentially painful procedures by trained study personnel. Specifically, animals will be observed for changes in appearance, respiration, and provoked and unprovoked behavior. If recovery is not complete by the end of two hours, observation will be continued until recovery is complete. If the animal is having difficulty moving about the cage, gel packs, which provide complete nutrition will be placed on the bottom of the cage to allow easier access to food. If the animal shows signs of unusually high levels of pain and discomfort, it will be euthanized

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: ALTBIB and PubMed

V.4.1.3.2. Date of Search: 8/27/15

V.4.1.3.3. Period of Search: all available

V.4.1.3.4. Key Words of Search:

Keywords for PubMed:

#1: Pain AND traumatic brain injury AND in vitro
#2: MRI AND traumatic brain injury AND in vitro
#3: MRI AND traumatic brain injury AND cell culture
#4: traumatic brain injury AND pain

V.4.1.3.5. Results of Search:

There is a small selection of brain injury research conducted in vitro and in computer models. These studies address specific bioengineering questions or specific cell injury questions. The only way to address the specific aims proposed in this study is to keep the cellular organization and biochemical interactions of the entire brain intact. This requires using an in vivo model and reducing the anesthetics and analgesics being administered. This search lead to a review article that discusses the need for in vivo experimental TBI and careful consideration of how anesthetic and analgesic agents can affect the experimental outcome (Rowe, et al 2013).

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

See CNRM SOP #1

The goal of the current study is aimed at understanding the benefits of n-3 fatty acid supplementation for brain development and recovery after traumatic brain injury. Analgesics and anesthetics commonly used during animal surgery have neuroprotective and anti-inflammatory side effects (Rowe, et al. 2013; Graham, et al. 2004; Zohar, et al. 2006; Zhang, et al. 2002). The efficacy of n-3 fatty acids after TBI may be a result of their effect on anti-inflammatory pathways (Calder 2005). N-3 fatty acids, specifically DHA, decreases the products of cyclooxygenase 1 and 2 (COX-1 and COX-2) (Norris, et al. 2012). The non-steroidal anti-inflammatory drugs (NSAIDs) typically used after animal surgery (i.e. meloxicam) work by inhibiting COX (Botting 2000; Tacca, et al. 2002). Another commonly used analgesic for animal surgeries is acetaminophen. Acetaminophen has been reported to inhibit prostaglandin synthesis, a prominent molecular product of the COX-inflammatory pathway (Graham and Scott, 2005). DHA and the former analgesics would exhibit similar biochemical footprints when investigating the COX inflammatory pathway. Opioids, like Buprenorphine, have been reported to show anti-inflammatory effects such as decreasing TNF (Walker 2003; Lufty, et al. 2004). Neuroinflammatory pathways are an important site of inquiry in this study. The neuroprotection and anti-inflammatory actions of additional post-operative analgesics may confound and mask n-3 fatty acid effects on brain biochemistry and repair. Avoiding additional analgesics is recommended until a better understanding of the effects of n-3 fatty acids on reparative brain mechanisms and development is acquired

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: see CNRM SOP #1
Mice will be fed a diet deficient in DHA. The present study will be conducted to establish the neuroprotective potential of n-3 fatty acids/DHA in traumatic brain injury model. Modified diet will be used as one of the sources to expose the animal to different levels of fatty acids. In these diet studies, the animals will be fed one of four defined diets (AIN-93) that consist of varying ratios of n3 and n-6 fatty acids prior to euthanasia and tissue collection. The supplementation entails only a substitution in fats, with the rest of the dietary composition maintained at normal levels. Since linoleic acid is considered an essential fatty acid, all diets will include at least 0.5 energy percent of this fatty acid. Therefore, animals will receive proper nutrition throughout the experiment and there will be no water restriction. Two-week pregnant dams will be placed on a special diet through the pregnancy as well as after delivery in the lactation period. After weaning, the male and female offspring will be placed on the same diet as had by the respective dams for a further 8-10 week period. For both series of experiments, the animals will be subjected to CCI when they are 8-10 weeks old and continued with their respective diet regime for about another week.

V.4.3.2. Procedure: see CNRM SOP #1 for

CCI: Mice will be induced in 100% oxygen and 4% isoflurane. Once mouse is unresponsive to toe pinch, the anesthesia will be maintained at 2.5% isoflurane. The head will be secured via ear bars and the top of the head will be shaved. Iodine will be

applied to the shaved area. A midline incision using a scapel will be performed to expose the parietal bone of the skull. The craniotomy will be 4 mm made over the left cerebral hemisphere between the lambda and the bregma marks about 1 mm from midline. An impact velocity of 3.5 m/s with a penetration depth at 1.5 mm and impactor dwell time of 500 ms will be used. After the injury, the wound will be covered with Surgicel (Johnson & Johnson, Arlington, TX, USA) and the craniotomy closed using cyanoacrylate. The incision will be sealed with non-absorbable nylon sutures (Ethilon Nylon Suture, Ethicon, Johnson & Johnson) and then the animal will be removed from anesthesia. Once the animal regains righting reflex, the animal will be returned to their home cage and monitored for excessive pain and discomfort. Any sutures that do not fall out naturally while the incision is healing post-surgery, will be removed when the incision is fully sealed (approx. 1 week post surgery.)

MRI: One set of mice will be housed at USUHS and taken to the USHUS Animal MRI facility directly for imaging. Another set of mice will arrive from Dr. (b)(6) (b)(6) . These mice will be transported to USHUS for MRI imaging via the LAM vehicle and euthanized immediately after imaging in the fume hood in Rm (b)(6) For MRI imaging, mice will be anesthetized in an induction chamber with 100% oxygen containing 4% isofluorane. After anesthesia is induced (indicated by loss of toe pinch reflex, decreased respiratory rate), the isofluorane is reduced to 1.5-2% and the animals will be maintained via nose cone in a stereotaxic setup. Core body temperature is monitored by a thermistor type rectal temperature probe (or an MR compatible fiber optic rectal probe) that is lubricated and inserted into the rectum. A target body temperature of 37 is maintained by a feedback controlled circulating warm water blanket. Plane of anesthesia will be monitored by anesthetic gas levels, and changes in physiological parameters that may include heart rate, respiratory rate, end tidal CO2 and body temperature (via rectal probe). ECG may be monitored if necessary.

For MR imaging of the brain, the head of the anesthetized mouse will be restrained with ear bars and a bite bar in a configuration similar to commercial stereotaxic devices. MR may be performed for up to 3 hours to include a T2 anatomical scan, Diffusion tensor imaging, relaxometry measures and other scans as deemed necessary.

V.4.3.3. Post-surgical Provisions: see CNRM SOP #1
The mouse will be recovered in a clean cage on a circulating warm water heating pad. It will be visually monitored until it is awake and ambulatory. Animals will be given ad lib access to food on the floor of their cage for 24 hours after anesthesia. Animals will be monitored daily for signs of pain and distress not associated with the surgical injury.

V.4.3.4. Location:
CCI will take place at the CCI apparatus in the LAM facilities at USUHS
MRI will take place at the animal MRI facility at USUHS
Cardial perfusion will take place in the fume hood in Rm (b)(6) USUHS campus

V.4.3.5. Surgeon:
(b)(6) Ph.D

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: See CNRM SOP #1
Using a 27 gauge needle, animals will receive an intraperitoneal (i.p.) injection (approximately 200-400 µL) of either DHA (16mg/kg), Synaptamide (5-20 mg/kg), DHA plus Synaptamide, or vehicle. DHA and Synaptamide are immiscible in saline, therefore the vehicle will be DMSO. A similar study in rat administered a daily dose i.p. injection of 16 mg/kg of DHA for just short of one month (21 days) (Harvey et al. 2014). No issues of toxicity relating to the DHA (16mg/kg) in DMSO injection were reported in that study. There is no literature available on the toxicity of synaptamide injections in vivo. Our collaborator, Dr. (b)(6) is working on toxicity and tolerability of synaptamide in vivo and will inform us of a safe and effective dose. Her lab has currently administered a daily synaptamide dose of 25 mg/kg to mice for 3 days without any signs of pain or distress and no mortalities (Desai, personal communication, Feb 2016). The synaptamide dose will be between 5-20 mg/kg. In our study, animals will initially be dosed 6 hours after injury and then once a day for the following 30 days. IP injection sites will alternate sides of the abdomen from day to day. Animals that do not receive injury (experiment 1) will begin injections at 3 months of age. For transcardial perfusions, animals will be injected IP with an overdose of Na Pentobarbital or Euthasol (50-70 mg/kg) before the non-survival transcardial perfusion procedure begins.

V.4.4.2. Biosamples:
All brains will be removed for ex-vivo imaging and histological characterization.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Animals will be identified via ear punch and hand written notes on cage cards regarding group and treatment.

V.4.4.6. Behavioral Studies: No behavioral studies will be performed for these experiments.

V.4.4.7. Other Procedures:
Transcardiac Perfusion: All mice will receive cardiac perfusions. Each mouse will be overdosed with Na Pentobarbital (50-70mg/kg) and be checked for reflex using a pinch test. In the instances when Na Pentobarbital is not available the commercially available euthanasia solution Euthasol® (50-70 mg/kg) will be used as an alternative. Once the animal is deeply anesthetized (as indicated by lack of response to pinch test), they will be cut from the bottom of the rib cage to the top of the sternum to expose thoracic organs. An appropriate size needle (26-27 G for mice) will be punctured through the left

ventricle of the heart into the right atrium. Saline will be pushed through the heart either by way of syringe or tubing. Once the blood has cleared, (measured by the aorta and liver color) then 4% buffered paraformaldehyde plus 4% glucose in phosphate buffer solution will be pushed through the heart. The brain will then be removed and immersed in paraformaldehyde solution or frozen. This procedure will occur in the fume hood in Rm (b)(6) USUHS campus.

Ear Punch: All mice will receive ear punch for identification in a cage. Mice in experiment 1 will be randomly assigned to experimental groups. Before drug administration begins, each mouse will be scruffed and quickly receive a 1 mm hole in their ear from a disinfected mouse ear punch in the appropriate identification location. The ear punch device will be disinfected initially in an autoclave and then rubbed down with 70% ethanol before the punch. Mice in experiment 2 will receive an ear punch for identification during the time of surgery, while the mouse is still anesthetized.

V.4.4.8. Tissue Sharing: N/A

V.4.5. Study Endpoint:
All animals will be euthanized at the time points indicated in the protocol. Any animal showing signs of excessive pain or distress will be euthanized before the experimental study endpoint and removed from the study. Signs of excessive pain or distress include hunched posture, self-mutilation, aggressive vocalization, ataxia, lethargy, soiled hair coat, and excessive body weight loss. Body weight will be measured once before surgery and then weekly after that. A total body weight loss of >10% loss from baseline weight for a sustained period of 1 week will constitute a sign of excess pain or distress. Any animal that shows these symptoms will be carefully observed for several days. If symptoms attenuate, the animal will be kept in the study. Excessive pain or distress will involve the any of the above symptoms lasting for more than a week and will necessitate removal from the study.

V.4.6. Euthanasia: Euthanasia for animals in the experiment will consist of transcardial perfusion as describe earlier. All animals needing to be euthanized for other reasons, such as excessive pain and distress, will be euthanized following AVMA guidelines and will consist of CO_2 inhalation from a 100% CO_2 compressed gas cylinder. Animals will be placed in an induction chamber and the tank will be filled with CO_2 gas at an approximate rate of 0.5-1 liter per minute. Gas will be maintained for 1 minute after respiration has stopped to prevent unintended recovery (approximately 3-4 minutes total.)

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:
Building(s) USUHS (b)(6) Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes	_____	No	X
Fluid Restriction:	Yes		No	X

V.5.1.3. Exceptions: Special Diet: The present study will be conducted to establish the neuroprotective potential of n-3 fatty acids/DHA in a traumatic brain injury model. Modified diet will be used as one of the sources to expose the animal to different levels of fatty acids. In these diet studies, the animals will be fed one of four defined diets (AIN-93) that consist of varying ratios of n3 and n-6 fatty acids prior to euthanasia and tissue collection. The supplementation entails only a substitution in fats, with the rest of the dietary composition maintained at normal levels. Since linoleic acid is considered an essential fatty acid, all diets will include at least 0.5 energy percent of this fatty acid. Therefore, animals will receive proper nutrition throughout the experiment and there will be no water restriction. Two-week pregnant dams will be placed on a special diet through the pregnancy as well as after delivery in the lactation period. After weaning, the male offspring will be placed on the same diet as had by the respective dams for a further 8-10 week period. For both series of experiments, the animals will be subjected to CCI when they are 8-10 weeks old and continued with their respective diet regime throughout the experiment.

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Veterinary staff at LAM will check on animal cages 2 times per day. Animal cages will be changed out for clean cages 2 times per week. Any animals that look ill or distressed will be reported to a Veterinarian for further assessment. The veterinarian's diagnosis of the animal will be reported and the animal will be monitored for worsening or improving of symptoms. If treatment of the animal is required, protocol staff will be notified for approval of the treatment.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekends and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions: None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Planning and interpreting CCI experiments; will not be handling animals	(b)(6)	Over 20 years of experience working in pre-clinical and clinical TBI	USUHS PI Training,
Planning and interpreting MRI experiments; will not be handling animals		Over 20 years of experience in MRI	Will take USUHS PI training
CCI		Postdoc, 5 yr experience performing CCI	Rodent Handling Training, 5 Nov 2015 USUHS PI Training, 5 Nov 2015
MRI		Postdoc, 5 yr experience handling rodents and trained on performing in vivo MRI by experienced technicians	MIF Safety Course, 2015 fMRI Summer Course, 2015
MRI		Postdoctoral fellow, 6+ years experience scanning various animals	Rodent Handling Training, 8 Jan 2013 USUHS PI Training, 6 Feb 2013
Perfusion		Postdoc, 5 yr experience in perfusion of rodents	Rodent Handling Techniques Course, 5 Nov 2015 USUHS PI Training, 5 Nov 2015
Intraperitoneal Injection		Postdoc, 5 yr experience performing IP injection	Rodent Handling Techniques Course, 5 Nov 2015 USUHS PI Training, 5 Nov 2015
CCI and Perfusion		Research Assistant, works on numerous projects in Dr (b)(6) (b)(6) lab, 2 years previous experience working	Rodent Handling Techniques Course, 4 February 2016 USUHS PI Training, 4 February 2016

		with mice	

VII. BIOHAZARDS/SAFETY:

PPE: All technicians will wear scrubs/lab coat, gloves, closed-toed shoes, and a mask while handling animals. When working with biologically hazardous materials, technicians will wear a lab coat, gloves, closed-toed shoes, goggles, and a mask.
Waste gas: Waste anesthetic gases will be exhausted using an active scavenging system.
Paraformaldehyde: Paraformaldehyde will be worked with in a fume hood. Waste will be collected and disposed of as chemical waste.

VIII. ENCLOSURES:

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IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) Sept 18, 2015

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

(b)(6) MD, PhD

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6)

Principal Investigator Signature

Sept 18, 2015
Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature

Sept 18, 2015
Date

X. PROTOCOL ABSTRACT:

A. **Animal Protocol Number**: To be assigned

B. **Animal Protocol Title**: Traumatic brain injury recovery with n-3 highly unsaturated fatty acids (HUFAs) in mice: A biomarker-driven approach

C. **Principal Investigator**: (b)(6)

D. **Performing Organization**: Uniformed Services University for the Health Sciences

E. **Funding**: Center for Neuroscience and Regenerative Mediicne

F. **Objective and Approach**: **Hypothesis**: Our hypothesis is that DHA supplementation after TBI is neuroprotective and this neuroprotection can be detected via blood biomarkers and advanced magnetic resonance imaging strategies. We will show this with the following specific aims:

Aim 1: Innovative imaging study, designed to determine whether advanced neuroimaging strategies, including diffusion imaging and relaxometry, are sensitive to the omega-3 fatty acid content of brain membranes. In this study we propose to use quantitative MRI techniques to identify potential biomarkers of brain changes induced by Omega-3 fatty acids in animals. We plan to investigate the effects of DHA intake on the brain in mice that are chronically depleted of Omega-3 FA vs. animals with an Omega-3 FA enriched diet, using a model based on the one developed by Desai et al. (2014) in Dr. (b)(6) lab. We will acquire MRI data of mice *in vivo* prior to DHA supplementation and then longitudinally. Ex-vivo, high resolution MRI scanning will be performed on the same brains studied with histological techniques.

Aim 2: Preclinical project designed to identify MRI biomarkers sensitive to omega-3 supplementation after TBI. Such MRI and blood biomarkers could readily be translated to clinical trials. These include measurements of bioactive lipids in plasma and RBC membranes such as synaptamide, 17-hydroxy-DHA, D-resolvins, endovanilloids, and endocannabinoids. These bioactive lipids have potent anti-inflammatory and pro-neurogenic effects that will simultaneously be tested in a parallel clinical study. Eight-10 weeks old male mice will be injured by an experimental TBI model, moderate controlled cortical impact in the parietal cortex. DHA and Synpatamide supplementation (40 mg/kg) will start 6 hours after injury and continue for 30 days {3136}. Accessible biological tissues (plasma, serum, RBC membranes, potentially others) will be assayed using sensitive HPLC-Mass Spectrometry (MS) assays in Dr (b)(6) lab. Our lab will acquire MRI data of mice *in vivo* prior to injury and then longitudinally after TBI induced by controlled cortical impact to investigate the effects of DHA treatment.

G. **Indexing Terms (Descriptors)**: Traumatic brain injury

Omega-3 fatty acids; DHA; Magnetic resonance imaging; Diffusion tensor imaging; Neuroprotective; Biomarker; Mouse/ Mice

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Institutional Animal Care and Use Committee
Phone: (b)(6)
Fax: (b)(6)

April 23, 2012

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF PATHOLOGY

SUBJECT: IACUC Approval of Protocol - Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on April 23, 2012:

Animal Protocol Title: "Regulation of Anti-Infectious Immunity (Mice)"

USUHS Protocol Number: (b)(6)

Expiration Date: April 22, 2015

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Regulation of anti-infectious immunity (mice)

GRANT TITLE (if different from above):
Dendritic and T cells in anti-bacterial Ig responses (NIH-R01) (b)(6)
Developing of Novel Adjuvants for Augmenting Humoral Immunity (USUHS) (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) M.D.

(b)(6)

Pathology (b)(6) 12/27/2011
Department Office/Lab Telephone Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)

Research Unit Chief/ Dept. Head Signature
Typed Name: (b)(6)

Chair (b)(6) 12/27/11
Title Telephone Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)

Statistician Signature
Typed Name: (b)(6)

PMB (b)(6) 1/4/12
Department Telephone Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)

Attending/Consulting Veterinarian Signature
Typed Name: (b)(6) DVM, MPH

LAM (b)(6) 12/28/2011
Department Telephone Date

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) M.D.

ANIMAL PROTOCOL TITLE: Regulation of anti-infectious immunity (mice)

GRANT TITLE (if different from above):
Dendritic and T cells in anti-bacterial Ig responses (NIH-R01) (b)(6)
Developing of Novel Adjuvants for Augmenting Humoral Immunity (USUHS) (b)(6)

CO-INVESTIGATOR(S):
(b)(6) Ph.D.-Research Assistant Professor, Dept. Pathology
(b)(6) Ph.D.-Research Assistant Professor, Dept. Pathology
(b)(6) M.D.-Research Associate, Dept. Pathology
(b)(6) Graduate Student, B.I.T.S., Pilani, India
(b)(6) -Graduate Student, B.I.T.S., Pilani, India

TECHNICIANS(S):
None.

I. NON-TECHNICAL SYNOPSIS:

Infections with extracellular bacteria are a major source of global morbidity and mortality. This fact, and the increasing incidence of antibiotic-resistance of bacterial strains make the development of vaccines against these agents of high clinical importance for both the civilian and military sectors. Immunity to this class of pathogens is mediated by antibody that facilitates the uptake and subsequent killing of bacteria by phagocytic cells such as neutrophils and macrophages. In order to optimize the design of effective vaccines against these agents, it is important to understand the basic immunologic parameters that regulate antibody production in vivo in response to immunization or infection with an intact bacterial pathogen.

Our laboratory studies the in vivo protein- and polysaccharide-specific immunoglobulin isotype response to intact Gram-positive (e.g. *Streptococcus pneumoniae, Streptococcus agalactiae, Staphylococcus aureus*) and Gram-negative (e.g. *Neisseria meningitidis, Acinetobacter baumannii*) extracellular bacteria or antigens derived from these pathogens, using the mouse as a model system. These bacteria continue to cause substantial global health problems. We currently have two major funding sources (see above) that collectively are integrated to explore this general issue in a complementary fashion. Hence, these respective projects, which are conducted in Rooms (b)(6) (b)(6) in the Department of Pathology, are subsumed under this one animal protocol, given that the methodologies related to the use of mice are widely overlapping among the projects.

Areas of study include the role of dendritic cells and macrophages, B and T lymphocytes, innate immunity including Toll-like receptors, cytokines, chemokines, and T cell costimulatory molecules, in the ability to elicit humoral immunity to intact extracellular bacteria, or purified bacterial antigens and novel adjuvants. Regarding the use of mice, methodologies include 1) utilizing athymic nude mice for a) making monoclonal antibody-containing ascites and b) as a model of T cell-deficiency for immunologic studies, 2) immunization of mice with heat-killed intact bacteria or bacterial antigens, by i.p., i.v. and

s.c. routes; in selected experiments alum or Toll-like receptor (TLR) ligand adjuvants are used, 3) tail vein bleeds to obtain sera for measurement of antigen-specific Ig isotype titers and cytokines by ELISA, and for obtaining circulating immune cells, 4) removal of lymphoid tissue for obtaining cells for various *in vitro* immunologic studies and for confocal fluorescence microscopy, 5) infection of mice with live extracellular bacteria for studies on immunologic protection or induction of humoral immunity, 6) use of various mouse strains as recipients in immune cell adoptive transfer experiments, and 7) gamma irradiation of immune cells to act as antigen-presenting cells for stimulating T cells, or of live mice to create bone marrow chimeras. Heavy use is made of transgenic and knockout mice (listed below) in which we have established breeding colonies for some strains, in LAM. As will be described, although these extracellular bacteria are human pathogens, their use experimentally at worst poses minimal health concerns that can easily be addressed using routine microbiologic precautions.

II. BACKGROUND:

II.1. Background:

Various extracellular bacteria, listed in this protocol, are major causes of global morbidity and mortality. Systemic adaptive immunity to these bacterial pathogens is mediated by antibody, especially IgG specific for the capsular polysaccharide (PS), but also for bacterial proteins. **Our long-term goal is to elucidate the cellular mechanisms that underlie the distinct differences that exist between *in vivo* anti-PS and anti-protein Ig responses between distinct extracellular bacteria, and between intact bacterial pathogens and bacteria-derived soluble vaccines, These studies will aid in the development of improved vaccines and to an understanding of physiologic humoral immune responses during bacterial infections.**

In contrast to the current dogma, based on using purified PS antigens, that IgG anti-PS responses are T cell-independent, we demonstrated that the IgG anti-PS responses to intact extracellular bacteria are heavily dependent on $CD4^+$ T cells. However, distinct bacteria elicit distinct types of T cell-dependent anti-PS responses in vivo. Further, IgG anti-PS responses to intact bacteria appear to be regulated by distinct cellular parameters relative to IgG anti-PS responses to soluble PS-containing vaccines. In light of these data, **the central hypothesis that underlies our proposed research is that distinct physical parameters and combinations of antigenic and immune-modulating moieties intrinsic to various intact bacteria and vaccines distinguish both *physiologic* anti-PS and anti-protein Ig responses to intact bacteria, as well humoral immunity to intact bacteria versus soluble bacterial vaccine antigens.** We will demonstrate that these parameters differentially impact on 1) the temporal usage of responding B cells, T cells, and APCs within the spleen, 2) the cellular interactions of the responding B cells with these other immune cell types, and the recruitment of regulatory innate immune processes. As a result we will provide a mechanistic basis that will elucidate the nature of the in vivo humoral immune responses observed.

II.2. Literature Search for Duplication:

II.2.1. **Literature Source(s) Searched**:
Biomedical Research Data Base (BRD)
Federal Research in Progress (FEDRIP)
Pub Med

II.2.2. **Date of Search**: December 23, 2011

II.2.3. **Period of Search**: 2001-2011 (FEDRIP); 2001-2011 (BRD)

II.2.4. **Key Words and Search Strategy**:
Search#1. Extracellular bacteria, mouse, and humoral immunity.
Search#2. Extracellular bacteria, mouse, and vaccine.
Search#3. Extracellular bacteria, mouse, and humoral immunity
Search#4. Polysaccharides, mouse, and antibody
Search#5. Extracellular bacteria, mouse, and polysaccharides
Search#6. Polysaccharides, mouse, and vaccine

II.2.5. **Results of Search**:
Although extensive work is being conducted in the more general areas subsumed under each search, and thus multiple hits too numerous to detail were obtained, the searches did not reveal any projects investigating the specific ideas outlined in this protocol including detailed studies investigating the differential regulation of anti-protein and anti-polysaccharide responses to intact extracellular bacteria or bacteria-derived antigens.

III. OBJECTIVE\HYPOTHESIS:

1. We will determine the nature and relationships of B cell and APC cell subsets, and CD4$^+$ T cells that differentially mediate *in vivo* anti-PS and anti-protein Ig isotype responses to systemic immunization with intact extracellular bacteria. We will utilize magnetic bead or high speed electronic cell sorting and adoptive transfer of wild-type and genetically altered immune cells or bacteria, and bacteria-derived soluble vaccine antigens, combined with ELISPOT and ELISA analyses of antigen-specific Ig isotype production to accomplish this aim. In addition, selective manipulation of immune cells in vivo will be accomplished by creation of bone marrow chimeric mice, in which mice are first irradiated to ablate the hematopoietic system, then reconstituted with bone marrow cells from genetically altered mice.

The bacteria selected for these studies are those that account for major morbidity and mortality in the human populations, and in which vaccines are either available (although requiring improvements) or being developed. The bacteria include 1) *Streptococcus pneumoniae* 2) *Streptococcus agalactiae* (Group B Strep [GBS]), 3) *Staphylococcus aureus*, 4/5) *Neisseria meningitidis*, group A and C, and 6) *Acinetobacter baumannii*. The justification for the use of these bacteria is that they include both Gram-positive and Gram-negative bacteria, which may behave differently in regard to the nature of the immune response elicited. **Thus, these studies represent a systematic approach to understanding intact bacterial pathogens as immunogens for elicitation of antibody responses specific for bacterial protein**

and polysaccharide antigens. For each bacteria, a corresponding conjugate vaccine is evaluated to further study if and how these conjugates differ from intact bacteria in eliciting humoral immunity. **Thus, the relatively large number of mice requested, reflects the systematic and comprehensive nature of these analyses, which have not previously been undertaken by others and which have potential clinical significance.** The time points chosen are based on our extensive experience on the kinetics of the immune response obtained from our previous studies on *Streptococcus pneumoniae*.

2. We will determine the *mechanism* by which B cells, APCs, and $CD4^+$ T cells differentially orchestrate anti-PS and anti-protein responses within a spatiotemporal context. We will utilize B cells from PS- and protein-specific B cell receptor transgenic mice and T cells from $CD4^+$ T cell receptor (TCR) transgenic mice with specificity for a bacteria-expressed peptide to accomplish this aim. These cells will be used to conduct *in vitro* functional studies and confocal microscopic analyses of splenic and lymph node tissue sections from mice immunized *in vivo*.

3. We will determine the mechanism by which distinct extracellular bacteria exert immunoregulatory effects on humoral immune responses to co-immunized soluble antigens. During infections with extracellular bacteria, the immune system likely encounters a variety of microbial components in soluble form, as well as those associated with the intact bacterium. Although soluble and particulate antigens may exhibit distinct immunologic properties, their potential cross-regulatory effects on the humoral immune response, following concomitant immunization, as might occur during bacterial infections, is unknown. We will determine the innate cellular mechanisms by which intact bacteria alter antibody responses to co-immunized proteins antigens, through the use of genetically altered bacteria and mice, immune cell adoptive transfer studies, and bone marrow chimeric mice.

These studies are the first to systematically determine the mechanisms that distinguish anti-PS from anti-protein responses to an *intact* bacterium, elucidate the differences between humoral immune responses to intact bacteria versus soluble bacterial antigen-derived vaccines, determine the mechanisms by which bacteria alter immune responses to heterologous antigens, and provide novel basic immunologic insights with direct relevance to the development of anti-bacterial vaccines.

IV. MILITARY RELEVANCE:

Infections with extracellular bacteria continue to exact significant morbidity and mortality among members of the military population. Although these infections can be treated with antibiotics, the increasing development of antibiotic-resistant strains has made necessary the development of new vaccines for host protection. Protective vaccines for extracellular bacteria necessitate the elicitation of high titers of antigen-specific IgG antibodies against select microbial antigens. The rational design of vaccines requires an understanding of the basic immunologic parameters that elicit antibody production in response to either an intact pathogen or purified soluble microbial antigens. The work subsumed under this protocol should provide useful new information on the basic humoral immune response to extracellular bacteria and their

relevant antigens, which will help guide the development of new anti-bacterial vaccines that will have immediate use among the military population that are at risk for these infections, both as natural pathogens, as well as potential bioterrorist agents. In addition, direct clinical applications will be explored through the development of novel adjuvants for stimulating immunity to various antigens derived from these bacterial pathogens.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

Two major types of experiments will be performed:

1) In vivo-in these experiments mice are immunized i.p., i.v., s.c., or i.n. with either intact bacteria, purified antigen, or specific types of purified immune cells (e.g. dendritic cells, macrophages, T cells, and B cells) with or without additional immunoreagents such as adjuvant (i.e. alum, TLR ligands), monoclonal antibodies or fusion proteins specific for soluble mediators or cell surface structures. At varying times thereafter, several parameters of the immune response will be determined such as;

a) blood levels of bacteria [counting on agar plates] and lethality,

b) serum titers of antigen-specific Ig isotypes [ELISA] or numbers of specific Ig-producing B cells [ELISPOT].

c) phenotypic changes and localization of various immune cell types found within intact lymphoid organs or freshly explanted [fluorescence confocal microscopy and flow cytometry],

d) cytokine and chemokine production [ELISA, ELISPOT, flow cytometry, immunohistochemistry, RT-PCR]

Generally, *in vivo* experiments are performed using 7 mice per experimental group based on our consultation with a professional statistician (see below: "V.2. Data Analysis"), as well as having long experience working in this research area. As such, this number of mice is crucial for meaningful statistics. The typical experiment covers 4-5 weeks (i.e. primary immunization followed by secondary immunization 2-3 weeks later, with an additional 1-2 weeks of follow-up analyses).

2) **In vitro**- Mice will also be used as a source of immune cell types for both in vitro stimulation and in vivo adoptive transfer experiments. Cells include:

a) dendritic cells and macrophages obtained from bone marrow, cultured initially in GM-CSF or M-CSF, respectively to increase cell numbers.

b) T cells, B cells, dendritic cells, or macrophages obtained from spleen or lymph node and then purified by either magnetic or electronic cell sorting and used either for in vitro studies or for adoptive transfer into new mice for in vivo studies.

c) Macrophages obtained via peritoneal lavage and used immediately.

The number of mice required for these experiments strictly depends on the nature of the experiment and the particular cell type or subset under study; different cells are found in different relative proportions and thus heavily influence the number of mice needed to obtain sufficient numbers for the experiment. Details of these experiments now follow:

V.1.1. Experiment 1: The T cell, costimulatory, and B cell subset requirements for induction of in vivo PS-specific and protein-specific IgG responses to intact bacteria and conjugate vaccines. We previously conducted a series of in vivo immunologic studies to determine the cellular and costimulatory parameters that mediated the antibody response to intact, heat-killed *Streptococcus pneumoniae*, capsular type 14 (Pn14) versus a soluble pneumococcal conjugate vaccine. To develop a broader view of how different Gram-positive and Gram-negative bacteria regulate an anti-capsular polysaccharide response in vivo, we will significantly extend our studies using the Gram-positive bacteria, *Streptococcus agalactiae* (Group B Streptococcus [GBS]) and *Staphylococcus aureus* (SA) and the Gram-negative bacteria, *Neisseria meningitidis* group A and group C (MenA and MenC, respectively) and *Acinetobacter baumannii* (AcB). Soluble conjugate vaccines utilizing the purified capsular PS from these bacteria, will also be evaluated in a similar fashion to better understand the differential parameters governing responses to particulate intact bacteria versus soluble bacterial conjugate vaccines.

The justification for these studies is the hypothesis that the unique structural and biochemical features between these bacteria, as well as conjugate vaccines, will result in distinctive anti-polysaccharide (PS) responses in vivo. All bacteria will be heat-killed and injected into mice i.p. and thus we anticipate no pain, suffering, or lethality from these series of studies, nor any risk to the investigator. 7 mice per group will be used to statistically power these studies. Serum will be obtained from tail vein bleeds at day 0 (pre-bleed), 21 (primary) and 28 (secondary) for determination of serum titers of PS-specific and whole bacterial protein-specific IgM and IgG.

a. Kinetics of the systemic primary and secondary IgM and IgG anti-PS responses to heat-killed bacteria. Wild-type (BALB/c, C57BL/6, FVB) mice will be injected i.p., i.v., or s.c. with 2 different doses (2×10^8 CFU/mouse and 1×10^9 CFU/mouse) of the respective bacteria in saline and boosted with the same dose on day 21. Following determination of serum titers of antigen-specific antibody, the mice will be euthanized. Thus, each experiment is expected to last ~35 days. All experiments will be planned to performed 3x for reproducibility, with the assumption that some experiments may fail and that at least 2 successful experiments are needed publishable data. On the basis of these studies we will choose a single dose of bacteria and a single route of immunization for subsequent studies. Since we plan to subsequently use genetically altered mice on either the BALB/c or C57BL/6 background

Total BALB/c: 5 types bacteria x 2 doses x 3 immunization routes x 7 mice x 3 experiments=630

Total C57BL/6: 5 types bacteria x 2 doses x 3 immunization routes x 7 mice x 3 experiments=630

Total FVB: 5 types bacteria x 2 doses x 3 immunization routes x 7 mice x 3 experiments=630

b. Kinetics of the systemic primary and secondary IgM and IgG anti-PS responses to soluble bacterial conjugate vaccine. A similar series of studies as described in 1.a. will be performed using a single standard dose (10 micrograms per mouse) of soluble conjugate vaccine containing a PS from the bacteria indicated above covalently attached to an immunogenic protein. Only two experiments each are proposed using C57BL/6 and FVB strains in this instance, since we consider that these will be largely confirmatory of the data obtained using the BALB/c strain.

Total BALB/c: 5 types conjugate x 1 dose x 3 immunization routes x 7 mice x 3 experiments=315

Total C57BL/6: 5 types conjugate x 1 dose x 3 immunization routes x 7 mice x 2 experiments=315

Total FVB: 5 types conjugate x 1 dose x 3 immunization routes x 7 mice x 2 experiments=315

c. PS-specific IgG memory response. To determine intact bacteria or conjugate vaccine induce memory B and/or CD4+ T cells specific for the IgG anti-PS and/or IgG anti-protein response, RAG-/- mice (BALB/c or C57BL/6 background) which lack B and T cells, will be used as recipients for adoptive transfer of B cells and CD4+ T cells from either naïve or bacteria/conjugate primed BALB/c mice or C57BL/6 mice. Reconstituted RAG-/- mice will be immunized with either intact bacteria or conjugate and serum titers of IgG anti-PS will be determined by ELISA. If memory cells for an IgG anti-PS response are produced by the initial immunization, then it is predicted that recipient RAG-/- mice will demonstrate substantially higher serum anti-PS titers when donor cells are from primed as opposed to naïve mice. Cells from one donor will be transferred into one recipient.

BALB/c, RAG-/-: 5 bacteria x 7 mice x 3 experiments=105 BALB/c, 105 RAG-/-

BALB/c, RAG-/-: 5 conjugate x 7 mice x 3 experiments=105 BALB/c, 105 RAG-/-

C57BL/6, RAG-/-: 5 bacteria x 7 mice x 3 experiments=105 C57BL/6, 105 RAG-/-

C57BL/6, RAG-/-: 5 conjugate x 7 mice x 3 experiments=105 C57BL/6, 105 RAG-/-

d. T cell-dependence of IgG anti-PS response to bacteria or conjugate vaccine. BALB/c or T cell-deficient (athymic nude) mice (7/group) will be immunized with heat-inactivated bacteria and boosted. Additional BALB/c mice will receive 1 mg of a depleting rat IgG2b anti-mouse CD4 mAb (clone GK1.5), rat IgG2b anti-mouse CD8 mAb (2.43) or species/isotype-matched negative control mAb (clone J1.2), 24h prior to immunization. Serum will be collected for primary and secondary IgM and IgG anti-PS and anti-protein responses by ELISA.

BALB/c mice: 5 types bacteria x 7 mice/group x 3 experiments x 3 Ab + 1 saline treatments=420

BALB/c mice: 5 types conjugate x 7 mice/group x 3 experiments x 3 Ab + 1 saline treatments=420

Athymic nude mice: 5 types bacteria x 7 mice/group x 3 experiments=105

Athymic nude mice: 5 types conjugate x 7 mice/group x 3 experiments=105

e. B cell subsets. Three complementary approaches will be utilized to determine the potential role of marginal zone B cell (MZB) and follicular B cells (FB) in the IgG anti-PS and anti-protein response to intact bacteria versus conjugate vaccine. **(i) Lsc-/- mice (ii) adoptive transfer of sort-purified FB or MZB cells, as well as CD4+ T cells, into RAG-/- mice**; 1 mouse as donor cells into 1 mouse as recipeint (41), and (iii) **selective depletion of MZB using anti-LFA-1 ($\alpha_L\beta_2$) + anti-α4-CD49d mAbs with rat IgG as control Ab.**

i. Lsc-/-: 5 types bacteria or conjugate (10) x 7 mice/group x 3 experiments=210

C57BL/6 control: 5 types bacteria or conjugate (10) x 7 mice/group x 3 experiments=210

ii. BALB/c: 5 bacteria or conjugate (10) x 7 mice [donor+recipient]/group (14) x 3 experiments=420

iii. BALB/c: 5 bacteria or conjugate (10) x 7 mice x 2 Abs or saline (3) x 3 experiments=630

f. T cell costimulation. If as expected, the IgG anti-PS response to intact bacteria and conjugate is dependent on CD4+ T cells, the T cell costimulatory requirements will be determined as follows: **(i) B7-dependence:** CD28-/- vs wild-type (WT) mice, and injection of CTLA4Ig or *control* L6 into WT mice, **(ii) ICOS-dependence:** ICOS-/- vs WT mice, and blocking rat IgG2a anti-mouse ICOSL (clone HK5.3) vs *control* rat IgG2a anti E coli β-galactosidase (clone GL117), and **(iii) CD40/CD40L**: CD40L-/- vs WT mice, and blocking hamster IgG anti-mouse CD40L mAb (clone MR-1) vs *control* hamster IgG. The use of knockout mice and corresponding blocking/depleting mAbs are important complementary, validating approaches.

i. C57BL/6: 5 bacteria/5 conjugate (10) x 7 C57BL/6 (WT) x 3 experiments=210
   CD28-/-: 5 bacteria/5 conjugate (10) x 7 CD28-/- x 3 experiments=210
   BALB/c: 5 bacteria/5 conjugate (10) x 7 BALB/c (CTLA4Ig) x 3 experiments=210
   BALB/c: 5 bacteria/5 conjugate (10) x 7 BALB/c (L6) x 3 experiments=210

ii. C57BL/6: 5 bacteria/5 conjugate (10) x 7 C57BL/6 x 3 experiments=210
   ICOS-/-: 5 bacteria/5 conjugate (10) x 7 ICOS-/- x 3 experiments=210
   BALB/c: 5 bacteria/5 conjugate (10) x 7 BALB/c (anti-ICOSL) x 3 experiments=210
   BALB/c: 5 bacteria/5 conjugate (10) x 7 BALB/c (control Ab) x 3 experiments=210

iii. CD40L-/-: 5 bacteria/5 conjugate (10) x 7 CD40L-/- x 3 experiments=210
   BALB/c: 5 bacteria/5 conjugate (10) x 7 BALB/c (WT) x 3 experiments=210
   BALB/c: 5 bacteria/5 conjugate (10) x 7 BALB/c (anti-CD40L) x 3 experiments=210
   5 bacteria/5 conjugate (10) x 7 BALB/c (hamster IgG) x 3 experiments=210

V.1.2. Experiment 2: Role of inflammatory monocytes and conventional dendritic cells (DC) in anti-PS responses to intact bacteria and conjugate vaccines. Transgenic mice expressing the Diphtheria toxin receptor (DTR) dependent on the activity of the CD11b or CD11c promoter (i.e. CD11b-DTR and CD11c-DTR mice, respectively) are used to selectively deplete a subset of CD11b+ (inflammatory monocytes) or total CD11c+ cells (conventional DC) following DT injection. To prevent lethality following DT administration, DT must be administered to bone marrow (BM) chimeric mice, made by injecting BM cells from either CD11b-DTR mice into lethally-irradiated wild-type FVB or C57BL/6 mice, or BM cells from CD11c-DTR mice into lethally-irradiated BALB/c mice, for reconstitution of the hematopoietic system expressing the respective transgene. C57BL/6 recipients will be used selectively, and thus in few numbers) for additional experiments involving other genetically altered cells from strain-matched mice. These BM chimeric mice can thus be used to study the role of inflammatory monocytes and conventional (c)DC in regulating anti-PS and anti-protein responses to intact bacteria and conjugate vaccines in adjuvant. Preliminary studies from our laboratory demonstrated that depletion of inflammatory monocytes markedly inhibits the anti-PS response to intact Pn14 (*Streptococcus pneumoniae*, capsular type 14) while enhancing the protein response. Further preliminary studies indicated that depletion of cDC results in an unexpected *enhancement* in both the anti-PS and anti-protein responses to intact Pn14.

In this series of studies we will determine (i.) whether the results observed for Pn14 apply to additional, distinct bacteria, as well as to different soluble conjugate vaccines. (ii.) whether inflammatory monocytes and or cDC play a role in innate host protection

against live bacteria by performing lethality studies using a dose of bacteria that we have determined is sub-lethal in WT mice. To minimize the use of mice in lethality studies, preliminary dosing to establish a sub-lethal challenge will entail the use of 3 mice per group to establish the dose of live bacteria of a given strain, that is minimally lethal as a prelude to performing more extensive lethality studies. Three-fold increments in bacterial dose will be tested based on historical doses previously used in published studies, so as to minimize the number of groups needed. For these experiments, two irradiated recipient mice can be reconstituted with BM from 1 CD11b-DTR or CD11c-DTR mice. Only 1 experiment is proposed using C57BL/6 mice since we consider this study relatively straightforward and confirmatory of the data using FVB, for the purpose of using the CD11b-DTR model,. Three experiments each are proposed for BALB/c mice since this strain is being used in conjunction with a different mouse model (i.e. CD11c-DTR).

The strains of *N. meningitidis* type A or C use in our curren studies are from ATCC and classified as BSL2 agents. However, in light of case studies (see below) reporting a risk of infection for laboratory workers with exposure to live *N. meningitidis* type A or C (used in our laboratory) [attack rate estimated at 13/100,000], we will take the following precautions: 1) The laboratory worker will be required to read the relevant published reports on potential risk. 2) The laboratory worker will be required to receive the tetravalent meningococcal conjugate vaccine before handling live meningococcus. If that individual refuses, they will not utilize these bacteria. 3) Handling of bacteria will require working both in a class II biological safety cabinet (currently in our lab) and wearing of a surgical mask in order to prevent exposure to droplets and aerosols, according to the published recommendations.

1. Kessler, A. T., D. S. Stephens, and J. Somani. 2007. Case Study. Laboratory-acquired serogroup A meningococcal meningitis. *J. Occup. Health*. 49: 399-401.

2. MMWR. 2002. Laboratory-acquired meningococcal disease---United States 2000. 51: 141-144.

3. Omer, H., G. Rose, K. A. Jolley, E. Frapy, J-R. Zahar et al. 2011. Genotypic and phenotypic modfications of *Neisseria meningitidis* after an accidental human passage. PloS ONE 6: e17145.

4. Sejvar, J. J., D. Johnson, T. Popovic, J. M. Miller, F. Downes et al. 2005. Assessing the risk of laboratory-acquired meningococcal disease. J. Clin. Microbiol. 43: 4811-4814.

(i.) FVB: 5 bacteria/5 conjugate (10) x 7 FVB + DT/7 FVB (without DT) x 3 experiments=420

C57BL/6: 5 bacteria/5 conjugate (10) x 7 C57BL/6 + DT/7 C57BL/6 (without DT) x 1 experiment=140

BALB/c: 5 bacteria/5 conjugate (10) x 7 BALB/c + DT/7 BALB/c (without DT) x 3 experiments=420

CD11b-DTR (FVB background): 210 (for BM donor cells)

CD11b-DTR (C57BL/6 background): 70 (for BM donor cells)

CD11c-DTR (BALB/c background): 210 (for BM donor cells)

(ii.) FVB: 5 bacteria x 7 FVB + DT/7 FVB (without DT) x 3 experiments=210

C57BL/6: 5 bacteria x 7 C57BL/6 + DT/7 C57BL/6 (without DT) x 1 experiment=70

BALB/c: 5 bacteria x 7 BALB/c + DT/7 BALB/c (without DT) x 3 experiments=210

CD11b-DTR (FVB background): 105 (for BM donor cells)
CD11b-DTR (C57BL/6 background): 35 (for BM donor cells)
CD11c-DTR (BALB/c background): 105 (for BM donor cells)

V.1.3 Experiment 3. **Regulation of in vivo antibody responses to soluble protein antigens by co-immunized distinct intact, heat-killed bacteria**. During infections with extracellular bacteria, the immune system likely encounters a variety of microbial components in soluble form, as well as those associated with the intact bacterium. Although soluble and particulate antigens may exhibit distinct immunologic properties, their potential cross-regulatory effects on the humoral immune response, following concomitant immunization, as might occur during bacterial infections, is unknown. Preliminary studies from our laboratory demonstrate that co-immunization (i.v.) of soluble chicken ovalbumin (cOVA) in alum adjuvant with intact, heat-killed unencapsulated *Streptococcus pneumoniae* (strain R36A) markedly inhibits the subsequent IgG anti-cOVA response, and that this depends on the expression of phosphorylcholine in the R36A cell wall. In contrast, unencapsulated Group B Streptococcus (GBS) or unencapsulated *Neisseria meningitidis*. group C (MenC), which do not express PC, significantly enhances the IgG anti-cOVA response.

We will conduct a series of experiments to better understand the mechanisms underlying these effects. We will determine: (i.) whether Toll-like receptors mediate the stimulatory effects of intact bacteria by using MyD88-/- mice; MyD88 is a critical adaptor molecule for TLR signaling, (ii.) the effects on CD4+ T helper cells specific for cOVA by co-immunized bacteria; cOVA-specific transgenic CD4+ T cells (D011.10 x RAG-/- [BALB/c background] or OT-II x RAG-/- [C57BL/6 background]) will be adoptively transferred into WT BALB/c or C57BL/6 mice for immunization studies; 1 transgenic mouse can be used as a donor for two WT recipients. Two different strains of mice are used (C57BL/6 and BALB/c) to accommodate the further use of additional strain-matched genetically altered immune cells. The transgenic CD4+ T cells will be evaluated by flow cytometry at days 0, 1, 2, 3, 7, 8, and 14 (7 time points) for T cell activation, proliferation, differentiation, and memory cell development, with each assay performed using 3 recipient mice each (iii.) the effect of TLR4 on the R36A-mediated inhibition of the IgG anti-cOVA response, in light of previous reports that the inhibitory action of phosphorylcholine (PC) may be mediated by TLR4; both TLR4-/- mice [C57BL/6 background] and C3H/HeJ (TLR4-mutated, C3H/HeN background) will be used in complementary studies (iv.) the role of suppressive, IL-10-producing B1 cells, which express specificity for PC, on the R36A-mediated inhibition of the IgG anti-cOVA response; x.i.d. mice which selectively lack B1 B cells will be used, (v.) the effects of bacteria on the IgG anti-cOVA utilizing the s.c. route (i.e. lymph node-mediated versus i.v. route which is mediated by the spleen), with splenectomy performed to rule out splenic contributions following s.c. immunization. The bacteria to be utilized will include R36A, R36A without PC (R36Apc-), GBS, and MenC.

i. MyD88-/-: 4 bacteria x 7 mice/group 2 immunizations (cOVA alone versus cOVA + bacteria) x 3 experiments=168
   C57BL/6: 4 bacteria x 7 mice/group x 2 immunizations x 3 experiments=168
ii. BALB/c: 4 bacteria x 3 mice/group x 2 immunizations x 7 time points x 3 experiments=504
   C57BL/6: 4 bacteria x 3 mice/group x 2 immunizations x 7 time points x 3

experiments=504
D011.10 x RAG-/-: 252 (donor cells)
OT-II x RAG-/-: 252 (donor cells)

iii. TLR4-/-: 4 bacteria x 7 mice/group x 3 experiments=84
C57BL/6: 4 bacteria x 7 mice/group x 3 experiments=84
C3H/HeJ: 4 bacteria x 7 mice/group x 3 experiments=84
C3H/HeN: 4 bacteria x 7 mice/group x 3 experiments=84

i.v. x.i.d.: 2 bacteria x 7 mice/group x 2 immunizations x 3 experiments=84
CBA/CaJ (control): 2 bacteria x 7 mice/group x 2 immunizations x 3 experiments=84

v. BALB/c: 4 bacteria x 7 mice/group x 2 immunizations [s.c] (cOVA alone versus cOVA + bacteria) x 3 mouse groups (WT, sham-splenectomized, splenectomized) x 3 experiments=504

V.1.4. Experiment 4. Adoptive transfer of transgenic B cells (PspA-specific and PPS14-specific [BALB/c background] and PC-specific (M167-Tg [C57BL/6 background]) and T cells (PspA-Tg, and D10.11 [cOVA-specific] (BALB/c background) and OT-II [cOVA-specific] (C57BL/6 background) into recipient mice (athymic nude, or BALB/c wild-type). In these studies, transgenic B cells and/or T cells will be transferred into recipients in order to study their ability to induce antigen-specific Ig isotype production (by ELISA and ELISPOT assay) in response to challenge with Pn (wild-type or expressing fusion PspA-cOVA) or conjugate vaccines, as well as transgenic B and T cell localization and migration within the spleen (by fluorescence confocal microscopy) following Pn and conjugate challenge. Isolation of adoptively transgenic B and T cells, by electronic cells sorting, following Pn and conjugate immunization for in vitro analyses will also be conducted. We anticipate using an average of 10 mice from each strain mentioned above every 1 month (6 strains) in order to obtain sufficient numbers of purified B and T cells for adoptive transfer and to have a sufficient number of recipient mice for analysis. In all adoptive transfer studies both donors and recipients must be on the same strain background (i.e. BALB/c or C57BL/6). These studies will be ongoing for upcoming 3 year period. Through the use of antigen-specific transgenic B and T cells we can transfer into recipients a sufficient number of antigen-specific cells that can then be visualized with confocal immunohistochemistry and flow cytometric analyses. These cells can be further isolated for the study of additional cellular as well as molecular and biochemical parameters in order to determine how their behavior differs when exposed to different forms of antigen (e.g intact bacteria, soluble protein or polysaccharide, or conjugate vaccine).

Total mice: 10/month x 9 strains x 36 months=3,240 mice (360 of each strain): 360 PspA-BCR-Tg,, 360 PPS14-BCR-Tg, 360 M167-Tg, 360 PspA-TCR-Tg,, 360 D10.11 x RAG-/-, 360 OT-II x RAG-/-, 360 athymic nude, 360 BALB/c 360 C57BL/6.

Mice in pain category "E" are in EXP2.ii as follows: FVB (210 mice), BALB/c (210 mice), and C57BL/6 (70 Mice).

Total mice required for upcoming 3 years=17,016 (see Table next page)

	EXP1										EXP2		EXP3					EXP4	
	a	b	c	d	e			f											
					i	ii	iii	i	ii	iii	i	ii	i	ii	iii	iv	v		
BALB/c	630	315	210	840		420	630	420	420	630	420	210		564			564	360	6513
C57BL/6	630	315	210		210			210	210		140	70	168	564	84			360	3111
FVB	630	315									420	210							1575
RAG-/- (BALB/c background)			210																210
RAG-/- (C57BL/6 background)			210																210
Athymic nude				210														360	570
Lec-/-					210														210
CD28-/-								210											210
ICOS-/-									210										210
CD40L-/-										210									210
CD11b-DTR (FVB)											210	105							315
CD11b-DTR (C57BL/6)											70	35							105
CD11c-DTR (BALB/c)											210	105							315
MyD88-/-													168						168
D011.10 x RAG-/-														282				360	642
OT-II x RAG-/-														282				360	642
TLR4-/-															84				84
C3H/HeJ															84				84
C3H/HeN															84				84
x.i.d																84			84
CBA/CaJ																84			84
PspA-BCR-Tg																		360	360
PPS14-BCR-Tg																		360	360
M167-Tg																		360	360
PspA-TCR-Tg																		366	366
																	Total		17016

V.2. Data Analysis:

We have consulted with a professional statistician (Dr. (b)(6) in the Department of Preventive Medicine and Biometrics, at USUHS. As per her advice, we applied a formula, obtained from "Van Belle Statistical Rules of Thumb" (Wiley 2002, page 35) to determine the number of mice needed per experimental group in order to generate 80% power at 0.05% significance for a 3-fold difference between control and experimental groups. 80% power indicates that 4 out of every 5 experiments will show a statistically significant difference, if in fact a difference exists. This determination is based on calculating a coefficient of variation that quantifies differences in serum titers of Ig between individual mice in the same experimental group. The formula is as follows: $N=8CV^2/FC^2$ $(1+ [1-FC]^2)$ where N=number of required mice, CV=coefficient of variation (standard deviation/mean), FC=fold-change. We calculated a set of CVs for 3 separate, *in vivo* experiments using intact Pn, involving several different (1) time points primary response (days 0, 7, 14) and secondary response (days 21 and 28), (2) antigens (PC, PPS14, and PspA), (3) isotypes (IgM, IgG) and (4) strains of mice (BALB/c, C57BL/6, and B6129). Collectively, the average CVs for the 3 experiments were 0.4 (using 5 mice per group, B6129), 0.51 (using 10 mice per group, BALB/c), and 0.53 (using 8 mice per group, C57BL/6). The number of required mice ('N") to generate 80% power to these studies was compatible with our typical use of 7 mice per group. Thus, these numbers clearly provides the power necessary to reproducibly detect significant changes anticipated in these studies, and is consistent with mouse numbers used by others for *in vivo* studies. Dr. (b)(6) agreed with our use of the student-T-test for determination of statistical significance, and the use of standard error of the mean for expressing variation, and suggested that geometric means would be preferable to arithmetic means, even though the latter are also acceptable.

V.3. Laboratory Animals Required and Justification:

An itemized justification of the number of animals required is found in section V.1. under the respective descriptions of each set of experiments, based on the internal logic of each subsection.

V.3.1. Non-animal Alternatives Considered:

As state in V.3.2 below the studies described herein cannot be performed using long-term cell lines or modeling, but must directly assess the reactivity of the intact host.

V.3.2. Animal Model:

Mice

Species Justification:

Collectively, these studies are designed to evaluate an immune response to either a whole pathogen or purified antigen in the intact host. In light of the vast complexity of an intact immune system, including the spatio-temporal organization of multiple cell types and cell surface and soluble mediators, a true understanding of the physiology of an immune response to a pathogen can only be studied in an intact host. The mouse model is ideal for studying the physiology of an in vivo immune response because: 1) it is the most widely studied animal model and hence a vast amount of information is already known in this species and 2) there are numerous immunoreagents available specific for use in the mouse.

Strain Justification:

1. **Conventional wild-type mice: BALB/c, C57BL/6, FVB, C3H/HeN and CBA/CaJ**. These mice will, in part, serve as controls for genetically altered mice that have been created on these respective strain backgrounds. Additionally, each of these wild-type strains have certain unique host response patterns to immunization that make them of interest for studies involving the intact immune system.

2. **Athymic nude mice (mice lacking T cells).** These mice have a natural mutation leading to the loss of thymic development. As a result, these mice are markedly deficient in T cells. Hence they serve as a convenient recipient for T cell transfer studies.

3. **RAG-/- [BALB/c and C57BL/6 background] (mice lacking both B cells and T cells).** RAG-/- mice are genetically deficient in a key protein involved in the formation of the antigen receptors for B cells and T cells. On this basis, RAG-/- mice fail to develop B cells and T cells, but otherwise develop in a relatively normal fashion. These mice thus serve as a convenient recipient for injecting B cells and/or T cells exhibiting specific

alterations, in order to determine if these alterations have an effect on an immune response.

4. **OVA-specific T cell receptor transgenic mice: D10.11 x RAG-/- (BALB/c background), OT-II x RAG-/- (C57BL/6 background.** These mice are transgenic for a rearranged T cell receptor that recognizes a chicken ovalbumin (cOVA) peptide in association with MHC class-II. As such, a large percentage or 100% (on RAG-/- background) of the T cells in these mice are specific for cOVA-MHC class-II on the surface of antigen-presenting cells. Since wild-type Pn does not express this peptide, these T cells will be used as negative controls in transfer experiments in which transgenic T cells with an anti-Pn peptide specificity (i.e. PspA-specific T cell transgenic mice are used to determine the role of such T cells in the anti-Pn humoral response. Alternatively, cOVA-specific T cells will be used to deliver specific cognate help to genetically altered Pn which expresses a PspA-cOVA fusion protein in its cell wall, but not wild-type Pn.

5. **Phosphorylcholine (PC)-specific B cell receptor transgenic mice: M167 Tg.** PC is a determinant on the cell wall C-polysaccharide (teichoic acid) of Pn. Thus, the B cells from M167 Tg mice will specifically recognize Pn through this determinant. B cells from these mice will be used to study the functional B cell response to Pn.

6. **Immunoglobulin transgenic mice: PPS14 light chain, PPS14 heavy chain, double PPS14 heavy/light chain, PspA light chain, PspA heavy chain, double PspA heavy/light chain.** These mice are currently being created in our lab. The immunoglobulin heavy chain and light chain encoding a B cell receptor specific for either the Pn cell wall protein, PspA, or the Pn capsular polysaccharide, PPS14 has been isolated and will be used to create BCR transgenic mice. This will lead to the creation of mice possessing large numbers of PspA-specific or PPS14-specific B cells. These B cells will be used in studies of the B cell functional response to intact Pn during an immune response.

7. **PspA-specific T cell receptor (TCR) transgenic (Tg) mice (PspA-TCR-Tg).** These mice are currently being created in our lab. They will posses rearranged T cell receptor genes encoding for a PspA peptide/MHC class-II specificity. These mice will possess a large number of CD4+ PspA-specific T cells useful for studying the T cell response to intact Pn during an immune response.

8. **Lsc-/- mice**. These mice lack the cytoplasmic signaling protein Lsc, which results in an inability of antigen-specific splenic marginal zone B cells (MZB) to migrate from the marginal zone to the T cell areas of the spleen following immunization. This results in a defect in MZB obtaining CD4+ T cell help during an immune response. Thus, these mice have been useful in defining B cell subset utilization in T cell-dependent humoral immune responses to intact bacteria versus soluble bacterial-derived antigens.

9. **MyD88-/-, TLR4-/- mice**. These mice are genetically deficient in the TLR adaptor protein MyD88 or the Toll-like receptor 4 [TLR4]. A role for these proteins in mediating in vivo innate and adaptive immune responses to Pn has been demonstrated.

10. **C3H/HeJ mice**. These mice have an inactivating mutation in the TLR4 gene leading to the expression of a non-signaling TLR4 molecule, and are used in a complementary fashion with TLR4-/- mice, which lack the TLR4 gene.

11. **CD11c-DTR and CD11b-DTR mice**. These mice express the Diphtheria toxin receptor in cells expressing either CD11c (dendritic cells) or CD11b (inflammatory monocytes), respectively. Injection of diphtheria toxin selectively depletes these cells allowing for studies on their functional role in humoral immunity.

Both male and female mice (6-12 weeks of age) will be used. The gender of the mouse has been shown by us previously not to significantly bias the phenomena under study and thus use of both genders represents a substantial savings in money. Nevertheless, when genetically-altered mice of one gender is used, the control mice will be of the same gender to maintain a strict control. The age range of the mouse is chosen in order to use mice with a mature and vigorous immune system, in a timely way.

Justification for performance of lethality studies:

Prevention of lethality from infectious agents following vaccination continues to be among the "gold-standards" of vaccine validation using mouse models. Introduction of live pathogens via the i.p., i.v., or i.n. route are considered standard, straightforward approaches. Below, are several representative samples of numerous recent citations in mainstream, peer-reviewed scientific journals in which lethality studies using extracellular bacteria were considered a major aspect of the reported project:

1. Cao, J., D. Chen, W. Xu, T Chen, S. Xu et al. 2007. Enhanced protection against pneumococcal infection elicited by immunization with the combination of PspA, PspC, and ClpP. *Vaccine* 25: 4996-5005.

2. Glover, D. T., S. K. Hollingshead, and D. E. Briles. 2008. Stretococcus pneumoniae surface protein PcpA elicits protection against lung infection and fatal sepsis. *Infect. Immun*. 76: 2767-2776.

3. N. Munoz, L. Van Maele, J. M. Marques, A. Rial, J. C. Sirard, J. A. Chabalgoity. 2010. Mucosal administration of flagellin protects mice from Streptococcus pneumoniae lung infection. *Infect. Immun*. 78: 4226-4233.

4. N. Cerca, T. Maira-Litran, K. K. Jefferson, M. Grout, D. A. Goldmann, and G. B. Pier. 2007. Protection against Escherichia coli infection by antibody to the Staphylococcus aureus poly-N-acetylglucosamine surface polysaccharide. *Proc. Natl. Acad. Sci. USA*. 104: 7528-7533.

V.3.3. Laboratory Animals

V.3.3.1. Genus & Species: Mouse

V.3.3.2. Strain/Stock: BALB/c, C57BL/6, FVB, RAG-/- (BALB/c background), RAG-/- (C57BL/6 background), Athymic nude, Lsc-/-, CD28-/-, ICOS-/-, CD40L-/-, CD11b-DTR (FVB), CD11b-DTR (C57BL/6), CD11c-DTR (BALB/c), MyD88-/-, D011.10 x RAG-/-, OT-II x RAG-/-, TLR4-/-, C3H/HeJ, C3H/HeN, x.i.d., CBA/CaJ, PspA-BCR-Tg, PPS14-BCR-Tg, M167-Tg, PspA-TCR-Tg.

V.3.3.3. **Source/Vendor**: (b)(4)

V.3.3.4. **Age**: 6-12 weeks
V.3.3.5. **Weight**: N/A
V.3.3.6. **Sex**: Male and Female
V.3.3.7. **Special Considerations**: None.

V.3.4. **Number of Animals Required (by species)**: 17,016 mice (see above)

V.3.5. **Refinement, Reduction, Replacement (3 Rs)**:

V.3.5.1. **Refinement**:

Strict endpoint criteria have been defined to euthanize mice at the earliest appropriate time in order to minimize pain and suffering. Mice will be examined three times daily (8 AM, 1 PM, and 6 PM). Mice will be infected ~6 PM since most mice that are not protected by vaccination will develop pre-terminal signs from 14h-24h post-infection. Thus, the majority of mice that will eventually die, can be identified when they are pre-terminal (as described above), in order to euthanize these mice to minimize distress. As discussed and cited in this protocol the use of anesthetics and analgesics is contraindicated for these studies, given the known effects of these agents on altering immune function and hence potentially obscuring the data obtained.

V.3.5.2. **Reduction**:

A minimal number of mice have been chosen for each experimental group based on the innate mouse-to-mouse variability of the humoral immune response to a bacterial agent in the context of maximizing our ability to discern statistical significance ($p \leq 0.05$). A further reduction is realized by using only 3 mice per group to establish the dose of live bacteria of a given strain, that is minimally lethal as a prelude to performing more extensive lethality studies.

V.3.5.3. **Replacement**:

As indicated in this protocol, the mouse is the lowest species on the phylogenetic scale in which to conduct detailed studies on the immunologic response of the intact host to a bacterial agent.

V.4. **Technical Methods**:

V.4.1. **Pain / Distress Assessment**:

V.4.1.1. **APHIS Form 7023 Information**:

V.4.1.1.1. **Number of Animals**:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	16,190	
V.4.1.1.1.2. Column D:	336*	
V.4.1.1.1.3. Column E:	490**	

*These mice (BALB/c) will be undergoing splenectomy, involving survival of a surgical procedure.

**These mice (FVB=210, C57BL/6=70, BALB/c=210) will be involved in lethality studies, in which mice not protected by vaccination and/or unvaccinated mice will die from the infection.

V.4.1.2. Pain Relief / Prevention: In bacterial lethality and other studies described above, no measures will be performed to relieve the distress the mice may encounter as this would represent a serious potential impediment to the interpretation of these studies, given that any such measure could impact on the ultimate functioning of the host immune system.

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: N/A
See justification in V4.1.3.5.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

Lethality studies-The end-point of lethality studies will be whether or not the mouse becomes moribund, a condition from which the mice do not recover. The moribund status will be defined by any of the following criteria: 1) inability to ambulate, 2) inability to eat or drink, 3) hunched posture, and 4) unresponsiveness to external stimuli. If any of these signs are present, the mouse will be euthanized by cervical dislocation as described in our protocol. Mice are checked 3x/day for moribund symptoms by (b)(6) (b)(6)

Mice will be monitored at 8:00 AM (14 h post-infection), 1:00 PM, and 6:00 PM. A log will be maintained documenting the date and time the mice were checked and by whom, the number of mice appearing moribund (including those which required euthanasia) and the number of mice found dead. When the lethality experiment is completed the findings will be reported to IACUC in order to evaluate the efficacy of this modified approach. If no improvement is observed in decreasing the number of mice found dead (versus moribund) we will consider other methods, including adding an additional time point for monitoring.

Mouse irradiation and establishment of bone marrow chimeric mice- Mice will be gamma irradiated at USUHS at 900-1000 Rads for the purpose of making bone marrow chimeras. Specifically, irradiation of wild-type mice will eliminate their hematopoietic cells, to be replaced with mutant donor (i.e. CD11b-DTR and CD11c-DTR) [DTR=Diphtheria toxin receptor] hematopoietic cells, via injection, i.v., of bone marrow cells from mutant mice. These "chimeric" mice will thus have a wild-type non-hematopoietic system and mutant hematopoietic system. This approach is being taken based on published observations that these mutant non-

hematopoietic phenotypes confer unwanted lethality under the experimental conditions being used (i.e. following DT [Diphtheria toxin] injection [see below]), which is designed to study the mutant hematopoietic cells.

Transfer of bone marrow cells rescues mice from the lethal effects of the irradiation. Chimeras are stably established and ready to use experimentally at 4-6 weeks. If donor cells don't repopulate bone marrow, mice will die in about 10 days. Donor bone marrow cells (10 million cells per mouse in 150 microliters) must be injected i.v. within 24h of irradiation. Mice are maintained on antibiotics in their drinking water (Ditrim [sulfadiazine/trimethoprim] or Sulfatrim [sulfamethoxazole/trimethoprim; 5ml/200ml) for 2 weeks following irradiation. Subsequently, mice will switched to regular water without antibiotics. The antibiotic treatment should have no effect on the data generated from these studies. No anesthesia nor other methods to alleviate pain/suffering are required. Identification of moribund mice (if, in the unlikely occasion, bone marrow cells fail to re-establish the hematopoietic system) with subsequent sacrifice of animals by cervical dislocation has already been described in our current protocol.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: Medline

V.4.1.3.2. Date of Search: 03/01/2012

V.4.1.3.3. Period of Search: 1980-2012

V.4.1.3.4. Key Words of Search: Animal, mouse, immunity, bacteria, pain, in vivo.

V.4.1.3.5. Results of Search:

opioids-opioids such as morphine have been reported to produce a number of immunomodulatory effects in both laboratory animals and humans. In vitro exposure of murine lymphocytes and macrophages to morphine and its metabolites at a wide range of concentrations resulted in suppressed B cell proliferation, suppression of IL-2, IL-4, and IL-6, and inhibited cytotoxic T lymphocytes function [(ref 1)]. Fentanyl and buprenorphine, both opioid analgesics, also have immunomodulatory attributes in mice. Both opioids have shown a dose dependent attenuation of the serum TNF-α response in mice as a result of exposure to LPS and results in a significantly lower LPS-induced serum TNF-α increase. Morphine also suppresses T and B cells, depresses NK cell activity, and decreases the primary antibody response[(ref 2)]. Narcotic analgesics can cause histamine release and respiratory depression which could alter the pathogenic and clinical response to infection[(ref 3)]

B. **Non-steroidal anti-inflammatories (NSAIDs)**-The cyclooxygenase inhibiting NSAIDs have been shown to produce progressive alterations of parameters of the thrombocyte vessel system of hemostasis, decreased ability of thrombocytes to aggregate, and activation of lipid peroxidation processes in rabbits injected with bacterial endotoxin[(ref 4)]

In summary-Steroidal and non-steroidal anti-inflammatories are contra-indicated in many protocols due to their interference with the inflammatory pathway which is critical in the normal pathogenesis of many physiological processes, including immunity.

1. Thomas PT, Bhargava HN, House RV. 1995. Immunomodulatory effects of in vitro exposure to morphine and its metabolites. Pharmacology, Jan;50(1):51-62.
2. Piersma, F.E., Daemen, M.A.R.C., vd Bogaard, A.E.J.M., and W.A. Buurman. 1999. Interference of pain control employing opioids in *in vivo* immunological experiments. Laboratory Animals, 33:328-333.
3. Soma, L.R. 1983. Anesthetic and analgesic considerations in the experimental animal. Ann N Y Acad Sci 406:32-47.
4. Malov VA, Studennikova LN, Pak SG, et al. 1991. The effect of cyclooxygenase inhibition on the indices of the thrombocyte-vascular link in hemostasis and on the free-radical processes of lipid oxidation in experimental Salmonella intoxication. Nauchnye Doki Vyss Shkoly Biol Nauki, (7):58-63.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

Pain will not be alleviated in the studies described in this protocol, except for splenectomized mice post-surgery (see below: V.4.3 "Surgery") since steroidal and non-steroidal anti-inflammatories are contra-indicated in these experiments due to their interference with the inflammatory pathway which is critical in the normal pathogenesis of many physiological processes, including immunity. However, animals listed under Category C that do show evidence of significant pain or distress, in the opinion of the attending veterinarian and after consultation with the PI or his staff if they can be reached, will be euthanized immediately". The projects described above are designed to study the immune response of the intact mouse to various types of immunization. These encompass essentially classical cellular immunologic approaches to studying the immune system including injections for immunization and bleeding to obtain sera for measuring antigen-specific Ig isotype titers. All members of the lab are trained and experienced in these procedures and this in turn minimizes the actual pain and discomfort inflicted.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: Splenectomy. The procedure is performed as follows (adapted from Griffith and Farris, 1942): Mice are anesthetized with a combination of ketamine, xylazine, and acepromazine, respectively at 30mg/kg BW IM, 6 mg/kg BW IM, and 1mg/kg BW IM (O'Rourke et al. 1994). The mouse is laid on its back with its tail towards the investigator. The abdominal cavity is exposed through a midline incision extending posteriorly for about half of the abdomen. Alternatively, a dorso-ventral incision can be made near to the costal border of the thorax, on the right of the investigator. The prominent splenic vein with an accompanying small artery courses through the pancreatic tissue and splits into several small vessels just before entering the spleen. A ligature is placed around the splenic blood vessels just before they divide; they are severed distal to the ligature and the spleen is removed before closing the incision.

Postoperative care includes analgesic given SC bid for 24 hrs (Buprenorphine at

0.05-0.10 mg/kg BW, as recommended by Flecknell, 1991). In addition, a single dose will be given during surgery. No adverse changes in body temperature were reported in the published protocol. Mice should remain healthy provided they are not infected with Bartonella muris, a blood parasite which produces a fatal microcytic anemia in splenectomized animals. If necessary, infection can be controlled with an i.m. injection of Neoarsphenamine at a dose of 140 mg/kg body weight. The pain level is "D".

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

Mice are immunized either s.c., i.p., or i.v. with either heat-killed or viable intact *S. pneumoniae*, soluble purified protein and/or polysaccharide antigens with or without alum or TLR adjuvant and/or immune cells. For immunizations, mice will be immobilized manually and 0.25-0.5 ml of PBS containing bacteria, antigen (+/- adjuvant) and/or cells will be injected using a 28 gauge needle. For i.v. injections mice will first be subjected to heating by infrared heat lamp, with close monitoring to prevent heat exhaustion, in order to increase blood flow to the tail vein to facilitate injections. After various time periods, sera may be collected by obtaining blood from the tail vein. The maximum number of blood collections from immunized mice is typically 3 per mouse. Approximately 0.2 ml of blood is collected from the tail vein of each mouse, 7 days apart. We have taken this approach for many years and have noticed no ill effects on mouse health. To accomplish this, a small transverse cut is made to puncture the tail vein allowing blood to drip into an eppendorf tube. Blood is collected until the bleeding stops spontaneously, which is not life threatening to the mouse. None of these procedures requires prior measures to relieve distress.

V.4.4.2. Biosamples:

Sera will be obtained by tail vein bleed and stored at –20°C. Spleen, lymph node, and bone marrow will be obtained after mouse euthanasia by cervical dislocation. These tissues will either be used immediately as a source of immunologic cells and/or frozen in liquid nitrogen for subsequent immunohistochemistry studies.

V.4.4.3. Adjuvants:

Alum (Aluminum hydroxide) or select Toll-like receptor ligands (e.g. CpG-ODN), will be used for enhancing immune responses. The use of these adjuvants historically has had no discernible adverse effects on mice at the dosages and frequencies described in this proposal.

V.4.4.4. Monoclonal Antibody (MAbs) Production:

1. All mAb production currently planned in-house will be done using in vitro methodologies. There are occasions when relatively large quantities of mAb are required necessitating the use of an outside vendor to make mAb using in vitro

bioreactors. These mAbs will be screened for pathogens prior to use in the USUHS animal facility as per IACUC policy 023.

V.4.4.5. Animal Identification:

In selected experiments individual mice are specifically identified using metal ear tags (purchased from the National Band and Tag Company). Otherwise all animals are identified by cage cards.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: N/A

V.4.4.8. Tissue Sharing:

Cells, tissue, and sera are available for sharing when appropriate.

V.4.5. Study Endpoint:

Bacterial lethality studies (Experiment: V.1.2.ii)-Mice will be infected i.p with viable extracellular bacteria that will be sub-lethal in a wild-type mouse. Changes in blood levels of live bacteria over time will be measured by counting colonies on agar plates, and the ability of the mice to recover from the infection will be graphed as number of deaths on the ordinate versus days after initial infection on the abscissa. The end-point of the study will be whether or not the mouse becomes moribund, a condition from which the mice do not recover. The moribund status will be defined by any of the following criteria: 1) inability to ambulate, 2) inability to eat or drink, 3) hunched posture, and 4) unresponsiveness to external stimuli. If any of these signs are present, the mouse will be euthanized by cervical dislocation as described in our protocol.

Mouse immunizations (Experiments: V.1.1-V.1.4)-Mice are immunized one or more times with either intact bacteria or purified bacterial antigens. At varying periods of time after immunization, as described in detail above, sera is obtained through tail vein bleeds for measurement of antigen-specific Ig isotype titers and/or cytokines. When the period of the immune response under study is completed, mice are euthanized by cervical dislocation. In other studies, mice are similarly immunized, and at select periods of time thereafter, are euthanized by cervical dislocation whereupon lymphoid tissue (i.e. spleen and lymph nodes) is removed for immunologic analyses. In some studies, naïve mice are euthanized by cervical dislocation and bone marrow cells are obtained for the in vitro generation of dendritic cells or macrophages, or as donor cells for bone marrow chimeric mice.

V.4.6. Euthanasia:

Mice will be euthanized by cervical dislocation.

Justification for select circumstance to use cervical dislocation instead of C02 asphyxiation for mouse euthanasia. A major concern of using CO_2 asphyxiation, which is not a concern when using cervical dislocation, is its potential effect on immune cell function. My laboratory routinely isolates immune cells from euthanized mice for further immunologic study in vitro or for adoptive transfer into naïve mice, and several studies indicate a potential for immune cell parameters to be altered by prior euthanasia with CO_2. In an article by Howard et al (ref 1) C02 euthanasia was associated with alterations in *in vitro* lymphoproliferation and cytotoxic T cell function, not observed after cervical dislocation. In an article by Butler et al (ref 2) CO_2 euthanasia was associated with alterations in prostacyclin production, an arachidonic acid metabolite with potential direct and indirect immune modulating properties (ref 3). Finally, a study by Pecaut et al (ref 4) demonstrated that CO_2 euthanasia can alter circulating CD8+ T cell numbers and spontaneous blastogenesis of blood and splenic leukocytes. The members of my laboratory have had prior experience in the procedure of cervical dislocation, which will assure the virtually instantaneous euthanization of the mouse, thus avoiding significant distress to the animal.

1. Howard, H. L., E. McLaughlin-Taylor, and R. L. Hill. 1990. The effect of mouse euthanasia technique on subsequent lymphocyte proliferation and cell-mediated lympholysis assays. *Laboratory Animal Science*. 40:519.
2. Butler, M. M., S. M. Griffey, F. J. Clubb Jr., L. W. Gerrity, and W. B. Campbell. 1990. The effect of euthanasia technique on vascular arachidonic acid metabolism and vascular and intestinal smooth muscle contractility. *Laboratory Animal Science* 40: 277.
3. Kumar, V., A. K. Abbas, N. Fausto. 2005. Pathologic Basis of Disease. 7th Edition, Elsevier Saudners.
4. Pecaut, M. J., A. L. Smith, T. A. Jones, and D. S. Gridley. 2000. Modification of immunologic and hematologic variables b method of C02 euthanasia. Comparative Medicine. 50: 595.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room: (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes	_____	No	X
Fluid Restriction:	Yes		No	X

V.5.1.3. Exceptions: None _____ _____

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:

All animals will be housed in LAM. The USUHS maintains a modern, AAALAC - accredited, Central Animal Facility of approximately 40,000 square feet. The facility and the University's animal care and use program, are managed by the Department of Laboratory Animal Medicine (LAM). LAM is directed by a veterinarian who is an ACLAM Diplomate and is staffed with two other veterinarians, a graduate of animal husbandry, and approximately 30 technicians. The University is able to provide appropriate care for a wide variety of laboratory animal species, from invertebrates to lower vertebrates, to higher vertebrates, including non-human primates and domestic livestock, as well as the more commonly used species such as rodents and rabbits."

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions: None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
iv (tail vein), ip, and s.c. injections, tail vein bleeds, lethality studies, and euthanasia.	(b)(6)	Associate Prof 16 yrs; 10 yrs doing lethality studies.	2000 rodent handling
iv (tail vein), ip, and s.c. injections, tail vein bleeds, lethality studies, and euthanasia.		Res Assoc 11 yrs; 6 yrs doing lethality studies. Will perform splenectomy according to published protocol and prior consultation with LAM. Has had extensive experience in mouse handling and invasive procedures.	2001 rodent handling
iv (tail vein), ip, and s.c. injections, tail		Prof 26 yrs	1989 rodent handling

vein bleeds, and euthanasia.			
iv (tail vein), ip, and s.c. injections, tail vein bleeds, lethality studies, and euthanasia.	(b)(6)	Grad Student 3 yr; 2 yrs doing lethality studies. Grad Student 3 yr; 2 yrs doing lethality studies Grad Student 1 yr; will be trained to do lethality studies.	2008 rodent handling and investigator training 2008 rodent handling and investigator training 2011 rodent handling and investigator training

VII. BIOHAZARDS/SAFETY:

Laboratory use of extracellular bacteria, discussed in this protocol, does not pose a significant health hazard for immunocompetent adults when standard laboratory precautions are taken as stated in the catalogue published by the American Type Culture Collection (ATCC, Manassas, VA). Indeed, most adults are already colonized with these bacteria. Hence, these bacteria are considered a "biohazard level 2" requiring investigator handling with gloves and a lab coat. Facial masks are to be worn upon entering animal rooms. The following types of individuals should not be exposed to these bacteria: 1) asplenic, 2) immunodeficient 3) children and elderly, 4) cancer. Areas in which these bacteria are stored should have appropriate biohazard labels. Mice receiving live, virulent extracellular bacteria are to be kept in filter-top cages as a precaution against transmission to other mice, although mouse to mouse transmission is "extremely rare". All cages that contained infected mice are to eventually be autoclaved before re-use. Dead mice are placed in a bag and either autoclaved immediately, or placed in the LAM cold room until autoclaved.

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

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C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 1/4/2012
Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____
_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 1/4/2012
Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) _____ 1/4/2012
Date

D. **Biohazard\Safety:** I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. **Training:** I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. **Training:** I verify that I have attended the USUHS Investigator/Animal User Training Course.

_____ _____

Principal Investigator Signature Date

G. **Training:** The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. **Responsibility:** I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

_____ _____

Principal Investigator Signature Date

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_____ _____

Principal Investigator Signature Date

X.

PROTOCOL ABSTRACT:

A. **Animal Protocol Number:** (b)(6)

B. **Animal Protocol Title**: Regulation of anti-bacterial immunity (mice)

C. **Principal Investigator:** (b)(6) M.D., Professor, Dept. of Pathology, USUHS.

D. **Performing Organization:** Uniformed Services University of the Health Sciences 4301 Jones Bridge Road; Bethesda, Maryland 0814

E. **Funding:** NIH, USUHS

F. **Objective and Approach:**

Infections with extracellular bacteria are a major source of global morbidity and mortality. This fact, and the increasing incidence of antibiotic-resistance of bacterial strains make the development of vaccines against these agents of high clinical importance for both the civilian and military sectors. Immunity to this class of pathogens is mediated by antibody that facilitates the uptake and subsequent killing of bacteria by phagocytic cells such as neutrophils and macrophages. In order to optimize the design of effective vaccines against these agents, it is important to understand the basic immunologic parameters that regulate antibody production in vivo in response to immunization or infection with an intact bacterial pathogen.

Our laboratory initially conducted extensive studies on the in vivo protein- and polysaccharide-specific immunoglobulin isotype response to intact *Streptococcus pneumoniae* or vaccine antigens derived from *S. pneumoniae*, using the mouse as a model system. Recently, additional extracellular bacterial species have been added to our scope of investigation, including *Staphylococcus aureus, Streptococcus agalactiae (Group B Streptococcus, GBS), Acinetobacter baumannii, and Neisseria meningitides, groups A and C.* These extracellular bacteria continue to cause substantial global health problems. We currently have two major funding sources (see above) that collectively are integrated to explore this general issue in a complementary fashion. Hence, these respective projects, which are conducted in Rooms (b)(6) in the Department of Pathology, are subsumed under this one animal protocol, given that the methodologies related to the use of mice are widely overlapping among the projects.

Areas of study include the role of dendritic cells and macrophages, B and T lymphocytes, innate immunity including Toll-like receptors, cytokines, chemokines, T cell costimulatory molecules, in the ability to elicit humoral immunity to distinct, polysaccharide-encapsulated extracellular bacteria, and conjugate vaccines containing specific capsular polysaccharide derived from these bacteria. Regarding the use of mice, methodologies include 1) utilizing athymic nude mice as a model of T cell-deficiency for immunologic studies, 2) immunization of mice with heat-killed intact bacteria or bacterial antigens, by i.p., i.v. and s.c. routes; in selected experiments alum or Toll-like receptor (TLR) ligand adjuvants are used, 3) tail vein bleeds to obtain sera for measurement of antigen-specific Ig isotype titers and cytokines by ELISA, and for obtaining circulating immune cells, 4) removal of lymphoid tissue for obtaining cells for various *in vitro* immunologic studies and for confocal fluorescence microscopy, 5) infection of mice with live extracellular bacteria for studies on immunologic protection, 6) use of $RAG^{-/-}$ (B cell-deficient + T cell-deficient) mice as recipients in immune cell adoptive transfer experiments, and 7) gamma irradiation of immune cells, to act as antigen-presenting cells for stimulating T cells, and gamma irradiation of mice for making bone marrow chimeras. Heavy use is made of transgenic and knockout mice in which, for some, we have established breeding colonies in LAM. As will be described, although the extracellular bacteria described in this protocol are human

pathogens, their use experimentally at worst poses minimal health concerns that can easily be addressed using routine microbiologic precautions.

G. Indexing Terms (Descriptors): Animal, bacteria, antibody, polysaccharide, mouse, dendritic cell, macrophage, T-cell, B-cell, innate, adaptive, costimulation, Toll-like receptor, oligodeoxynucleotide, vaccine.

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Institutional Animal Care and Use Committee
Phone: (b)(6)
Fax: (b)(6)

October 9, 2013

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF PATHOLOGY

SUBJECT: IACUC Approval of Protocol - Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on October 9, 2013:

Animal Protocol Title: "Role of MicroRNAs in Mild Traumatic Injury and Posttraumatic Stress Disorder: Identification of Biomarkers and Therapeutic Targets in Mice"

USUHS Protocol Number: (b)(6)

Expiration Date: October 8, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Role of MicroRNAs in Mild Traumatic Injury and Posttraumatic Stress Disorder: Identification of Biomarkers and Therapeutic Targets in Mice

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: DMRDP

EARLIEST ANTICIPATED FUNDING START DATE:

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6)	PAT	(b)(6)	7/15/2013
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	Chair	(b)(6)	7/15/13
Research Unit Chief / Dept. Head Signature	Title	Telephone	Date

Typed Name: (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

PAT (b)(6)

(b)(6)

Statistician Signature

PAT
Department

Telephone

Date 7/15/2013

Typed Name (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)

Attending/Consulting Veterinarian Signature

LAM
Department

(b)(6)
Telephone

20 Aug 13
Date

Typed Name (b)(6) ~~DVM, MPH~~
(b)(6) DVM

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6)

ANIMAL PROTOCOL TITLE: Role of MicroRNAs in Mild Traumatic Injury and Posttraumatic Stress Disorder: Identification of Biomarkers and Therapeutic Targets in Mice

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER:

CO-INVESTIGATOR(S):

(b)(6) PhD
(b)(6) MS,
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(b)(6) MS
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(b)(6) PhD

TECHNICIANS(S): TBD

I. **NON-TECHNICAL SYNOPSIS:** Traumatic brain injury (TBI) is described as an insult to the head by a violent force. Mild traumatic brain injury (mTBI) is one of the most common types of injury sustained in the war zones. mTBI is also called signature injury of war in Iraq and Afghanistan. Recent advancement in the knowledge of mTBI has shed light on the role of immune and repair response generated by the body in response to mTBI. Dysregulated inflammatory responses following mTBI are increasingly being discovered at the core of the mTBI induced brain injury resulting in the loss of cognitive skills and neurobehavioral disorders. mTBI injury usually go unnoticed due to lack of any outward sign of injury. There are no diagnostic markers for identifying mTBI injury at early stages. In the proposed study we will sought to determine early serum based biomarkers to detect mTBI. In this regard we will evaluate the expression pattern of microRNAs (miRNA) in the blood of the animals that sustain mTBI. MiRNA are a class of small oligonucleotide molecules that have come to be known as key regulators of gene expression and thereby cellular processes. Their presence in blood has been reported after organ injury such as of liver and muscle and in cancer such as of colon and pancreas. mTBI injury will be induced by an free falling impactor (333gm) from a height of 2 cm. This has been demonstrated to induce a clinically relevant mTBI injury that results in cognitive and neurobehavioral deficits. MiRNA expression will be evaluated in the blood and the brain of these animals and their expression and significantly modulated miRNAs will be determined. These significantly modulated miRNA will then be restored to their normal levels either by using a mimic (a molecule similar to the miRNA made by the body) or an antagonist (a molecule that will stop the miRNA made by the body). Restoration to normal levels of

miRNA will then be correlated with improvement in the cognitive and neurobehavioral deficits.

II. BACKGROUND:

II.1. Background:

Traumatic Brain Injury (TBI) is defined as a blow or jolt to the head or a penetrating head injury resulting in the disruption of brain function. Mild TBI (mTBI) is generally referred to as the minor injury caused to the head. This is defined as alterations or loss of consciousness lasting 30min to 24hr post injury (Vasterling et al 2009, www.dvbic.org). Mild TBI is much more difficult to define and diagnose and early identification usually involves a history of direct trauma to the head and brief loss of consciousness. Clinical symptoms of mTBI include headache, dizziness, inability to concentrate, loss of memory and other neuropsychiatric and cognitive complaints. Most of the symptoms associated with mTBI resolve within days or weeks of injury with substantial recovery in most cases. However approximately 10-20% of patients reported continued problems and 44-50% experienced three or more symptoms in a year post injury (Bigler 2008, Wood 2004, Vasterling et al 2009). These kind of reoccurring symptoms are termed as persistent post concussive syndrome (Bigler 2008).

mTBI often goes undiagnosed because of the lack of visible wound and lack of diagnostic markers (Belanger et al 2005, 2007 and 2009). Traditionally, neuroimaging techniques i.e. computed tomography (CT) And magnetic resonance imaging (MRI) is used to diagnose the brain injury. In case of mTBI these two diagnostic tools have limitations due to sensitivity of detection of minor brain tissue alterations (Le et al 2009). Development of minimally invasive assays for biomarkers capable of detecting and managing mTBI is urgently needed to reduce high morbidity associated with it.

MicroRNAs (miRNAs) are a class of small (19-28nt) endogenous RNA molecules that regulate gene expression at post transcriptional level either by translational repression. MicroRNA binds to the complementary sequences in the mRNA and blocks its translation and accelerates mRNA decay (Brown et al 2009). MiRNAs play important role in development and functioning of the brain. More than 1000 miRNA have been identified in the brain and have been shown to differential expressed in brain during the developmental stages (Forero et al 2010, Shao et al 2010, Sempre et al 2004, Nelson et al 2006, Krichevsky et al 2003, Kosik and Krichevsky 2005, Liu et al 2011). MiRNA have also been shown to regulate memory and learning by modulating dendrite spine formations and synaptic plasticity (Bredy et al 2011, Konopka et al 2010, 2011, Edbauer et al 2010, Rajasetjupathy et al 2009). Addiction to drug such as cocaine and its effect on cognition, memory and learning has also been suggested to be mediated by miRNAs and miRNAs have also been shown to regulate learning and memory associated genes (Chandrasekar and Dreyer et al 2011, Gao et al 2010, Spadaro et al 2012).

Biomarker is "a measurable substance in an organism whose presence is indicative of some phenomenon such as disease, infection, or environmental exposure".. MiRNAs have emerged as novel diagnostic biomarkers for various diseases. MiRNA changes in the serum have been suggested as a potential marker of disease and injury (Mayr et al 2013, Allegra et al 2012, Wahid et al 2010). MiRNA modulations in patients with TBI have also been reported (Redell et al 2009). Earlier we have reported that miRNA modulation occurs in the serum of rats exposed to repetitive blasts (Balakathiresan et al

2012). Serum miRNA expressions can also reflect the miRNA modulation that may occur in the CNS following TBI, which was indicated by the simultaneous up-regulation of Let-7i in the blood and brain of rats with TBI (Balakathiresan et al 2012).

As mentioned earlier, mTBI is under diagnosed at present; therefore a non invasive test for biomarker of mTBI is needed for the diagnosis of mTBI. The presence of specific miRNAs in blood following organ injury including brain facilitates the use of miRNAs a biomarker of mTBI. Simple and non invasive tests such as blood test will prove extremely useful in diagnosis of mTBI. We hypothesize that upon induction of mTBI, miRNAs will be modulated which result in inflammatory response in the brain. These miRNAs will cross the BBB to enter the blood and hence can be detected in blood plasma and utilized as biomarkers for mTBI.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: Medline, Defense Technical Information Center (DTIC), DoD Biomedical Research Database (BRD) and Research Portfolio Online Reporting Tools (RePORT).

II.2.2. Date of Search: June 28-July1 2013

II.2.3. Period of Search: 1980 – current

II.2.4. Key Words and Search Strategy: Traumatic brain injury (TBI), microRNA, biomarkers, Post traumatic stress disorders (PTSD), War and TBI, Operation Enduring Freedom (OEF), Operation Iraqi Freedom (OIF), miRNA and brain injury, miRNA and blood, miRNA and plasma, circulating miRNA and brain injury, brain injury and blood brain barrier (BBB), brain and miRNA antagomirs, brain and miRNA mimics, miRNA as biomarkers etc.

II.2.5. Results of Search: No duplication of the proposed study was found in the above mentioned database. Below is the summary of the search results:

1.) Incidence of TBI has increased significantly in the current conflicts of OEF and OIF over the previous wars. Untill Q1 of 2013 the total incidence of TBI in US troops is 273, 859.

2.) mTBI is a signature injury of OEF and OIF.

3.) There is no reliable diagnostic test available to detect mTBI at early stages though some putative protein based biomarkers such as S100B, UCH-L1, GFAP and its breakdown products and neuronal elonases etc have been described but there use in diagnosing the mTBI and its prognosis is unknown.

4.) Circulating miRNAs have been describes as potential biomarkers in various organ injury and cancers.

5.) BBB is breached after mTBI and brain specific miRNAs have been detected in the circulation when an injury results in BBB breach.

6.) miRNA expression pattern signature may be used as a potential biomarkers after an injury.

7.) RePORT database search with key words "traumatic brain injury" return 1310 results, further filtering using key word "biomarker" returned 51 results. Combination of TBI and microRNA return 6 results. Careful examination of these

results revealed funded projects in microRNA and brain function but no duplication of proposed research effort was found.

III. OBJECTIVE\HYPOTHESIS:

The central hypothesis of the proposed study is that miRNA profile of the serum will change after mTBI. This altered pattern of serum miRNAs may then be used as diagnostic marker of mTBI injury. Some of the identified miRNA can also be used as therapeutic molecules for treating mTBI. The overall objective of the proposed study is to establish miRNA as diagnostic markers for mTBI and to develop miRNAs as therapeutic moleculaes for treating mTBI.

The first objective is to evaluate the expression profile of miRNAs in the blood of mouse following an mTBI. Evidence in the literature suggests that miRNA modulation occur upon injury to brain either caused by stroke or by traumatic events (Liu et al 2009, Lei et al 2009). Our working hypothesis is that, a mild injury to the brain is going to cause a focused as well as diffused injury which will cause a modulation of expression of miRNAs which then results in an inflammatory response throughout the brain. Mild TBI also cause disruption of BBB which would allow the miRNAs of brain to enter the blood stream. These circulating miRNAs in blood plasma can be used as a biomarker for mTBI.

From previous studies on mTBI, we know that inflammation plays a major role in the pathophysiology of mTBI. Gene involved in inflammatory response would be modulated and along with them the miRNAs which regulate them would also be modulated. We expect to find significant modulation of miRNAs in brain which are involved in regulating the inflammatory and neuronal repair and regeneration pathways.

Reconstitution of aberrantly expressed miRNAs in diseased conditions using antagomirs and expressing miRNA through plasmid vector "mimic" has been successfully shown in many studies as discussed before. The working hypothesis here is that due to mTBI, miRNA expression is altered which results in a persistent inflammation and reduced neuronal repair and development. Restoring the altered levels of miRNAs expression may be important in reducing the inflammation in the brain and would enhance repair process in damaged neurons. In this study, we will design specific antagomirs and plasmid vector expressing pre-miRNAs for the miRNA over expressed and repressed during mTBI.

IV. MILITARY RELEVANCE:

Traumatic brain injury (TBI) is a significant concern amongst military personnel who are currently or were previously deployed in Iraq and/or Afghanistan. Among the causes of war-related TBI are exposures to blast, gunshot and shrapnel wounds, falls and motor vehicle accidents. Mild TBI (mTBI) that is also commonly referred to as concussions occurs when the head is hit or shaken leaving a person dazed, confused or with loss of consciousness for a short period of time. About 22 % of the wounded soldiers in the current conflicts have sustained TBI and approximately 43,000 and 39,000 cases of TBI and PTSD have been recorded by military health system (MHS) in the year 2008. The cost associated with the diagnosis and treatment of TBI and posttraumatic stress disorder (PTSD) is huge and about $2 billion was spent in the FY

2010 on the diagnosis, treatment and care recent veterans with TBI and PTSD (A CBO Study: The Veterans Health Administration's Treatment of PTSD and Traumatic Brain Injury Among Recent Combat Veterans, February 2012)).

Symptoms of mild TBI can appear in days, weeks to months post injury. Clinical symptoms of mTBI are wide ranging and include sleep disturbances, fatigue, trouble in decisions making, headaches, depression, memory loss, short attention span, difficulty organizing and completing the tasks, irritability, anger, impulsive behavior and sensitivity to lights and sound. These conditions not only degrade the quality of life among veterans it also have detrimental effect on their family social and mental health. Morbidity due to mTBI also affects a soldiers potential for redeployment and the ever growing numbers of TBI may also affect the military operational readiness.

Mild TBI often goes undiagnosed because of the lack of visible wound and lack of diagnostic markers. Traditionally, neuroimaging techniques have been used for the diagnosis of TBI which have several limitations for the detection of mTBI. MicroRNAs have been successfully shown as circulating biomarkers in various neurodegenerative disease models and tissue injury models. Diagnosis of mTBI using miRNAs as biomarker will be an easy, non invasive and inexpensive method. Upon successful evaluation of miRNAs in this study, it can be easily tested in mTBI patients and integrated in military for diagnosis in a significantly short period of time.

The experimental models to create mTBI injury in mice that will be employed in the proposed study have been well characterized. This model replicates all the pathophysiological changes such as cerebral edema and decreased blood pressure that is seen in the soldiers sustaining mTBI injury. Secondary neuronal damage such as diffuse axonal injury and excitotoxicity and inflammation that is implicated in the brain tissue damage following mTBI is also reproduced in this model. Cognitive and neurobehavioral deficits associated with mTBI are also reproduced in this model. Further, we are using a closed head injury model which also replicates the kind of injury which is often associated the soldiers and war veterans who experience mTBI.

The proposed study has significant military relevance and importance as there is no specific biomarker for diagnosis and effective treatment available for mTBI and PTSD. There is a greater prevalence of PTSD in veterans who had suffered mTBI. Both the conditions contribute towards the morbidity and loss of quality life in veterans returning from the war zones and also have significant financial burden on the military in terms of diagnosis, treatment and care of veterans. Social and mental stress on veterans and their families is far greater than the civilian population. This project therefore addresses the task area "diagnosis and treatment of brain injury" where we will evaluate the capability of miRNAs as biomarkers for mTBI and treatment of mTBI by targeting aberrantly expressing miRNAs during mTBI. We have assembled a team of experienced investigators with significant expertise and capabilities, as documented in their biographical sketches, to investigate miRNA as biomarker for mTBI and to evaluate the neuroprotection by targeting modulated miRNAs in brain upon mTBI. The environment, resources and various centers involved in TBI and PTSD research at USUHS offer an added advantage and will enhance the chances of success.

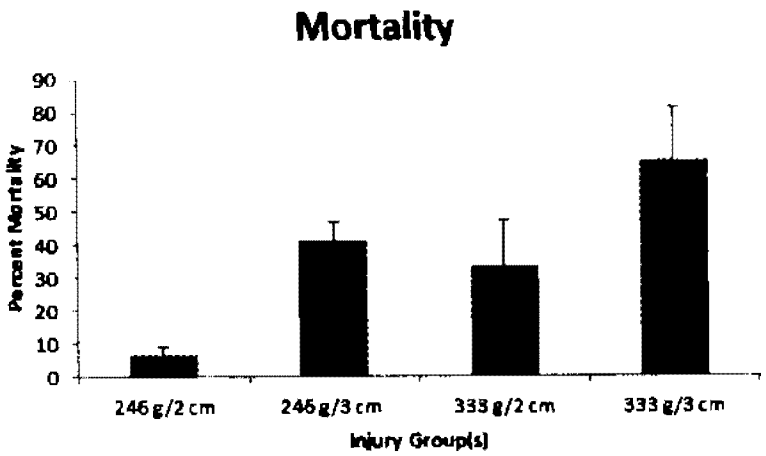
V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

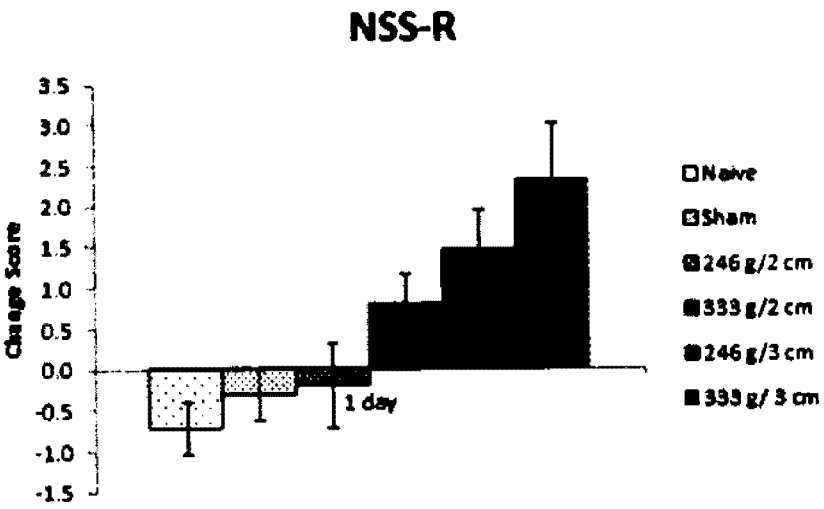
V.1.1. Experiment 1: To evaluate the mild TBI in mouse induced with the injury using a free falling weight that is restricted to stop at the depth of 4-5 mm from the point of impact.

From our previous experiments, it was observed that mild injury with 333g, weight and falling height of 2 cm and 3cm result in high percentage of mortality (Fig 1A). Resulting injury causes mild injury with acute neurological and behavioral alterations at day 1 (Fig 1B). This may be due to compression of brain in between the hard surface of the falling rod and solid plastic base. Therefore, in the proposed experiment we will restrict the falling weight to stop at the depth of 4-5mm from the point of contact (Fig 2). In a preliminary experiment, the mortality associated with the injury was reduced to 20% in 333g/3cm weight injury. Further contusion on the brain that result in a mild injury may not cause a full skin depth cut wound, therefore in one subset of animals injury will be induced on the head over the left parietal lobe area without the scalp incision. For this subset, skin over the head will be stretched to minimize the slipping of falling rod.

A



B



Groups	mTBI by weight Drop method	# iteration/ group/ method	Total animals	Pain category
No Injury with scalp incision	6	3	18	E
Injury (333g/3cm) with scalp incision	6	3	18	E
No Injury without scalp incision	6	3	18	C
Injury (333g/3cm) without scalp incision	6	3	18	E
Total animals required			72	

Total animals required [6 animals/group x 4 groups x 3 iterations/ group/ method =72 mice]

Anticipated results/problems and alternative approach: Based on our previous experience we expect that the described model (333g/3cm) will produce a mild traumatic brain injury while significantly reducing the associated mortality that is seen if weight is dropped unrestricted under gravity. If, mTBI is not reproduced as per the described parameters we will increase the height of the falling weight by 0.5 cm until we achieve the desired parameters of mTBI. The above table includes the number of mice (iteration #) that may be needed for the standardization of injury. This pilot study will also predict the percent survival of the mice after receiving the injury.

V.1.2. Experiment 2: **To evaluate the miRNA expression profile in mouse blood and brain upon mTBI using 333g weight falling from a height of 3cm.**

In this experiment animals will be subjected to single mTBI as described above in experiment 1 (please also see Section V.4.3.2 page 24). miRNA expression profile in the brains and blood of mice subjected to mTBI will be evaluated at different time points as described below. A correlation will be done to identify blood miRNA that are common, or not, with the miRNA expressed in the brain. Target prediction to predict the genes that may be modulated by the miRNAs will also be done using Target Scan software.

NOTE: From our previous experiments observation, 333g weight and 3cm height should reproduce the mild TBI. This combination of weight and height will be used in this and rest of the experiments. However, restricted fall may change the outcome. In that case the parameters determined in the experiments 1 will be used to create injury in this and the rest of the experiments.

Test and controls: Following test and control groups will be taken: 1.) No injury-naive control; 2.) mTBI-3hr; 3.) mTBI-1day; 4.) mTBI-4day; 5.) mTBI-7day; mTBI will be induced using weight drop method as described in section V4.3.2. Sham controls (with only the scalp incision) for each of the above time point will also be taken. Mice will be euthanized after the indicated time point post injury and blood and brain samples will be collected as described below.

Groups	No. of Animals	# iteration/group	Total animals	Pain Category
No injury control-naive	6	2	12	C
mTBI-3hr	6	2	12	E
mTBI-Day 1	6	2	12	E
mTBI-Day 4	6	2	12	E
mTBI-Day 7	6	2	12	E
mTBI-Day 14	6	2	12	E
mTBI-Day 30	6	2	12	E
Sham (3hr, Day 1, Day 4, Day 7, Day 14 and Day 30)	6 each group	1	36	E
Total animals required	48	2 (except sham)	**120**	

Total animas required [{6 animals /group x 7 groups x 2 iterations/group = 84 mice} + {6 animals/group x 6 groups x 1 iterations= 36mice} = 120 mice]

Sample collection: Animals will be deeply anesthetized using isoflurane and blood will be collected from animals by cardiac puncture (terminal procedure) into the collection vials (MiniCollect Serum Clot Activator Tubes with Gel Separator (0.8 ml), Greiner Bio-One) and serum will be separated by incubating at RT for 40 min followed by centrifugation at 3000g for 10min. Serum will then be stored at -20°C. Five out of 10 animals will be perfused with 4% neutral buffered formalin and brains will be collected for histological evaluation. Other five animals will be euthanized without perfusion and brains will be collected and freeze immediately for RNA isolation. Other tissues *viz* spleen and liver will also be collected from all the animals and banked for use if need arises.

miRNA analysis: cDNA synthesis for the miRNA will be done using stem-loop multiplex miRNA primer pool (ABI Inc). Analysis of miRNA expression profile will be done using Taqman low density array cards (ABI Inc) in a 384 well format, as per manufacturer's protocol. Total of 10 biological replicates will be used for each time point after the injury for the analysis. The results will be analyzed using Statminer software (Integromics, Inc) for determining the statistically significant miRNAs modulated upon mTBI. We will use Benjamin Hochberg algorithm for adjusting the false discovery rate (less than 5%) to reduce the number of false positive results.

Anticipated results/problems and solution: From the literature we know that miRNAs are candidate biomarkers in various tissue injury models in blood plasma. Since in mTBI blood brain barrier is compromised, we expect to find miRNAs in plasma which have entered the circulation from the brain. The miRNAs expressed exclusively in animals with mTBI and not in the plasma of animals with no injury would be the candidates for biomarkers of mTBI. Further miRNA expression profile of the brain will give us insights

of the dysregulation of miRNA during mTBI. This will enable us to elucidate the role of these miRNAs in various regulatory pathways important with regard to mTBI.

V.1.3. Experiment 3: To reconstitute the expression of specific miRNAs in mouse brain with mTBI by deploying miRNA inhibitors and/or mimics.

Reconstitution of aberrantly expressed miRNAs in diseased conditions using antagomirs and expressing miRNA through plasmid vector "mimic" has been successfully shown in many studies (Thum et al 2008, Krutzfeldt et al 2005). The working hypothesis here is that due to mTBI, miRNA expression is altered which results in a persistent inflammation and reduced neuronal repair and development. Restoring the altered levels of miRNAs expression may be important in reducing the inflammation in the brain and would enhance repair process in damaged neurons. In this study, we will design specific antagomirs and plasmid vector expressing pre-miRNAs for the miRNA over expressed and repressed during mTBI.

Design and synthesis of antagomirs and mimics: From the Experiment#2, we will get the data for specific miRNAs which gets modulated upon induction of mTBI. We will custom design locked nucleic acid (LNA) antagomirs (Exiqon Inc) specific for these miRNAs which are upregulated. LNA oligonucleotides contain one or more of the 2′-O, 4′-C-methylene-β-D-ribofuranosyl nucleosides which results in a conformational lock of the molecule into a near perfect N-type conformation. LNA modified antagomirs are more stable, less toxic and highly specific. MicroRNA mimics will be ordered for specific miRNAs in the form of precursor molecules (Pre-miR™ miRNA Precursor Molecule, Ambion Inc).

Treatment with antagomirs and/ or mimics: Animals will be treated with antagomirs or mimics following the mild TBI (as determined in experiment #2) via intravenous injections in the tail vein. LNA antagomirs will be dissolved in Phosphate buffer saline (PBS) buffer and would be administered at a dose of 80mg/kg of body weight (Krützfeldt et al, 2005). MicroRNA mimics in the form of precursor molecules will be administered at a dose of 50ug/mice in PBS buffer via tail vein injections (Takeshita et al 2009). This treatment will be repeated after 24 and 48hrs post injury.

Following parameters will be evaluated;

(A) Determine the delivery of the miRNA antagomir or mimic: Cy3 labeled LNA antagomirs and/or mimics will be administered intravenously in a separate group of mice to verify the delivery of antagomirs and mimics to brain post mTBI. Brain sections from these mice will be studied using a laser scanning microscope to detect the localization of cy3. Mice will be evaluated every day for 3 days for localization of cy3 signal.

Group	# of animals	Pain Category
Cy3 labeled LNA antagomir and mimic with no injury	18 (n=6/each time point)	C
Cy3 labeled LNA antagomir or mimic with injury	18 (n=6/each time point	E
Total animals required	36	

Total of animals required [18 animals /group x 2 groups = 36 mice]

(B) Effect of miRNA antagomir or mimics treatment on cognitive and neurological dysfunctions following mTBI:

Animals will be treated as described above. Following groups will be taken:

Groups	# of Animals	# of iterations/ group/ function	Total animals	Pain Category
Saline control with Sham (without injury)	6	3	18	E
Saline control with injury	6	3	18	E
Antagomirs / mimics control with sham (without injury)	6	3	18	E
Treatment with antagomirs and/or mimics following mTBI. We anticipate selecting 4 miRNA targets. Represents 4 groups	24 (n=6 for each of the 4 miRNA target groups)	3	72	E
Total Animals	42	3	126	

Number of animals required-

[6 animals/group x 8 groups x 3 iterations/group = 144 mice].

Evaluation of neuro-behavioral deficits: On day 1 post injury three different tasks will be evaluated. Animals will be first evaluated for NSS-r in the morning followed by evaluation of OFL and then ASR in the afternoon. On day 14, only OFL and ASR test will be sequentially done in the afternoon

The above three tests and time points were selected based on our experience with this model. However, it may turn out that as the experiments progress we may have to do specific test for memory, depression or motor activity. Therefore, we have added the Barnes Maze Test, the Morris Water Maze test, Forced Swimming Test, and the Rotarod test, and the under section V4. In case we decide that a specific test is needed, animals under those experiments will undertake only that particular test in addition to NSS-r.

(C) Effect of miRNA antagomir and mimic treatment on the biochemical and histological changes in the brain. This experiment will be undertaken only with the

most effective miRNA antagomir or mimic as determined from the experiment in part B. Animals will treated with the miRNA antagomir or mimic will be euthanized at 3hr, Day 1, 4, and 7 after the last treatment with antagomirs or/mimics as described above. Brains will be isolated from one group of animals and immediately stored at -80°C for biochemical parameter analysis. Another group of animals will be perfused with the 4% formaldehyde solution and brains will be collected for histological studies. Serial sagittal sections of the 1mm thickness will be cut and placed on slides from all parts of the brain.

Groups	# of animals for biochemical parameters	# of animals for histological parameters	# of iterations/ group/ parameter	Total animals	Pain Category
Injury + Treatment with antagomirs and/or mimics following mTBI (For histological and biochemical parameter analysis)	24 (n=6/each time point)	24 (n=6/each time point)	2	96	E
Treatment with antagomir or mimic without injury	24 (n=6/each time point)	24 (n=6/each time point)	2	96	C
Total animals required				192	

Number of animals required- [24 animals/group x 2 groups x 2 iterations/group = 240 mice]

V.2. <u>Data Analysis and Statistics</u>: The statistical framework for designing experiment and estimating the sample size considers the magnitude of the effect in the control group the "additional effect" (over and above the control effect) expected in the treatment group, statistical power to detect this desired difference between the treatment and control, and the alpha level. The laboratory and clinical experiments will be designed with a statistical power of at least 80%. The alpha level is traditionally set at 5%. To observe a treatment effect of 60% with 80% power and 5% alpha level a sample size of 6 mice per group would be necessary and therefore 6 mice per group will be taken in all the experiments. All qualitative variables will be compared between treatment and control groups using Fisher's exact test. Since several variables will be measured in each experiment, analyses of all variables at 5% alpha level will result in inflation of P-value. This inflated P-value may increase the probability of false positive result. Therefore to maintain the experiment wise alpha level of 5% and to control for multiple comparisons in the experiment, we will adjust the P- values using statistical procedures of Holm 1979 and Hochberg 1988. This will rule out any false positive results. USUHS has a Biostatistics Consulting Center (BCC) that provides help with the statistical and graphical analysis of the data. BCC provides advice at all stages of the research process from study design to presentation of results. BCC will be extensively consulted while analyzing the data and its presentation.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: Due to the complex nature of the traumatic brain injury and especially in mTBI where there are no outward sign of trauma there are no non-animal alternatives to the experiments involving TBI experiments. Further in the proposed study biomarkers in the circulating blood post mTBI will be evaluated which will require use of animal model and there are no non-animal alternative to this study.

V.3.2. Animal Model and Species Justification: Several animal models such as mouse and rat have been described for studying TBI injury and effect of therapeutic drugs in TBI. We have taken 10-12 week old (weighing 28 gm), male C57BL/6 mice (Jackson Labs) for the proposed study. C57BL/g mouse model have been well characterized for creating mTBI (Flierl et al 2009). Male mice are preferred as progesterone has been shown to have protective effect on TBI in humans (Beauchamp et. al., 2008). Further, other mouse species will require re characterization of the TBI model as the parameters such as weight and the height from which it is dropped will change with the species. Therefore, we have chosen male C57BL/g mouse for the proposed study.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	Mouse	
V.3.3.2. Strain/Stock:	C57BL/6	
V.3.3.3. Source/Vendor:	(b)(4)	
V.3.3.4. Age:	10-12 week	
V.3.3.5. Weight:	28 gm	
V.3.3.6. Sex:	Male	

V.3.3.7. Special Considerations:
No special considerations are needed.

V.3.4. Number of Animals Required (by Species): 546

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: The free fall weight drop model originally described in Flierl et.al 2009 caused significant mortality in our hands. Therefore, we will modify the free fall in such a way that the falling weight stops after impacting the brain (please see figure 2). This will help in reducing the compression of brain tissue between the falling weight and the solid surface of the platform on which animals are placed. We expect that such refinement will result in significant reduction in the mortality. Further if any animal show symptom of sever disease such as loss of consciousness for prolonged time (>30min), NSS score of >5 and paralysis, it will be removed from the study and will be humanely euthanized. Animals will be anesthetized before performing any brain injury.

We have deliberated use of rat model in the proposed study but mouse model provided us with greater advantages. Since mouse is much smaller than the rat handling and induction of mTBI will be faster and far easier in this model. Further the miRNA work relating to use of antagomirs and mimics has been extensively carried out and validated *in-vivo* in the mouse model and only a few study has shown this approach in rat. Consideration was given to the number of known miRNA in mouse and their similarity with the known human miRNAs. As listed in the microRNA resource database (www.mirbase.org) number of known mouse microRNAs (579) is much higher than in the rats (325). The number of known human microRNAs is 721. Several microRNAs that are known in both human and mouse are not discovered in the rat yet. Therefore, we believe the advantages of using mouse in the proposed studies outweighed the use of rat model.

V.3.5.2. Reduction: The number of animals to be used is the minimum number of animals needed to achieve scientifically sound conclusions for the experimental objectives. In order to reduce the number of animals in case the defined parameters do not produce the desired injury, we will perform a small pilot study upfront to establish the induction of desired mTBI using the weight drop method. This will significantly reduce the number of animals that otherwise may be needed for successful completion of this study.

V.3.5.3. Replacement: Due to the nature of the study, *in-vitro* and/or computer modeling systems are not available. A whole live animal model is necessary to study and possibly identify specific biomarkers after TBI.

V.4. Technical Methods:

Procedure for induction of mTBI by weight drop method: Please refer to section V4.3.2.

Neurological Severity Scale Revised (NSS-R) (b)(6) Laboratory Version. The Neurological Severity Scale – Revised (NSS-R) is currently being used to characterize traumatic brain injury in rats. Using this assessment will allow for comparisons both between multiple behavioral tests as well as behavioral comparisons between strains.

The Neurological Severity Score (NSS) is a battery of motor and reflex tests used to assess the extent of brain injury in experimental rodents (Shohami et al., 1995; Hamm, 2001; Mahmood et al., 2001). The tests assess reflex suppression, general movement, and postural adjustments in response to a challenge. The NSS includes observations of behaviors and performance measures. The Revised Neurological Severity Scale (NSS-R) is a specific, continuous sequence of behavioral tests and observations. The NSS-R is based on several previous reports but modified to increase standardization.

NSS-R tasks 2, 3, and 10 are based on the methods of Shohami et al. (1995); tasks 1, 3, 6, 8, and 10 are based on the methods of Mahmood et al. (2001); tasks 6 and 10 are based on methods described by Hamm (2001); task 4 is based on the methods of Marti et al. (2005); tasks 7 and 9 are new. The testing is conducted using two empty "guinea pig" polycarbonate cages (46 cm x 36 cm x 20 cm) with no bedding or lid.

Scoring:

In the previous version of the NSS, behavior was scored in a binary fashion with the absence of behavior assigned one point; higher total scores indicated a greater extent of injury. The Shohami system allowed for a total of 24 points and the Mahmood system allowed for a total of 14 points. By contrast, a three-point Likert scale is used in the NSS-R to increase sensitivity, in which a normal, healthy response is assigned a "0," a partial or compromised response is assigned a "1," and the absence of a response is assigned a "2." Therefore, the NSS-R has a scoring range of 0-30, which is designed to increase sensitivity.

In the NSS-R, the mice are scored as follows:

0-presence of reflex or response that is comparable to a normal mouse

1-reflex or response is present but impaired/attenuated

2-absence of reflex or response

Higher scores will reflect greater extent of injury, with the highest score possible being the total number of measures multiplied by three. Following the methods of Shohami et al., two terms will be used to describe the clinical status of the mice:

1. NSS – the total score for performance on a particular test

2. Δ NSS – the difference between the total score immediately post-injury and any subsequent scores.

NSS-R Tests:

1. General balance test

The mouse's ability to walk on a balance beam is assessed. The balance beam is placed lengthwise at above Cage A by placing the ends of the balance beam on top of two pairs of stacked cages. The mouse is gently placed onto the balance beam and observed. The mouse's movement on the balance beam is given a score of 0, 1, or 2. A score of 0 is given if the mouse maintains balance and walks successfully on the beam. A score of 1 is recorded if the mouse balances on the beam but does not walk along the beam. A score of 2 is given if the mouse does not balance or walk on the beam at all. The task is repeated three times and the score is based on the "best" performance. The second test begins immediately after test #1.

2. *Landing test*

In this test, the mouse lands on a flat surface after being dropped from the cage height where the balance beam is placed as described above, and the reflexes of its paws and body posture are observed. The landing test occurs immediately following the general balance test, as the mouse lands on the cage floor. The mouse's landing is observed and rated. A score of 0 is given if the mouse shows normal reflexes when landing. A

score of 1 is given if the mouse shows partial reflexes that are compromised in some way. A score of 2 is given if the mouse does not use landing reflexes when falling off the balance beam, and instead falls flatly onto the cage floor.

3. Tail raise test

Immediately following the landing test, the mouse will be gently lifted by the base of the tail (dorsal side up). When the mouse is lifted, the reflexes of its forelimbs and hind limbs will be observed and scored for the proper flexion and extension. A score of 0 indicates normal reflexes, a score of 1 indicates partial reflexes, and a score of 2 indicates that no reflexes were observed (e.g., the mouse was limp when lifted by the tail). After the tail raise test, the mouse is brought to the second cage, Cage B, to conduct the drag test.

4. Drag test

While continuing to hold the mouse by the base of the tail, the mouse is placed on the floor of Cage B, with the mouse facing and close to one of the shorter walls. Allowing only the mouse's forepaws to remain on the floor of the cage, the mouse is gently dragged backwards at a constant speed (about 20 cm/sec) across the length of the cage. The mouse's behavior while being dragged is observed. A score of 0 indicates extension of forepaws and effort to slow down the drag or to pull away from the drag. A score of 1 indicates some effort to slow down the drag or to pull away. A score of 2 indicates no resistance.

5. Righting Reflex

After the conclusion of the drag test, the mouse remains in Cage B for the Righting reflex test, as well as for the remainder of the reflex tests. The mouse is placed on its back and the mouse is observed. A score of 0 indicates that the mouse gets onto four paws without difficulty. A score of 1 indicates partial "righting" or complete righting with difficulty. A score of 2 indicates inability to right completely.

6. Face Reflex

The face reflex will be tested by lightly touching the head with a long Q-tip or eraser end of a pencil and observing responses. A score of 0 indicates a head shake or movement away from the stimulus. A score of 1 indicates partial response and a score of 2 indicates no response.

7. Eye Reflex

The eye reflex is tested by lightly touching the eye with a Q-tip and observing the response. The response will be scored according to the following criteria: 0 indicates a complete and immediate eye blink; 1 indicates a delayed eye blink; and 2 indicates no response.

8. Sound Reflex

This reflex is a movement in response to the noise of a short, sharp clap of the experimenter's hands. The reflex is observed and rated as follows: 0 for a quick jumpy movement followed by freezing; 1 for a slow movement and/or no freezing; and 2 for no response.

9. *Tail reflex*

This reflex is tested by applying a brief pinch using the experimenter's fingers to the tail for a marked and immediate squeak; 1 for a delayed or weak squeak; and 2 for a no response.

10. Paw flexion reflex

This reflex is tested by briefly applying a pinch with the experimenter's fingers to the hind paw and observing whether a withdrawal response is elicited. The test will be scored as follows: 0 for a limb withdrawal reflex; 1 for a partial or delayed withdrawal reflex; and 2 for no response

Other Neurological behavior tests:

Barnes Maze test: Spatial memory task in rodents after TBI will be evaluated using Barnes Maze test (Barnes 1979, Harrison et al 2006, Doll et al 2009, Maegele et al 2005). The arena consists of a circular platform (122 cm diameter) with 20 holes around the perimeter. One hole is designated as the "escape hole" and has a long box underneath it that extends under the platform. The other 19 holes can be used with or without the false boxes directly underneath the holes, but do not extend out underneath the platform. Bright light is pointed towards the maze to act as an aversive stimulus and induce mice to find the "escape hole". The time line for training and testing using the Barnes Maze test consists of 4-5 consecutive training days and multiple testing days. On the first day, three trials (one acclimatization and two training trials) are given to each animal. Next for four consecutive days each animal receive two training trails. Following the induction of mTBI each animal will be given two test trails each day for seven days to evaluate the spatial memory task. Training is conducted by placing the animals at the center of the platform and ends after the animal has escaped from the "escape hole" or the predetermined time of 300 sec has ended. After the animal has escaped from the maze it is covered for 30sec to reinforce the escape from the aversive stimuli. If the animal fails to escape the maze then it will be led to the escape hole and will be covered for 60sec to reinforce the escape from the aversive stimuli. Platform will be cleaned with 35% isopropyl alcohol in between the training and or testing of each animal to remove any olfactory cues.

Rotarod test: The rotarod test measures the motor skills of an animal. It also measures learning behavior as the animal improves the balancing act by practice (Chen et al 2007, Fujimoto et al 2004). The rotarod treadmill (Med Association) consists of a motor-driven drum divided into four stations. When the animal falls off the rotating drum it breaks a photobeam stopping the timer associated with that chamber. The animals will have three consecutive trials on this device with speed of rotation increasing with each trial from 0 to 35 revolutions per minute for a maximum of 3 min. Average time from three trails will be expressed in seconds and will be recorded for scoring each animal.

Open field locomotion: Open field locomotion will be measured during the dark cycle i.e. the active period of the animals using an Omnitech/Accusan Electronics Digiscan infrared photocell system (Elliott and Grunberg 2005). The system consists of a clear box with infrared beams to measure horizontal and vertical movement of the animal. Activity for each animal will be measured for one hour.

Startle acoustic test: The acoustic startle reflex test in animal will be conducted with a Startle Response Acoustic Test System (Coulbourn Instruments, Columbus, Ohio, USA) (Faraday and Grunberg 2000). The system consists of eight weight sensitive platforms

in a sound-attenuated chamber. A subject's movements in response to stimuli will be transduced into analog signals by the platforms. These signals will then be digitized and stored by a computer. All acoustic stimuli will be given by a loudspeaker mounted 24 cm above the test cage. To minimize the effects of handling and stress on drug testing, animals will be allowed to acclimate to the startle chamber for 2 consecutive days. During acclimation animals are in the chamber for 20 min/each session. The startle baselines will then be tested for each animal the next day on Day -1 before their drug administration or experimental manipulations. During testing mice will be individually placed in an animal holder (E05-15, Coulbourn Instruments, Columbus, Ohio, USA), which will be then placed on a weight-sensitive platform within the chamber. A ventilating fan built into the chamber provides background noise. Following 3-min acclimation period animals will be exposed to six types of sound stimulus trials: 100 and 110 dB alone, both 100 and 110 dB with pre-pulse (84dB), pre-pulse alone and no stimulus. Each trial type will be presented eight times. Trial types will be presented in random order to avoid order effects and habituation. Inter-trial intervals ranged randomly from 15 to 25s. Animals will be tested in the dark cycle; during the experiments the red light will be provided in the dark room. Each animal's responses will be averaged within trial type. Trials during which no stimuli presented will be used as control for normal subject movements on the platform. Amplitudes of each trial type will be derived by subtracting grams (g) of platform displacement on the no-stimulus trials (i.e., body weight of each subject) from g of platform displacement in response to specific stimuli. The remainder resulting from this calculation represented absolute amplitude of ASR to the stimulus (e.g., 100 dB, 100 dB with pre-pulse, 110 dB, 110 dB with pre-pulse). The ASR amplitude tested each time was finally represented as "% of baseline", which was calculated using the equation: % of baseline= (absolute amplitude /baseline absolute amplitude) 100%. For each test day, ANOVAs for repeated measures will be performed on startle amplitudes with factors of stress status and drug dosage. Tukey or Bonferroni test was used to assess significant post-hoc differences between individual groups. The data will be represented as mean ± S.E.M.

Forced swimming test: Forced swim test consists of two phases, a training phase and a test phase on two successive days (Carlezon et al 2002, Pliakas et al 2001). For the training phase animals are placed into a 65cm tall x 25cm diameter cylinders filled with tepid water up to 48 cm for 15 minutes. For testing phase animals are similarly placed into the water for 5 min. Animal is considered immobile in water if 75% of the animal's body is still for at least 5 seconds (as analyzed by video recording software). Total time of immobility, latency to first instance of immobility and number of immobile periods will be recorded for each animal. Animals under stress or showing inability or difficulty in swimming will be immediately removed from the water. All mice will be dried off using paper towels after the test and before placing them back in their respective cages.

Morris Water Maze (MWM) test: Our current neurobehavioral experiments such as open field locomotion and rotarod test indicate that there may be a learning behavior deficit in the mild TBI group as compared to the control. Published literature on mild TBI suggest that Morris Water Maze test may be more sensitive to bring out the learning behavior deficits in mouse as compared to the other neurobehavioral tests. Therefore we would like to conduct MWM test in our studies details of which are given below:

Ten days (day 1 of the training) after TBI or sham procedures, a Stoelting Morris water maze is filled with tap water (25°C) to a depth of 20-30 cm. A clear plastic platform is placed in one of the "quadrants" of the tank floor. On Day 1, a pre-training trial takes place where the mouse is gently placed on the platform in the tank and the mouse remains there for 15 seconds. If the mouse jumps into the water during this time, it is allowed a brief swim (< 60 seconds) before being guided back to the platform. On Days 2-5, training trials take place in which the mouse is placed near the side of the tank in a location away from the platform, and is allotted 60 seconds to swim to the platform, where it remains for 15 seconds before being dried and placed into the home cage under a heating lamp. If the mouse does not reach the platform in the allotted 60 seconds, they are gently guided to the platform. A total of four trials per mouse, separated by 1-2 minutes, are performed each day and the latency to find the platform is recorded for each trial. Finally, on the final day of training (Day 5) about one hour after the last training trial, a single trial is performed with the platform removed from the tank. The time spent in the pool quadrant where the platform was located is measured, compared to time spent in the other three quadrants. The time to reach the platform, distance traveled, and time in each quadrant of the tank is recorded by the Any-Maze video tracking program.

Twenty six days (day one of the 2nd round of training) after the TBI or sham procedure above procedure will be repeated again with following differences. The platform will be moved to a different quadrant of the Stoelting Morris water maze. Training will done for 3 days i.e. day 26, 27 and 28 post TBI or sham procedure. Day 26 training will be similar to day 1 of first round of training as described above. On day 27 and day 28 training will be similar to day 4 and 5 of the 1st round of training respectively. On day 28, a single trial with platform removed will be conducted 1 hour after the last training trail. Data will be measured and analyzed as described above.

Ear punch identification: We have initially proposed in our protocol that a temporary marking on tail with a sharpie marker will be used to identify animals. We have increasingly face problem with this mode of animal identification as many times due to water bottle leak or form other reasons the marking on the tail fades away and it becomes extremely difficult to identify the animals. Therefore we would like to request a more reliable animal identification technique of ear punching. Ear punches will be made on the day of surgery when the injury or sham group of mice will be under anesthesia (as described in the protocol). Scissor style ear punch (Kent Scientific, cat# INS500076) will be used to make ear punch. Animals will be identified in the following manner:

Mouse #1-No holes.
Mouse #2-One hole on the right ear.
Mouse #3-One hole on the left ear.
Mouse #4-One hole on each ear.
Mouse #5-Two holes on the right ear.
Mouse #6-Two holes on the left ear.
Mouse #7-Two holes on the right and one hole on the left.
Mouse #8-Two holes on the left and one hole on the right.
Mouse #9-Two holes on each ear.

Mostly numbering from 1 to 5 five will be required and further identification will be done based on the cage number followed by the animal number.

Perfusion and collection of brain: Animals will be perfused as described before (Sharma et. al., 2009). Briefly, each animal will be euthanized using over dose of isoflurane and will be perfused first with 15ml of ice cold IX PBS (GIBCO Phosphate-Buffered Saline, Invitrogen) containing 12.5U of heparin followed by 15ml of 10% neutral buffered formalin (NBF) (VWR, West Chester, PA). Whole brain will be carefully removed and fixed in the 10% NBF solution.

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	144	
V.4.1.1.1.2. Column D:	0	
V.4.1.1.1.3. Column E:	402	

Please refer to section V.4.1.4 for details regarding grouping of animals under each category.

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

Anesthesia: Animals will be anesthetized by inhalation anesthetic immediately prior to the induction of TBI. General anesthesia will be induced by 2-4 % isoflurane + 100% oxygen using a precision vaporizer via a rodent anesthesia machine in a clear chamber. Mice will then be removed from the chamber and maintained under anesthesia on 0.5-3% isoflurane + 100% oxygen using a nose cone during the procedure. All the procedures such as induction of TBI, euthanasia and perfusion will be performed on mice with no pedal withdrawal reflex. Mice will also be carefully monitored for a stable rate of respiration. Waste anesthetic gases will be scavenged either through the chemical fume hood or passively by the charcoal filter. Anesthetics will be administered by the PI or his staff or by LAM personnel.

Analgesia: None

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

Animals will be kept at the animal housing facility of LAM for 10-14 days prior to the induction of mTBI and will receive standard care. Acclimation and baseline measurements for the behavior test will be done in the week preceding the day of injury. Post injury animals will be monitored for any distress. If animal is found to be distressed for reasons other than the mTBI it will be removed from the study and humanly euthanized.

V.4.1.2.3. Paralytics: none

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: Medline (PUBMED), defense technical information center biomedical research database (BRD) and research portfolio online reporting tools (RePORT)

V.4.1.3.2. Date of Search: June 1-8, 2013

V.4.1.3.3. Period of Search: 1970-current

V.4.1.3.4. Key Words of Search: Traumatic brain injury, mild traumatic brain injury, traumatic brain injury + pain, mild traumatic brain injury + pain, mild traumatic brain injury + distress + pain, mild traumatic brain injury + alternative, mild traumatic brain injury + pain + alternative, mild traumatic brain injury + analgesic, mild traumatic brain injury + analgesic + microRNA, perfusion and tissue fixation, perfusion+ pain, perfusion + pain + alternative, cardiac puncture blood collection, cardiac puncture + pain, tail vein injection, tail vein injection + pain, tail vein injection + pain + alternative.

V.4.1.3.5. Results of Search:

Use of the various key words and their combinations returned several results. These results showed that induction of mTBI by weight drop method, formalin perfusion by cardiac puncture, tail vein injection and blood collection by tail vein nick and cardiac puncture are routinely used technique and are used as per the need of the experiment. We could not find any alternative to the weight drop method to induce mTBI that is minimally invasive in producing contusion. Further the blood collection by cardiac puncture will give greater blood volume as compared to the tail vein nick with minimum stress to the animal as it will be done under anesthesia. Below is the summary of the literature search:

1.) Search using the term "traumatic brain injury" returned 54467, 4825 and 626 results in PUBMED, BRD and RePORT database respectively. Adding "mild" to this search reduced the numbers to 2961, 1316 and 211 in PUBMED, BRD and RePORT database respectively. Search with "mild traumatic brain injury + pain" returned 141, 504 and 171 results in PUBMED, BRD and RePORT database respectively. "Mild traumatic brain injury + alternative" returned 28, 399 and 103 results in PUBMED, BRD and RePORT database respectively. These results show that there is no painless alternative to study mild traumatic brain injury. Most of the results indicate interventions to reduce the associated pain after the injury has happened.

2.) Use of analgesic: Pubmed search of the analgesic (NSAIDS such as aspirin, cyclooxygenase 2 blocker, ibuprofen, Sildenafil, acetaminophen etc) and gene expression showed that analgesics modulate gene expression in the host.

3.) Perfusion: Pubmed search of "perfusion by cardiac puncture" returned 123 results, where as "cardiac perfusion" returned 42445 results. Search for formalin cardiac perfusion returned 80 results. These results showed that perfusion with formalin through cardiac puncture is a well establish methodology. Search for "alternative to formalin perfusion" returned 6 results. All these results showed alternative to use of formalin with some other fixative such as acrolein but did not indicate any painless alternative to the perfusion technique.

4.) Blood collection: Search with key words "blood collection" returned 26909 results which reduced to 507 when the term "cardiac puncture" was added to the first search.

These results indicate that blood collection by cardiac puncture is a well established method.

5.) Tail vein injections: Search for "tail vein injection + mouse" returned 1114 results and addition of microRNA to the above keywords retuned only two results. These results show that tail vein injection is a well established method of administering drugs/ dyes and microRNA can also be delivered by tail vein injection.

V.4.1.4. <u>Unalleviated Painful or Distressful Procedure Justification</u>: All the animals in the experiment 1, 2 and 3, that will be induced with the TBIhave been placed under the unalleviated painful or distress category, category E. Animals that will undergo only the scalp incisions have also been placed under the category E. Total number of animals under this category are 402. This is done due to the concern that analgesics (NSAIDS such as aspirin, cyclooxygenase 2 blocker, ibuprofen, Sildenafil, acetaminophen etc) results in modulation of gene expression in the host. Buprenorphine is a mu-opiod receptor partial agonist and kappa-opiod receptor agonist (Sanchez et al 2008). Detailed molecular mechanism of Buprenorphine action is not fully understood. It has been shown to induce dose dependent affect on myelin associated glycoprotein expression and glycosylation. Buprenorphine engages both mu-opiod receptor and nociceptin/orphanin FQ receptor (NOP receptor) in the oligodendrocytes presumably via modulating activity of Src-family tyrosine kinase Fyn (Sanchez et al 2008, Eschenroeder eta l 2012). Buprenorphine also induce low level of interleukin 4 response in T lymphocytes and has also been reported to induce apoptosis in NG108-15 nerve cells (Borner et al 2013, Kugawa et al 1998). Studies on molecular mechanism of Buprenorphine action are limited. Therefore, it is also not know if buprenorphine treatment will affect the microRNA expression in the serum and brain of mice. The main objective of the proposal is to evaluate mTBI induced miRNA expression in brain and blood so as to use that information as diagnostic marker of mTBI. It is well establish that microRNA modulate the gene expression by acting upstream of the mRNA translation step. Since the analgesics modulate gene expression it will be safe to assume that microRNA expression will also be modulated by these analgesics. Analgesic, acetaminophen is known to modulate the miRNA expression in the serum (Wang et al 2009). Therefore, use of analgesic may interfere with the outcome of the proposed study i.e. identification of microRNA in serum post mTBI and their establishment as mTBI biomarkers, in a way that may result in false identification of miRNAs or inaccurate correlation of miRNA with mTBI. **However, to reduce the pain at the time of injury or only scalp incisions, all procedures will be done under continuous inhalation anesthesia.**

Animals that are placed under the category C (n=144) are the animals that will either be naïve subjects or receive momentary discomfort due to injections of saline or miRNA antagomirs/mimics.

Treatment with miRNA mimics/antagmoirs is expected to alleviate the pathology associated with the induction of mTBI therefore will be expected to alleviate the pain associated with the mTBI. If treatment with microRNA mimic(s) or antagomir(s) would be found effective in treating the mild TBI, then the above categories will be changed as deemed necessary. Such observations will be recorded and reported in the annual report to IACUC.

V.4.2. Prolonged Restraint: None

V.4.3. Surgery: Following section have been adopted from the SOP provided by the CNRM under brain injury models (b)(4)

V.4.3.1. Pre-surgical Provisions: Animals will be kept at the animal housing facility of LAM for 10-14 days prior to the induction of mTBI and will receive standard care.

V.4.3.2. Procedure: All the procedures will be carried out under aseptic conditions at the CNRM's TBI surgical suite in DLAM. Bench space and instrument to be used will be cleaned with 70% ethanol. Following two procedures will be done under the proposed study:

Weight drop induced mTBI: Mice (~28gms) will be anesthetized by inhalation anesthesia as described in section **V.4.1.2.1**. Skin will be cleaned with betadine and a midline scalp incision (~1.0-1.5 cm) will be made in a single cut exposing the skull. Mild closed head injury (CHI) will be induced by weight drop method. 333 gm weight will be dropped from a height of 3 cm causing a mild CHI. An impactor tip of 3 mm diameter at the end of the rod will be used to create the blunt end injury at the predetermined left anterior frontal area. Rod will be retracted immediately to prevent and "rebound injury" that may otherwise result in multiple contusions. Such injury causes a NSS-r) score of 3-4 at Day 1 post injury (figure 1B), which is consistent with the mTBI (Flierl et al 2009, Brody et al 2007). Following injury scalp will be closed with tissue glue. Sham-treated controls will be treated similarly except the impact with the free falling weight.

Controlled cortical impact TBI: This method has been incorporated as a backup to weight drop mTBI method. In case if weight drop mTBI model fails to provide any measurable alteration (biochemical/molecular/behavioral), CCI model for inducing mTBI, which is well established and characterized in the CNRM, will be used to undertake miRNA mimic/antagomir experiments. Mice will be induced and maintained on isoflurane anesthesia as described in section **V.4.1.2.1.** Mouse will be placed in the injury device stereotaxic frame at 0° angle. Skin will be cleaned with Betadine and a small surgical incision in the scalp will be made to expose the skull overlying the right lateral ventricle. A 5.0mm burr hole will be drilled into the skull of the right hemisphere at anterior-posterior = 0 mm, medial-lateral = 2.0 mm from bregma with a hand-held trephine to expose the dura mater. After removal of the bone flap, injury will be produced with the CCI device operated and maintained under the Center for Neuroscience and Regenerative Medicine (CNRM) core services in DLAM. The impact tip (3 mm in diameter) will be slowly lowered to the surface of the dura and a low-voltage detector indicates when the tip contacts the dura (contact will be visually verified). A single contusion will be made and target insult parameters will be a penetration depth of 0.5 mm (mild injury) with a stroke velocity of 5 m/s (Brody et al., 2007). Following CCI, the burr hole filled with bone wax and incision is closed with a Vicryl absorbable suture or with tissue glue. Sham-treated controls will be treated similarly, but the impactor will not be activated. Body temperature will be maintained with the use of a warming pad.

V.4.3.3. Post-surgical Provisions:

Animals will be allowed to recover from the anesthesia on a hot water circulating pad. No analgesic will be given to the animal as it may interfere with the inference of results. Animals will be monitored for 4 hours after the induction of surgery and will be housed singly in their cages. There after animals will be checked once a day by PI's staff. A cage card indicating the date and time of the TBI and the responsible person will be placed on each of the animal cage. Animals will also be assessed for weight loss, absence of grooming and undergo neurological tests to assess the absence/presence of impairment. Animals shown signs of distress not typically associated with the TBI such as muscle rigidity or lack of muscle tone, twitching, trembling, tremor, self-mutilation, or unkempt appearance (erect, matted, or dull hair coat) will be identified. PI will consult with DLAM veterinarians on a case by case basis to determine pain and distress in all animals. Animals deemed to be in distress will be removed from the study and euthanized by DLAM personnel, PI or her/his staff following consultation by LAM vet staff and the PI or his staff.

V.4.3.4. Location: (b)(6) USUHS.

V.4.3.5. Surgeon: PI, (b)(6)

V.4.3.6. Multiple Major Survival Operative Procedures: none

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: Animals in the experiment #3 will receive three intravenous injections of miRNA antagomirs in the tail vein starting just after the injury, at 24 and 48 hour post injury. LNA Antagomirs (dose of 80 mg/kg) or miRNA mimics (50μg/mice) will be dissolved in PBS and a final volume of 50μl will be injected in the tail vein using a 26(1/2)-27 gauge needle. Control mice will receive only PBS in similar fashion.

V.4.4.2. Biosamples: Animals will be anesthetized as described in the section V.4.1.2.1. Blood samples will be collected from the animals by cardiac puncture while under anesthesia or by tail vein nick. Brains will be isolated after the perfusion (for histology studies) or after the removal of the head (for biochemical and miRNA studies). Other tissues such as spleen and liver will also be harvested and banked for future use is the necessity arises.

V.4.4.3. Adjuvants: none

V.4.4.4. Monoclonal Antibody (MAbs) Production: none

V.4.4.5. Animal Identification: Cage cards, temporary markers and ear punches will be used for animal identification.

V.4.4.6. Behavioral Studies:

V.4.4.7. Other Procedures: NA, no other procedures will be performed on the animals.

V.4.4.8. Tissue Sharing: none

V.4.5. Study Endpoint:

For experiment #1: 4 day post injury.

For experiment #2: 3hr, Day1, 3, 4, 7, 14 & 30 post injury.

For experiment #3: (a): 3days post injury.

(b): 7 day post injury.

(c): 3hr, Day 1, 4 and 7 post last treatment with the antagomir/mimic.

In addition to the above time points animals found to be under duress for reason other than the TBI such as, extensive bite marks and skin lesions from fighting, will be removed from the study and humanely euthanized.

V.4.6. Euthanasia:

Animals will be euthanized at the study end points according to the 2013 AVMA Guidelines on Euthanasia. Mice will be deeply anesthetized as described in section V 4.1.2.1. Deeply anesthetized mice (slow rhythmic breathing with no response to foot pinch) will be euthanized either by cervical dislocation, decapitation or exsanguination during perfusion.

Animals that become paralytic or sustain severe injury due to the creation of TBI will be removed from the study and will be euthanized by CO_2 inhalation by DLAM staff (explanation of this procedure has been adapted from the IACUC policy#13: Rodent Anesthesia with CO2 Prior to Euthanasia (09/08)). Without pre-charging the chamber, animal(s) will be placed in the chamber and 100% carbon dioxide will be introduced. A fill rate of higher than 20% of the chamber volume per minute with CO2, added to the existing air in the chamber will cause rapid unconsciousness with minimal distress to the animals. The expected time to unconsciousness and death is about 2 minutes. Mice will be observed for lack of breathing and pale eye color. If both signs are observed, CO2 will be allowed to flow for another 1 minute. Mice will be removed and will be verified for death after euthanasia and prior to disposal and cervical dislocation and bilateral thoracotomy may be used to ensure death.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) TBD

V.5.1.2.
Special Husbandry Provisions:

Food Restriction: Yes _____ No X

Fluid Restriction: Yes _____ No X

V.5.1.3. Exceptions: none _____ _____

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Mice will be checked on daily basis by the LAM veterinarian and PI staff. All the animals will be checked by the PI's staff twice a day for the first two days after the injury. After that animals will be checked once a day till the end of the study. Routine care such as plentiful chow, water and change of beds will be provided by the DLAM staff. Mice that show increased distress as determined by the veterinarian and described in section 4.3.3 will be removed from the study and will be humanely euthanized by the DLAM staff as described in the section V.4.6.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions: None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
TBI, anesthesia, and injections.	(b)(6)	PI, >30 years of experience with the mice model. PI in several IACUC rodent protocols at USUHS.	Rodent handling training class 2006, USUHS Investigator Training Course PI training 2003
Neurobehavioral and cognitive functions.		PI in IACUC mice protocols at USUHS evaluating the traumatic brain injury in rodent model.	USUHS Investigator Training Course. PI training 2005
Neurobehavioral and cognitive functions.		PI in several IACUC mice and rat protocols at USUHS evaluating neurobehavioral and cognitive functions in rodent model.	USUHS Investigator Training Course. PI training 2003
Neurobehavioral and cognitive functions.		PI in IACUC rat protocols at USUHS evaluating the neurobehavioral and	USUHS Investigator Training Course PI training 2003

		cognitive functions in rodent model.	
TBI, anesthesia, tail vein injections, perfusions/fixation, serum collection/ tissue harvest	(b)(6)	Research associate, > 10 years of experience with the mice model studying viral disease.	Rodent handling training class 2002 and 2006, USUHS Investigator Training Course (2006),
TBI, anesthesia, tail vein injections, perfusions/fixation, serum collection/ tissue harvest		Graduate student, 5 years of experience handling mice.	Rodent handling training class 2008, USUHS Investigator Training Course 2007,
TBI, anesthesia, injections, Neurobehavioral and cognitive functions.		Graduate student, 3 years of experience handling mice.	Rodent handling training class held on 08/17/2010, USUHS Investigator Training Course 08/17/2010
TBI, anesthesia, injections, Neurobehavioral and cognitive functions.		Graduate student, 3 years of experience handling mice	Rodent handling training class held on 08/17/2010, USUHS Investigator Training Course 08/17/2010
Neurobehavioral and cognitive functions.		3+ years of experience with mice	USUHS Investigator Training Course July 2009.
Neurobehavioral and cognitive functions.		3+ years of experience with mice	USUHS Investigator Training Course 2009.
Neurobehavioral and cognitive functions		2 years experience working with mice and rats	USUHS investigator training course in 2008
Weight drop injury and surgery		>3 year of experience in mice and rat TBI surgery related to her clinical practice as a microsurgeon.	USUHS Investigator Training Course on September 9, 2008. Rodent Handling and Procedural Techniques on September 24th, 2009.
Weight drop injury and surgury		15 years of experience in mice and rat	USUHS Investigator Training Courses on

	(b)(6)	handling, aseptic surgery, and rodent behavior testing	October 28, 2009. Rodent Handling and Procedural Techniques on November 19th, 2009.
TBI, anesthesia, Blood and tissue collection.		Graduate student, 5 years of experience handling mice.	Rodent handling training class 2008, USUHS Investigator Training Course 2007.

VII. BIOHAZARDS/SAFETY: None

VIII. ENCLOSURES:

References:

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IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

_____ _____

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

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(b)(6) _____ 7/15/2013

Principal Investigator Signature Date

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_____ _____

_____ _____

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(b)(6) [redacted] 7/15/2013

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) [redacted] 7/15/2013

Principal Investigator Signature Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number: (b)(6)

B. Animal Protocol Title: Role of MicroRNAs in Mild Traumatic Injury and Posttraumatic Stress Disorder: Identification of Biomarkers and Therapeutic Targets in Mice

C. Principal Investigator: (b)(6)

D. Performing Organization: USUHS, Bethesda, MD

E. Funding: DMRDP

F. Objective and Approach: The overall goal of this study is to determine the miRNA modulation in serum and brain post mTBI, to establish miRNAs as early diagnostic markers of mTBI and to determine use of miRNA mimics and antagomirs as therapeutic molecules for treating mTBI. To achieve the proposed objectives we will use a free falling weight drop model to create mild traumatic brain injury (mTBI) in mice. Mice brain and serum samples will be evaluated for changes in the miRNA expression patterns to establish the miRNA expression signature post mTBI. Once these miRNA expression signatures will be established we will determine the significantly modulated miRNAs and will test for their therapeutic potential. This will be achieved by restoring the normal expression pattern (as determined from the sham controls) of the significantly modulated miRNA by using miRNA antagomirs (for inhibiting miRNA) and or mimics (to restore the suppressed miRNA). This will determine the therapeutic potential of these miRNAs. Changes in the miRNA expression pattern will be correlated to the cognitive and neurological dysfunctions that are observed post mTBI. Also the treatment with the miRNA antagomirs and mimics will be correlated with cognitive and neurological behavior changes to establish any beneficial effect of these miRNA antogomirs or mimics.

G. Indexing Terms (Descriptors): mild traumatic brain injury, microRNA, biomarkers, mice, antagomirs and mimics.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

Institutional Animal Care and Use Committee
Phone: (b)(6)
Fax: (b)(6)

May 7, 2014

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF PATHOLOGY

SUBJECT: IACUC Approval of Protocol - Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on May 7, 2014:

Animal Protocol Title: "Post-transcriptional Silencing of Venezuelan Equine Encephalitis Virus (VEEV) RNA for Developing an Antiviral Therapy against Exposure with VEEV, a Bio-warfare Agent (Mice)"

USUHS Protocol Number: (b)(6)

Expiration Date: May 6, 2017

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc: Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Post-transcriptional silencing of Venezuelan Equine Encephalitis Virus (VEEV) RNA for developing an antiviral therapy against exposure with VEEV, a bio-warfare agent (in mice).

GRANT TITLE (if different from above): Medical countermeasures against alphaviruses.

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Defense Threat Reduction Agency (DTRA)

EARLIEST ANTICIPATED FUNDING START DATE: 10/30/2013

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6)	PAT	(b)(6)	3/28/14
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	Vice Chair	(b)(6)	30 Mar 2014
Research Unit Chief / Dept. Head Signature Typed Name:	Title	Telephone	Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)	PAT	(b)(6)	3/28/14
Statistician Signature Typed Name:	Department	Telephone	Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress,even if relieved by anesthetics or analgesics.

(b)(6)	LAM	(b)(6)	2 April 2014
Attending/Consulting Veterinarian Signature	Department	Telephone	Date

Typed Name: (b)(6) DVM, MPH

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6)

ANIMAL PROTOCOL TITLE: Post-transcriptional silencing of Venezuelan Equine Encephalitis Virus (VEEV) RNA for developing an antiviral therapy against exposure with VEEV, a bio-warfare agent (in mice).

GRANT TITLE (if different from above): N/A

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S):

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS: Alphaviruses have caused widespread outbreaks resulting in significant morbidity in humans and mortality in equines. Currently, there is no safe and effective vaccine or a therapeutic drug against the two important New and Old World alphaviruses, Venezuelan equine encephalitis virus (VEEV) and Chikungunya virus (CHIKV) respectively. The proposed study addresses the research gap that exists to develop effective anti-alphavirus therapeutic drugs. In our earlier studies, we had tested small interfering RNAs (siRNA) for their efficacy against VEEV replication. Two lead siRNA molecules were selected based on the experiments in the cell culture. One hurdle of using the siRNA duplexes in the animals is that these molecules elicit innate immune response of the host after administration. A chemical modification of the siRNA, however, attenuates this property of siRNA. Another problem of using the siRNA in animals is a very short life of the siRNA in the blood circulation. This is addressed by encapsulation of complexing of siRNA molecules to lipid based vesicles that protect the siRNA from the harsh degrading environment of the blood. In this study, we will evaluate the immunostimulatory activity of the non-modified and 2`O methyl modified siRNA duplexes in mice to establish the loss of immunomodulatory activity of modified siRNAs. SiRNA duplexes will be injected either encapsulated/complexed with PEGylated DOTAP vesicles or without any encapsulation in the tail vein of mice. Serum will be isolated from the blood to test for the innate immune response markers. This knowledge will provide information regarding potential use of the modified siRNAs as anti-alphavirus therapy agent. In the second objective, we will evaluate the miRNA changes in the blood of the alphavirus infected mice at the non-symptomatic stages of infection. The miRNA expression will then be analyzed for their use as non-symptomatic and/or prognostic marker of alphavirus exposure. The proposed study, therefore, will address two main research gaps i.e. developing an effective anti-alphavirus therapeutic drugs and diagnostic markers for alphavirus exposure and prognosis.

II. BACKGROUND:

II.1. Background:

Venezuelan Equine Encephalitis Virus (VEEV): VEEV is a New World alphavirus classified under family Togaviridae [Schlesinger 1998]. Genome of 11.7kb single strand message RNA is packed within an icosahedral protein capsid composed of repeated copies of protein C [Strauss et al 1969]. 5' capped two third genome encodes for non-structural polyproteins nsP1-4 (200-300kDa), including the precursor of RNA polymerase required for message amplification. The terminal one third of the genome encodes for structural proteins that are translated from a subgenomic mRNA copy (26S RNA) of full lengths minus strand intermediate. The genomic replication strategy involves a minus strand replicative intermediate and is similar to the strategies used by Picornaviruses, Flaviviruses and Calciviruses [Schlesinger 1998, Flint et al 1969]. Viral RNA dependent RNA polymerase (rdrp) is encoded by the nsp-4 gene of the virus and is central to viral replication in the cells. In this study VEEV rdrp will be targeted using the anti-sense RNA technology.

TC83, a live attenuated strain of VEEV will be used in the proposed experiments. TC83 is highly attenuated and have been **excluded from select agent regulations**. Because of the low level of pathogenicity, this strain can be **safely handled under BSL-2 conditions without vaccination or additional personal protective equipment** (Page 243; Appendix B: BMBL5-section VIIIf).

Chikungunya virus (CHIKV): CHIKV is an Old World alphavirus in the family Togaviridae. Genome organization of CHIKV is same as that of the VEEV with 4 non-structural genes nsp1-4 and 5 structural proteins capsid, 6K, and E1-3. Similar to VEEV Viral RNA dependent RNA polymerase (rdrp) is encoded by the nsp-4 gene of the virus and is central to viral replication and in this study CHIKV rdrp will be targeted using the anti-sense RNA technology.

CHIKV181/25, which is a live attenuated strain of CHIKV, will be used in this study. The United States army had developed and tested CHIKV181/25 for vaccine application. CHIKV181/25 demonstrated an excellent immunogenic profile in Phase II clinical trials (98% seroconversion (Edelman et al 2000). CHIKV is **not a** CDC select agent. **CHIKV181/25 strain can be safely handled under BSL-2 conditions** (see attached document, Doc-2: BMBL5-section VIIIf; Page 235 and Doc-3).

Antiviral potential of RNA Interference Technology: RNA interference (RNAi) or post transcriptional gene silencing (PTGS) was first discovered in plants and later successfully tested in *Drosophila*, *Caenorhabditis elegans*, mammalian cells and against viruses [Bann and Puri 2004]. PTGS is a phenomenon where introduction of double stranded RNA (dsRNA) in the cell system results in a target specific degradation of mRNA resulting in gene silencing. Synthetic short 21-nt RNA duplexes (siRNA) are introduced into the cell. Once inside the cell, siRNAs associate with the RNA –induced silencing complex (RISC), which catalyzes the degradation of complementary cellular mRNA in a highly specific manner. As a result gene expression is reduced both at mRNA and protein level- the hallmark of RNAi. This technique has tremendous potential to be used as a therapeutic strategy in treating the disease involving altered gene expression [Forte et al 2005, Stein et al 1999, Zentilin et al 2004, Xia et al 2002]. Human immunodeficiency virus (HIV), polio virus, hepatitis B virus, hepatitis C virus , West Nile virus, severe acute respiratory syndrome associated coronavirus (SARS-CoV), foot and mouth disease (FMD) virus and Influenza virus (including pandemic

subtypes H5 and H7 and adenovirus infections) have been inhibited both *in vitro* and *in vivo* using virus specific siRNAs [Jacque et al 2002, Ge 2003, Hui et al 2004, Tompskins et al 2004, Wang et al 2004, Giladi et al 2003, Klein et al 2003, Chen et al 2004, Mohanpatra et al 2005, Kahana et al 2004, Sanchez-Vargas et al 2004, Kaliq et al 2010, Eckstein et al 2010). We and others have also shown that siRNA and miRNA targeting the viral nsp4 gene can inhibit VEEV replication *in vitro* [Bhomia et al 2013, O'Brien 2007]. These results suggest that RNA interference may provide a viable therapeutic approach to treat viral infection. Viral RNA polymerase is our target of interest as it is critical for the amplification of VEE viral genome.

Modified SiRNA and Immune Response: Native siRNA duplex when administered in the host is treated as foreign moiety and host demonstrate an innate immune response to the siRNA duplex. 2'O'Me modification of the U and G residues in the sense strand of the siRNA duplex does not interfere with the siRNA efficacy, however, inhibit the innate immune response against the siRNA even when encapsulated with in the SNALPs [Judge et al 2006, Ursic-Bedoya et al 2014].

Encapsulation of siRNA: Naked siRNA show rapid degradation in the blood with in 4hr after injection. However, lipid encapsulated siRNA are protected for more than 24hr at 37^0C. is small nucleic acid lipid particle (SNALP)/lipid-nano particle (LNP) given via systemic administration has been shown to accumulate in the liver and have deliver siRNA to the liver to treat hepatic carcinoma, and in one study a modified SNALP was used to treat dermal carcinoma. Systemically administered SNALP encapsulated siRNA has also been shown to inhibit EBOLA and Marbug infection. Liver is the primary site of virus replication or both EBOLA and Marbug virus [Geisbert et al 2006, Ursic-Bedoya et al 2014].

DOTAP (cationic lipid) liposomes have been used to deliver the siRNA/peptide to dendritic cells after sub-cutaneous injection. These DCs then migrate to the draining lymph nodes (DLN). Particle size of DOTAP based liposomes have been described to be in the range of 100-267 nm. Other studies (non-DOTAP based) have shown that desirable size for lymph node specific delivery of drug range from 20-80nm. However, DOTAP based liposomes have been repeatedly shown to sequester in the DLNs. Further, DOTAP based cationic liposomes have been found to be less toxic than the lipofectamine/SiPORT NeoFX/SiPORT amine based transfection reagents. Incorporation of DOTAP liposomes with DSPE-PEG2000; enhances liposome uptake by resident antigen-presenting cells, accelerates the drainage of DOTAP liposomes into draining LNs, increases deeper distribution of liposomes in lymph nodes and spleen, and prolong their LN retention. DOTAP encapsulated siRNA (non-modified) induce type I and II interferon. DOTAP by itself also act as adjuvant and facilitate CD8+ T cell induction. Cholesterol:DOTAP based cationic lipid liposomes have been used to inhibit HBV and HCV infections *in-vivo*.

MicroRNA and Biomarker: Biomarker is "a measurable substance in an organism whose presence is indicative of some phenomenon such as disease, infection, or environmental exposure". MiRNAs have emerged as novel diagnostic biomarkers for various diseases. MiRNA changes in the serum have been suggested as a potential

marker of disease and injury [Wahid et al 2010]. Earler we have also described the microRNA changes in the brains of mice infected with VEEV [Bhomia et al 2010].

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: Medline, Federal Research in Progress, and Defense Technical Information Center and BRD

II.2.2. Date of Search: ~~March~~ April 28~~20~~, 2014.

II.2.3. Period of Search: 1950-2014

II.2.4. Key Words and Search Strategy: Venezuelan equine encephalitis, Venezuelan equine encephalomyelitis, VEEV, CHIKV, Chikungunya virus, siRNA, ~~antiviral treatment~~, innate immune response, 2`O Methyl modification, modified siRNAs, ~~therapy, therapeutic,~~ mice, EMCV, alphavirus, biomarkers, miRNA and biomarkers. One or combination of these keywords was used.

II.2.5. Results of Search:

VEEV/Venezuelan equine encephalitis/Venezuelan equine encephalitis virus + siRNA: Five results: 2 relevant to siRNAs: One published by (b)(6) and one published by another groups; both deal with the anti-VEEV siRNA in-vitro work only. No work with modified siRNA and effect on immunomodulation.

Chikungunya virus+ siRNA: 10 results: One deal with host siRNA response to chikungunya virus; 3 deals with anti-CHIKV activity of siRNAs. None discuss evaluation of modified siRNA against Chikungunya virus and or evaluation of immune modulation by native and chemically modified siRNA sequences.

2`O Methyl modification + siRNAs: 25 results: Non-modified siRNA induces innate immune response via TLR7 activation; 2`O Methyl modification in G and U residues of the siRNAs inhibit host innate immune response; 2`O Methyl modification of G and U residue in sense strand does not effect the anti-gene activity of siRNAs duplex, however, modification in anti-sense strand intereferes with the anti-gene activity of siRNAs. Ebola virus, Marbug virus, hepatitis virus and various host cell genes have been successfully targeted using the 2`O Methyl modified siRNAs.

MicroRNA and VEEV/Venezuelan equine encephalitis virus : 2 results, both from (b)(6) (b)(6) one describe anti-VEEV activity of miRNAs and other described host miRNA response to VEEV in brain. No results describing microRNA as VEEV infection biomarker.

MicroRNA + alphavirus: 10 results including above 2 from (b)(6) Alphaviruses have not been shown to encode for microRNAs. Sindbis virus, eastern equine encephalitis virus and semliki forest virus modulate host miRNA responses in various cell lines. Alphavirus based replicon particles have been utilized to express miRNAs in host cells. No study was reposrted to utilize microRNA as marker of alphavirus infection. No study has proposed usage of host microRNA response to alphaviruses as survelliance biomarker at non-symptomatic stages of infection.

EMCV/ encephalomyocarditis virus + microRNA: 4 results: 2 are relevant to EMCV and microRNA research which deals with mechanistic study regarding the miRNA mediated translation inhibition. EMCV internal ribosome entry sites (IRESes) was used to

describe sensitivity and resistant to miRNA mediated inhibition of gene translation. O study was found to describe host miRNA response to EMCV infection in serum.
MicroRNA + biomarker: 3076 results.
MicroRNA + alphavirus + biomarker: No results. There are two research publications describing the use of siRNA and miRNA for VEEV replication inhibition. One of these is from (b)(6) describing the use of anti-VEEV siRNA *in-vitro* (b)(6),(b)(4) Other paper describes the use of siRNAs to inhibit multiple strains of VEEV *in vitro* [O'Brien 2007]. No literature was found describing use of siRNA mediated interference to inhibit VEEV replication in animals. Ten publications were found to describe microRNA in response to alphaviruses. MicroRNA modulation in response to alphavirus infections have been described however, no literature was found to describe use of microRNA as biomarker of alphavirus infection.

III. **OBJECTIVE\HYPOTHESIS**: The objectives of this proposal are:

1) To evaluate the loss of immunomodulatory activity of 2'O Methyl modified anti-VEEV and CHIKV siRNA duplexes.

2) To evaluate the blood miRNA expression in the mice post alphavirus exposure.

The hypotheses of this project are:

1. *2'O Methyl modified siRNA duplexes will not elicit non-specific innate immune response in the host that is otherwise seen with the non-modified siRNA duplexes.* Non-specific immune response may itself have some protective effect against the alphaviruses and therefore, will hamper the conclusion of any effect by siRNA duplexes. The long term goal of the project is to identify the siRNA duplexes that will inhibit the VEEV replication in the host and any non-specific inhibitory effect will interfere with such interpretation.

2. *Circulatory miRNA expression profile of the host will change after alphavirus exposure. Specific changes in the miRNA expression may predict the type of alphaviral infection.* Since non-symptomatic individuals may act as carriers, a diagnostic marker may help in identifying such individuals and introduction of preventive medical measures can be employed.

IV. **MILITARY RELEVANCE**: VEEV is a CDC category B agent. VEEV has been developed as a biowarfare agent and is infectious through aerosol distribution. Both civilians and soldiers are vulnerable to VEEV exposure as VEEV is endemic in United States and can be potentially used as bio-terrorist agent. Current experimental vaccine is not licensed by FDA and has many non-responders (approximately 20%). Similarly CHIKV is an emerging pathogen that has caused very large outbreaks with significant morbidity in humans. Though CHIKV is not endemic in the continental USA now, states such as Florida with tropical climates and *Ades albopictus* as endemic mosquito population are at high risk of CHIKV spread. Currently there is no antiviral therapy for either VEEV or CHIKV infection. Testing novel strategies like RNA interference may

help in developing treatment for VEEV and CHIKV infection for both civilians and armed forces.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

Two siRNAs targeting VEE nsp4 mRNA and the scrambled siRNA that will be used are as following:

1. **Veev siRNA 1**
   Sense: 5'GCUGCGCAGCUUUCCAAAGUU3'
   Antisense: 5'CUUUGGAAAGCUGCGCAGCUU3'

2. **Veev siRNA 2**
   Sense: 5'AAAGAAAUUGCAACGUCACUU3'
   Antisense: 5'GUGACGUUGCAAUUUCUUUUU3'

Two siRNA targeting the CHIKV nsp4 region of the viral genome will be identified in the *in-vitro* experiments

3. **CHIKV siRNA 1**
   Sequence: to be determined (TBD)

4. **CHIKV siRNA 2**
   Sequence: TBD

5. **ScambledScrambled RNA sequence** 4G 7T 4C 6A (21 nt)
   5' TAGAGCGTAT TACGTATACC T 3'
   Only 76% identical to 2 oryza sativa (rice) genes AP006523 AND AP008208

V.1.1. Experiment 1: To evaluate the loss of immunomodulatory activity of 2'O Methyl modified siRNA duplexes in mice.

SiRNA Formulations:

1.) Without encapsulation: HPLC purified lyophilized siRNA re-suspended in nuclease free autoclaved PBS.

2.) With PEG-DOTAP encapsulation/complex: HPLC purified lyophilized siRNA will be complexed with PEG-DOTAP liposomes in our collaborators laboratory (Dr. (b)(6) NCI, Frederick, MD).

Doses: siRNA dose of 2.5mg/kg body weight will be administered vial the tail vein. This dose is based on the available data in the literature where 2 to 3mg/kg body weight of siRNA has been successfully used in animal studies [Zimmerman et al 2006].

Mice: Male Balb/c, 6 – 8 week-old, 12-16 grams mice will be used in the study.

Sample collection: Blood will be collected at 6hr post siRNA administration via the cardiac puncture. Animal will be deeply anesthetized using cylinderized CO_2 and will be closely observed for respiration to cease; after a period of 10 seconds with no breaths, thoracic cavity will be opened to expose the heart and blood (700-900 µl) will be collected using a 26-1/2 gauge needle mounted on a 1ml syringe. After blood collection cervical dislocation will be performed to ensure the death of animal. Serum will be isolated and tested for an array of innate immune inflammatory cytokines (SABioscience, Qiagen, Frederick, MD).

Animal housing: Animals will be housed in LAM. For this experiment BSL1 housing will be needed for the animals.

Groups	No. of Animals	# iteration/group	Total animals	Pain Category
Control				
Naive	6	2	12	C
Saline	6	2	12	C
Empty PEG-DOTAP Liposomes	6	2	12	C
Non-encapsulated siRNAs				
Veev siRNA 1	6	2	12	C
Veev siRNA 2	6	2	12	C
Chikv siRNA 1	6	2	12	C
Chikv siRNA 2	6	2	12	C
Scrambled siRNA	6	2	12	C
PEG-DOTAP Encapsulated/Complexed siRNAs				
PEG-DOTAP Veev siRNA 1	6	2	12	C
PEG-DOTAP Veev siRNA 2	6	2	12	C
PEG-DOTAP Chikv siRNA 1	6	2	12	C
PEG-DOTAP Chikv siRNA 2	6	2	12	C
PEG-DOTAP Scrambled siRNA	6	2	12	C
Training				
Tail vein injection training using sterile saline solution	20	1	20	C

Total animas required

[{(6 animals /group x 13 groups x 2 iterations/group)} = 156] + [Training animals (20 animals x 1 iteration)=20] = 176

Animals in pain category "C"= ~~156~~176

Experiment 1: In the first experiment none of the mice (n=156) are expected to experience any disease as they will either receive saline or siRNA injections and therefore, are kept under category C.

As tail vein injections are not routinely done in PI's laboratory, a group of 20 mice has been added as training animals. Two personnel from the PI's laboratory will train for tail vein injections using 10 animals each.

V.1.2. Experiment 2: To evaluate the blood miRNA expression in mice post alphavirus exposure.

Mice: Male Balb/c and C57BL/6J, 6 – 8 week-old, 12-16 grams mice will be used in the study.

Virus Infections: TC83, CHIKV181/25 and Encephalomayocarditis virus (EMCV) will be used in this experiment. 1000 PFU of the virus will be injected sub-cutaneous at the back of animal neck. Time of injection will be considered 0 hr.

Sample collection: Blood will be collected at 12hr, 24hr, 48hr and 72hr post infection with the respective viruses. Blood will be collected via the terminal cardiac puncture, therefore, different set of mice (n=6/group) will be used at each of the above indicated time points. at which time animal will be euthanized. At each time point the aanimals will be deeply anesthetized using cylinderized CO_2 and will be closely observed for respiration to cease; after a period of 10 seconds with no breaths, thoracic cavity will be opened to expose the heart and blood (700-900 µl) will be collected using a 26-1/2 gauge needle mounted on a 1ml syringe.will be deeply anesthetized using cylinderized CO_2 and blood will be collected via cardiac puncture. After blood collection at each time point, cervical dislocation will be performed to ensure the death of animal. No repeat cardiac puncture will be performed on the same mouse.

Animal housing: Animals will be housed in LAM. All injection with the viruses will be done in (b)(6) in biosafety cabinet. Animals will return to LAM after the injection procedure and will be housed in LAM for the remainder of the experiment. After virus injections, animals will need BSL-2 housing conditions for the rest of the experiment.

Groups	No. of Animals	# of time points	# iteration/group	Total animals	Pain Category
Uninfected control groups:					
Naïve (Balb/c)	6	4	2	48	C
Saline (Balb/c)	6	4	2	48	C
Naïve (C57BL/6J)	6	4	2	48	C
Saline (C57BL/6J)	6	4	2	48	C
Non-alphavirus infection control group:					
EMCV (Balb/c)	6	4	2	48	E
Alphavirus infection test groups:					

TC83 (Balb/c)	6	4	2	48	~~D~~C
CHIKV181/25 (C57BL/6J)	6	4	2	48	~~D~~C

Total animals required

Balb/c = [6 animals /group x 4 groups x 4 time points x 2 iterations/group] = 192.

C57BL/6J = [6 animals /group x 3 groups x 4 time points x 2 iterations/group] = 144.

Animals in Pain Category C= 288

Animals in Pain Category E= 48

Experiment 2: 192 animals in experiment 2 will either be under naïve group (n=96) or receive saline injection (sub cutaneous) (n=96). Therefore, these animals have been kept under category C.

Two groups will receive live TC83 (n=48) or CHIKV181/25 (n=48) via subcutaneous injection. These two virus strains are live attenuated vaccine strains of the VEEV and CHIKV respectively, and do not cause disease in mice. Therefore, these mice have also been placed under category C.

One group under non-alphavirus control will receive EMCV infection. EMCV causes full blown disease and in our earlier experience cause 100% mortality in mice in 5-6 days post infection. The experiment will be terminated at 3 day post infection and therefore, animals should not suffer with the full blown encephalitis. However, since no analgesic or anti-pyretic drugs will be used, there animals have been kept under category E.

V.2. Data Analysis: The statistical framework for designing experiment and estimating the sample size considers the magnitude of the effect in the control group, the "additional effect" (over and above the control effect) expected in the treatment group, statistical power to detect this desired difference between the treatment and control, and the alpha level. The laboratory and clinical experiments are designed with a statistical power of at least 80%. The alpha level is traditionally set at 5%. To observe a treatment effect of 60% enhancement with 80% power and 5% alpha level a sample size of 6 mice per group would be necessary and therefore 6 mice per group were chosen. To achieve maximum power, animals are randomly allocated to treatment and control groups in equal numbers. Since several variables are measured in each experiment, analyses of all variables at 5% alpha level will result in inflation of P-value. This inflated P-value may increase the probability of false positive result. Therefore, to maintain the experiment wise alpha level of 5% and to control for multiple comparisons in the experiment, we will adjust the P- values using statistical procedures of Tukey's post hoc multiple comparison. Means of quantitative variables will be compared across treatments and time points using 2-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparisons. This will minimize the likelihood of any false positive results.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: The potency of siRNA to silence/inhibit VEEV replication have been evaluated *in-vitro* and two siRNA duplex were selected out of seven siRNA sequences that were tested. Similar approach will be taken for the anti-CHIKV siRNA and two lead molecules will be selected for *in-vivo* testing. Similarly, for serum miRNA evaluation study, miRNA changes against the CHIKV 181/25 and TC-83 strains were evaluated to determine that viral infection indeed induced differential miRNA expression. However, *in-vivo* environment is more variable as compared to the *in-vitro* cultures and thus *in-vivo* miRNA expression changes will be evaluated in mice.

V.3.2. Animal Model and Species Justification:

Experiment 1:

> **Balb/c mice**: Aim of the study is to evaluate the innate immune response of the mice against the siRNA molecules. Balb/c mice are immunocompetent mice and have been used in similar studies to evaluate the immunomodulatory effect of siRNA [Robbins et al 2007].

Experiment 2:

> **Balb/c mice**: VEEV is endemic in rodents [Day et 1996, Bigler et al 1974] VEE infection in mice induces the bi-phasic disease observed in humans [Jackson A et al 1991, Grieder et al 1995]. Because mice mimic the biphasic disease observed in the humans it is a model of choice to study VEEV pathogenesis. Several murine strains have been described as models such as BALB/c, C3H/Hen and Swiss CD-1 [Paessler et al 2003, Schoneboom et al 2000, Hart et al 1997, Grieder et al 1995, Reynolds et al 1980]. Both BALB/c and CD-1 mice initiate humoral antibody response following subcutaneous vaccination with TC-83 vaccine. Balb/c has also been used to study EMCV infection and prophylaxis studies [Jeoung et al 2011]. Therefore, balb/c will be used to study miRNA expression following TC83 (VEEV) and EMCV infection.
>
> **C57BL/6J mice**: CHIKV infection in adult C57BL/6J mice has been described to mimic the self-limiting arthritis, tenosynovitis, and myositis seen in humans [Gardner et al 2010]. This model is useful in studying the pathogenesis and host responses. Therefore, C57BL/6J mice will be used to study miRNA expression following CHIKV181/25 (CHIKV) infection.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	Mouse (mus musculus)	Mouse (mus musulus)
V.3.3.2. Strain/Stock:	Balb/c	C57BL/6J

V.3.3.3. Source/Vendor:	(b)(4)	
V.3.3.4. Age:	6-8 weeks	6-8 weeks
V.3.3.5. Weight:	10-12 gm	10-12gm
V.3.3.6. Sex:	Male	Male
V.3.3.7. Special Considerations:	All animals will be free of common laboratory pathogens, such as MHV.	

V.3.4. Number of Animals Required (by Species): ~~348~~ ——— 368 144

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: *In vitro* experiment models do not replicate the comprehensive host responses to either siRNAs or alphaviruses. We are using the lowest animal in the phylogenetic scale that serves well as an animal model to study alphavirus pathogenesis. Experiments are designed to use noninfectious strain of alphaviruses, thereby reducing the morbidity associated with the alphavirus infection. Further, the study end points are designed to be in the non-symptomatic stage of infection, which will further reduce the morbidity in the animals.

V.3.5.2. Reduction: The number of animals to be used is the minimum number of animals needed to achieve scientifically sound conclusions for the experimental objectives. In order to reduce the number of animals in the study, we tested the efficacy of siRNA to inhibit VEEV replication in cell culture. Only the most effective siRNA duplexes will be used in the proposed animal studies. For biomarker studies, we have confirmed modulation of miRNAs post alpahvirus infection *in vitro*, which has shown differential modulation of miRNAs against TC83 and CHIKV181/25, thereby establishing the proof of concept before beginning the animal experiments.

V.3.5.3. Replacement: We have extensively researched for an alternative to the animal model for studying immune and miRNA modulation by siRNA and alphaviruses, respectively. Due to the nature of the study, *in vitro* experiments will not yield the desired data and animal experiments are required to achieve the successful completion of the study.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. **Column C:**	~~300~~320	144
V.4.1.1.1.2. **Column D:**	0	0
V.4.1.1.1.3. **Column E:**	48	0

Experiment 1: In the first experiment none of the mice (n=~~156~~176) are expected to experience any disease as they will either receive saline or siRNA injections and therefore, are kept under category C.

Experiment 2: 192 animals in experiment 2 will either be under naïve group (n=96) or receive saline injection (sub cutaneous) (n=96). Therefore, these animals have been kept under category C.

Two groups will receive live TC83 (n=48) or CHIKV181/25 (n=48) via subcutaneous injection. These two virus strains are live attenuated vaccine strains of the VEEV and CHIKV respectively, and do not cause disease in mice. Therefore, these mice have also been placed under category C.

One group under non-alphavirus control will receive EMCV infection. EMCV causes full blown disease and in our earlier experience cause 100% mortality in mice in 5-6 days post infection. The experiment will be terminated at 3 day post infection and therefore, animals should not suffer with the full blown encephalitis. However, since no analgesic or anti-pyretic drugs will be used, there animals have been kept under category E.

V.4.1.2. Pain Relief / Prevention: None

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: None

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Animals will be kept at the animal housing facility of LAM for 5-7 days prior to the start of experiments and will receive standard care. Since siRNA duplexes are not toxic by themselves we do not anticipate any adverse effect of siRNA injection in the tail vein or in the brain. We do anticipate a transient localized discomfort due to tail vein injection. Post inoculation, animals will be monitored for any distress. If animal is found to be distressed post siRNA injection, animal will be humanely euthanized. TC83 and CHIKV181/25 are attenuated strain and does not cause disease in mice therefore we do not anticipate any adverse reaction to these virus infections. EMCV causes disease in mice; however, study end point of 3 day post infection is before the symptoms of encephalitis develop. Animals will be humanely euthanized post EMCV injection if any of the following symptoms is observed before the study end point; excitability to sound, more than 20% body weight loss, paralysis or non-responsiveness to touch. A momentary discomfort is expected at the time of virus injections.

V.4.1.2.3. Paralytics: None

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PubMed (National Library of Medicine)

V.4.1.3.2. Date of Search: March 26 2014

V.4.1.3.3. Period of Search: 1985-2014

V.4.1.3.4. Key Words of Search: Individual and combination of following words was used; pain, analgesic, mice, alternative, procedure, justification, VEEV, EMCV, CHIKV181/25.

V.4.1.3.5. Results of Search: In regards to alternative to animals for determining loss of immune activity of modified siRNAs, peripheral blood mononuclear cells (PBMCs) have been successfully used. PBMCs demonstrate differential gene expression response to non-modified and 2' O Methyl modified siRNAs. However, to determine the complete host innate immune response, final screening for immune stimulation has been done *in vivo*.

In regards to the alternative to study host miRNA response to alphavirus exposure several models of VEEV infection such as guinea pig, mouse, hamsters and non-human primates. Of these, the mouse model mimics the human biphasic infection of VEEV and the arthralgia of CHIV infection. No alternatives to animal model were found that can comprehensively replicate the host response to alphavirus infection/exposure. Search for EMCV animal model returned around 337 published articles. These describe the use of mouse model for studying EMCV pathogenesis and also for vaccine evaluation. No alternatives to evaluation of host response to EMCV infection other than animal models were found.

Animal model alternatives: Venezuelan equine encephalitis virus + alternate + animal model: 1 result describing adaptation and evolution of VEEV in the mosquitoes and obligate hosts. No *in-vitro* alternative models to mouse and monkey models of VEEV pathogenesis were found. Venezuelan equine encephalitis + animal model + alternative: 4 results; none relevant to alternatives to animal model. Chikungunya virus + animal models + alternatives: No result. EMCV + animal model + alternatives: No results.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: Majority of the animals in the proposed study are either under category C or D. Only one group of animals that may develop disease is the EMCV group in experiment # 2. The main objective of the exp#2 is to determine miRNA expression changes in the blood of mouse exposed to alphaviruses. EMCV is included as a non-alphavirus control group to increase the sensitivity of the miRNA correlations to alphavirus exposure. EMCV cause disease in mice with a mean survival time of 5-6 days. The end point of the experiment is day 3 post infection, therefore, it is highly unlikely that these mice will experience full blown EMCV disease. However, out of an abundance of caution, these mice have been placed under category E.

No analgesics will be used in the study due to the concern that analgesics (NSAIDS such as aspirin, cyclooxygenase 2 blocker, ibuprofen, Sildenafil, acetaminophen *etc.*) results in modulation of gene expression in the host. Buprenorphine is a mu-opiod

receptor partial agonist and kappa-opiod receptor agonist (Sanchez et al 2008). Detailed molecular mechanism of Buprenorphine action is not fully understood. It has been shown to induce dose dependent effect on myelin associated glycoprotein expression and glycosylation. Buprenorphine engages both mu-opiod receptor and nociceptin/orphanin FQ receptor (NOP receptor) in the oligodendrocytes presumably via modulating activity of Src-family tyrosine kinase Fyn (Sanchez et al 2008, Eschenroeder eta l 2012). Buprenorphine also induce low level of interleukin 4 response in T lymphocytes and has also been reported to induce apoptosis in NG108-15 nerve cells (Borner et al 2013, Kugawa et al 1998). Studies on molecular mechanism of Buprenorphine action are limited. Therefore, it is also not know if buprenorphine treatment will affect the microRNA expression in mice. The main objective of the study is to evaluate host miRNA expression in blood so as to use that information as diagnostic marker of alphavirus exposure. It has been well establish that microRNA modulate the gene expression by acting upstream of the mRNA translation step. Since the analgesics modulate gene expression it will be safe to assume that microRNA expression may also be modulated by these analgesics. In fact analgesic, acetaminophen is known to modulate the miRNA expression in the serum (Wang et al 2009). Therefore, use of analgesic may interfere with the outcome of the proposed study i.e. identification of microRNA in serum post alphavirus exposure and their establishment as biomarkers, in a way that may result in false identification of miRNAs or inaccurate correlation of miRNA with alpahvirus exposure.

Pain + Venezuelan equine encephalitis virus: 4 results: All described VEEV infection in human population. Venezuelan equine encephalitis virus + analgesics: 6 results: None of the six results describe use and effect of analgesic on VEEV pathogenesis and symptoms. MicroRNA + analgesic drugs: 210 results; microRNA + NSAIDS: 105 results; microRNA and opioid receptors: 18 results. Morphine, δ-Opioid receptor activation, cocain, μ-opioid receptor, opioid tolerance and mu-Opioid receptor agonists, have been shown to modulate or modulated by microRNAs. MicroRNAs have also been shown to play role in chronic pain development by regulating nociception and were proposed as biomarker of pain for better patient stratification. Anti-inflammatory molecules such as resveratrol, EGCG and curcumin have also been reported to modulate miRNA expression.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: N/A

V.4.3.1. Pre-surgical Provisions:

V.4.3.2. Procedure:

V.4.3.3. Post-surgical Provisions:

V.4.3.4. Location:

V.4.3.5. Surgeon:

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures:

V.4.3.6.2. Scientific Justification:

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

- Experiment # 1: siRNA duplexes with or without encapsulation will be dissolved in nuclease free sterile water and will be injected one time in tail vein using 30 gauge needle in a maximum volume of 25µl.

- Experiment # 2: Virus will be suspended in sterile 1XPBS and will be inoculated subcutaneously using 26 gauage needle in maximum volume of 100 µl dorsally at the back of neck of the mouse.

V.4.4.2. Biosamples:

- Experiment # 1: Blood will be collected at 6hr post siRNA inoculation via the cardiac puncture.

- Experiment # 2: Blood will be collected at 12hr, 24hr, 48hr and 72hr post infection via cardiac puncture.

V.4.4.3. Adjuvants: None

V.4.4.4. Monoclonal Antibody (MAbs) Production: None

V.4.4.5. Animal Identification: Cage cards

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: N/A

V.4.4.8. Tissue Sharing: N/A

V.4.5. Study Endpoint:

- Experiment # 1: 6hr post inoculation of siRNA duplexes at which time animals will be euthanized and blood will be collected via cardiac puncture.

- Experiment # 2: 12hr, 24hr, 48hr and 72hr post infection at which time animals will be euthanized and blood will be collected via cardiac puncture.
  For animals infected with EMCV, maximum time animal will be held is 72hr post infection. However, if symptoms such as ruffled fur, lethargy, sensitivity to sound, shivering, and paralysis are observed in infected animals, animals will be euthanized and removed from the study.

V.4.6. Euthanasia: Animals will be euthanized at the study end points according to the 2013 AVMA Guidelines on Euthanasia. For terminal blood collection via cardiac puncture animals will be sedated using cylinderized CO_2, after the blood collection cervical dislocation will be done to ensure the death of animal. All euthanasia procedures will be performed in LAM. PI or (b)(6) and authorized LAM personnel will coordinate and will be responsible for the euthanasia. Disposal of BSL-2 carcasses will be handled in the manner described in VII Biohazard/Safety.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. Animals under experiment #1 will be housed in BSL-1 conditions. Animals under experiment # 2 will be housed in BSL-2 housing conditions.

V.5.1.1. Study Room: LAM
Building(s) (b)(6) Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes	_____	No	X
Fluid Restriction:	Yes		No	X

V.5.1.3. Exceptions: _____ _____

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be monitored for health, humane treatment, and husbandry considerations, twice daily by LAM veterinary technicians during routine weekday rounds and at least once on weekends/holidays. This is in addition to, and not in place of, the monitoring that is done by the principal investigator and their staff. In the event of a debilitating illness or adverse reaction, the decision to treat or euthanize an animal will be made by either the veterinarian and/or principal investigator. If mice are found showing increased distress such as ruffled fur, hunched posture, weight loss and lethargy, animals will be removed from the study and will be humanely euthanized as described in the section V.4.6.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays. In the case of an emergency health problem, if the responsible person (e.g. PI) is not available or if the investigator and veterinary staff cannot reach consensus on treatment, the veterinarian has the authority to treat the animal, remove it from the experiment, institute appropriate measures to relieve severe pain or distress, or perform euthanasia if necessary.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions:

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Injections, euthanasia, blood collection.	(b)(6)	PI, >30 years of experience with the mice model. PI in several IACUC rodent protocols at USUHS.	Rodent handling training class 2006, USUHS Investigator Training Course PI training 2003
Tail vein and sub cutaneous injections, injections, euthanasia, blood collection		Research associate, > 10 years of experience with the mice model studying viral disease.	Rodent handling training class 2002 and 2006, USUHS Investigator Training Course (2006).
Tail vein and sub cutaneous injection, euthanasia, blood collection		Post-Doctoral fellow, 7 years of experience handling mice.	Rodent handling training class 2008, USUHS Investigator Training Course 2007.
Injection, euthanasia, blood collection		Post-Doctoral fellow, 7 years of experience handling mice.	Rodent handling training class 2008, USUHS Investigator Training Course 2007.
Injections, euthanasia, blood collection		Graduate student, 3 years of experience handling mice	Rodent handling training class held on 08/17/2010, USUHS Investigator Training Course 08/17/2010

VII. BIOHAZARDS/SAFETY: TC83 and CHIKV181/25 strain of VEEV and CHIKV respectively, are attenuated and have been excluded from select agent regulations.

Because of the low level of pathogenicity, TC83 and CHIKV181/25 can be safely handled under BSL-2 conditions without vaccination or additional personal protective equipment (Page 243; Appendix B: BMBL5-section VIIIf). All the injections with the viruses will be done in the biosafety cabinet in the BSL2 lab (b)(6)

Transport of animals between BSL-2 rooms AND the PI laboratory or LAM Euthanasia room will be carried out as follows. The research staff will decontaminate cages and carts with an appropriate disinfectant (e.g. MB-10) before moving animals though command access areas. Cages will be double-bagged and taped closed in the clear biohazard waste bags kept in the BSL-2 room. The bags will be sprayed with an appropriate disinfectant. Carts will be sprayed down as well, especially the wheels. Allow disinfectant contact time of 3-5 minutes.

Procedures after euthanasia of BSL-2 animals will be carried out as follows. Animal carcasses will be doubled bagged and taped (include burn card) in clear autoclavable biohazard bags. Cages will be double bagged and tapped in clear autoclavable biohazard bags as well, but separate from the carcasses. The downdraft table, C02 lid, instruments and any other surfaces that became contaminated will be disinfected by the person performing the euthanasia (e.g. research staff) and allowed contact time of 3-5 minutes. The double bagged cages and carcasses will be dropped off in autoclave room (b)(6) The bagged carcasses will be placed in the white biohazard container. The bagged cages can be left on the floor. LAM will autoclave the carcasses/cages and dispose of them.

VIII. ENCLOSURES: Appendix A: References
Appendix B: BMBL5-section VIIIf

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 3/28/14

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 3/28/14

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature

3/28/14

Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: Post-transcriptional silencing of Venezuelan Equine Encephalitis Virus (VEEV) RNA for developing an antiviral therapy against exposure with VEEV, a bio-warfare agent (in mice).

C. Principal Investigator: (b)(6)

D. Performing Organization: USUHS

E. Funding: Defense Threat Reduction Agency (DTRA)

F. Objective and Approach: The objectives of this proposal are:

1) To evaluate the loss of immunomodulatory activity of 2'O Methyl modified anti-VEEV siRNA duplexes in mice.
2) To determine the miRNA expression in the blood of mice exposed to alphaviruses.

Earlier report have shown that 2'O Methyl modified siRNA duplexes do not elicit a non-specific innate immune response in the host that is seen with the non-modified siRNA duplexes. Non-specific immune response may itself have some protective effect against the VEEV and therefore, will hamper the conclusion of any protective effect by siRNA duplexes. The long term goal of the project is to identify the siRNA duplexes that will inhibit the VEEV replication in the host, any non-specific inhibitory effect thus will interfere with such interpretation. Host miRNAs have emerged as novel diagnostic biomarkers for various diseases. MiRNA changes in the serum have been suggested as a potential marker of disease and injury. In this study, host blood miRNA profile will be determined to identify potential diagnostic and prognostic biomarkers.

G. Indexing Terms (Descriptors): mice, RNA interference, siRNA, VEEV, CHIKV, alphaviruses, biomarkers, miRNAs, diagnostic markers.

Appendix A: References

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Appendix B: Please see attached document regarding safety of TC83 and CHIKV181/25.

Enclosure(s)
(1)

Section VIII-F: Arboviruses and Related Zoonotic Viruses

In 1979, the American Committee on Arthropod-Borne Viruses (ACAV) Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations for each of the 424 viruses then registered in the International Catalogue of Arboviruses, including Certain Other Viruses of Vertebrates.[1] Working together, SALS, the CDC and the NIH have periodically updated the catalogue by providing recommended biosafety practices and containment for arboviruses registered since 1979. These recommendations are based, in part, on risk assessments derived from information provided by a worldwide survey of laboratories working with arboviruses, new published reports on the viruses, as well as discussions with scientists working with each virus.

Table 6, located at the end of this Section, provides an alphabetical listing of 597 viruses and includes common name, virus family or genus, acronym, BSL recommendation, the basis for the rating, the antigenic group[2] (if known), HEPA filtration requirements, and regulatory requirements (i.e., import/export permits from either the CDC or the USDA). In addition, many of the organisms are classified as select agents and require special security measures to possess, use, or transport. (See Appendix F.) Table 4 provides a key for the SALS basis for assignment of viruses listed in Table 6.

Agent summary statements have been included for certain arboviruses. They were submitted by a panel of experts for more detailed consideration due to one or more of the following factors:

- at the time of writing this edition, the organism represented an emerging public health threat in the United States;
- the organism presented unique biocontainment challenge(s) that required further detail; and
- the organism presented a significant risk of laboratory-acquired infection.

These recommendations were made in August 2005; requirements for biosafety, shipping, and select agent registration can change. Please be sure to confirm the requirements with the appropriate Federal agency. If the pathogen of interest is one listed in Appendix D, contact the USDA for additional biosafety requirements. USDA guidance may supersede the information found in this Chapter.

Recommendations for the containment of infected arthropod vectors were drafted by a subcommittee of the American Committee on Medical Entomology (ACME), and circulated widely among medical entomology professionals. (See Appendix E.)

Some commonly used vaccine strains for which attenuation has been firmly established are recognized by SALS. These vaccine strains may be handled safely at BSL-2 (Table 5). The agents in Table 4 and 5 may require permits from USDA/DOC/DHHS.

Table 4. Explanation of Symbols Used in Table 6 to Define Basis for Assignment of Viruses to Biosafety Levels

Symbol	Definition
S	Results of SALS survey and information from the Catalog [1]
IE	Insufficient experience with virus in laboratory facilities with low biocontainment
A	Additional criteria
A1	Disease in sheep, cattle or horses
A2	Fatal human laboratory infection—probably aerosol
A3	Extensive laboratory experience and mild nature of aerosol laboratory infections justifies BSL-2
A4	Placed in BSL-4 based on the close antigenic relationship with a known BSL-4 agent plus insufficient experience
A5	BSL-2 arenaviruses are not known to cause serious acute disease in humans and are not acutely pathogenic for laboratory animals including primates. In view of reported high frequency of laboratory aerosol infection in workers manipulating high concentrations of Pichinde virus, it is strongly recommended that work with high concentrations of BSL-2 arenaviruses be done at BSL-3.
A6	Level assigned to prototype or wild-type virus. A lower level may be recommended for variants with well-defined reduced virulence characteristics.
A7	Placed at this biosafety level based on close antigenic or genetic relationship to other viruses in a group of 3 or more viruses, all of which are classified at this level.
A8	BSL-2 hantaviruses are not known to cause laboratory infections, overt disease in humans, or severe disease in experimental primates. Because of antigenic and biologic relationships to highly pathogenic hantaviruses and the likelihood that experimentally infected rodents may shed large amounts of virus, it is recommended that work with high concentrations or experimentally infected rodents be conducted at BSL-3.

Table 5. Vaccine Strains of BSL-3 and BSL-4 Viruses that May Be Handled as BSL-2

Virus	Vaccine Strain
Chikungunya	181/25
Junin	Candid #1
Rift Valley fever	MP-12
Venezuelan equine encephalomyelitis	TC83 & V3526
Yellow fever	17-D
Japanese encephalitis	14-14-2

Based on the recommendations listed with the tables, the following guidelines should be adhered to where applicable.

Viruses with BSL-2 Containment Recommended

The recommendation for conducting work with the viruses listed in Table 6 at BSL-2 are based on the existence of historical laboratory experience adequate to assess the risks when working with this group of viruses. This indicates a) no overt laboratory-associated infections are reported, b) infections resulted from exposures other than by infectious aerosols, or c) if disease from aerosol exposure is documented, it is uncommon.

Laboratory Safety and Containment Recommendations

Agents listed in this group may be present in blood, CSF, various tissues, and/or infected arthropods, depending on the agent and the stage of infection. The primary laboratory hazards comprise accidental parenteral inoculation, contact of the virus with broken skin or mucous membranes, and bites of infected laboratory rodents or arthropods. Properly maintained BSCs, preferable Class II, or other appropriate personal protective equipment or physical containment devices are used whenever procedures with a potential for creating infectious aerosols or splashes are conducted.

BSL-2 practices, containment equipment, and facilities are recommended for activities with potentially infectious clinical materials and arthropods and for manipulations of infected tissue cultures, embryonate hen's eggs, and rodents.

Large quantities and/or high concentrations of any virus have the potential to overwhelm both innate immune mechanisms and vaccine-induced immunity. When a BSL-2 virus is being produced in large quantities or in high concentrations, additional risk assessment is required. This might indicate BSL-3 practices, including additional respiratory protection, based on the risk assessment of the proposed experiment.

Viruses with BSL-3 Containment Recommended

The recommendations for viruses listed in Table 6 that require BSL-3 containment are based on multiple criteria. SALS considered the laboratory experience for some viruses to be inadequate to assess risk, regardless of the available information regarding disease severity. In some cases, SALS recorded overt LAI transmitted by the aerosol route in the absence or non-use of protective vaccines, and considered that the natural disease in humans is potentially severe, life threatening, or causes residual damage.[1] Arboviruses also were classified as requiring BSL-3 containment if they caused diseases in domestic animals in countries outside of the United States.

Laboratory Safety and Containment Recommendations

The agents listed in this group may be present in blood, CSF, urine, and exudates, depending on the specific agent and stage of disease. The primary laboratory hazards are exposure to aerosols of infectious solutions and animal bedding, accidental parenteral inoculation, and contact with broken skin. Some of these agents (e.g., VEE virus) may be relatively stable in dried blood or exudates.

BSL-3 practices, containment equipment, and facilities are recommended for activities using potentially infectious clinical materials and infected tissue cultures, animals, or arthropods.

A licensed attenuated live virus is available for immunization against yellow fever. It is recommended for all personnel who work with this agent or with infected animals, and those entering rooms where the agents or infected animals are present.

Junin virus has been reclassified to BSL-3, provided that all at-risk personnel are immunized and the laboratory is equipped with HEPA-filtered exhaust. SALS also has reclassified Central European tick-borne encephalitis (CETBE) viruses to BSL-3, provided all at-risk personnel are immunized. CETBE is not a registered name in *The International Catalogue of Arboviruses* (1985). Until the registration issue is resolved taxonomically, CETBE refers to the following group of very closely related, if not essentially identical, tick-borne flaviviruses isolated from Czechoslovakia, Finland and Russia: Absettarov, Hanzalova, Hypr, and Kumlinge viruses. While there is a vaccine available that confers immunity to the CETBE group of genetically (>98%) homogeneous viruses, the efficacy of this vaccine against Russian spring-summer encephalitis (RSSE) virus infections has not been established. Thus, the CETBE group of viruses has been reclassified as BSL-3 when personnel are immunized with CETBE vaccine, while RSSE remains classified as BSL-4. It should be noted that CETBE viruses are currently listed as select agents and require special security and permitting considerations. (See Appendix F.)

Investigational vaccines for eastern equine encephalomyelitis (EEE) virus, Venezuelan equine encephalitis (VEE), western equine encephalomyelitis (WEE) virus, and Rift Valley fever viruses (RVFV), may be available in limited quantities and administered on-site at the Special Immunization Program of USAMRIID, located at Ft. Detrick, Frederick, MD. Details are available at the end of this section.

The use of investigational vaccines for laboratory personnel should be considered if the vaccine is available. Initial studies have shown the vaccine to be effective in producing an appropriate immunologic response, and the adverse effects of vaccination are within acceptable parameters. The decision to recommend vaccines for laboratory personnel must be carefully considered and based on an risk assessment which includes a review of the characteristics of the agent and the disease, benefits versus the risk of vaccination, the experience of the laboratory personnel, laboratory procedures to be used with the agent, and the contraindications for vaccination including the health status of the employee.

If the investigational vaccine is contraindicated, does not provide acceptable reliability for producing an immune response, or laboratory personnel refuse vaccination, the use of appropriate personal protective equipment may provide an alternative. Respiratory protection, such as use of a PAPR, should be considered in areas using organisms with a well-established risk of aerosol infections in the laboratory, such as VEE viruses.

Any respiratory protection equipment must be provided in accordance with the institution's respiratory protection program. Other degrees of respiratory protection may be warranted based on an assessment of risk as defined in Chapter 2 of this manual. All personnel in a laboratory with the infectious agent must use comparable personal protective equipment that meets or exceeds the requirements, even if they are not working with the organism. Sharps precautions as described under BSL-2 and BSL-3 requirements must be continually and strictly reinforced, regardless of whether investigational vaccines are used.

Non-licensed vaccines are available in limited quantities and administered on-site at the Special Immunization Program of USAMRIID. IND vaccines are administered under a cooperative agreement between the U.S. Army and the individual's requesting organization. Contact the Special Immunization Program by telephone at (301) 619-4653.

Enhanced BSL-3 Containment

Situations may arise for which enhancements to BSL-3 practices and equipment are required; for example, when a BSL-3 laboratory performs diagnostic testing on specimens from patients with hemorrhagic fevers thought to be due to dengue or yellow fever viruses. When the origin of these specimens is Africa, the Middle East, or South America, such specimens might contain etiologic agents, such as arenaviruses, filoviruses, or other viruses that are usually manipulated in a BSL-4

(2)

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PHASE II SAFETY AND IMMUNOGENICITY STUDY OF LIVE CHIKUNGUNYA VIRUS VACCINE TSI-GSD-218

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Abstract. We conducted a phase II, randomized, double-blind, placebo-controlled, safety and immunogenicity study of a serially passaged, plaque-purified live chikungunya (CHIK) vaccine in 73 healthy adult volunteers. Fifty-nine volunteers were immunized one time subcutaneously with the CHIK vaccine and 14 were immunized with placebo (tissue culture fluid). Vaccinees were clinically evaluated intensively for one month, and had repeated blood draws for serological assays (50% plaque-reduction neutralization test) for one year. Except for transient arthralgia in five CHIK vaccinees, the number and severity of local and systemic reactions and abnormal laboratory tests after immunization were similar in CHIK vaccinees and placebo recipients. Fifty-seven (98%) of 58 evaluable CHIK vaccinees developed CHIK neutralizing antibody by day 28, and 85% of vaccinees remained seropositive at one year after immunization. No placebo recipients seroconverted. This promising live vaccine was safe, produced well-tolerated side effects, and was highly immunogenic.

INTRODUCTION

Chikungunya virus (CHIK) is an alphavirus borne by *Aedes* mosquitoes that produces a dengue-like illness in humans, characterized by fever, rash, painful arthralgia, and sometimes arthritis.[1–5] The virus is widely disseminated throughout sub-Saharan Africa, Southeast Asia, India, and the Western Pacific, and numerous epidemics have been reported in these areas.[6–11] The widespread geographic distribution, recurrent epidemics, and infection of military personnel, travelers, and laboratory staff working with CHIK have indicated the need for a safe and efficacious vaccine.[12–15] Individual strains of CHIK are closely related antigenically,[8,16,17] and infection with one CHIK strain leads to protection against all strains.[18] Reciprocal cross-protection after infection with other alphaviruses occurs in animal models,[19,20] although it is unclear if similar cross protection occurs in humans sequentially exposed to natural infection or live alphavirus vaccines.[21]

An isolate from a patient in Thailand, CHIK strain 15561, was used to develop a small lot of vaccine first passaged in green monkey kidney (GMK) cells and then formalin-inactivated before administration to 16 volunteers.[22] The vaccine produced no untoward reactions and was highly immunogenic. The current live vaccine (Lot 1-85, TSI-GSD-218) was developed at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) and was produced at the Salk Institute, Swiftwater, PA, from a lot of the GMK-passaged strain 15561 inactivated vaccine by subsequent serial passage in MRC-5 cells.[23] The live vaccine proved to be safe and immunogenic in a phase I trial in 15 alphavirus-naive volunteers.[21] The current phase II, randomized, double-blind, placebo-controlled trial was designed to provide additional safety and immunogenicity data for live CHIK vaccine TSI-GSD-218.

MATERIALS AND METHODS

Vaccine. The live, attenuated, TSI-GSD-218, Lot 1-85, chikungunya vaccine is a lyophilized supernatant from human lung cell cultures (Medical Research Council-5 [MRC-5] cells, certified for vaccine use) infected with an attenuated strain, CHIK 181/Clone 25. This attenuated vaccine seed was originally derived from a serum isolate (CHIK strain 15561) obtained from an infected patient during the 1962 outbreak of CHIK disease in Thailand. Chikungunya strain 15561 was subjected to 18 plaque-to-plaque passages in MRC-5 cultures before CHIK 181/Clone 25 was selected as the vaccine seed.[22,23] The CHIK vaccine elicited neutralizing antibody and protected mice and monkeys against challenge.[23] The vaccine was shown to be safe and immunogenic in humans in phase I trials[21] (Malinowski FJ, unpublished data). The placebo was MRC-5 tissue culture fluid manufactured concurrently with the CHIK vaccine, and diluted identically as the CHIK vaccine. The vaccine and placebo fluids contained less than 0.02 μg neomycin base and 0.25% human serum albumin per ml.

Volunteers. Seventy-three adult volunteers (47 men, 26 women) were recruited by poster, published advertisement, and by word-of-mouth at the University of Maryland, Baltimore, MD and at the University of Maryland, College Park, MD. Healthy volunteers, 18–40 years of age, were screened and included if they had no clinically significant history of medical or psychiatric disease (including Lyme disease and personal and family history of arthritis). Additional screening consisted of a standard but brief physical examination and the usual serum chemistries, urinalysis, and complete blood count. Negative results of serum HIV, hepatitis virus B and C, and alpha virus serology ($PRNT_{50}$ <1:10 for CHIK, Venezuelan equine encephalitis virus [VEE], western equine encephalitis virus [WEE], and eastern equine encephalitis virus [EEE]) were required. In addition, we documented that female volunteers were not pregnant within 48 hours of vaccine administration.

The study was approved by the Institutional Review Boards of the University of Maryland at Baltimore and the University of Maryland at College Park, and by the Human Subjects Research and Review Board, Office of the Surgeon General, United States Army. Informed consent was obtained from all volunteers.

Study design. Volunteers were randomized in blocks of 5 (4 vaccine and 1 placebo recipient) using a computer random-number generator. For logistic reasons, volunteers were

TABLE 1
Number of volunteers developing clinical reactions during the first 28 days after immunization with the chikungunya (CHIK) vaccine

Reaction	CHIK vaccinees* n = 59 No. showing reaction (%)	Placebo recipients n = 14 No. reaction (%)
Local symptoms and signs†	12 (20)	3 (21)
Any systemic symptoms and signs‡	34 (58)	9 (64)
Systemic symptoms possibly associated with immunization§	19 (32)	4 (29)
Arthralgia in isolation	5 (8)	0
Flu-like symptom(s)	13 (22)	4 (29)
Urticaria	1 (2)	0

* All differences between CHIK and placebo groups were NS ($P > 0.58$, Fisher's exact test). Clinical reactions were graded and associated with immunization in a blinded fashion before vaccine code was broken.
† Pain, tenderness, erythema, induration, and restricted arm motion.
‡ Malaise, fever, chills, headache, photophobia, myalgia, arthralgia, anorexia, nausea, vomiting, pruritis, and rash.
§ Based on temporal association with immunization 28 days before onset of symptom, and by the absence of another identifiable condition that may have accounted for symptom or symptoms.

divided into five cohorts. In total, 59 volunteers (35 men, 24 women) received the CHIK vaccine, and 14 volunteers (12 men, 2 women) received the placebo preparation. Sixty (82%) of the 73 volunteers were Caucasian, and 13 were either African-American (5 persons), Asian (5 persons), or Hispanic (3 persons).

The vaccine or placebo was administered as a 0.5 ml dose of either the reconstituted vaccine ($\sim 10^5$ plaque-forming units [pfu]/dose) or placebo by injection subcutaneously over the deltoid muscle. Neither the volunteer nor the investigators knew whether virus or placebo fluid was administered until after the completion of the study. After inoculation, all volunteers remained in the vicinity of the clinic for 30 minutes for observation of immediate reactions. Volunteers subsequently returned for clinical checks on study Days 1–4, 10, 14, and 28. Parameters examined and recorded at these times included local pain, tenderness, warmth, erythema, and induration at the inoculation site, and limited range of arm movement. Systemic signs and symptoms noted included temperature, subjective fever or chills, malaise, arthralgia, arthritis, myalgia, rash, pruritus, sore throat, photophobia, headache, anorexia, nausea, and vomiting. Reactions were quantitatively scored on the basis of severity of symptoms: (0 = none, 1 = mild, symptom can be ignored; 2 = moderate, symptom affects activity; and 3 = severe, symptom requires analgesics). Volunteers were given a disposable thermometer and instructed to report to the research nurse or physician whenever they suspected a reaction to the vaccine. A physician investigator was available on a 24-hour basis throughout the study to screen any subject who reported suspected vaccine side effects.

Blood for the safety screen included complete blood counts with differential, platelet count, chemistry panel (electrolytes, glucose, blood urea nitrogen, creatinine, glucose, calcium, phosphorus, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, and alkaline phosphatase) and urinalysis obtained on Days 0, 14, and 28 after inoculation. Clinical laboratory tests were performed by licensed, commercial pathology laboratories approved by the American College of Pathology. Blood for CHIK serology was obtained on Days 0, 14, 28, 42, 180, and 365 after vaccination.

Serologic procedures. Sera were assayed for 50% plaque-reduction neutralization test ($PRNT_{50}$) antibody,[26] modified for use with the vaccine strain of CHIK.[20] The assays were performed by the Diagnostic Systems Division at USAMRIID. A positive antibody titer was considered to be ≥1:20 by the $PRNT_{50}$ assay. The end-point titer was expressed as a reciprocal of the highest initial dilution that demonstrated ≥50% reduction in the number of plaques for the average viral dose. Less than 50% neutralization by the initial serum test dilution of 1:10 was reported as 5.

Statistical methods. Each volunteer's clinical reactions and serological results were entered into the computer before the study code was broken. Both the clinical and the clinical laboratory responses to vaccine versus placebo were compared using Fisher's exact tests, evaluated at two-tailed ($P = 0.05$).

RESULTS

Volunteer retention. One volunteer whose serum interfered non-specifically with the antibody assay was not included in the serological analysis. Thus, 72 volunteers (58 of 59 CHIK vaccinees and all 14 placebo vaccinees) were evaluated serologically and all 73 volunteers were evaluated for side effects. Only three of 365 scheduled blood specimens were obtained off schedule, each from a different volunteer. One Day 180 serum was donated on Day 270, one Day 365 serum was donated on Day 385, and one Day 365 serum was not obtained.

Local reactions. The number of volunteers who experienced one or more local symptoms or signs was 12 (20%) of 59 CHIK vaccinees, and 3 (21%) of the 14 placebo controls (Table 1). The few reactions noted at the immunization site were invariably mild, except one CHIK volunteer who developed moderate intermittent pain in her inoculated arm between 12 and 48 hours after immunization that was associated with a mildly pruritic eczema-like rash at the vaccination site.

Systemic reactions. The CHIK vaccine was well tolerated. Except for transient arthralgia, systemic symptoms were no more frequent or severe in CHIK vaccinees than in placebo recipients (Table 1). The only severe systemic symptoms reported after CHIK vaccination occurred in two individuals who treated their headaches with analgesics for one day. Nineteen (32%) of CHIK vaccinees had symptoms judged to be possibly associated with immunization, compared to 4 (29%) of placebo recipients. Of these immunization-associated reactions, no CHIK vaccinee developed a

TABLE 2
Arthralgia in volunteers immunized with the chikungunya vaccine

Volunteer no.	Description of arthralgia
11	Mild intermittent pain in the left elbow beginning Day 4 (lasted 24 hours)
19	Moderate pain in the left wrist on Day 1 (lasted 10 minutes)
22	Mild arthralgia in the left hand on Day 13 (lasted 3 hours)
32	2–3 bouts of episodic pain lasting 15 minutes in left elbow and wrist on Days 1, 2, and 4 that limited typing
43	Mild left knee and thigh pain on Day 1 (lasted 6 hours)

temperature elevation > 100.0°F, and afebrile, flu-like symptoms occurred with similar frequency in vaccinated and placebo recipients (Table 1). Five (8%) of CHIK vaccinees (3 men, 2 women) had transient arthralgia without arthritic signs or associated flu-like symptoms (Table 2). The most severely affected volunteer (No. 32, Table 2) suffered pain in her left elbow and wrist which required her to stop typing episodically over three days. No placebo recipient reported arthralgia at any time, and no CHIK vaccinee reported joint symptoms between Day 14 and Day 360, the period of passive surveillance.

Clinical laboratory results. No clinically significant abnormal laboratory tests were noted in any volunteer. No significant difference existed in the percentage of CHIK and placebo-group volunteers who had abnormal laboratory tests at baseline or at post-vaccination (data not shown).

Serological results. Fifty-seven (98.3%) of 58 Chik volunteers seroconverted (≥1:20 by $PRNT_{80}$) by Day 28, and although antibody levels declined somewhat over time, 85% of CHIK vaccinees were still seropositive at one year (Figure 1A). The highest geometric mean titer (GMT) (1:582) was reached at Day 28 and declined slowly to 1:105 on Day 360 (Figure 1B). Maximum titers reached 1:10,240 on Days 28 and 42, and declined to 1:1,280 on Days 180 and 360. No placebo recipient seroconverted.

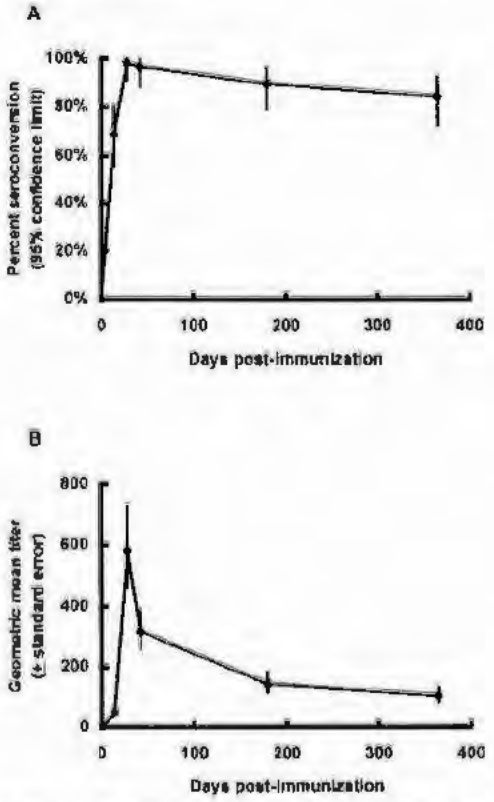

FIGURE 1. Chikungunya virus (CHIK) 80% plaque reduction neutralization test ($PRNT_{80}$) antibody titers in the sera of 58 alphavirus-naive volunteers after immunization with attenuated CHIK vaccine. Serum was obtained on Days 0, 14, 28, 42, 180, and 360. (A) Percent seroconversion (≥1:20 $PRNT_{80}$), 95% confidence intervals. (B) Geometric mean titer ± standard error. No placebo recipients seroconverted (data not shown).

DISCUSSION

This phase II, double-blind study was designed to examine the safety and immunogenicity of the live CHIK vaccine TSI-GSD-218 in healthy adult volunteers. Side effects experienced by vaccinee and placebo volunteers who were followed actively and passively for one year were compared. The neutralizing antibody response was also studied for one year. This study confirms the encouraging clinical reaction profile and antibody response noted in a phase I study of 15 alphavirus-naive volunteers immunized with this vaccine,[7] and in several phase I trials conducted at USAMRIID (Malinoski FJ, unpublished data). In the current blinded phase II study, the number and severity of adverse clinical signs and symptoms were similar in the vaccine-immunized and placebo-inoculated volunteers. No CHIK volunteer developed clinically important reactions to the vaccine. Clinical laboratory abnormalities were also comparable in both volunteer groups in the current study, although minor differences in hematologic values were noted in the previous phase I trial.[7]

Because virulent chikungunya virus can cause acute arthralgia and arthritis,[1,3,21,26] which on occasion may persist for months or years, we were particularly interested in enumerating joint symptoms. Five of 59 CHIK vaccinees developed transient arthralgia without other known cause (Table 2), while none of 14 controls developed arthralgia ($P = 0.33$). The incidence of arthralgia in vaccine recipients compared to controls may have reached statistical significance had we conducted a larger study. Three of 5 volunteers noted arthralgia one day after immunization, which is earlier than predicted based on the fact that CHIK vaccine viremia occurred in previous trials on Day 2–4 as reported by McClain and others[21] and noted by FJ Malinoski, (unpublished data). Nevertheless, it is plausible medically that some of the arthralgia episodes were caused by the attenuated CHIK virus. No volunteer reported symptoms of arthralgia or arthritis during passive surveillance conducted between 1 and 12 months after immunization. One Army volunteer complained of mild, intermittent small-joint pains without evidence of arthritis or abnormal laboratory studies after long-term follow-up (Malinoski FJ, unpublished data). The sum of the safety data in the current phase II trial and in the phase I

Army trials summarized above strongly suggest that the live CHIK vaccine is safe and well-tolerated and that it produces no more severe or more frequent symptoms than found in placebo recipients.

Mild hemorrhage limited to petechia, epistaxis, and bleeding gums, or severe hemorrhage characterized by melena, hematemesis, hemoptysis, or hematuria, have occurred in up to 10% of patients during outbreaks of CHIK infection, depending of the locale.[1,11,27,28] Some of the more severe cases of hemorrhage may have been caused by dengue virus infection.[27] Rarely, death in children and the aged has been associated with outbreaks of CHIK;[28] CHIK was isolated from the blood of one Ceylonese child who died.[29] Nevertheless, because of the rarity of CHIK isolations and because the live CHIK vaccine is highly attenuated for man, hemorrhagic disease and death are not considered to be vaccine risks.

Although chikungunya viral disease is not known to adversely affect the pregnant woman or fetus and no reports of teratogenicity or increased abortion have been associated with massive epidemics of CHIK, there is a theoretical potential that any replicating vaccine virus will adversely affect the developing fetus. This vaccine has not been tested for teratogenicity or abortogenicity in animal models.

The theoretical risk of transmission of the attenuated CHIK vaccine virus to mosquitoes is considered to be remote. Because of the transient low level of viremia produced in inoculated volunteers, it is unlikely that mosquitoes would become infected by feeding on a person inoculated with the CHIK vaccine.[30] Moreover, although the vaccine was successfully transmitted by mosquitoes after intrathoracic inoculation, there was no evidence of reversion to a virulent phenotype.[30] Studies to detect possible revertant isolates from vaccine recipients indicated that most isolates retain the biological characteristics of the vaccine virus. One isolate from a volunteer in an Army study had suckling mouse lethality intermediate between the vaccine (0%) and parent (100%) viruses and induced viremia in monkeys that was higher than that produced by the parent vaccine virus. However, the viremia in the volunteer was probably too low to infect a feeding mosquito (Malinowski FJ, unpublished data).

The CHIK vaccine was highly immunogenic. Forty vaccinees (69%) seroconverted by Day 14, and 57 vaccinees (98%) seroconverted by Day 28 (Figure 1A). Neutralizing antibody was still detected in 85% of volunteers after 12 months. Long-term follow-up studies would be needed to determine the durability of the immune response. The GMT of the vaccinees was high (Figure 1B). All 15 alphavirus-naive individuals seroconverted in the single published phase 1 trial.[31] By contrast, seroconversion in laboratory workers who had previously been immunized with the VEE TC-83 vaccine was only 36%,[21] suggesting that the VEE TC-83 vaccine interferes immunologically with the live CHIK vaccine. These and other investigations suggest that sequential attenuated alphavirus immunization is detrimental to the development of neutralizing antibody and may impair the development of protective responses to subsequent CHIK immunization. It is unknown if previous attenuated alphavirus vaccines can interfere with subsequent infection and illness caused by virulent CHIK.[2]

In summary, the live CHIK vaccine was safe, produced well-tolerated side effects such as transient arthralgia in 5 (8%) of 59 volunteers, and was highly immunogenic. It is, therefore, a promising vaccine for use in alphavirus naive individuals.

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June 1, 2016

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF PATHOLOGY

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on June 1, 2016:

Title of Application: "Deinococcus Radiodurans Mn2+Complexes: A Revolutionary Approach to Radioprotection and Vaccine Production (in mice)"

USUHS Protocol Number: (b)(6)

Expiration Date: May 31, 2019

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6), Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: *Deinococcus radiodurans* Mn^{2+} Complexes: A Revolutionary Approach to Radioprotection and Vaccine Production (in mice).

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Defense Threat Reduction Agency

EARLIEST ANTICIPATED FUNDING START DATE: Oct 30th 2015

PRINCIPAL INVESTIGATOR:

(b)(6)	DAT	(b)(6)	3/22/16
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) (b)(6)	Chair	(b)(6)	03/24/2016
Research Unit Chief / Dept. Head Signature Typed Name:	Title	Telephone	Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)	DAT	(b)(6)	3/22/16
Statistician Signature Typed Name: (b)(6)	Department	Telephone	Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and

manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)	LAM	(b)(6)	24 Mar 16
Attending/Consulting Veterinarian Signature	Department	Telephone	Date

Typed Name (b)(6) DVM

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: Dr. (b)(6)

ANIMAL PROTOCOL TITLE: *Deinococcus radiodurans* Mn^{2+} Complexes: A Revolutionary Approach to Radioprotection and Vaccine Production (in mice).

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6)
(b)(6)

TECHNICIANS(S):

I. NON-TECHNICAL SYNOPSIS:

Studies have found that the resistance towards radiation, an ability to grow in a high radiation environment, in the bacteria called *Deinococcus radiodurans* comes from high concentration of Manganese (Mn^{2+}) complexes found inside this bacteria. These Mn^{2+} complexes work by protecting the bacterial machinery which repair its genome damaged by the radiations, thus helping the bacteria to survive in the high radiation environment. In the proposed animal studies we will utilize this protein protecting ability of the Mn^{2+} complexes to 1) to test the safety and potential of viruses inactivated in presence of Mn^{2+} complexes for usage as vaccine candidates; and 2) determine the efficacy of topical application of Mn^{2+}-complex to improve wound healing post irradiation. Response to vaccine administration that results in the production of protective antibodies against a pathogen is a complex process. It requires several different processes inside the body to work together. Similarly, process of wound healing is also a complex processes that require a cascade of tightly controlled events to occur for normal healing. These processes cannot be truly replicated in the culture therefore, require use of animals. In the proposed experiments we will use small animal model i.e. mouse for the above mentioned studies. Mouse models for these studies are well established and have been routinely used. This multi-disciplinary research holds the prospect of formulating novel radio protectors, with applications ranging from pre-exposure prophylactic interventions to post-exposure therapeutics, and expediting vaccine production during epidemic outbreaks, bioterrorist attacks and other biothreats.

II. BACKGROUND:

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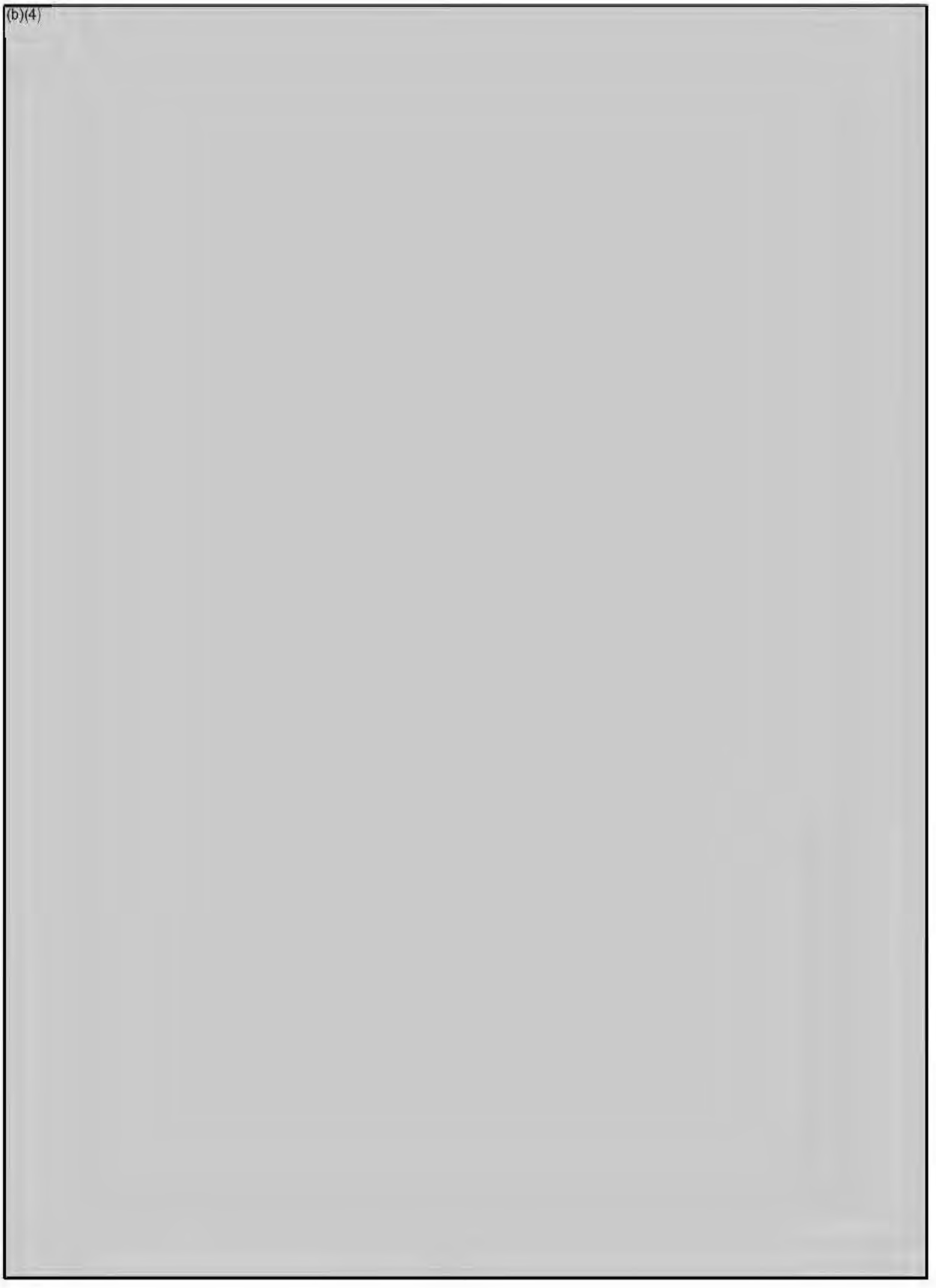

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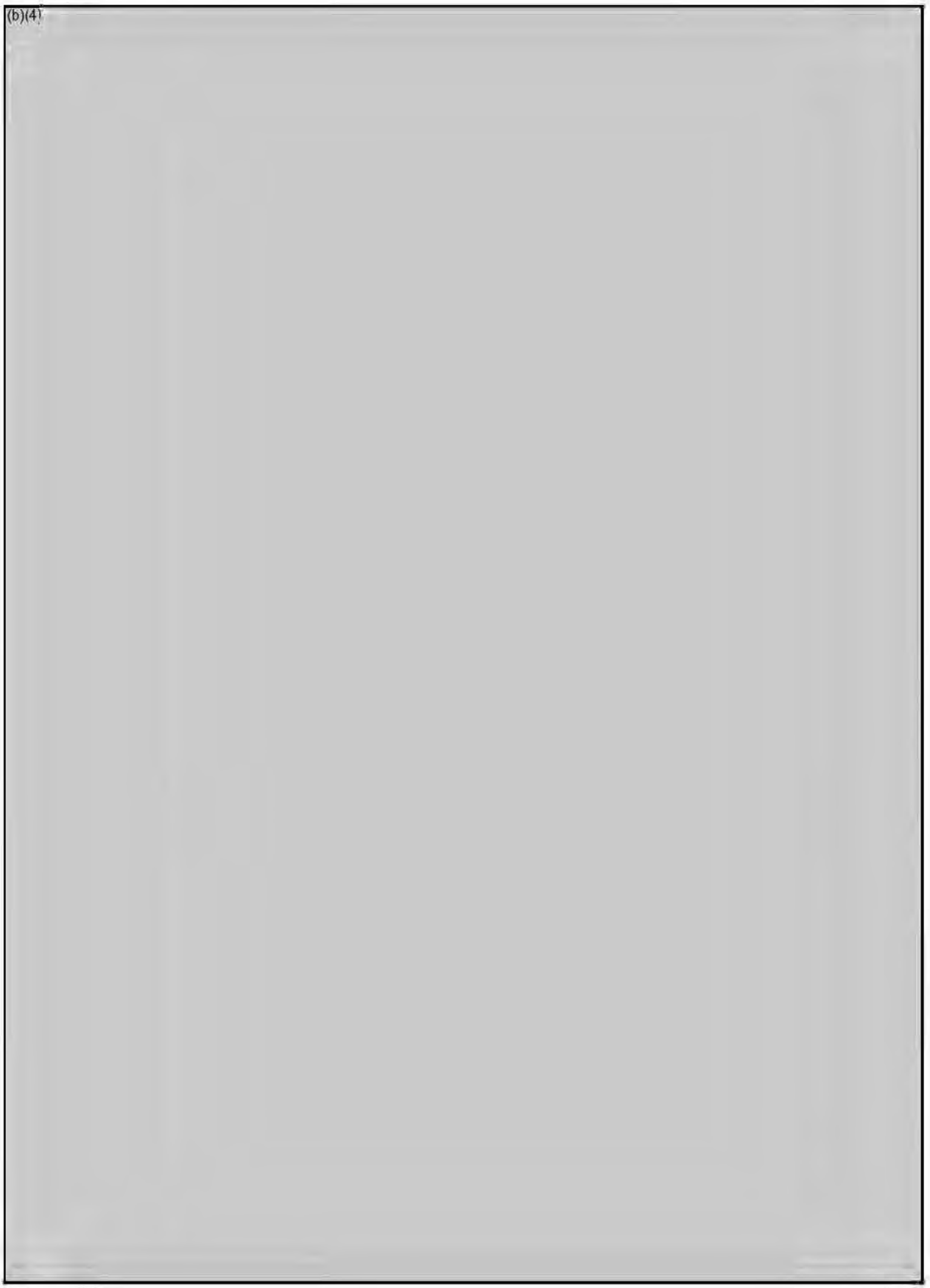

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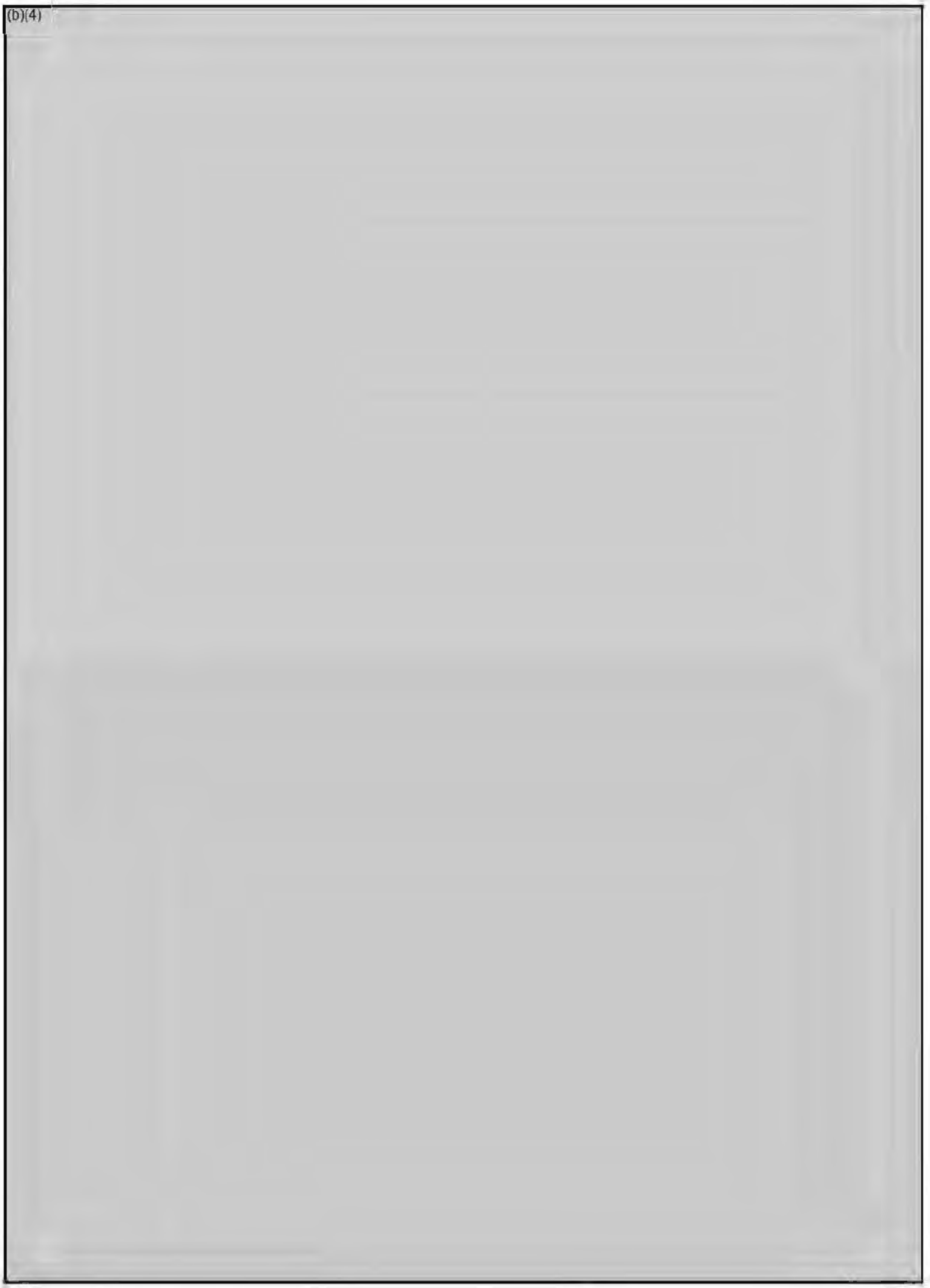

IV. MILITARY RELEVANCE:

Successful completion of the proposed study holds the prospect of identifying and characterizing highly radioprotective compounds against acute and chronic forms of ionizing radiation, thereby helping to sustain military operations in "WMD environments". Dr. (b)(6) previous and current work clearly demonstrates that Mn2+ complexes of *D. radiodurans* are protective against acute exposures to γ-rays (acute and chronic) when applied *ex vivo* to mammalian cells and bacteria. Most importantly, once fully characterized, these Mn2+ complexes could be further developed into novel radioprotective drugs used both by military and domestic authorities in the event of WMD incidents.

Another important potential application of *D. radiodurans* Mn2+ complexes is the production of irradiated viral vaccines. To date, γ-irradiation is the most effective way of sterilizing materials – hence, the widespread use and success of industrial-scale 60Co-sterilization technologies. Other approaches of chemical inactivation such as formalin have resulted in incomplete inactivation and also loss of immunogenicity, which also occurs with inactivation by γ-irradiation. Utilizing *D. radiodurans* Mn2+ complexes for protecting irradiated viral envelope proteins, while at the same time achieving complete inactivation, would have a profound impact on generating inactivated viral vaccines in general. If successful, it would avoid the major disadvantage of epitope loss that occurs during γ-irradiation. CHIKV is an emerging infectious agent and poses great threat to

our troops deployed in CHIKV endemic areas. Additionally, several recent cases of locally transmitted CHIKV infection have also been detected in US. Currently, there is no FDA approved licensed vaccine for prophylaxis against CHIKV. The proposed research would identify and characterize novel Mn^{2+} complex formulations that preserve virus immunogenic epitopes during high-dose irradiation, thus reducing the loss of antigenicity. Prospectively, this approach would help reduce the amount of virus needed for vaccination and thereby reduce the time required to produce mass vaccine in the event of a CHIKV infection outbreak.

V. MATERIALS AND METHODS:

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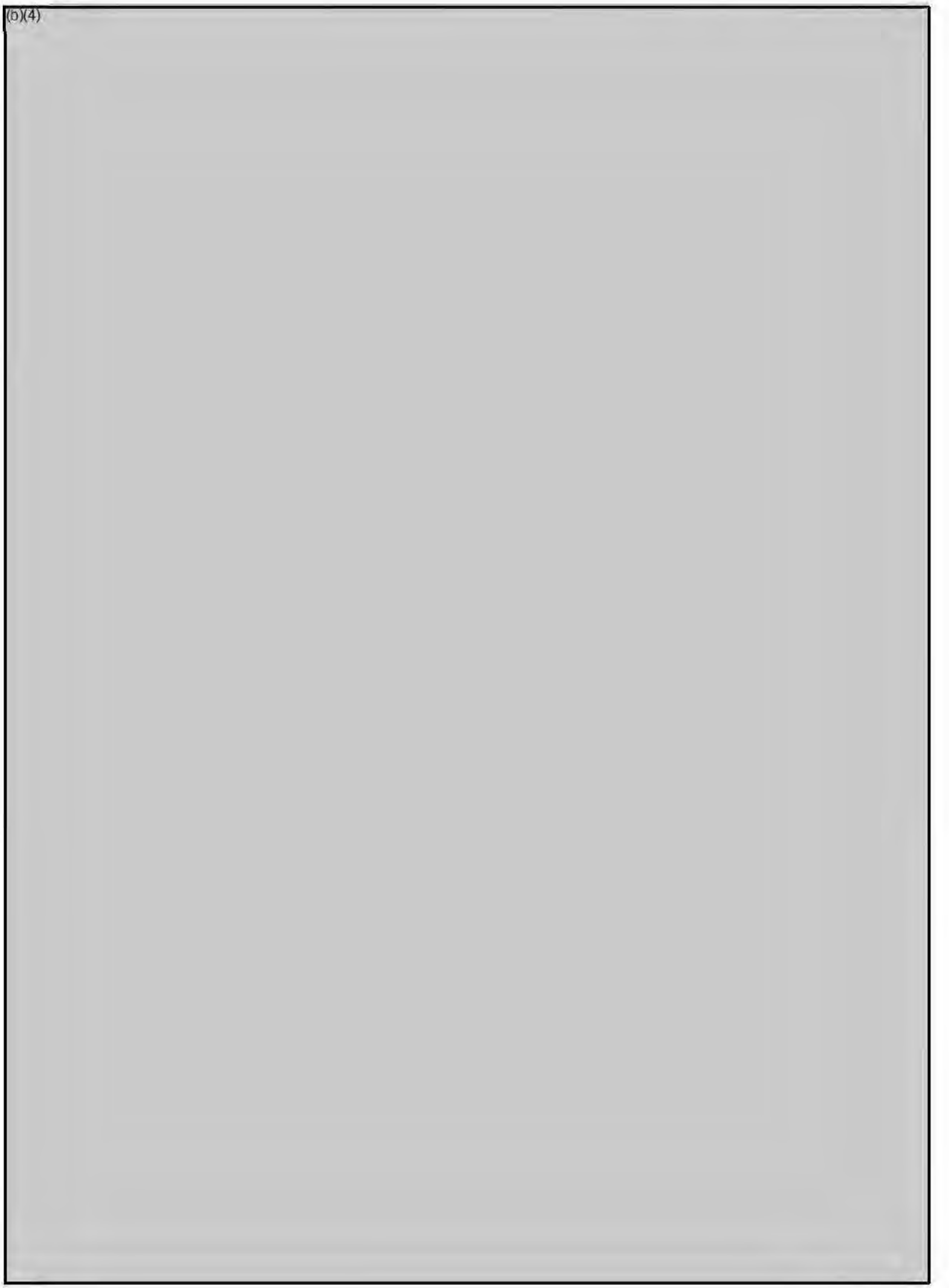
(b)(4)



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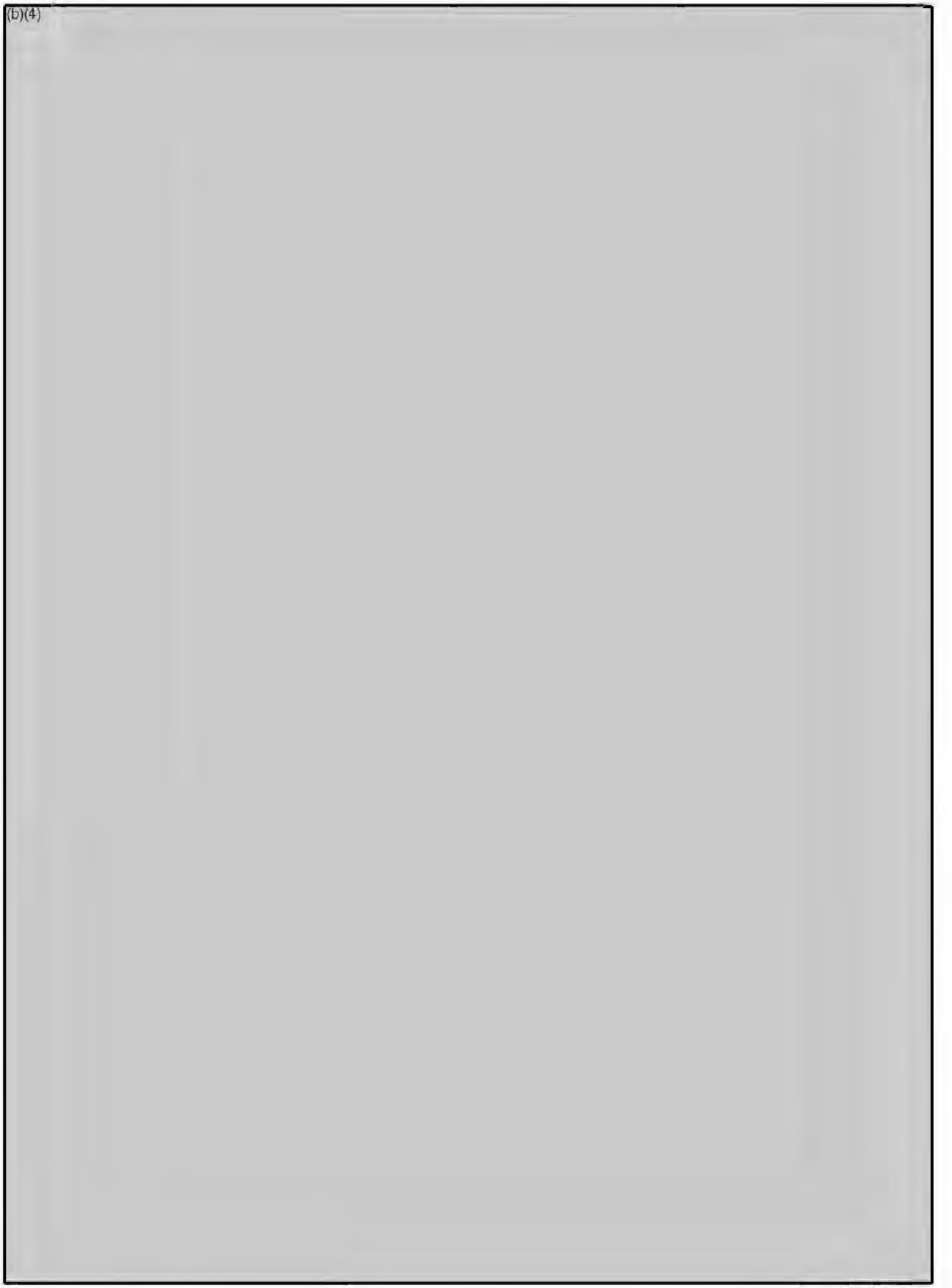

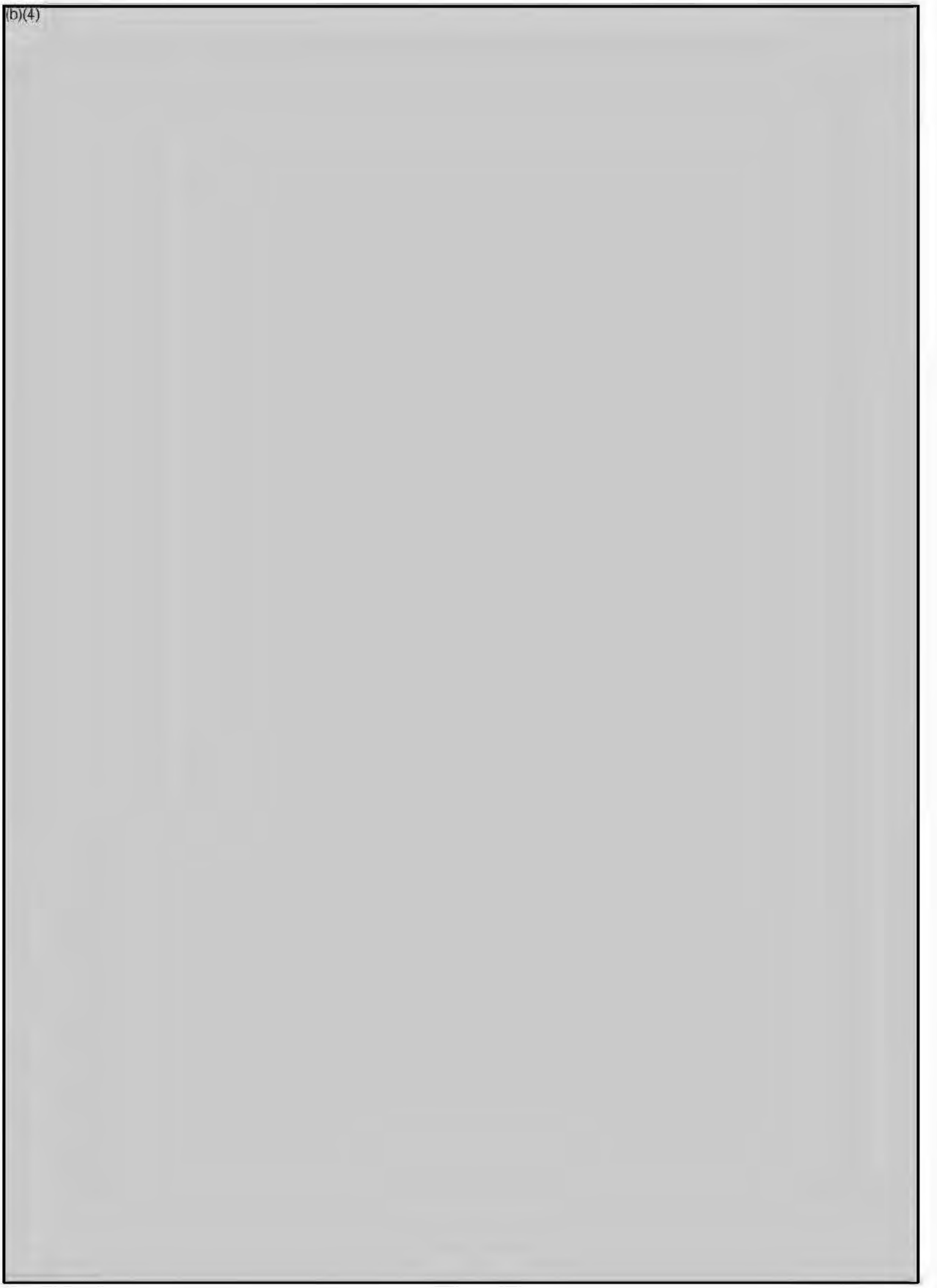
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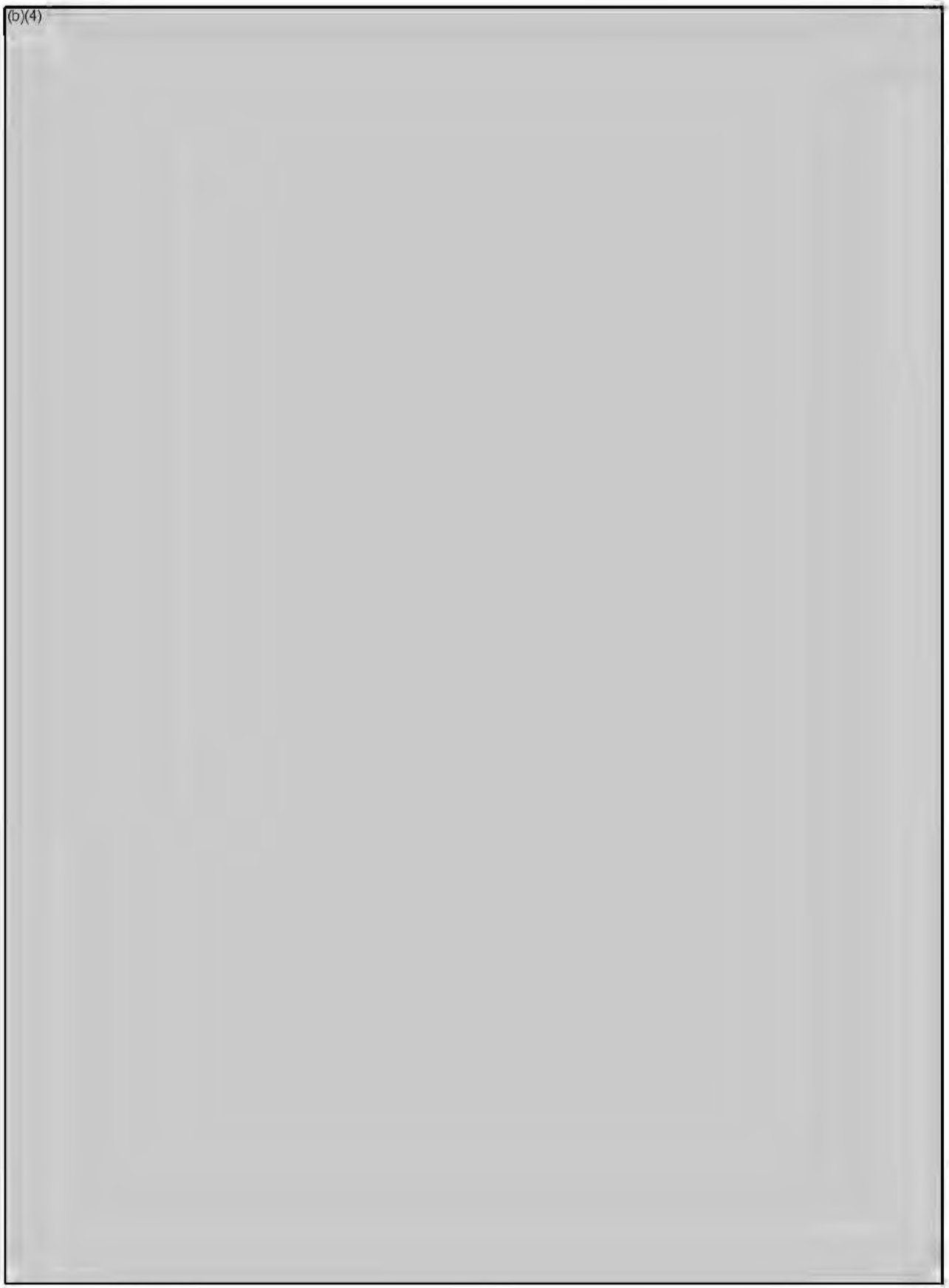

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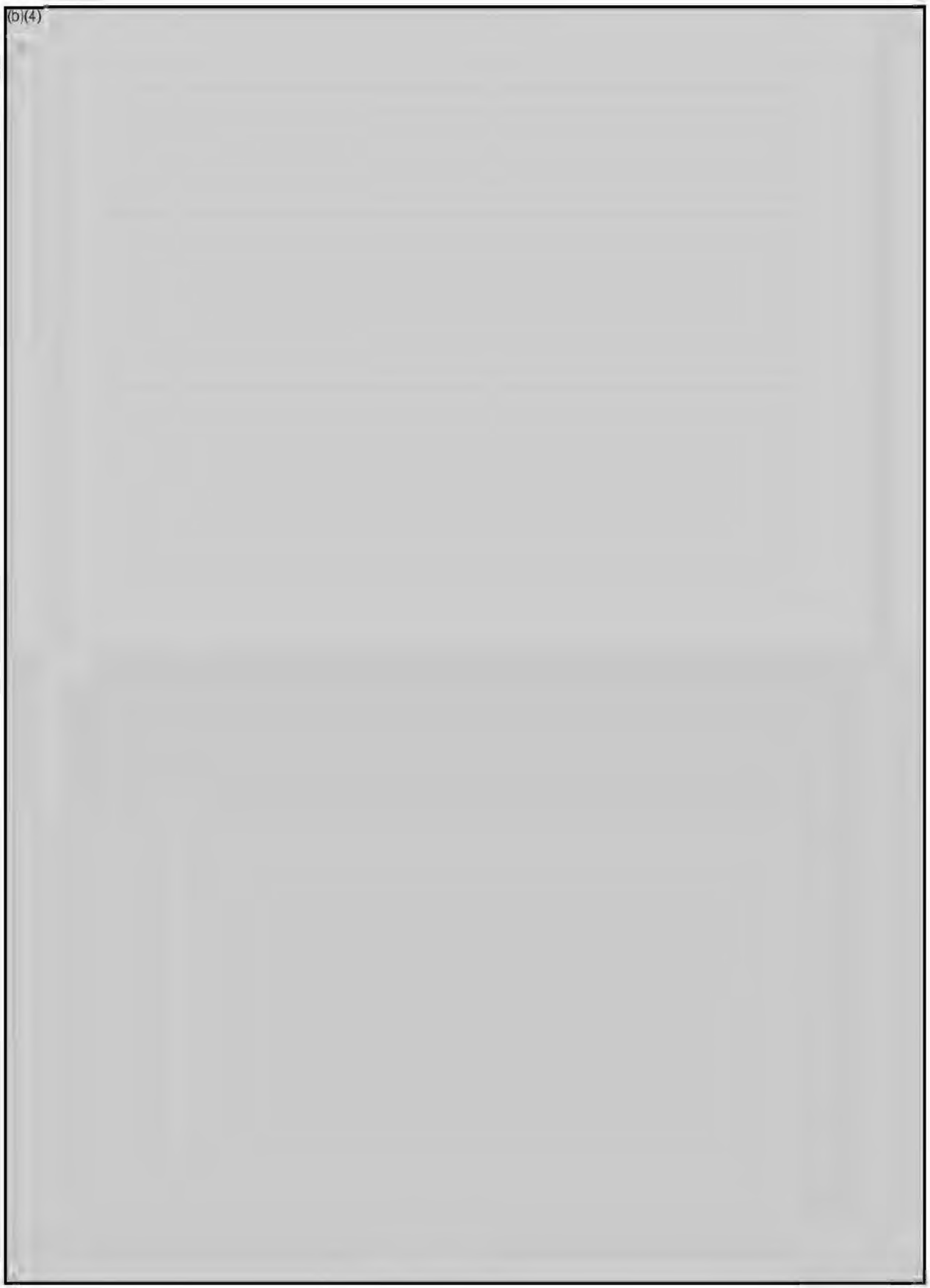

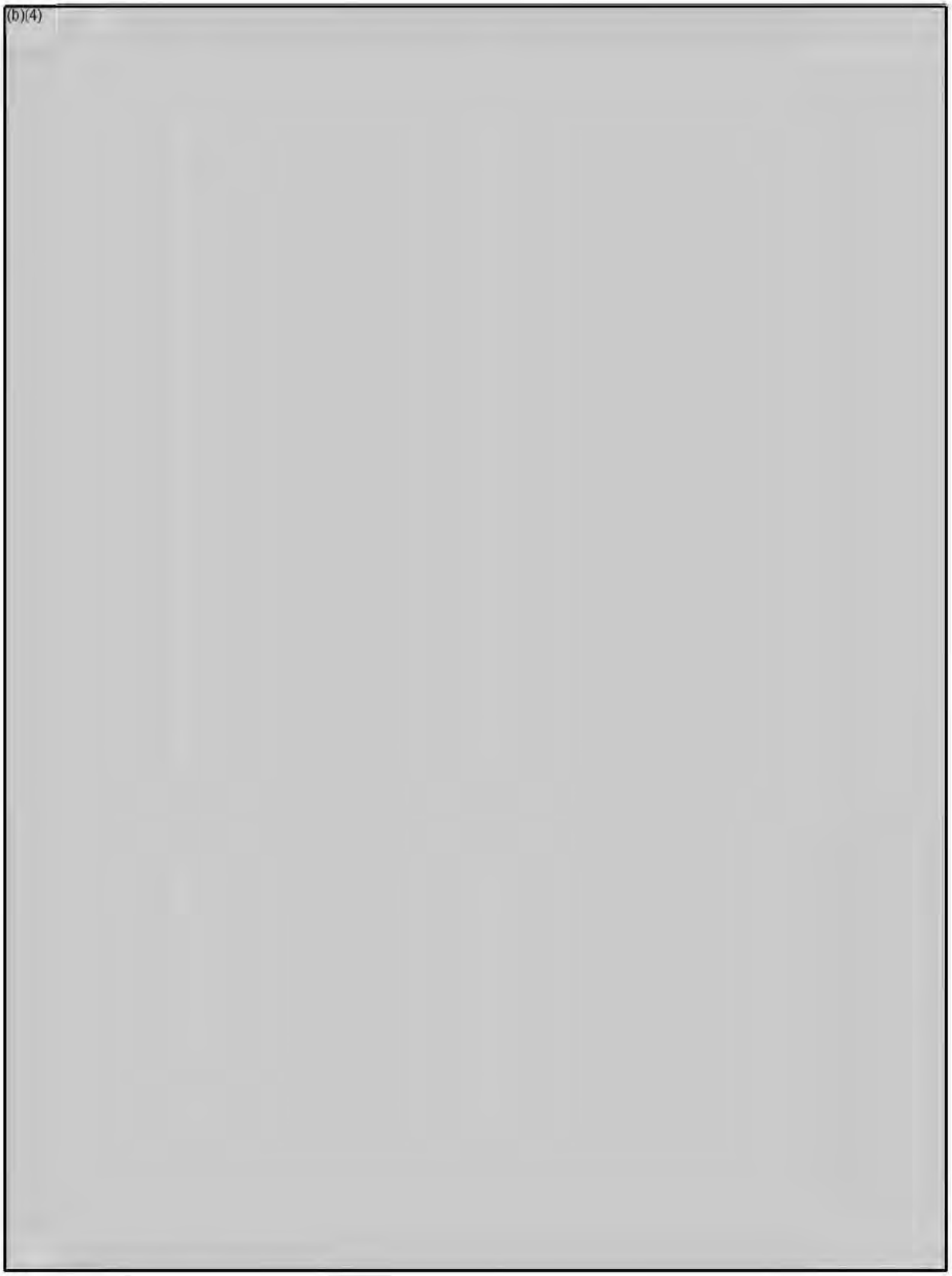
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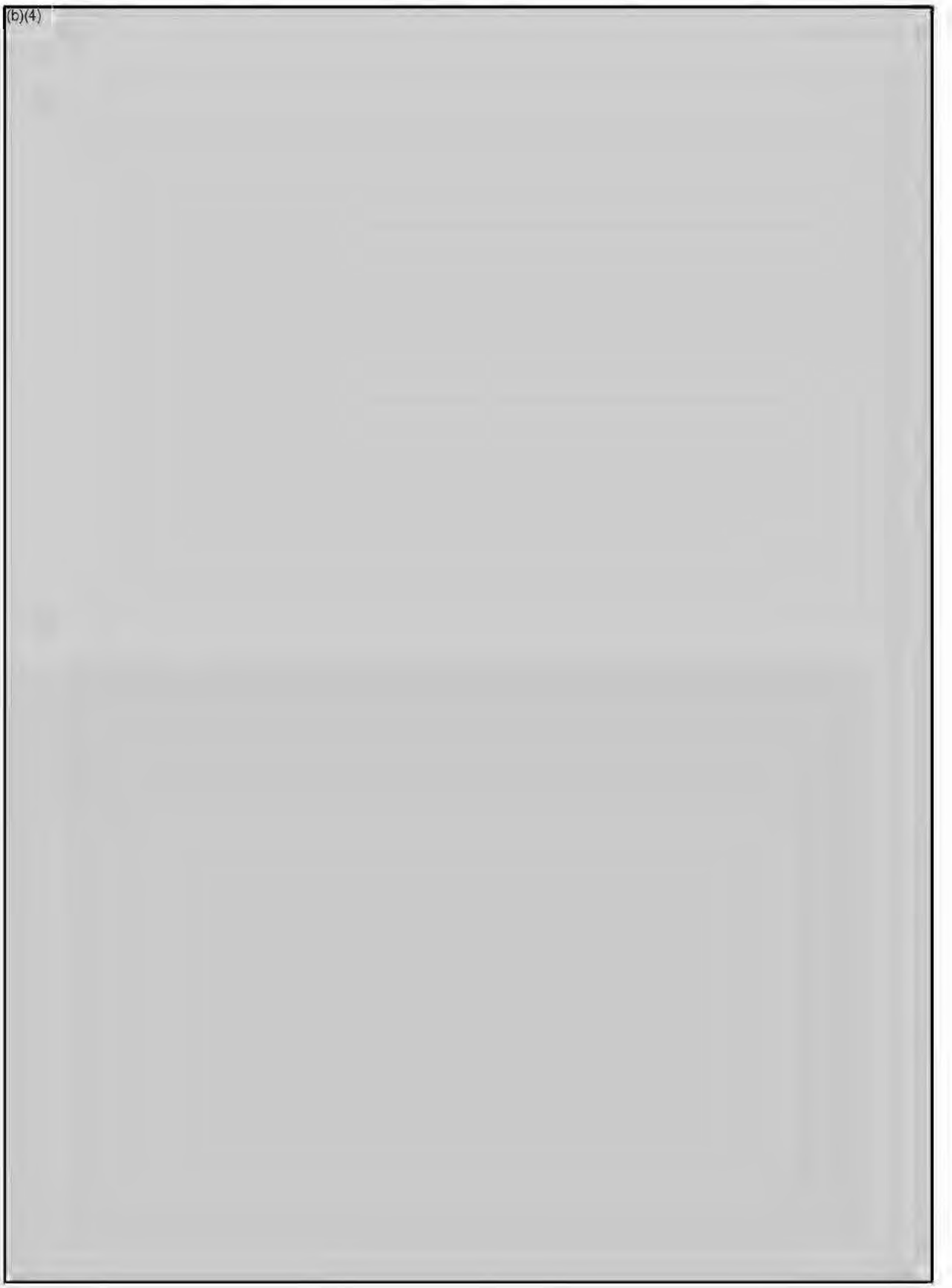

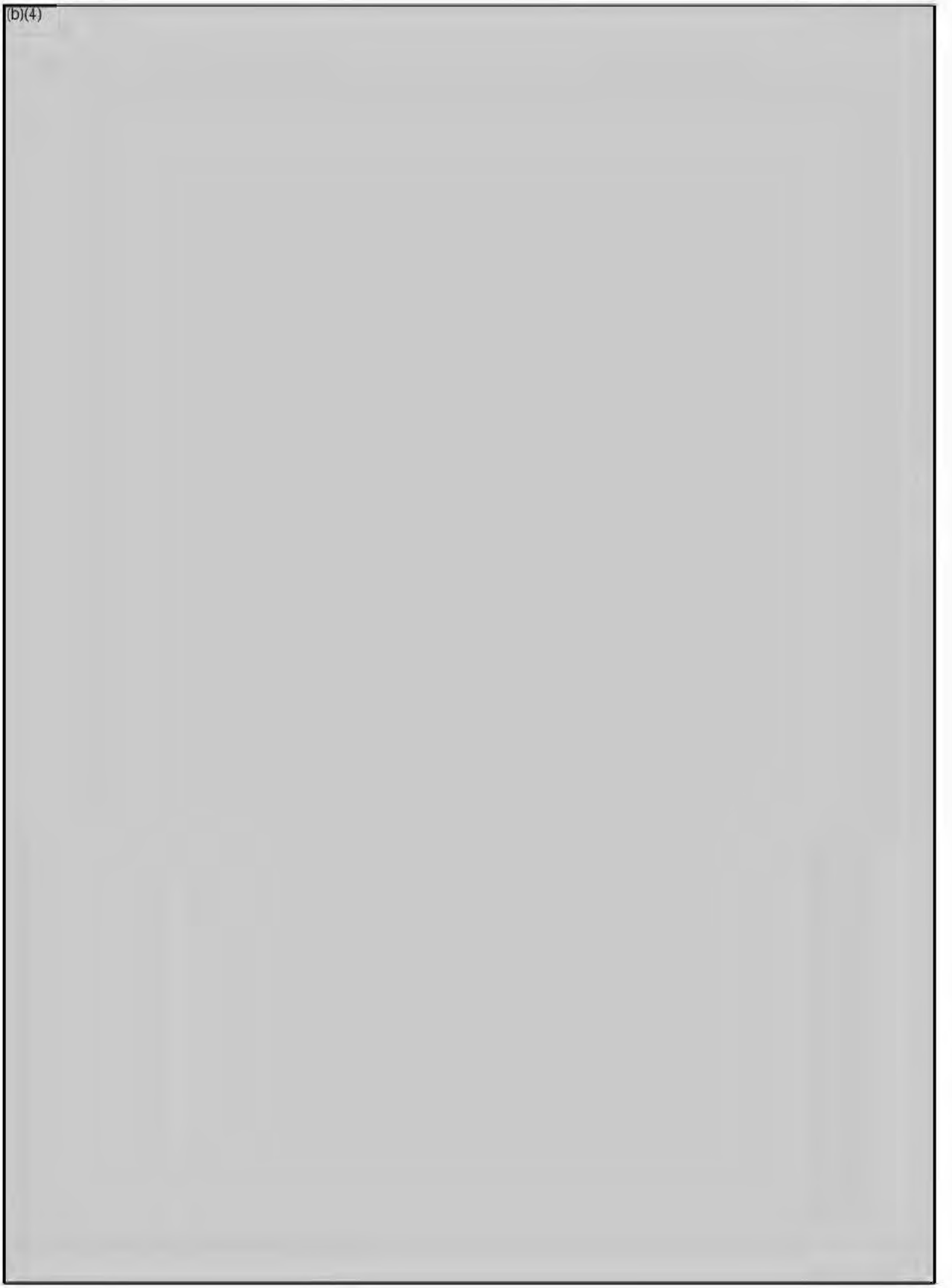
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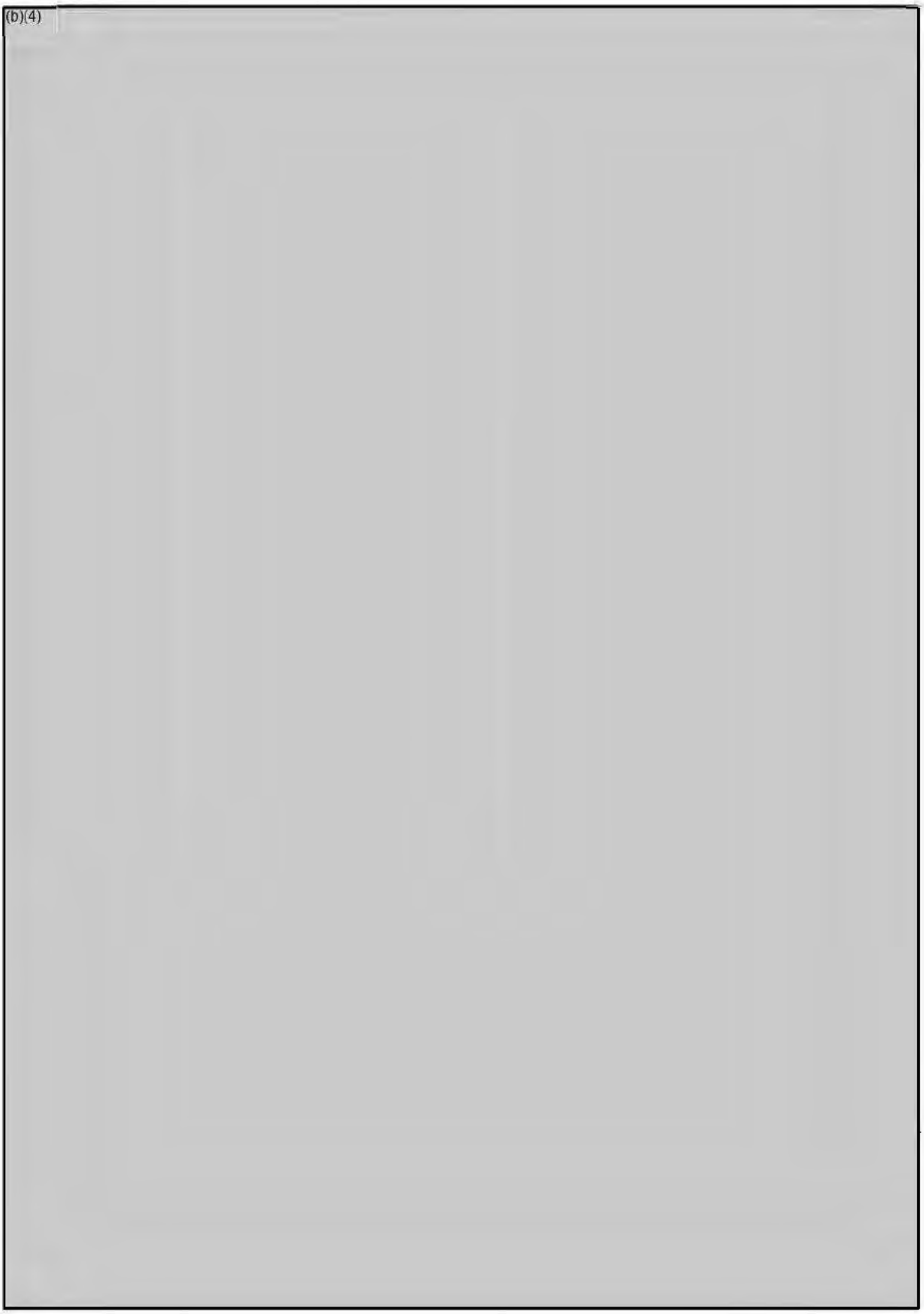

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VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Injections, euthanasia, blood and tissue collection.	(b)(6)	Staff Scientist, > 10 years of experience with the rodent model studying viral disease, TBI and wound healing.	Rodent handling training class 2002 and 2006, USUHS Investigator Training Course (2006)
Injection, euthanasia, blood and tissue collection.		Research associate, > 8 years of experience handling animal samples	
Injection, euthanasia, blood and tissue collection.		Staff Scientist, 8 years of experience handling animal samples	Rodent handling training class 2008, USUHS Investigator Training Course 2007,
Injection, euthanasia, blood and tissue collection.		Senior post-doctoral fellow, 8 years of experience handling animal samples	Rodent handling training class 2008, USUHS Investigator Training Course 2007,
Injections, euthanasia, blood and tissue collection.		Post-doctoral fellow, 5 years of experience handling animal samples	Rodent handling training class held on 08/17/2010, USUHS Investigator Training Course 08/17/2010

VII. BIOHAZARDS/SAFETY:

According to BMBL 5th edition CHIKV181/25 shall be handled at BSL2 conditions. We would like to emphasize that no live attenuated virus will be used in the proposed experiments. Only gamma irradiation inactivated viruses will be used to immunize the animals.

Standard BSL2 precautions will be taken while performing the experiments utilizing the viruses. In accordance to BMBL5 recommendation the personal protective equipment worn at the time of handling the live attenuated virus (CHIKV181/21) or handling the mice given the vaccination are lab coats, gloves and face mask. Live attenuated virus will be handled inside a certified biosafety cabinet in a BSL2 laboratory. Irradiation of the virus will be done in an enclosed airtight tube (screw cap tubes) incubated on ice. All the trash generated such as pipette tips, used tubes *etc.* will be autoclaved before discarding. Animal carcasses will be disposed as per LAM SOP. NOTE that none of the animals in the proposed experiments will receive any live attenuated virus all the animals will receive inactivated attenuated virus. Sharps such as needles will be disposed in the designated sharp containers. No recapping of the needles will be done. All the biohazard material generated from the animals work except the animal carcasses will be packaged in the red biohazard bag, autoclaved and packaged in the designated biohazard box and disposed as biohazard material.

VIII. ENCLOSURES:

1. Doc-1: BMBL 5th edition section VIIIf. This section specifically state that V3526 and CHIK181/25 can be handled at BSL2 level.
2. Doc-2: Research publication outlining the phase-II trail of CHIKV181/25.

   Edelman R, Tacket CO, Wasserman SS, Bodison SA, Perry JG, Mangiafico JA. Phase II safety and immunogenicity study of live chikungunya virus vaccine TSI-GSD-218. Am J Trop Med Hyg. 2000 Jun;62(6):681-5.

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 3/22/16

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 3/22/16

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) [redacted]

Principal Investigator Signature

3/22/16

Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: Deinococcus radiodurans Mn^{2+} Complexes: A Revolutionary Approach to Radioprotection and Vaccine Production (in mice).

C. Principal Investigator: Dr. (b)(6)

D. Performing Organization: USUHS, Bethesda, MD.

E. Funding: Defense Threat Reduction Agency.

F. Objective and Approach:

The specific objectives of the proposed study are:

1. To determine the immunogenic potential of the γ-MDP-CHIK181/25.
2. To determine the protective efficacy of topical application of MDP complex on full thickness cutaneous wound post irradiation.

Recently, we along with Dr (b)(6) group (Gaidamakova et al 2012) have shown the protective efficacy of the peptide (DEHGTAVMLK) isolated from the bacteria *Deinococcus radiodurans* against exposure to high level radiations for inactivating VEEV. This peptide preserves the activity of multimeric proteins at doses that obliterate the nucleic acid. This property of the peptide will be used to inactivate CHIKV181/25. The hypothesis is that viruses treated with the above peptide and irradiated at 50,000Gy will retain the structural protein; however, the viral genome will be destroyed. Therefore, such virus particles will not be infectious but should elicit protective antibody response. We will also test the radioprotective potential of this complex when administered *in vivo* against radiation induced delay in wound healing

G. Indexing Terms (Descriptors): *Deinococcus radiodurans,* live attenuated chikungunya virus, radioprotection, Manganese(Mn^{2+})-Decapeptide-Phosphate complex, antibody response, immune response, protein protection.

XI. References:

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40.

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4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

June 1, 2016

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF PATHOLGY

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on June 1, 2016:

Title of Application: "Deinococcus radiodurans Mn2 Complexes: A Revolutionary Approach to Radioprotection (in mice)"

USUHS Protocol Number: (b)(6)

Expiration Date: May 31, 2019

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: *Deinococcus radiodurans* Mn^{2+} Complexes: A Revolutionary Approach to Radioprotection (in mice).

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)
This is the number assigned by REA when a research grant proposal is submitted.
Example: G170AB-01
FUNDING AGENCY: Defense Threat Reduction Agency.

EARLIEST ANTICIPATED FUNDING START DATE: Oct 30th 2015

PRINCIPAL INVESTIGATOR:

(b)(6)	PAT	(b)(6)	4/21/16
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) MD	Chair, DAT	(b)(6)	04/22/2016
Research Unit Chief / Dept. Head Signature Typed Name:	Title	Telephone	Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)	PAT	(b)(6)	4/21/16
Statistician Signature Typed Name:	Department	Telephone	Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)	LAM	(b)(6)	25 April 16
Signature	Department	Telephone	Date

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

final 6/6

IACUC Date Stamp

PROTOCOL NUMBER:

PROTOCOL TITLE: *Deinococcus radiodurans* Mn^{2+} Complexes: A Revolutionary Approach to Radioprotection (in mice).

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ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

Attending/Consulting Veterinarian Signature Typed Name: (b)(6)	LAM Department	(b)(6) Telephone	Date

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: Dr. (b)(6)

ANIMAL PROTOCOL TITLE: *Deinococcus radiodurans* Mn^{2+} Complexes: A Revolutionary Approach to Radioprotection (in mice).

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6) (b)(6)

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

Development of effective therapies for treatment or protection from radiation injury has been a long-sought goal. The potential threat of the wide spread destruction that can be caused by the use of dirty bombs by rogue nations or terrorists, and accidents like the recent release of nuclear material from the Fukushima Daiichi nuclear power plant in Japan, reflects the urgency to develop safe and efficient radiation countermeasures.

Deinococcus radiodurans, is a microorganism that can survive high doses of radiation. Its radiation-resistance is attributed to high intracellular concentration of manganese present in complex with phosphate and other biological materials such as peptides or nucleosides present inside the cell.

We have recently shown that when artificially reconstituted, such a complex, called MDP complex, can protect mice against lethal radiation exposure. An MDP complex consists of Manganese + decapeptide + phosphate and is made spontaneously when the three constituents are mixed together in a test tube. Under the proposed studies, we plan to further investigate the radioprotective efficacy of MDP complex in a mouse model. Following parameters will be investigated: the maximum radiation that MDP complex can protect from; the route of treatment *i.e.*, oral consumption vs injection under the skin, the changes occurring in blood cell count, cytokine, protein and tissue pathology upon treatment with MDP complex. These experiments will help in understanding the cellular and molecular mechanisms involved in MDP mediated radioprotection.

II. BACKGROUND:

II.1. Background:

Proteins in *D. radiodurans* are exceptionally resistant to oxidation, but they are not inherently radiation-resistant. When purified from *D. radiodurans*, proteins are rendered as susceptible to γ-radiation-induced ROS as proteins in naturally radiation-sensitive cells. Proteome protection in *D. radiodurans* and other resistant prokaryotes is mediated

by the accumulation of small-molecule Mn antioxidants which can functionally substitute enzymes such as superoxide dismutase and catalase, which scavenge superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), respectively. In *D. radiodurans*, Mn-antioxidants make up approximately 70% of the cytosolic Mn^{2+} (0.2 - 1 mM), forming complexes with orthophosphate (Pi) (~12 mM) and peptides (~3 mM). These complexes specifically protect proteins from ROS generated by abiotic and biotic processes arising from exposure to γ-radiation, ultraviolet (UV) radiation, and desiccation. When applied ex vivo, naturally-occurring Mn-peptide-Pi complexes in protein-free cell extracts of *D. radiodurans* protected cultured human cells and Escherichia coli bacteria from extreme cellular insults caused by γ-rays, and preserved the activities of enzymes irradiated or desiccated in vitro. Subsequently, under this project, rational design of the peptide-component of *D. radiodurans* Mn antioxidants yielded a synthetic complex named Mn-DP1-Pi (referred to here simply as MDP, or MDP complex), which forms spontaneously when the decapeptide-1 (b)(4) Pi and Mn^{2+} are combined. Under aqueous conditions, MDP preserved the structure and function of irradiated proteins exposed to massive doses of γ-radiation, but did not significantly protect DNA or RNA. For example, when applied in vitro at physiologically-relevant concentrations, (b)(4)

(b)(4)

and massively increased the in vitro inactivation-dose of the DNA repair enzyme T4 DNA ligase from ~200 Gy to >60,000 Gy; but MDP did not protect DNA or RNA in vitro or in vivo.

The trends in prokaryotes which support the primacy of function (proteome) over information (genome) in radiation toxicity parallel several trends emerging for irradiated mammalian cells. For example, the relationship between DSB yields and γ-ray dose in human cells is about the same as in all other cell-types (0.005 DSBs/Gy/Mbp). In cultured mouse cells exposed to γ-rays, protein oxidation precedes DNA damage, and is implicated as a critical and very early event in radiotoxicity. Moreover, mouse cells which maintain low levels of ROS, either naturally or by treatment with antioxidants, are consistently more resistant to ionizing radiation than cells with high ROS levels, but with no overt effects on DSB yields. However, the degree to which protein oxidation is expected to influence recovery of irradiated mammalian cells is greater than for bacteria because of the impact of genome size. For example, 8 Gy does not cause genomic DSBs in radiation-sensitive bacteria such as *E. coli* (~4.6 Mbp), and any oxidative damage to DSB repair proteins in *E. coli* exposed to 8 Gy would be inconsequential. Not so for most human cells, where a typically lethal dose of 8 Gy is expected to cause approximately 120 DSBs per haploid genome (0.005 × 8 Gy × 3,000 Mbp), and where any oxidative damage to DSB repair proteins is expected to lower DNA repair efficiency. We have shown that proteome activity in human Jurkat T cells exposed to 200 Gy in vitro is protected by MDP administered pre- or post-irradiation, and that mice treated with MDP and exposed to 9.5 Gy all survived. These results give realistic hope that *Deinococcus* Mn-antioxidants will confer radiation resistance on humans, and that antioxidant treatments based on MDP which target the proteome of mammalian cells may be the basis for countermeasures development against acute radiation syndrome

(ARS), a constellation of health effects which present within 24 hours of exposure to acute doses of ionizing radiation.

Preliminary Studies:

Surplus Mn^{2+} in *D. radiodurans* forms ROS-scavenging complexes which protect proteins from ionizing radiation [redacted] studies on protein-free cell extracts of *D. radiodurans* [redacted] have shown that they are armed with low-molecular-weight (<3 kDa) ROS-scavenging Mn^{2+} complexes which consist mainly of peptides bound to Mn^{2+} and orthophosphate (Pi). When reconstituted *in vitro* at physiologically relevant concentrations, Mn^{2+}, Pi, and peptides display multifactorial antioxidant synergism. Using an *in vitro* approach based on the ROS-sensitive enzyme glutamine synthetase (GS) [redacted] demonstrated distinct antioxidant mechanisms involved in protein protection - where Mn^{2+}-Pi alone, or organic metabolites alone (*e.g.*, peptides, amino acids, nucleosides) provided moderate levels of radioprotection [redacted] However, when peptides, Mn^{2+} and Pi were combined, the complexes were substantially more protective [redacted] Yet, the same reconstituted Mn^{2+} complexes afforded little protection on irradiated DNA preparations [redacted] In summary, surplus Mn^{2+} (*i.e.*, the portion of a cell's Mn^{2+} budget which is not bound to proteins) appear to form ROS-scavenging complexes with various metabolites (Daly et al 2010), which provide global protein protection and preserve the quaternary structures and activities of irradiated enzymes (Daly et al 2010).

Protein-free cell extracts of D. radiodurans are radioprotective of human Jurkat T cells: [redacted] recently demonstrated that protein-free cell extracts of *D. radiodurans* are radioprotective of human Jurkat T cells grown and then irradiated in liquid culture [redacted] In collaboration with [redacted] have shown that the same *D. radiodurans* decapetide-Mn^{2+}-Pi complex which is highly protective of glutamine synthetase is also extraordinarily protective of irradiated human Jurkat T cells.

The MDP complex is non-toxic in vivo: Several in vitro assays [Daly et al., 2010, and Patent Publication # WO/2013/098743] have shown that the MDP complex is non-toxic to mammalian cell cultures and when applied to rats and mice, at concentrations much higher than what would be used in the studies proposed here. In the third year of this project, we showed that there is no toxicity when the [redacted] complex was administered intracranial or intramuscular to mice; i.e. [redacted] The MDP-treated mice developed normally, like the saline-treated mice. We also determined the toxicity profile of the MDP complex in our mouse model via subcutaneous (s.c.) and oral (p.o.) routes of administrations for up to 7 days. This confirmed that MDP is not toxic to mice. Indeed, all the MDP-treated animals gained weight and developed normally. A detailed evaluation of the extent of toxicity/injury from MDP in mice was conducted in serum-samples of the mice on day-21, using a hepatic injury marker panel consisting of ALT, AST, ALKP, GGT, total bile and total protein; a renal injury marker panel consisting of creatinine, phosphorus, sodium, potassium, chlorine, calcium, carbon dioxide, urea

nitrogen and glucose; and another injury marker panel consisting of albumin, LDH, CK, amylase, lipase, cholesterol, direct HDLC, triglycerides and uric acid. Importantly, no significant adverse effects of MDP administration were observed in comparisons to the vehicle-treated mice. These observations demonstrate that the MDP complex (300 mg/kg) is safe *in vivo*.

MDP complex protected mice against radiation injury: (b)(6) recently shown that a synthetic *Deinococcus* decapeptide, which spontaneously forms antioxidant complexes with Mn2+ and inorganic phosphate (MDP) is nontoxic and protected B6D2F1/J mice from acute radiation syndromes when administered at 300 mg/kg. 100% of the mice exposed to 9.5 Gy and treated with MDP pre- and post-irradiation survived 30 days with a significantly lower clinical score, versus the irradiated untreated control group which displayed 63% lethality, significant morbidity and loss of body weight (Figure-1). Moreover, our evaluation indicates that the MDP plays an important role in the early protection of white blood cells, and in the attenuation of radiation-induced bone marrow damage, splenomegaly and hematopoietic damage via G-CSF and GM-CSF modulation (data not shown).

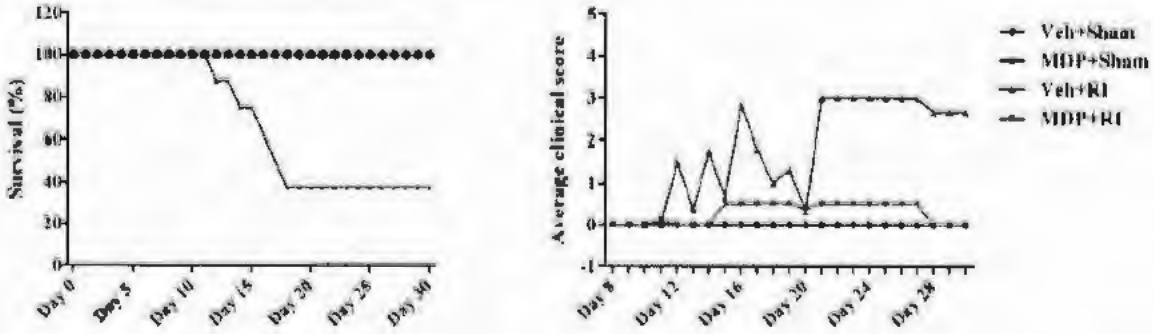


Figure 1. MDP complex confers radio-protection *in vivo*: A) All the mice treated with MDP complex survived a 9.5 Gy dose of irradiation in comparison to a 37.5% survival without MDP treatment and displayed a significantly lower **B)** clinical score.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: PubMed, Defense Technical Information Center (DTIC), and **NIH RePORTER.**

II.2.2. Date of Search: 4/10/2016

II.2.3. Period of Search: 1986-2016

II.2.4. Key Words and Search Strategy: Following key words and their combinations were used to perform the literature search for any duplication. *Deinococcus radiodurans*, radioprotective Manganese complex, radioprotection in mice, gamma irradiation, lethal dose in mice.

II.2.5. Results of Search: In summary no duplication effort was found for the proposed study.

NIH RePORTER: Search for key words D. radiodurans, Manganese complex and radioprotection returned "0" match. Search with key words manganese complex and radiation returned "2" results with studies on manganese superoxide dismutase and its effect on ischemia reperfusion. Search with key words *Deinoccocus* and radiation returned 0 results. Therefore, no duplicating effort was found in the NIH RePORTER system.

PubMed search for manganese complex, radiation injury returned 13 publications which reduced to "0" results when decapeptide word was added to the search terms. Search with the key words *Deinoccocus* and radiation returned more than 500 publication which reduced to 5 articles when radiation was replaced with radiation injury. None of these articles had any duplication of the proposed studies. Search with the key words, radiation injury and treatment returned more than 43000 publications which reduced to 16 articles when manganese peptide was added to the search terms. None of these articles had any duplication of the proposed studies. DTIC search for manganese complex and radiation injury as well as *Deinoccocus* and radiation returned "0" results. Therefore, no duplication effort was found.

III. OBJECTIVE\HYPOTHESIS:

The recent Fukushima nuclear disaster in Japan has brought sharp focus on the urgent need for research on the prevention of acute and chronic radiation effects. Radiological terrorist "dirty bombs" and nuclear explosive devices also are among the most devastating potential threats facing Americans and their allies. *Deinococcus radiodurans*, a bacterial species, has evolved remarkable defenses against, and repair of, damage from radiation, effectively neutralizing all measurable biological damage caused by acute and chronic radiation exposures. The extreme radiation resistance of *D. radiodurans* is dependent on exceptionally efficient forms of protein protection based on intracellular Mn^{2+} complexes. Those Mn^{2+} complexes specifically prevent protein oxidation, and they have been reconstituted *in vitro* by Daly's group [Daly et al 2010].

(b)(6) evaluated the radio-protective potential of MDP complex in B6D2F1/J female mice against a whole-body ^{60}Co γ-radiation exposure at a dose of 9.5 Gy. MDP complex (6.6 mg peptide/mouse) was administered once daily starting from 24h pre-irradiation until day-7 post-irradiation. The MDP treatment resulted in 100% survival (8 mice survived out of 8) in comparison to a 37.5% survival (3 mice survived out of 8) in the absence of treatment. Such remarkable radio-protection on mice conveyed by treatment with MDP supports that the MDP complex of *D. radiodurans* would likely also confer radiation resistance to humans. Notably, MDP treatment in our study also significantly prevented radiation-induced weight loss in mice and depletion of bone marrow cells. Moreover, MDP-treatment prevented splenomegaly in irradiated mice. Based on results from our previous experiment, we will evaluate the radio-protective efficacy of the MDP complex at higher doses of radiation exposure, and to identify the host cellular mechanisms contributing to survival. We will also evaluate the radio-protective efficacy of the MDP complex when administered either only before (prophylactic) or only after (therapeutic) radiation exposure.

(b)(6) hypothesize that a catalytic antioxidant compound such as the MDP complex, which specifically protects proteins from oxidative damage during and after irradiation, can be used in reducing the cell injury/death associated with high radiation exposures. The hypothesis will be tested under the following specific aims.

To test this hypothesis following are the objectives of the study:

1. Evaluate the efficacy of treatment with the MDP complex on survival after RI and evaluate the underlying cellular changes during the process.
2. Evaluate the efficacy of prophylactic and therapeutic treatment with MDP complex on survival after RI.
3. Evaluate the effect of different doses of radiation exposure on the efficacy of radio-protection by MDP complex treatment.

The proposed study has significant military and civilian application as it would characterize (structurally and functionally) the radioprotective Mn^{2+}-peptide complexes of *D. radiodurans*, and would demonstrate if such Mn^{2+}-peptide complexes can protect higher animals from high doses of chronic or acute forms of ionizing radiation (γ-rays).. The major thrust of the proposed study is to exploit the mechanisms of radioprotection conferred by the cytosolic Mn^{2+}-peptide complexes of *D. radiodurans*, which would spur the development and design of novel metallo-organic drugs for prophylaxis and treatment against exposure to ionizing radiation and radiomimetic agents.

IV. MILITARY RELEVANCE:

With the rapidly growing technology today, there is an even greater risk of nuclear sources being misused for destructive purposes by the terrorist groups. There has been a great deal of awareness towards the destructive potential, and the chaos and casualty that can be caused from the use of radiological weaponry from dirty bombs to atomic/nuclear weapons. Any radiation injury scenario during such an event will not only affect military operations but also significantly impact civilian population. Such a possibility presents an area of great concern to the national security and public health. Additionally, the recent incidence of reactor meltdown at Fukushima, Japan and the wide spread suffering caused to the local civilian population, has re-emphasized the requirement for immediate attention.

The proposed study is highly innovative in its approach for C-WMD. One of the main goals of C-WMD is to minimize WMD effects in order to sustain military operations in a "WMD environment". Upon successful completion of the proposed studies, we would have a radio-countermeasure candidate ready to be tested for improved pharmacokinetics and then taken into clinical trials. This would thus be a significant step forward towards developing countermeasures against potential bioterrorist acts causing mass radiation exposure and helping to sustain military operations in "WMD environments" by minimizing the effects of WMD. We anticipate that the information generated by evaluating the hematological and pathological changes as well as protein expression will provide a better understanding of the underlying mechanisms contributing to radioprotection or radiotherapy by MDP complex in the host and have far-reaching scientific implications on how to counter and/or reduce the effects of ionizing radiations.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1:

Evaluate the effect of different routes of MDP complex treatment on the radio-protection efficacy.

We have already seen that MDP complex confers 100% protection against RI at an exposure dose of 9.5 Gy when administered in a combination of oral and subcutaneous (s.c.) injection. However, to identify the best route of MDP treatment, protective efficacy using individual routes of treatment will be evaluated. Mice will be randomly divided into 4 groups (n=10 per group with one iteration) as described in the table below.

Treatment: MDP complex (6.6 mg peptide complex in 200 µl PBS) will be administered either orally or subcutaneously starting 24 hr before irradiation followed by two doses 14-15 hr before and at the time of irradiation. Thereafter, animals will be administered with MDP complex once daily for 7 days. Corresponding vehicle groups will receive PBS instead of MDP treatment.

	Treatment	Route of treatment	N	Iterations /grp	Total animals / grp	Pain category
1.	Vehicle	Oral	10	2	20	E
2.	Vehicle	Subcutaneous	10	2	20	E
3.	MDP	Oral	10	2	20	E
4.	MDP	Subcutaneous	10	2	20	E
		Total			80	

Irradiation: Radiation exposure dose will be 9.5 Gy.

Sample collection and study end point: Water consumption will be monitored for the first 7 days; and total body weights and survival will be monitored for 30 days post irradiation. Blood, spleen, bone marrow, and ileum will be collected from surviving mice on day 30 from all the groups and used for measuring total blood cell counts, bone marrow cell counts, histological evaluation of ileum and sternum etc.

Pain category for the animals (see also section V.4.1.1.1): In this experiment, all animals will receive radiation exposure. Although based on the results from our previous study, we expect that the animals treated with MDP will have significantly higher survival rate and reduced morbidity. However, since they may experience some clinically adverse symptoms initially, all the animals have been placed under category E (n=40 x 2=80). If MDP treated animals consistently show improved clinical score and protection against radiation exposure, it will be reported in the annual report and pain category can be changed to D after consultation with LAM veterinarian and IACUC.

V.1.2 Experiment-2. Evaluate the effect of different doses of radiation exposure on the efficacy of radio-protection by MDP complex treatment.

To test the protective potency of MDP complex treatment against RI, dose reduction factor (DRF) will be determined by studying the survival of MDP complex treated animals against various doses of radiation. Mice will be randomly divided into 8 groups (n=10 per group with one iteration) as described in the table below.

Treatment: MDP treatment will be given as described in experiment-1. Based on the results from Experiment-1, the route of MDP administration resulting in higher protection efficacy will be chosen for this experiment.

Irradiation: Radiation exposure dose of 10.5 Gy generally results in 100% mortality without any treatment. We have already seen that MDP complex confers 100% protection against RI at an exposure dose of 9.5 Gy (resulting in 67.5% mortality without treatment). Thus, under this aim, mice will be exposed to the radiation doses of 10.0 Gy, 10.5 Gy, 11.0 Gy, or 11.5 Gy as described in Table 2.

Table 2: Experimental set-up for Exp-2

	Injury group	Treatment	N	Iterations /grp	Total animals /grp	Pain category
1.	Radiation 10.0Gy	Vehicle	10	2	20	E
2.	Radiation 10.5Gy	Vehicle	10	2	20	E
3.	Radiation 11.0Gy	Vehicle	10	2	20	E
4.	Radiation 11.5Gy	Vehicle	10	2	20	E
5.	Radiation 10.0Gy	MDP	10	2	20	E
6.	Radiation 10.5Gy	MDP	10	2	20	E
7.	Radiation 11.0Gy	MDP	10	2	20	E
8.	Radiation 11.5Gy	MDP	10	2	20	E
		Total			160	

Sample collection and study end point: Water consumption will be monitored for the first 7 days; and gain in total body weights and survival will be monitored for 30 days post irradiation. Blood, spleen, bone marrow, and ileum will be collected from surviving mice on day 30 from all the groups and used for measuring total blood cell counts, bone marrow cell counts, histological evaluation of ileum and sternum etc.

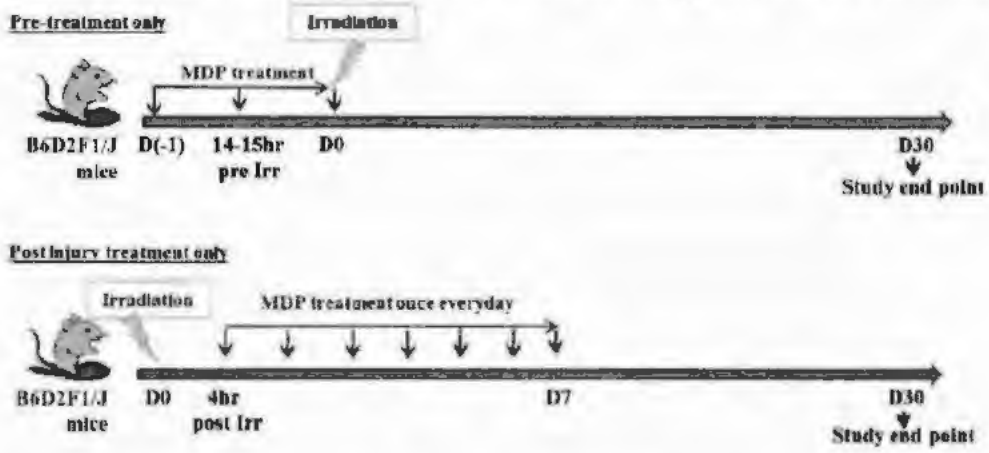
Pain category for the animals (see also section V.4.1.1.1): In this experiment, all animals will receive radiation exposure. Although based on the results from our previous study, we expect that the animals treated with MDP will have significantly higher survival rate and reduced morbidity. However, since the animals will experience some disease symptoms initially and protective effect of MDP against higher doses of radiation exposure is not known, all the animals have been placed under category E (n=160).

V.1.3 Experiment-3. Evaluate the efficacy of prophylactic and therapeutic treatment with MDP complex on survival after RI.

To test the prophylactic and mitigating potentials of MDP complex, this task will evaluate the radio-protection conferred by the MDP complex when given only before and at the time of radiation exposure or only after exposure. Mice will be randomly divided into 8 groups (n=20 per group) as described in Table 3.

Treatment: MDP complex will be administered either three times for the pretreatment only group i.e. 24h and 12h before and at the time of irradiation; or starting 4h post-irradiation until day-7 to the animals in the 4h post-injury group (a total of 7 administrations).

Irradiation: The irradiation dose will be decided based on the results from Exp-2.
Sample collection and study end point: Water consumption will be monitored for the first 7



days; and gain in total body weights, and survival will be monitored for 30 days post-irradiation. Blood, spleen, bone marrow, and ileum will be collected from surviving mice on day 30 from all the groups and used for measuring total blood cell counts, bone marrow cell counts, histological evaluation of ileum and sternum etc.

Table 3: Experimental set-up for Exp-3

	Injury group	Treatment	N	Iterations/ grp	Total animals/ grp	Pain category
1.	Sham	Vehicle (pretreatment only)	5	2	10	C
2.	Sham	MDP complex (pretreatment only)	5	2	10	C
3.	Sham	Vehicle (4h post-injury)	5	2	10	C
4.	Sham	MDP complex (4h post-injury)	5	2	10	C
5.	Radiation	Vehicle (pretreatment only)	10	2	20	E
6.	Radiation	MDP complex (pretreatment only)	10	2	20	E
7.	Radiation	Vehicle (4h post-injury)	10	2	20	E
8.	Radiation	MDP complex (4h post-injury)	10	2	20	E
		Total			**120**	

Pain category for the animals (see also section V.4.1.1.1): In this experiment, the animals receiving radiation exposure have been placed under category E (n=80). Although in previous experiments MDP complex protected animal against radiation exposure, treatment included both pre- and post-exposure treatment. As this experiment tests the prophylactic and therapeutic approach separately effect of which is not known at this time, these animals are placed in pain category E. The animals in the sham groups will not receive any irradiation but will receive PBS or MDP via either oral or subcutaneous route and thus have been placed under category C (n=40).

V.1.4 Experiment-4. Evaluation of effect of radiation exposure on blood cells, bone marrow and ileum after prophylactic and therapeutic treatment with MDP

complex.

To test the effect of prophylactic and mitigating potentials of treatment with MDP complex on blood, bone and marrow cells and various tissues against RI a time-course analysis will be done. Briefly, mice will be randomly divided into 8 groups (n=15 per group) as described in Table 4.

Treatment and irradiation: Radiation exposure doses and MDP treatment regime will be chosen based on the results from experiment-1, 2 and 3.

Sample collection and study end point: Water consumption will be monitored for the first 7 days; and gain in total body weights. Blood, spleen, bone marrow, and ileum will

Table 4: Experimental set-up for Exp-4

	Injury group	Treatment	N (D1, D3 and D7)	Iterations/ grp	Total animals/ grp	Pain category
1.	Sham	Vehicle (pretreatment only)	15	1	15	C
2.	Sham	MDP complex (pretreatment only)	15	1	15	C
3.	Sham	Vehicle (4h post-injury)	15	1	15	C
4.	Sham	MDP complex (4h post-injury)	15	1	15	C
5.	Radiation	Vehicle (pretreatment only)	15	1	15	E
6.	Radiation	MDP complex (pretreatment only)	15	1	15	E
7.	Radiation	Vehicle (4h post-injury)	15	1	15	E
8.	Radiation	MDP complex (4h post-injury)	15	1	15	E
		Total			**120**	

be collected on day 1, 3 and 7 from all the groups (5 mice/group at each time point) and used for measuring total blood cell counts, bone marrow cell counts, serum cytokine levels, histological evaluation of ileum and sternum etc.

Pain category for the animals (see also section V.4.1.1.1): In this experiment, the animals receiving radiation exposure have been placed under category E (n=60). If MDP show protection in animals exposed to irradiation and reduction in clinical score in experiment # 3, then these mice will be moved to category D after consultation with LAM veterinarian and IACUC. The animals in the sham groups will not receive any irradiation but will receive PBS or MDP via either oral or subcutaneous route and thus have been placed under category C (n=60).

V.2. Data Analysis:

Survival in Experiment # 1, 2, and 3 will be described using Kaplan-Meier curves and compared between groups using the logrank test. We anticipate 30% survival in the control group. A sample size of 20 per group (pooled over two iterations) will have 80% power to detect a significant difference in survival between groups if survival in the experimental group is at least 80%. The calculation is based on a 5% two-sided significance level.

In experiment # 4 two-way ANOVA, one-way ANOVA, post-hoc test, chi-square test, and student's will be used for statistical analysis of non-survival outcomes. The required sample size for these outcomes is only 5 per group. This sample size will have 80% power to detect differences of 2 standard deviations between groups based on

Student's t test for independent samples with 5%, two-sided significance level. Note that in experiment 3, only 5 animals per group are requested for sham conditions. 100% of these animals are expected to survive, and the primary comparisons will be for the non-survival outcomes.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

Drug manufacturers use computer modeling – structure function relationship – and screening *in vitro* by cell cultures during the development of candidate drugs. Computer modeling and cell cultures are insufficient to determine whether or not a given regimen (prophylactic or therapeutic) is effective in promoting survival, resisting infection, or enhancing hemopoiesis and biochemical interruption. An integrated, functional, computer model of a mammal has not yet been achieved for the purpose of examining intricate physiological interactions among cells, tissues, and organs that occur after exposure to ionizing radiation. The resiliency of organs, such as the gastrointestinal system or hematopoietic system, to ionizing radiation depends on a complex network of interactive signaling systems to sense the magnitude of tissue damage, and to initiate repair, recovery, and other defense processes. The physiology of these interacting networks is altered by radiation in ways that cannot yet be modeled. In fact, the proposed studies will provide data, which will contribute directly to future models to predict the progress of radiation injury and appropriate medical treatments.

V.3.2. Animal Model and Species Justification:

B6D2F1/J female mice, 12-20 weeks of age, will be housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Animal rooms will be maintained at 22° ± 4°C with 50% ± 20% relative humidity on a 12-hr light/dark cycle. Animals will be randomized prior to each experiment and housed in small shoebox cages (four animals per cage). Commercial rodent ration (Harlan Teklad Rodent Diet 8604) will be freely available as will acidified (pH=2.5 - 3.0) water to control opportunistic infections. All animal-handling procedures will be performed in compliance with guidelines from the National Research Council (1996). The B6D2F1/J female mouse was chosen as the best animal model to use for radiation therapy protocol because:

(1) It is one of the least sentient mammalian species that will permit us to achieve our research objectives.
(2) The cellular proliferative characteristics of gastrointestinal tract and hematopoietic tissues in the mouse are qualitatively similar to those in the human.
(3) Studies in mice have had a direct impact on work with larger animals and humans, as well as clinical treatment.
(4) There are extensive published reports available for comparison, review, and analysis of major biological findings.

V.3.3. Laboratory Animals

	Species#1 (Exp 1, 2, 3 and 4)
Genus & species	Mouse
Strain/stock	B6D2F1/J
Source/ Vendor	(b)(4)
Age	12-20 weeks
Weight	20-30 gm
Sex	Female
Special considerations	BSL-2
Number of animals required	480

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:

The animals will also be humanely euthanized if they become moribund and show signs such as ruffled fur, weakness, lethargy, difficulty breathing, weight loss of more than 20%, or loss of appetite, are cool to the touch, show inappetance, or diarrhea, earlier than the proposed study end points. In our earlier pilot study, MDP complex protected mice from radiation induced death, which was also accompanied by improvement in the clinical score as compared to the sham treated animals. Therefore, MDP complex itself may present as a refinement that protect animals and reduce radiation exposure-induced morbidity and mortality.

V.3.5.2. Reduction:

The number of animals to be used is the minimum number of animals needed to achieve scientifically sound conclusions for the experimental objectives. In order to reduce the number of animals in the study we have kept the number of control animals as low as possible to achieve statistical significance for the data obtained. Experiments are designed in a progressive way so as to reduce the number of groups (e.g. first route of treatment then prophylactic vs therapeutic approach) needed to ascertain the protective effect of MDP complex both on the survival as well as molecular pattern).

V.3.5.3. Replacement:

Extensive *in-vitro* testing of the MDP complex for determining the radiation protection activity. Radiation protection property of the MDP complex was tested on biologically active enzymes and human Jurkat T cells. We have extensively researched for an alternative to the animal model for studying the radiation injury but, no alternative to *in vivo* experiments are available at this time. We are using the lowest animal in the phylogenetic scale that serves well as an animal model to study the radioprotective potential of MDP and its underlying mechanism.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1
V.4.1.1.1.1. Column C:	100
V.4.1.1.1.2. Column D:	
V.4.1.1.1.3. Column E:	380

In experiments 1-4, a total of 380 mice will receive radiation exposure to sub-lethal/lethal doses and thus may become moribund. Some of these animals will receive MDP treatment and others will receive PBS as the vehicle after radiation injury. Although, MDP treatment is expected to alleviate the morbidity caused by radiation injury and enhance the survival, these animals have been placed under category E. A total of 100 mice under exp# 3 and 4 will not be irradiated and will only receive momentary distress due to subcutaneous or oral administrations. These animals will not develop any disease or experience any morbidity. Therefore, they have been placed under category C.

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

Anesthesia: Blood collection: At days 1, 3, and 7 for Exp-4 and on day 30 for the rest of the experiments, animals will be anaesthetized appropriately under deep CO_2 inhalation (fill rate of 10-30% of the chamber volume per minute). Blood will be collected via terminal cardiac puncture with 23 G 5/8 - 28G 1/2 needle and will be used for CBC analysis and serum cytokine level evaluation. Tissues will also be collected and frozen at -80°C until use.

Analgesics: No analgesics will be used.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

Animals will be kept at the animal housing facility of LAM for 5-7 days prior to the irradiation procedure and will receive standard care. Post irradiations, animals will be monitored for any distress. Mice will be monitored at least twice a day (i.e., first thing in the morning and then again in the afternoon) during the experiment. If animal is found to be distressed such as ruffled fur, lethargic and weight loss post immunization, animal will be humanely euthanized. For experiment # 1 most morbidity and mortality is expected between days 10-21 post irradiation. For experiment # 2 the period of most morbidity and mortality is expected to occur earlier than is exp#1 as higher doses of radiation will be used. Expected period of most morbidity and mortality is between days 7-15. For experiment #3 the expected range of days when expected morbidity and mortality is high will be based on observation in experiment #2 and is expected to lie

between days 7-15. For Experiment # 4 no such period is expected as animals will be sacrificed by day 7. Observation of animals during this heightened period of morbidity and mortality will be done at least 3 times (early morning, noon, and late afternoon) a day by the investigative staff (twice) and LAM personnel (once). If, during any of the checks, it is suspected that the animals may succumb before the next observation period, the animals will either be checked again before the next scheduled observation period or be euthanized immediately as in section V.4.5.

We will follow the **Rodent Intervention Score Sheet** (see section VIII) to track morbidity and moribund state in injured group of mice. If any single category is above 12 (i.e., the mouse cannot stand or breathe normally, right within 5 seconds when placed gently on side, or respond when the paws are pinched gently), the animal will be euthanized immediately. Mice that score greater than 10 for all categories combined will be considered to be moribund and humanely euthanized. Morbidity signs usually appear much earlier than body weight loss for more than 20%. If there are no above mentioned signs appearing and the body weight loss is an only concern, then mice with body weight loss more than 20% will be euthanized immediately. These mice will be monitored very closely for their appearance, general behavior, respiratory rate, and provoked behavior prior to making a determination of euthanasia. Weights taken 2-24 hours prior to irradiation will be used as a baseline reference.

V.4.1.2.3. Paralytics: NONE

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PubMed (National Library of Medicine).

V.4.1.3.2. Date of Search: 4/10/2016

V.4.1.3.3. Period of Search: 1986-2016

V.4.1.3.4. Key Words of Search: Individual and combination of following words was used; pain, analgesic, mice, alternative, procedure, justification, radiation injury.

V.4.1.3.5. Results of Search:

There are no alternative procedures for irradiation because its effects cannot be otherwise duplicated.

Review of several research article for irradiation protocol and post treatment did not reveal any usage of any separate drug specifically for alleviating the pain associated with the irradiation.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

All the animals will be treated with local application of antibiotic ointment (over the counter available Neosporin) after the ear punching. Analgesics such as opioids, local anesthetics and non-steroidal anti-inflammatory drugs (NSAIDS) cause alteration in the host inflammation response. Several research articles have been published

looking at the beneficial effect of NSAIDs after radiation exposure and their use has been recommended post irradiation. Since alteration in inflammation hemostasis play a crucial role in radiation injury, administration of these drugs may alter the host response to irradiation and negatively affect determining the radioprotective efficacy of MDP complex. Therefore, these drugs will not be used in the studies (experiment 1-4). We have previously conducted toxicity study for MDP and it was found to be completely safe *in vivo*. Thus, we don't expect any side effects from MDP treatment. Regarding pain, there is no data or indication that it will enhance pain. A logical guess may be that due to its free radical scavenging properties it may reduce the pain via controlling the inflammation, however that is speculative.

However, sufficient care will be given to ensure frequent monitoring of the animals and performing early euthanasia as soon as the disease symptoms (as explained in section V.1.1 and V.4.5) are visible to alleviate unnecessary pain and distress to the animals.

V.4.2. Prolonged Restraint: NONE

V.4.3. Surgery: NONE

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

Subcutaneous injections (a total volume of 200µl/ mouse) will be administered on the back of the animals for MDP treatment using 23G 5/8 - 28G 1/2 needles.

V.4.4.2. Biosamples:

For experiment#1, 2 and 3, blood will be collected on day 30 and for experiment#4, blood will be collected on day1, day3 and day7 via cardiac puncture. Animals will be deeply anesthetized with CO_2 (fill rate of 10-30% of the chamber volume per minute),

and thoracic cavity will be opened to gain access to the heart and blood will be drawn using 26 gauge needle on a 1cc syringe from the right ventricle. Spleen, bone marrow, and ileum will be collected from mice for experiment# 1-4.

V.4.4.3. Adjuvants: NONE

V.4.4.4. Monoclonal Antibody (MAbs) Production: NONE

V.4.4.5. Animal Identification: Ear punching will be used to identify the animals within each cage, if needed. Ears will be punched at least 2 days prior to the initiation of the experiments.

V.4.4.6. Behavioral Studies: NONE

V.4.4.7. Other Procedures:

Irradiation procedure:

Animals will be exposed to whole body gamma radiation using the USUHS cesium-137 source (Gammacell 40 or JLS Mark 1 irradiators) at ambient temperature. The irradiators are located inside the USU irradiator facility (b)(6) and access is restricted. Drs. (b)(6) are approved as authorized irradiator operators and authorized for unescorted access into the irradiator facility. They will perform the irradiations. During irradiation mice will be kept in a circular well ventilated covered container which will then be placed in the irradiator on a turn table to ensure uniform whole body irradiation. The amount of time for radiation exposure will be calculated based on the current dose rates of the sources and the final desired dose (as listed in the experiments above). Control animals will be sham-irradiated and will receive the same handling and procedures as the irradiated mice except the radiation exposure. There after animals will be housed back in their original room in a fresh cage containing sterile bedding. Animals will be fed water and food at libitum. We have also consulted with Mr. (b)(6) Assistant RSO, EHS Radiation Safety regarding the irradiation of mice using the Cs^{137} irradiator.

Oral administration of MDP complex: Oral (p.o.) administration of MDP complex will be done using round tipped 20G-1&1/2 disposable animal feeding needles (Fisherbrand, Cat# 01-208-87). 200 ul of MDP complex in saline will be slowly released inside the mouth along the cheek lining and mouse will be allowed to swallow the fluid. Complete oral gavage administering the fluid in mouse stomach is not being done due to issues that the procedure may bruise the esophageal lining resulting in a combined injury (bruising + radiation). Combined injury (wound + radiation) has been shown to reduce the survival in mouse over irradiation alone in B6D2F1/J mice that are proposed in this protocol (Kiang and Fukumoto (2014) Health Phys. 2014 Jun; 106(6): 720–726).

Ear punching: Ear punches will be made 2 days prior to the initiation of the experiments. Scissor style ear punch (Kent Scientific, cat# INS500076) will be used to make ear punch. Animals will be identified in the following manner:

Mouse #1-No holes.
Mouse #2-One hole on the right ear.
Mouse #3-One hole on the left ear.
Mouse #4-One hole on each ear.
Mouse #5-Two holes on the right ear.
Mouse #6-Two holes on the left ear.
Mouse #7-Two holes on the right and one hole on the left.
Mouse #8-Two holes on the left and one hole on the right.
Mouse #9-Two holes on each ear.

Mostly numbering from 1 to 5 five will be required and further identification will be done based on the cage number followed by the animal number. A topical application of Neosporin ointment will be applied over the punch before returning animals to their cage.

V.4.4.8. Tissue Sharing: Tissues and samples collected under study will be stored as appropriate and will be available to share if required and useful in reducing any additional animal work.

V.4.5. Study Endpoint:
For Experiment#1-3 study end point will be day 30 and for experiment#4, study end point will be on day 1, 3 and 7 from the day of irradiation. If an animal is found to exhibit significant adverse clinical symptoms such as ruffled fur, weakness, lethargy, difficulty breathing, weight loss of more than 20%, or loss of appetite, are cool to the touch, show inappetance, or diarrhea, such animals will be identified in consultation with the LAM veterinarian and will be humanely euthanized by LAM staff or PI staff. Experimental mice that are found morbid, but not moribund, based on the scoring criteria listed under section VIII, will be allowed to continue in the experimental protocol in order to obtain an optimal number of specimens for evaluation.

V.4.6. Euthanasia: All animals will be humanely euthanized using deep CO_2 anesthesia (flow rate of 10-30% of the chamber volume per minute) followed by cervical dislocation. Animals will be euthanized at the predetermined end point of each experiment except for the animals that are found moribund as described in section V.4.1.2.2 for which euthanasia will be done as required. In experiments requiring terminal blood collection, blood will be collected via cardiac puncture after deep CO2 anasthesia following which cervical dislocation will be performed as described in V.4.4.2.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs and by LAM personnel for each species in this protocol. Animals will be housed in autoclaved cages and will be provided with acidified water. If the cages are found dirty during inspection by the PI staff, they will also change the bedding as needed. Graduated water bottles will be used

to determine the water consumption per cage per day for 10 days after the irradiation procedure.

V.5.1.1. Study Room: Barrier housing is required for ultra clean condition for immunocompromised animals.. Room will be designated by LAM as per the availability of the room.

Building(s) (b)(6) Room Number(s) TBD

V.5.1.2. Special Husbandry Provisions: Animals will be housed and handled according to LAM SOPs for BSL2 handling.

Food Restriction:	Yes		No	X
Fluid Restriction:	Yes		No	X

V.5.1.3. Exceptions: For experiment # 1-4, mice will be group housed except during the irradiation procedure as described above or in the event that their cage mates have had to be removed from the study; under these circumstances they will be housed individually.

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Mice will be checked on daily basis by the LAM veterinarian and PI staff. Routine care such as plentiful chow, water and change of beds will be provided by the DLAM staff. However, LAM staff will be requested (a note will be posted in the room) not to change the water bottles during initial 7 days after irradiation for experiment# 1-4 since water consumption will be monitored by the PI staff.
Mice that show increased distress as determined by the veterinarian and described in section V.4.5 will be removed from the study and will be humanly euthanized by the DLAM or the PI staff as described in the section V.4.6.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays. Additional two checks will be conducted by the PI staff during the heightened observation period as described in section V.4.1.2.2.). Moribund animals meeting the study endpoint criteria in section V.4.5 will be euthanized as per section V.4.6.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM

SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions: None.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Subcutaneous injections, oral administration, euthanasia, blood and tissue collection.	(b)(6)	Staff Scientist, > 12 years of experience with the rodent models. SC injections, 12 years; cervical dislocation 12 years; CO_2 anesthesia 12 years; cardiac blood collection, 10 years, oral gavage: 4 years.	Rodent handling training class 2002 and 2006, USUHS Investigator Training Course (2006)
Injection, euthanasia, blood and tissue collection.		Research associate, > 8 years of experience handling animal samples. SC, 7 yrs, CO_2 anesthesia, 7 yrs; and oral gavage: 7 yrs.	
Injection, euthanasia, blood and tissue collection.		Staff Scientist, 8 years of experience handling animal samples. SC injections, 2 years; cervical dislocation 2 years; CO_2 anesthesia 2 years; and cardiac blood collection, 2 years.	Rodent handling training class 2008, USUHS Investigator Training Course 2007,
Injection, euthanasia, blood and tissue collection, and		Post-doctoral fellow, 8 years of experience handling animal samples.	Rodent handling training class 2008, USUHS Investigator Training Course

Irradiation procedure.	(b)(6)	SC injections, 2 years; cervical dislocation 5 years; CO_2 anesthesia 5 years; and cardiac blood collection, 2 years.	2007, Irradiator user training 2012
Injections, euthanasia, blood and tissue collection, and Irradiation procedure.		Post-doctoral fellow, 5 years of experience handling animal samples. SC injections, 2 years; cervical dislocation 2 years; CO_2 anesthesia 2 years, tail vein nick blood collection, 2 years; and tissue collection, 2 years.	Rodent handling training class held on 08/17/2010, USUHS Investigator Training Course 08/17/2010, Irradiator user training 2012

VII. BIOHAZARDS/SAFETY:

Drs (b)(6) listed in the present IACUC protocol have been trained either as radiation users or as general radiation users on a regular basis and will perform the irradiations. Radiation dosimeters will be worn as required during the irradiation procedure and exposure will be minimized in accordance with ALARA principles of radiation safety. Exposure doses will be monitored for each individual as per the USU radiation biosafety program. All investigators and technicians will use appropriate procedures which will include wearing of mask, bonnet, gown, booties, and gloves during observation of mice and terminal sample collections. All PI staff involved in the study with animal contact will undergo annual health survey. All sharps such as needles, surgical blades etc., will be discarded in the red sharps containers. No recapping of needles will be practiced. All formalin requiring procedures such as storing of tissues in formalin for histology will be performed under a chemical hood with specific filters for handling formalin. Formalin waste will be discarded in clearly labeled containers accordance with the USU chemical waste pickup guidelines. All investigators and technicians are current in their safety training and medical surveillance.

VIII. ENCLOSURES:

Rodent Intervention Score Sheet.

Rodent Intervention Score Sheet		
PARAMETER	**DESCRIPTION**	**SCORE**
Appearance	Normal (coats smooth, eyes/nose clear)	**0**
	Reduced grooming OR minor hunching	**1**

	Ocular/nasal discharge AND/OR rough coat and hunching OR facial edema	**3**
	Emaciated, dehydrated, OR soft stools (fecal matter around anus)	**5***
	Presence of bloody diarrhea	**9**
General behavior	Normal	**0**
	Minor changes – writhe or grimace, slightly less active than baseline	**1**
	Moderately less mobile and alert	**2**
	Ataxia, wobbly, appearing weak	**6***
	Unable to stand	**12**
Respiratory Rate	Normal breathing	**0**
	Increased (double) breathing rate, rapid or shallow	**6**
	Abdominal breathing (gasping +/- open mouth breathing)	**12**
Provoked behavior	Normal	**0**
	Subdued or weak, but moves away when stimulated	**1**
	Subdued even when stimulated (moves away slowly).	**3**
	Unresponsive when stimulated, weak, precomatose	**6***
	Does not right when placed gently on side within 5 seconds, or no response when pinch the paws	**12**
***Notify LAM immediately – may need to euthanize**		
<6 – Normal		
6-9 – Morbid, some pain/distress, monitor at least three times a day.		
>10 - Moribund. Either euthanize or notify LAM. If any single category is at 12, euthanize animal immediately.		

Notes: Mice found to have lost greater than 20% of their body weight (relative to weight at start of experiment) will be brought to the attention of LAM staff or euthanized immediately.

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) 4/21/16

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) 4/21/16

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) 4/21/16

Principal Investigator Signature Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: *Deinococcus radiodurans* Mn^{2+} Complexes: A Revolutionary Approach to Radioprotection (in mice).

C. Principal Investigator: (b)(6)

D. Performing Organization: USUHS, Bethesda, MD.

E. Funding: Defense Threat Reduction Agency.

F. Objective and Approach:

The specific objectives of the proposed study are:

1. Evaluate the efficacy of treatment with the MDP complex on survival after RI and evaluate the underlying cellular changes during the process.
2. Evaluate the efficacy of prophylactic and therapeutic treatment with MDP complex on survival after RI.
3. Evaluate the effect of different doses of radiation exposure on the efficacy of radio-protection by MDP complex treatment.

Recently, (b)(6) have shown the protective efficacy of the peptide (b)(4) isolated from the bacteria *Deinococcus radiodurans* against exposure to sub-lethal radiation exposure. Now, we want to further evaluate its radioprotective potential at higher doses of radiation exposure and at different treatment doses. Additionally, we will explore the underlying mechanism of MDP induced radioprotection *in vivo*.

G. Indexing Terms (Descriptors): *Deinococcus radiodurans*, radioprotection, Manganese(Mn^{2+})-Decapeptide-Phosphate complex, radiation injury, protein protection.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
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BETHESDA, MARYLAND 20814-4712
http://www.usuhs.mil

July 19, 2013

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF PEDIATRICS

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on July 19, 2013:

Animal Protocol Title: "Generation and Characterization of Genetically Engineered Mouse Strains Carrying Setbp1 Mutations"

USUHS Protocol Number: (b)(6)

Expiration Date: July 18, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Generation and characterization of genetically engineered mouse strains carrying Setbp1 mutations

GRANT TITLE (if different from above): Characterization of molecular mechanisms underlying cancer development using mouse models

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Pediatrics, USUHS

EARLIEST ANTICIPATED FUNDING START DATE: 7/16/2013

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6)
Principal Investigator Signature

Pediatrics
Department

(b)(6)
Office/Lab Telephone

5/31/13
Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)
Research Unit Chief / Dept. Head Signature

Chair
Title

(b)(6)
Telephone

5/31/13
Date

Typed Name: (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)
Statistician Signature

PMB
Department

(b)(6)
Telephone

5/23/13
Date

Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)
Attending/Consulting Veterinarian Signature

LAM
Department

(b)(6)
Telephone

6/3/13
Date

Typed Name: (b)(6) VMD

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) Department of Pediatrics

ANIMAL PROTOCOL TITLE:
Generation and characterization of genetically engineered mouse strains carrying Setbp1 mutations

GRANT TITLE (if different from above): Characterization of molecular mechanisms underlying cancer development using mouse models

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): N/A

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS: This study is designed to use mice as a model to characterize the function of a new gene called Setbp1 during normal human development. We will generate genetically engineered mice carrying mutations in Setbp1, which will be used to characterize the effects of these mutations on blood generation and neural development. For some studies, immature blood cells from these mutant mice will be transplanted into normal mice to better characterize their regeneration properties. Transplant recipient mice will be irradiated before transplantation to enhance the establishment of the transplant donor cells in their bone marrow. Irradiation at our proposed dosage is lethal to mice if not treated afterwards. However, these recipient mice in our study will be fully rescued by the subsequent transplantation of donor immature blood cells.

II. BACKGROUND:

II.1. Background: Hematopoietic development is a critical process that starts from the hematopoietic stem cells (HSCs) and produces all essential cell types in the blood [1]. HSC is the only cell type in the system capable of extensive self-renewal. The self-renewal of HSCs is essential for sustaining the entire hematopoietic system, and its deregulation has severe consequences. Decreased HSC self-renewal could cause bone marrow failure while increase in self-renewal could lead to leukemia development. However, the critical factors that control HSC self-renewal are still largely unknown. Setbp1 is a novel transcription factor found in our recent mutagenic screens capable of conferring self-renewal capability to hematopoietic progenitor cells that normally lack such capability [2,3], and was also indicated in human leukemia development [4], suggesting that it may play an important role in the (b)(4) and (b)(4). To test this hypothesis, we generated a (b)(4) a (b)(4) and a (b)(4) allele for Setbp1 in mice. (b)(4) (b)(4) Additional studies have been proposed in this protocol to further characterize this (b)(4) due to Setbp1 loss. The (b)(4) reporter transgene driven

by the endogenous (b)(4) promoter and will be used to characterize the normal cell type specific (b)(4) The knowledge that we learned about the function of Setbp1 in the (b)(4) should also help us gain insights into its involvement in leukemia development.

Preliminary studies on (b)(4) embryos also suggest that (b)(4) may cause defects in the (b)(4) (b)(4) knockout versus wild-type embryos. Therefore, studying the function of Setbp1 in (b)(4) may help uncover new molecular mechanisms (b)(4) cells maintenance, which remains poorly understood. However, (b)(4) (b)(4) preventing us from fully assessing their (b)(4) (b)(4) In order to circumvent this problem, we have generated (b)(4) (b)(4) which express a neural specific Cre recombinase resulting (b)(4) (b)(4)

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:
BRD, NIH RePORTER, and PubMed

II.2.2. Date of Search: 5/18/2013

II.2.3. Period of Search:
BRD: 1998 – 2013
NIH RePORTER: 1988 – 2013
PubMed: 1950 – 5/30/2013

II.2.4. Key Words and Search Strategy: This study is mainly designed to use knockout mouse models to characterize the function of Setbp1 in (b)(4) (b)(4) Therefore, "Setbp1" in combination with "mouse" was used as the search term.

II.2.5. Results of Search:
Four projects were returned on BRD and they are (b)(6) protocols from 2008 and 2009. (b)(6) project entitled "Role of Setbp1 in leukemic stem cell self-renewal" was the only project returned on NIH RePORTER. However, this project focuses on overexpression of *Setbp1* in leukemia development and does not address the function of (b)(4) Two articles[5,6] were returned on PubMed. However, both of them tried to study effects of Setbp1 overexpression and none addressed the normal function of Setbp1 during development using a (b)(4) (b)(4) mouse model.

III. OBJECTIVE\HYPOTHESIS: The objective of this protocol is to understand the role of Setbp1 during development, especially its function in the self-renewal of hematopoietic stem cells and neural stem cells, through characterizing the phenotypes

of mice carrying different mutations of Setbp1. By carrying out this study, we also hope to gain insights into the mechanism through which overexpression of Setbp1 may cause leukemia development.

IV. MILITARY RELEVANCE:

Military personnel on the battlefield could be exposed to radiation or toxic substances that could induce leukemia development later in life. Understanding the mechanisms underlying leukemia development should help find preventive measures and better treatments for this devastating disease. The focus of this study involves characterizing the molecular mechanisms controlling self-renewal of normal hematopoietic stem cells, however deregulation of the same mechanisms is also likely responsible for the uncontrolled self-renewal by malignant stem cells in leukemias. Therefore, accomplishing this study has the potential to identify novel pathways that could be targeted for treating leukemias.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1:

In our previous studies, we have found that (b)(4) (b)(4) (b)(4) We will test in this study whether this defect can be reversed by expression of exogenous Setbp1in more differentiated hematopoietic progenitors. Fetal liver cells from E18.5 (b)(4) will be used to carry out this study. These cells will be infected with a replication incompetent retroviral vector expressing the Setbp1 cDNA or control empty vector, and subsequently transplanted into irradiated B6-LY5.2/NCr recipient mice (7-12 weeks old) along with supporting bone marrow cells from un-irradiated B6-LY5.2/NCr mice. These recipient mice will be aged for 4 months for the assessment of LT-HSC rescue.

Setbp1and empty virus will be collected from culture medium of cell lines designed to package viral particles in ecotropic envelops. After initial infection these viruses are no longer infectious and are replication deficient. Prior to and during infection, these fetal liver cells may be cultured in the presence of recombinant murine growth factors including interleukin-3 (IL-3), IL-6, and stem cell factor (SCF) to stimulate their proliferation (In-vitro procedures only) B6-LY5.2/NCr mice (7-12 weeks old females purchased from (b)(4) will be used as donors for the competing bone marrow cells as well as transplant recipients. These mice are immune-compatible with our (b)(4) (b)(4) mice and no immune rejection between donor and host cells after transplantation is expected. The hematopoietic cells of B6-LY5.2/NCr mice also carry the LY5.2 marker that is used to distinguish them from the donor (b)(4) cells expressing a different LY5.1 marker. Before transplantation, B6-LY5.2/NCr recipients will be irradiated with1,100 rads of total body irradiation from a ^{137}Cs source located at room (b)(6) which eliminates the endogenous HSCs and enables efficient engraftment of the transplanted cells. The mixture of fetal liver cells and supporting bone marrow cells will

be subsequently introduced into each recipient in 200ul volume in PBS by tail vein injection. The dose of irradiation used is expected to be lethal within 10 days in the absence of bone marrow reconstitution by transplanted cells. Following transfer of the hematopoietic cells mice will be monitored for signs of distress at least twice daily. Since, in our past experience, morbidity due to reconstitution failure usually occurs between 7 and 10 days after irradiation, an additional daily inspection will be carried out during this period to catch distressed animals as soon as possible. Any recipients found morbid will be euthanized immediately. Successful recolonization of the recipient bone marrow in transplanted animals results in restoration of constitutive hematopoiesis over the normal life span of the recipient. Past experience has shown that lethally irradiated mice can suffer from infection. To prevent infection, all recipient mice will receive amoxicillin at a concentration of 500ug/ml in the drinking water from 3 days prior to irradiation to 10 days post irradiation.

Each transplantation experiment consists of 2 experimental groups and requires a total of 11 B6-LY5.2/Cr animals as listed below: 1. ko/ko fetal liver cells transduced by Setbp1 virus(5 recipients); 2. ko/ko fetal liver cells transduced by empty virus (5 recipients). One animal will also be needed to provide supporting bone marrow cells for transplantation along with indicated fetal liver cells. In addition, 3 fetal livers will be needed to provide sufficient donor cells for infection and transplantation into 10 recipients. All recipients should survive for at least 6 months after transplantation. Two of the recipients of each group will be euthanized at 2 month and the rest at 4 month after transplantation. Bone marrow will be collected at euthanization for analysis of reconstitution of long-term hematopoietic stem cell compartment by transduced donor cells by FACS. Above described transplantation experiment will need to be repeated 3 times in order to draw any conclusions. Since this fetal liver cell transduction and transplantation procedure contains several sensitive and error prone steps such as preventing stem cell differentiation during in vitro culturing and ensuring their high transduction efficiency, one additional experiment is requested for personnel training and potential experimental failures due to human errors. Therefore, **the total number of B6-LY5.2/NCr mice required for this study is: [11 animals/experiment (5,5,1) x 4 experiments = 44]. A total of 12 (3 donors/experiment x 4 repeats) E18.5 (b)(4) embryos will be needed as fetal liver cell donors.**

Summary of required animal numbers for Experiment 1:

Strains	Experimental groups		Supporting B.M.	Total
	Setbp1 virus	Empty virus		
B6-LY5.2/NCr	20	20	4	44
(b)(4) (liver cell donor)	12			12

V.1.2. Experiment 2:
We will also determine whether Setbp1 is required for known oncogene (b)(4) to induce leukemia. Fetal liver cells from (b)(4) (b)(4) will be used to carry out this study. These cells will be infected with a replication incompetent retroviral vector expressing the (b)(4) cDNA, and subsequently transplanted into irradiated B6-LY5.2/NCr recipient mice (7-12 weeks old)

along with 5 x 10^5/recipient supporting bone marrow cells from un-irradiated B6-LY5.2/NCr mice. Same infection, irradiation, and transplantation steps as described in Experiment 1 will be followed. Recipient mice will be aged for 4 months for the assessment of leukemia development. Recipients for infected (b)(4) cells are expected to develop leukemia in 3 to 4 weeks and are included as positive controls.

Each transplantation experiment consists of 2 experimental groups and requires a total of 11 B6-LY5.2/Cr animals as listed below: 1 (b)(4) fetal liver cells transduced by (b)(4) virus(5 recipients); 2 (b)(4) fetal liver cells transduced by (b)(4) virus (5 recipients). One animal will also be needed to provide supporting bone marrow cells for transplantation along with fetal liver cells. In addition, 4 fetal livers (2 of each genotype) will be needed to provide sufficient donor cells for infection and transplantation. All recipients should survive for at least 6 months after transplantation. Any transplant recipients showing signs of leukemia development including rough coat, listlessness, decreased body weight, and hunched appearance, will be euthanized immediately and fresh tissues including bone marrow, spleen, thymus, liver, and kidney will be collected and examined by flow cytometry, tissue culturing and pathology. Any remaining mice 4 month after transplantation will be euthanized. Above described transplantation experiment will need to be repeated 3 times in order to draw any conclusions. Since this fetal liver cell transduction and transplantation procedure contains several sensitive and error prone steps such as preventing stem cell differentiation during in vitro culturing and ensuring their high transduction efficiency, one additional experiment is requested for personnel training and potential experimental failures due to human errors. Therefore, **required B6-LY5.2/NCr animals: 11 animals/experiment x 4 experiments = 44 mice. A total of 16 (4 donors/experiment x 4 experiments (b)(4) (b)(4) embryos will be needed as fetal liver cell donors.**

Summary of required animal numbers for Experiment 4:

Strains	Experimental groups (b)(4)		Supporting B.M.	Total
B6-LY5.2/NCr	20	20	4	44
(b)(4) (liver cell donor)	8	8		16

V.1.3. Experiment 3:

We will also determine whether Setbp1 is required for the maintenance of leukemia induced by (b)(4) For this purpose, bone marrow progenitors from (b)(4) mice (2-4 months old) carrying a (b)(4) allele of Setbp1 (b)(4) (b)(4) will be infected with the same (b)(4) virus described in Experiment 2, and subsequently transplanted into irradiated B6-LY5.2/NCr recipient mice (7-12 weeks old) along with 5 x 10^5 supporting bone marrow cells from un-irradiated B6-LY5.2/NCr mice. Once leukemia develops in these mice, they will be euthanized to purify leukemia stem cells ($lin^-Sca\text{-}1^+c\text{-}kit^+$) from their bone marrow. These leukemia stem cells will be subsequently transplanted into irradiated secondary recipient mice (B6-LY5.2/NCr) along with 5 x 10^5 supporting bone marrow cells from un-irradiated B6-LY5.2/NCr mice. At 2 weeks after transplantation, all secondary recipients will be injected with poly I:C, which (b)(4) leukemia

cells and no changes in [(b)(4)] leukemia cells, and subsequently aged for assessment of leukemia development. All retroviral infection, irradiation, transplantation, and monitoring steps will be carried out as described in Experiment 1. Any remaining mice 4 months after transplantation will be euthanized.

In order to achieve statistical significance, 3 primary leukemias of each genotype [(b)(4)] will be tested. To generate these leukemias, 20 [(b)(4)] [(b)(4)] donor mice (10 each genotype) will be needed to extract sufficient bone marrow for infection. 2 additional B6-LY5.2/NCr mice will be needed to provide supporting bone marrow cells. **Therefore, a total of 8 B6-LY5.2/NCr mice (6 as recipients and 2 as supporting marrow donors) and 20 [(b)(4)] mice are needed for leukemia generation.** For secondary transplantations to assess leukemia maintenance, each leukemia will be transplanted into 5 secondary recipients and 6 additional B6-LY5.2/NCr will be needed to provide supporting bone marrow cells. Therefore, **a total of 36 B6-LY5.2/NCr mice (5 recipients/leukemia x 6 leukemias + 6 supporting marrow donors) are required for secondary transplantations.**

Summary of required animal numbers for Experiment 3:

Strains	Experimental groups [(b)(4)]		Supporting B.M.	Total
B6-LY5.2/NCr	18	18	8	44
[(b)(4)] (BM donor)	10	10		20

V.1.4. Experiment 4:

Our previous studies suggest that [(b)(4)] [(b)(4)] To test this idea, [(b)(4)] will be challenged with 5-FU treatments in a competitive reconstitution assay in comparison to wild-type HSCs. The 5-FU treatments deplete the number of more mature progenitor cells in the bone marrow and induce proliferative stress on HSCs. Reduced reconstitution capability of Setbp1 null cells after the treatments will suggest that Setbp1 help protect HSCs under stressful conditions. For this purpose, 5 x 10^5 bone marrow cells from [(b)(4)] mice (2-4 months old) carrying an polyinosinic:polycytidylic acid (poly I:C) inducible [(b)(4)] will be mixed with 5 x 10^5 competing wild-type bone marrow cells from B6-LY5.2/NCr and then transplanted into lethally-irradiated B6-LY5.2/NCr recipient mice. [(b)(4)] will be induced in the transplanted [(b)(4)] cells by poly I:C injections of recipient mice one month after transplantation. Two weeks after poly I:C injections, all recipients will receive two intraperitoneal injections of 5-fluorouracil (5-FU) at a dosage of 150 mg/kg with a 2-week interval. This dosage of 5-FU is not expected to cause animal morbidity and illness[15]. Reconstitution by the donor cells will be determined at one week post poly I:C injections and at 1, 2, and 4 months after the last 5-FU injection by retro-orbital bleeding and FACS. All animals will be euthanized after the last retro-orbital bleeding, and hematopoietic tissues including bone marrow, thymus, and spleen will be collected after euthanasia and analyzed by FACS. Same calculation of animal numbers as in Experiment 1 applies here. 11 B6-LY5.2/NCr mice will be required for each

transplantation experiment containing 2 experimental groups (one group for each of the 2 genotypes, 5 animals per group, 1 competing marrow donor) and 3 experiments in total will be conducted. **The minimum number of B6-LY5.2/NCr mice required for this study is: [11 animals/experiment (5,5,1) x 3 experiments = 33]. A total of 6 (b)(4) mice (1 donor/ transplanted group x 2 groups x 3 repeats) will be needed as bone marrow cell donors.**

Summary of required animal numbers for Experiment 4:

Strains	Experimental groups (b)(4)		Competing B.M.	Total
B6-LY5.2/NCr	15	15	3	33
(b)(4) (BM donor)	3	3		6

V.1.5. Experiment 5:

In order to understand the mechanisms through which Setbp1 (b)(4) (b)(4) we will carry out expression profiling studies to identify targets of (b)(4) Since Setbp1 deletion in (b)(4) mice induced by poly I:C is not specific to the hematopoietic system, to exclude potential effects from Setbp1 deletion in the microenvironment, we will first transplant their bone marrow cells into irradiated B6-LY5.2/NCr recipient mice (5 x 10^6 cell/recipient) and then (b)(4) (b)(4) by injecting the recipient mice with poly I:C. at 4 weeks after transplantation. Irradiation, transplantation, and monitoring of recipients will be carried out as described in Experiment 1. The (b)(4) will be purified from the recipient mice at 2 weeks after the last poly I:C injection by FACS and used for RNA extraction for microarray-based expression profiling experiments. Due to extreme scarcity of (b)(4) in the bone marrow (0.01-0.02%), **10 recipient mice for each genotype (b)(4) will be needed** to obtain sufficient cells for expression profiling in one experiment. As such, **2 (b)(4) mice of each genotype will be needed to provide sufficient donor cells** for transplantation in each experiment. In order to achieve statistical significance, such experiment will be repeated 3 times. **Total required B6-LY5.2/NCr animals: 20 animals/experiment x 3 experiments = 60 mice. A total of 12 (2 donors/genotype x 2 genotypes x 3 experiments) (b)(4) mice will be needed as bone marrow donors.**

Summary of required animal numbers for Experiment 5:

Strains	Experimental groups (b)(4)		Total
B6-LY5.2/NCr	30	30	60
(b)(4) (BM donor)	6	6	12

V.1.6. Experiment 6:

Preliminary studies on Setbp1 null E18.5 embryos suggest that Setbp1 loss may cause (b)(4) (b)(4) are significantly reduced in size in

knockout versus wild-type embryos. Therefore, studying the function of Setbp1 in [redacted] may help uncover new molecular mechanisms controlling [redacted] which remains poorly understood.

For this experiment, age- and sex-matched littermates of [redacted] mice consisting of two genotypes: [redacted] will be euthanized at approximately 2 months of age and their brains will be used to characterize neural stem cell function in the absence of Setbp1. [redacted] will be collected from these animals after euthanasia and subjected to 4 different types of experiments including neural stem cell isolation for neurosphere assay, RNA and protein extraction from neurogenic regions, immunohistochemistry analysis for different neural markers, and also histological examinations. Each type of experiment will need to be repeated on 5 animals per genotype to reach any conclusions. So the minimum animal number for each genotype is: 4 experiment types x 5 animals/genotype = 20. In addition, 3 animals for each genotype are also requested as a preventive measure for potential experimental failures due to unpredictable factors such as human error and equipment breakdowns, and also for technique development/personnel training. Therefore, **for these studies, the requested animal number is: (5 animals/genotype x 2 genotypes x 4 experimental evaluations) + 3 extra mice x 2 genotypes = 46 mice.** In addition, 15 animals of each genotype will also be aged for 12 months to study long-term effect of Setbp1 deletion in the neural system. **The total requested animal number is: 46 mice + (15 animals/genotype x 2 genotypes) = 76 mice.**

Summary of required animal numbers for Experiment 6:

Strains	Experimental groups [redacted]		Total
[redacted]	38	38	76

V.1.7. Experiment 7:

Seb-LacZ mice of 2 genotypes [redacted] will be used to determine the normal expression pattern of Setbp1 [redacted] Hematopoietic tissues including bone marrow, spleen, and thymus will be collected from these animals after euthanasia and analyzed by FACS and also histological examinations. Each type of experiment will need to be repeated on 5 animals per genotype to reach any conclusions. So the minimum animal number for each genotype is: 2 experiment types x 5 animals/genotype = 10. In addition, 3 animals for each genotype are also requested as a preventive measure for potential experimental failures due to unpredictable factors such as human error and equipment breakdowns, and also for technique development/personnel training. **The total requested animal number is: [5 animals/genotype x 2 genotypes x 2 experimental evaluations + 3 extra mice x 2 genotypes = 26 mice].**

Summary of required animal numbers for Experiment 7:

Strains	Experimental groups [redacted]	Total

Seb-LacZ	13	13	26

V.2. Data Analysis: For all 7 experiments, gene expression, contribution of cell populations, colony/cell counts will be compared across genotypes using analysis of variance (ANOVA), student's t test, or nonparametric tests as appropriate. A sample size of 5 in each group will have 80% power to detect a difference of 2 standard deviations between groups using a 2-group t test with a 5%, two-sided significance level.
For aging studies in Experiment 2-4, log-rank tests will be used to compare time to development of sickness such as leukemia across groups. When the sample size in each group is 15, a 5% two-sided log-rank test for equality of survival curves will have 80% power to detect a difference in proportion surviving of at least 54 percentage points (e.g. 99% vs. 45% surviving)

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: Tissue culture was considered, however hematopoietic development is a complex process that requires proper interactions among different tissue and cell types within the context of a whole animal, and currently there is no tissue culture system capable of recapitulating this environment [8]. In addition, hematopoietic cell lines used in tissue culture studies are different from normal hematopoietic cells in that they have acquired mutations that allow them to replicate indefinitely in culture, and results from these studies may not apply to normal cells. Computer modeling was considered as well, but the regulation of hematopoietic development is still largely unknown and there is no system available in the hematopoiesis field that can be used to predict gene function and consequences of gene mutations.

V.3.2. Animal Model and Species Justification: I have chosen to use mice for my studies because they are genetically well-characterized mammalian organism that has been shown to have a very similar hematopoietic system to that of humans, and more and more studies have been carried out in mice to understand human hematopoiesis and leukemia development [9-11]. In addition, there are many tools available in mice but not in other mammalian models to genetically alter the germline and study the consequences of the mutation on multiple organ systems simultaneously. I also happen to have extensive experiences working with mouse hematopoietic system as a research fellow in the Mouse Cancer Genetics Program at National Cancer Institute. Lower organisms such as Zebrafish and Drosophila were also considered, however both species are very different from mammals and do not represent good models for studying human hematopoiesis.

V.3.3. Laboratory Animals

Species #1 **Species #2**

V.3.3.1. **Genus & Species:** Mus musculus

V.3.3.2. **Strain/Stock:** Seb-LacZ
Seb-KO
(b)(4)
B6-LY5.2/NCr

V.3.3.3. **Source/Vendor:** In-house (Seb-LacZ, Seb-KO, (b)(4) (b)(4) (B6-LY5.2/NCr)

V.3.3.4. **Age:** B6-LY5.2/NCr (7-12 weeks) (b)(4) (2 months); Seb-KO (E18.5); (b)(4) 1 mice (2-4 months); (b)(4) mice (2-12 months)

V.3.3.5. **Weight:** N/A

V.3.3.6. **Sex:** Male: 84
Female: 309

V.3.3.7. **Special Considerations:** Specific pathogen free

V.3.4. **Number of Animals Required (by Species):** Mice: 393 (B6-LY5.2/NCr, 225; (b)(4) 28; (b)(4) (b)(4) 38; (b)(4) (b)(4) 76; Seb-LacZ, 26)

V.3.5. **Refinement, Reduction, Replacement (3 Rs):**

V.3.5.1. **Refinement:**
All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels. All animals subjected to irradiation will be treated with amoxicillin 3 days prior to irradiation to 10 days post irradiation to reduce distress caused by potential infection.

Animals observed to be ill or showing signs of pain or distress including 30% reduction in body weight (compared to before transplantation), hunched posture, ruffled fur, and decreased activity will be evaluated by veterinary staff to be treated or be euthanized for relief of suffering.

V.3.5.2. Reduction: In order to reduce the animal numbers for this study, experiments have been designed to include tissue sharing among different experimental procedures including DNA extraction, RNA extraction, and protein extraction. Also, sample size calculations were performed to use least number of animals to achieve statistical significance.

V.3.5.3. Replacement: Tissue culture and computer models were considered but not adopted. Detailed explanations are provided in V.3.1.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	187	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	206	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: For retro-orbital bleeding, 3.5-4% Isoflurane will be applied before the procedure through induction chamber of an anesthesia machine at LAM until animals are fully anesthetized (judging by lack of movement and decrease in respiratory rate to about once every 4 seconds and a negative pedal withdrawal reflex).

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: N/A

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures: N/A

V.4.1.3.1. Sources Searched: N/A

V.4.1.3.2. Date of Search: N/A

V.4.1.3.3. Period of Search: N/A

V.4.1.3.4. Key Words of Search: N/A

V.4.1.3.5. Results of Search: N/A

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: Whole body irradiation of recipient mice is essential for successful mouse bone marrow transplantation as it is the only way to efficiently deplete host bone marrow stem cells and to greatly increase donor cell engraftment in mice.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: Transplantation of fetal liver and bone marrow cells will be carried out by one time tail vein injection of cells resuspended in 200ul of PBS using 28 Gauge x ½ inch needles. Since transplanted cells are from mice congenic to the recipients, no immune rejection is expected after transplantation. Injection of hematopoietic cells through the tail vein is not known to cause any toxic effects to mice. All cells for injection will be obtained from mice housed in USU colony. Injection of all biological materials will be in accord with USU IACUC Policy #23 on the use of biological in experimental animals.

Transplant recipient mice in Experiment 3, 4 and 5 will also receive 5 intra-peritoneal injections with 2 day intervals of 200 ul poly I:C (a chemical used to induce Cre gene expression) at a concentration of 2 ug/ul diluted in sterile phosphate buffered saline. 28 Gauge x ½ inch needles will be used for the injections. Injection of poly I:C at this dosage is safe for mice[12].

V.4.4.2. Biosamples: Retro-orbital bleeding is chosen for the protocol based on the blood sample size required for our study and available in-house expertise. Given the relatively large sample size (200 ul of blood from each animal each time) that is needed for our study to perform FACS analysis, 2 other blood collection procedures including from the saphenous vein and jugular vein were considered as suitable alternatives.[13,14] Saphenous bleeding is the only procedure that is less painful than retro-oribal bleeding and does not require anesthesia. However, this procedure requires extensive experience and has not been performed on mice by any LAM technicians previously. In contrast, retro-orbital bleeding has been routinely carried out by staff in my laboratory under previously approved protocols and will be done under isoflurane anesthesia.

In this protocol, 200ul blood will be obtained each time by retro-orbital bleeding at one week after poly I:C injections and at 1, 2 and 4 month after last 5-FU injection as described in Experiment 4. IACUC policy on retro-orbital bleeding (POLICY 001) will be strictly followed when performing this procedure. Mice will be anesthetized with Isoflurane. When the animal is no longer moving voluntarily it is placed on a paper towel. The forefinger of the operator's nondominant hand is used to pull the facial skin taut and cause the eyes to protrude slightly while the skin at the back of the neck is grasped by the thumb and remaining fingers to restrain. Breathing and color are monitored throughout the procedure to ensure that the restraint does not compromise the airway. The tip of a capillary tube is gently inserted below the eye at approximately a 45 degree angle into the space between the globe and the lower eyelid. When the tip of the pipette contacts the boney floor of the orbit it is gently twisted between thumb and forefinger to rupture the capillary sinus. Blood is allowed to flow by capillary action into the pipette. Care is taken not to take any more blood than is needed. At the conclusion of the blood withdrawal tension on the animal is released and a gauze pad is gently pressed over the eye for about 30 seconds until the bleeding has stopped. A drop of tetracaine is added in the eye to reduce any postprocedural discomfort that may occur. Normal color and respiration are reconfirmed and the animal is returned to its cage for recovery. Alternate eyes are used for successive bleeds. A maximum of two bleeds per eye (four bleeds total) will be done. In addition, hematopoietic tissues including bone marrow, spleen, thymus, lymph nodes and control tissue brain, lung, and kidney will be extracted after euthanasia.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Cage cards will be used to distinguish different study groups.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: Recipient B6-LY5.2/NCr mice will be irradiated on the day of transplantation with1100 rads of total body irradiation from a ^{137}Cs source located at room (b)(6) Mice will be transported and irradiated at room temperature in irradiator disks (purchased from Braintree Scientific) that maintain a filter protected sterile environment and hold up to 20 mice at each time. The entire process will last no more than 30 minutes and during which time no food or water will be provided for the animals.

Hematopoietic cells resuspended in PBS in a volume of 200 ul will be introduced by tail vein injection. Following transfer of the hematopoietic cells mice will be monitored twice daily and three times between days 7-10 after irradiation for signs of distress including rough coat, listlessness, 30% reduction in body weight (compared to before transplantation), or hunched posture. Any recipients displaying any combination of two or more signs of distress above will be euthanized immediately by PI staff. The dose of irradiation used is expected to be lethal within 10 days in the absence of bone marrow reconstitution by transplanted cells. Successful recolonization of the recipient bone marrow in transplanted animals results in restoration of constitutive hematopoiesis over the normal life span of the recipient. Past experience has shown that lethally irradiated mice can suffer from infection. To prevent infection, all recipient mice will receive amoxicillin at a concentration of 0.5mg/ml in the drinking water from 3 days prior to irradiation to 10 days post irradiation, and sterile feed and autoclaved caging will be provided.

V.4.4.8. Tissue Sharing: Tissues may be shared with other investigators upon request.

V.4.5. Study Endpoint:
The study endpoint for mice is between 2 to 16 months of age as specified in the experimental design and general procedures section. Animals could be euthanized earlier due to leukemia development or engraftment failure of transplanted hematopoietic cells after irradiation. To capture and euthanize animals in distress as soon as possible, irradiated mice will be monitored at least twice daily by PI staff. Since morbidity due to bone marrow failure usually occurs between 7 and 10 days after irradiation in our past experience, one additional inspection daily will be added during this period to catch any possible distressed animals. Signs of these conditions include rough coat, listlessness, 30% reduction in body weight (compared to before transplantation), or hunched posture. Any animals displaying any combination of two or more symptoms will be euthanized immediately.

V.4.6. Euthanasia:
Animals under this protocol will be euthanized by research personnel. Mice will be euthanized using cylinderized CO2. These animals will be placed in a cage or chamber that is not crowded and exposed to a mix of CO2 and oxygen. The CO2 flow rate will displace at least 20% of the chamber volume per minute. Flow will continue for at least one minute after visible sign of death. CO2 euthanasia will be followed by cervical dislocation to ensure death. Gestation day 18.5 embryos will be first anesthetized by placing them in a Petri dish that is placed on top of ice for 15 minutes and then euthanized by decapitation using surgical scissors. Both procedures are consistent with the 2013 AVMA Guidelines for the Euthanasia of Animals.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. **Study Room:**

Building(s) _____ Room Number(s) (b)(6)

V.5.1.2. **Special Husbandry Provisions:**

Sterile micro-isolator cages, feed (9% fat), and bedding will be used.

Food Restriction: Yes _____ No X

Fluid Restriction: Yes _____ No X

V.5.1.3. **Exceptions:** N/A

V.5.2. **Veterinary Medical Care:**

V.5.2.1. **Routine Veterinary Medical Care:**

Animals will be observed at least once daily by Center for LAM personnel. PI group will also observe the colony twice daily except during the period 7-10 days post-irradiation during which they will be monitored three times daily. Animals or pups observed to be ill or showing signs of pain or distress including weight loss, hunched posture, ruffled fur, and decreased activity will be evaluated by veterinary staff to be treated or be euthanized. PI should be contacted for potential tissue collection needs if sick animals are to be euthanized by LAM personnel.

V.5.2.2. **Emergency Veterinary Medical Care:** All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays. Sick or moribund animal will be evaluated by veterinary staff to be treated or be euthanized. PI should be contacted for potential tissue collection needs if euthanasia is necessary. Euthanasia by LAM personnel should proceed if PI can not be reached.

V.5.3. **Environmental Enrichment:**

V.5.3.1. **Enrichment Strategy:** Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. **Enrichment Restrictions:** N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Animal monitoring, euthanasia, tissue	(b)(6)	PI, 12 years of experience	Animal Care and Use Introductory

extraction, intra-peritoneal injection			Training Course at NCI, 2001; Animal Care and Use Training Course at NCI, 2007; Investigator training course at USUHS, 2007.
Tail vein injection, animal monitoring, Euthanasia, tissue extraction	(b)(6)	Technician, 5 years of experience	Investigator training course at USUHS, 2007; Rodent Handling Training by PI, 2007
Euthanasia, tissue extraction	(b)(6)	Technician, 5 years of experience	Investigator training course at USUHS, 2007; Rodent Handling course at USUHS, 2007

VII. BIOHAZARDS/SAFETY: N/A

VIII. ENCLOSURES:

References:

1. Morrison SJ, Uchida N, Weissman IL. The biology of hematopoietic stem cells. Annu Rev Cell Dev Biol. 1995;11:35-71.

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13. Hem A, Smith AJ, Solberg P. 1998. Saphenous vein puncture for blood sampling of the mouse, rat, hamster, gerbil, guinea pig, ferret and mink. Lab Anim. 32:364-8.

14. Hoff J. 2000. Methods of blood collection in the mouse. Lab Anim. 29:47-53.

15. Lerner C, Harrison DE. 1990. 5-Fluorouracil spares hematopoietic stem cells responsible for long-term repopulation. Exp Hematol. 18:114-8.

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 5/31/13

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 5/31/13

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL** or **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature

5/31/13

Date

X. PROTOCOL ABSTRACT:

A. **Animal Protocol Number:**

B. **Animal Protocol Title**: Generation and characterization of genetically engineered mouse strains carrying Setbp1 mutations

C. **Principal Investigator:** (b)(6) Department of Pediatrics

D. **Performing Organization**: Uniformed Services University of the Health Sciences

E. **Funding**: Department of Pediatrics

F. **Objective and Approach**: The objective of this protocol is to characterize the function of a novel gene Setbp1 during normal mammalian development, with a focus on hematopoietic development. Different genetically engineered mutant mouse strains for Setbp1 will be generated. Developmental defects in these mutant animals will be characterized comparing to wild-type littermates. The three R's (reduction, replacement, and refinement) of animal usage will be employed.

G. **Indexing Terms (Descriptors)**: Animals, mice, Setbp1, and knockout

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

August 5, 2013

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF PEDIATRICS

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on August 5, 2013:

Animal Protocol Title: "Generation and Characterization of Genetically Engineered Mouse Strains Carrying Prdm16 Mutations"

USUHS Protocol Number: (b)(6)

Expiration Date: August 4, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET
IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Generation and characterization of genetically engineered mouse strains carrying Prdm16 mutations

GRANT TITLE (if different from above): Characterization of molecular mechanisms underlying cancer development using mouse models

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Pediatrics, USUHS

EARLIEST ANTICIPATED FUNDING START DATE: 7/16/2013

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6) — Principal Investigator Signature

Pediatrics — Department

(b)(6) — Office/Lab Telephone

5/31/13 — Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) — Research Unit Chief / Dept. Head Signature

Chair — Title

(b)(6) — Telephone

5/31/13 — Date

Typed Name: (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) — Statistician Signature

PMB — Department

(b)(6) — Telephone

5/23/13 — Date

Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) — Attending/Consulting Veterinarian Signature

LAM — Department

(b)(6) — Telephone

6/3/13 — Date

Typed Name: (b)(6) VMD

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR (b)(6) Department of Pediatrics

ANIMAL PROTOCOL TITLE:
Generation and characterization of genetically engineered mouse strains carrying Prdm16 mutations

GRANT TITLE (if different from above): Characterization of molecular mechanisms underlying cancer development using mouse models

USUHS PROJECT NUMBER:

CO-INVESTIGATOR(S): N/A

TECHNICIANS(S): (b)(6)

I. **NON-TECHNICAL SYNOPSIS:** This study is designed to use mice as a model to characterize the function of a new gene called Prdm16 during normal human development. We will generate genetically engineered mice carrying mutations in Prdm16, which will be used to characterize the effects of these mutations on blood generation. For some studies, immature blood cells from these mutant mice will be transplanted into normal mice to better characterize their regeneration properties. Transplant recipient mice will be irradiated before transplantation to enhance the establishment of the transplant donor cells in their bone marrow. Irradiation at our proposed dosage is lethal to mice if not treated afterwards. However, these recipient mice in our study will be fully rescued by the subsequent transplantation of donor immature blood cells.

II. BACKGROUND:

II.1. Background: Hematopoietic development is a critical process that starts from the hematopoietic stem cells (HSCs) and produces all essential cell types in the blood [1]. HSC is the only cell type in the system capable of extensive self-renewal. The self-renewal of HSCs is essential for sustaining the entire hematopoietic system, and its deregulation has severe consequences. Decreased HSC self-renewal could cause bone marrow failure while increase in self-renewal could lead to leukemia development. However, the critical factors that control HSC self-renewal are still largely unknown. Prdm16 is a novel transcription factor found in our recent mutagenic screens capable of conferring self-renewal capability to hematopoietic progenitor cells that normally lack such capability [2,3], and was also indicated in human leukemia development [4], suggesting that it may play an important role in the (b)(4) and (b)(4) To test this hypothesis, we generated a (b)(4) (b)(4) for Prdm16 in mice. (b)(4) (b)(4) (b)(4) Additional studies have been proposed in this protocol to further characterize this (b)(4) due to Prdm16 loss. The knowledge that we learned about the function of Prdm16 in the (b)(4)

(b)(4) should also help us gain insights into its involvement in leukemia development.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:
BRD, NIH RePORTER, and PubMed

II.2.2. Date of Search: 5/30/2013

II.2.3. Period of Search:
BRD: 1998 – 2013
NIH RePORTER: 1988 – 2013
PubMed: 1950 – 5/30/2013

II.2.4. Key Words and Search Strategy: This study is mainly designed to use knockout mouse models to characterize the function of Prdm16 in normal hematopoietic stem cells. Therefore, "Prdm16" in combination with "mice" and "hematopoietic" were used as the search term.

II.2.5. Results of Search:
Two projects were returned on BRD and they are (b)(4) protocols from 2008 and 2009. A total of 2 projects were returned on NIH RePORTER, entitled respectively "Hematopoietic stem cells and longevity" and "Mechanism of action of Prdm16 in hematopoietic stem cells". While the first project investigates the difference of different naturally occurring alleles of Prdm16 in affecting HSC function, the second project is the only one studying Prdm16 loss of function in mice. However, the mouse model employed is a germline knockout model which only allows them to study fetal liver hematopoietic stem cells as Prdm16 knockout mice die shortly after birth. (b)(4) (b)(4) (b)(4) A total of 9 articles were returned on PubMed [15-23]. Only two of them studied Prdm16 loss of function in mice[18,19]. However, both used the same Prdm16 germline knockout model and (b)(4) that we created.

III. OBJECTIVE\HYPOTHESIS: The objective of this protocol is to understand the role of Prdm16 during hematopoietic development, especially its function in the self-renewal of hematopoietic stem cells, through characterizing the phenotypes of mice carrying different mutations of Prdm16. By carrying out this study, we also hope to gain insights into the mechanism through which overexpression of Prdm16 may cause leukemia development.

IV. MILITARY RELEVANCE:
Military personnel on the battlefield could be exposed to radiation or toxic substances that could induce leukemia development later in life. Understanding the mechanisms underlying leukemia development should help find preventive measures and better treatments for this devastating disease. The focus of this study involves characterizing the molecular mechanisms controlling self-renewal of normal hematopoietic stem cells,

however deregulation of the same mechanisms is also likely responsible for the uncontrolled self-renewal by malignant stem cells in leukemias. Therefore, accomplishing this study has the potential to identify novel pathways that could be targeted for treating leukemias.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1:

In our previous studies, we have found that (b)(4) (b)(4) (b)(4) We will test in this study whether this defect can be reversed by expression of exogenous Prdm16in more differentiated hematopoietic progenitors. Fetal liver cells from E18.5 (b)(4) will be used to carry out this study. These cells will be infected with a replication incompetent retroviral vector expressing the Prdm16 cDNA or control empty vector, and subsequently transplanted into irradiated B6-LY5.2/NCr recipient mice (7-12 weeks old) along with supporting bone marrow cells from un-irradiated B6-LY5.2/NCr mice. These recipient mice will be aged for 4 months for the assessment of LT-HSC rescue.

Prdm16and empty virus will be collected from culture medium of cell lines designed to package viral particles in ecotropic envelops. After initial infection these viruses are no longer infectious and are replication deficient. Prior to and during infection, these fetal liver cells may be cultured in the presence of recombinant murine growth factors including interleukin-3 (IL-3), IL-6, and stem cell factor (SCF) to stimulate their proliferation (In-vitro procedures only) B6-LY5.2/NCr mice (7-12 weeks old females purchased from (b)(4) will be used as donors for the competing bone marrow cells as well as transplant recipients. These mice are immune-compatible with our (b)(4) (b)(4) mice and no immune rejection between donor and host cells after transplantation is expected. The hematopoietic cells of B6-LY5.2/NCr mice also carry the LY5.2 marker that is used to distinguish them from the donor (b)(4) cells expressing a different LY5.1 marker. Before transplantation, B6-LY5.2/NCr recipients will be irradiated with1,100 rads of total body irradiation from a ^{137}Cs source located at room (b)(6) which eliminates the endogenous HSCs and enables efficient engraftment of the transplanted cells. Supporting bone marrow cells will be extracted from un-irradiated B6-LY5.2/NCr mice after their euthanization. The mixture of fetal liver cells and supporting bone marrow cells will be subsequently introduced into each recipient in 200ul volume in PBS by tail vein injection. The dose of irradiation used is expected to be lethal within 10 days in the absence of bone marrow reconstitution by transplanted cells. Following transfer of the hematopoietic cells mice will be monitored for signs of distress at least twice daily. Since, in our past experience, morbidity due to reconstitution failure usually occurs between 7 and 10 days after irradiation, an additional inspection will be carried out during this period to catch distressed animals as soon as possible. Any recipients found morbid will be euthanized immediately. Successful recolonization of the recipient bone marrow in transplanted animals results

in restoration of constitutive hematopoiesis over the normal life span of the recipient. Past experience has shown that lethally irradiated mice can suffer from infection. To prevent infection, all recipient mice will receive amoxicillin at a concentration of 500ug/ml in the drinking water from 3 days prior to irradiation to 10 days post irradiation.

Each transplantation experiment consists of 2 experimental groups and requires a total of 11 B6-LY5.2/Cr animals as listed below: 1. ko/ko fetal liver cells transduced by Prdm16 virus(5 recipients); 2. ko/ko fetal liver cells transduced by empty virus (5 recipients). One animal will also be needed to provide supporting bone marrow cells for transplantation along with indicated fetal liver cells. In addition, 3 fetal livers will be needed to provide sufficient donor cells for infection and transplantation into 10 recipients. All recipients should survive for at least 6 months after transplantation. Two of the recipients of each group will be euthanized at 2 month and the rest at 4 month after transplantation. Bone marrow will be collected at euthanization for analysis of reconstitution of long-term hematopoietic stem cell compartment by transduced donor cells by FACS. Above described transplantation experiment will need to be repeated 3 times in order to draw any conclusions. Since this fetal liver cell transduction and transplantation procedure contains several sensitive and error prone steps such as preventing stem cell differentiation during in vitro culturing and ensuring their high transduction efficiency, one additional experiment is requested for personnel training and potential experimental failures due to human errors. Therefore, **the total number of B6-LY5.2/NCr mice required for this study is: [11 animals/experiment (5,5,1) x 4 experiments = 44]. A total of 12 (3 donors/experiment x 4 repeats) E18.5 (b)(4) fetuses will be needed as fetal liver cell donors.**

Summary of required animal numbers for Experiment 1:

Strains	Experimental groups		Supporting B.M.	Total
	Prdm16 virus	Empty virus		
B6-LY5.2/NCr	20	20	4	44
(b)(4) (liver cell donor)	12			12

V.1.2. Experiment 2:

We will also determine whether Prdm16 is required for known oncogene (b)(4) to induce leukemia. Fetal liver cells from (b)(4) (b)(4) will be used to carry out this study. These cells will be infected with a replication incompetent retroviral vector expressing the (b)(4) cDNA, and subsequently transplanted into irradiated B6-LY5.2/NCr recipient mice (7-12 weeks old) along with 5 x 10^5/recipient supporting bone marrow cells from un-irradiated B6-LY5.2/NCr mice. Same infection, irradiation, and transplantation steps as described in Experiment 1 will be followed. Recipient mice will be aged for 4 months for the assessment of leukemia development. Recipients for infected (b)(4) cells are expected to develop leukemia and are included as positive controls.

Each transplantation experiment consists of 2 experimental groups and requires a total of 11 B6-LY5.2/Cr animals as listed below: 1. (b)(4) fetal liver cells transduced by (b)(4) virus(5 recipients); 2 (b)(4) fetal liver cells transduced by (b)(4) virus (5

recipients). One animal will also be needed to provide supporting bone marrow cells for transplantation along with fetal liver cells. In addition, 4 fetal livers (2 of each genotype) will be needed to provide sufficient donor cells for infection and transplantation. All recipients should survive for at least 6 months after transplantation. Any transplant recipients showing signs of leukemia development including rough coat, listlessness, decreased body weight, and hunched appearance, will be euthanized immediately and fresh tissues including bone marrow, spleen, thymus, liver, and kidney will be collected and examined by flow cytometry, tissue culturing and pathology. Any remaining mice 4 month after transplantation will be euthanized. Above described transplantation experiment will need to be repeated 3 times in order to draw any conclusions. Since this fetal liver cell transduction and transplantation procedure contains several sensitive and error prone steps such as preventing stem cell differentiation during in vitro culturing and ensuring their high transduction efficiency, one additional experiment is requested for personnel training and potential experimental failures due to human errors. Therefore, **required B6-LY5.2/NCr animals: 11 animals/experiment x 4 experiments = 44 mice. A total of 16 (4 donors/experiment x 4 experiments) (b)(4) (b)(4) embryos will be needed as fetal liver cell donors.**

Summary of required animal numbers for Experiment 4:

Strains	Experimental groups (b)(4)		Supporting B.M.	Total
B6-LY5.2/NCr	20	20	4	44
(b)(4) (liver cell donor)	8	8		16

V.1.3. Experiment 3:

We will also determine whether Prdm16 is required for the maintenance of leukemia induced by (b)(4) For this purpose, bone marrow progenitors from (b)(4) mice (2-4 months old) carrying a (b)(4) allele of *Prdm16* (b)(4) (b)(4) will be infected with the same (b)(4) virus described in Experiment 2, and subsequently transplanted into irradiated B6-LY5.2/NCr recipient mice (7-12 weeks old) along with 5 x 10^5 supporting bone marrow cells from un-irradiated B6-LY5.2/NCr mice. Once leukemia develops in these mice, they will be euthanized to purify leukemia stem cells ($lin^-Sca\text{-}1^+c\text{-}kit^+$) from their bone marrow. These leukemia stem cells will be subsequently transplanted into irradiated secondary recipient mice (B6-LY5.2/NCr) along with 5 x 10^5 supporting bone marrow cells from un-irradiated B6-LY5.2/NCr mice. At 2 weeks after transplantation, all secondary recipients will be injected with poly I:C, which (b)(4) leukemia cells and no changes in (b)(4) leukemia cells, and subsequently aged for assessment of leukemia development. All retroviral infection, irradiation, transplantation, and monitoring steps will be carried out as described in Experiment 1. Any remaining mice 4 months after transplantation will be euthanized.

In order to achieve statistical significance, 3 primary leukemias of each genotype (b)(4) will be tested. To generate these leukemias, 20 (b)(4) (b)(4) donor mice (10 each genotype) will be needed to extract sufficient bone marrow for infection. 2 additional B6-LY5.2/NCr mice will be needed to provide

supporting bone marrow cells. **Therefore, a total of 8 B6-LY5.2/NCr mice (6 as recipients and 2 as supporting marrow donors) and 20 (b)(4) mice are needed for leukemia generation**. For secondary transplantations to assess leukemia maintenance, each leukemia will be transplanted into 5 secondary recipients and 6 additional B6-LY5.2/NCr will be needed to provide supporting bone marrow cells. Therefore, **a total of 36 B6-LY5.2/NCr mice (5 recipients/leukemia x 6 leukemias + 6 supporting marrow donors) are required for secondary transplantations.**

Summary of required animal numbers for Experiment 3:

Strains	Experimental groups (b)(4)		Supporting B.M.	Total
B6-LY5.2/NCr	18	18	8	44
(b)(4) (BM donor)	10	10		20

V.1.4. Experiment 4:

Our previous studies suggest that (b)(4) (b)(4) To test this idea (b)(4) will be challenged with 5-FU treatments in a competitive reconstitution assay in comparison to wild-type HSCs. The 5-FU treatments deplete the number of more mature progenitor cells in the bone marrow and induce proliferative stress on HSCs. Reduced reconstitution capability of Prdm16 null cells after the treatments will suggest that Prdm16 help protect HSCs under stressful conditions. For this purpose, 5 x 10^5 bone marrow cells from (b)(4) mice (2-4 months old) carrying an poly I:C inducible (b)(4) (b)(4) will be mixed with 5 x 10^5 competing wild-type bone marrow cells from B6-LY5.2/NCr and then transplanted into lethally-irradiated B6-LY5.2/NCr recipient mice. (b)(4) will be induced in the transplanted (b)(4) cells by poly I:C injections of recipient mice one month after transplantation. Two weeks after poly I:C injections, all recipients will receive two intraperitoneal injections of 5-FU at a dosage of 150 mg/kg with a 2-week interval. This dosage of 5-FU is not expected to cause any animal morbidity and illness. Reconstitution by the donor cells will be determined at 1 week after poly I:C injections and at 1, 2, and 4 months after the last 5-FU injection by retro-orbital bleeding and FACS. All animals will be euthanized after the last retro-orbital bleeding, and hematopoietic tissues including bone marrow, thymus, and spleen will be collected after euthanasia and analyzed by FACS. Same calculation of animal numbers as in Experiment 1 applies here. 11 B6-LY5.2/NCr mice will be required for each transplantation experiment containing 2 experimental groups (one group for each of the 2 genotypes, 5 animals per group, 1 competing marrow donor) and 3 experiments in total will be conducted. **The minimum number of B6-LY5.2/NCr mice required for this study is: [11 animals/experiment (5,5,1) x 3 experiments = 33]. A total of 6 (b)(4) (1 donor/ transplanted group x 2 groups x 3 repeats) will be needed as bone marrow cell donors.**

Summary of required animal numbers for Experiment 4:

Strains	Experimental groups (b)(4)		Competing B.M.	Total
B6-LY5.2/NCr	15	15	3	33
(b)(4) (BM donor)	3	3		6

V.1.5. Experiment 5:
In order to understand the mechanisms through which Prdm16 (b)(4) (b)(4) we will carry out expression profiling studies to identify targets of (b)(4) Since Prdm16 deletion in (b)(4) mice induced by poly I:C is not specific to the hematopoietic system, to exclude potential effects from Prdm16 deletion in the microenvironment, we will first transplant their bone marrow cells into irradiated B6-LY5.2/NCr recipient mice (5 x 10^6 cell/recipient) and then (b)(4) (b)(4) by injecting the recipient mice with poly I:C. at 4 weeks after transplantation. Irradiation, transplantation, and monitoring of recipients will be carried out as described in Experiment 1. The (b)(4) will be purified from the recipient mice at 2 weeks after the last poly I:C injection by FACS and used for RNA extraction for microarray-based expression profiling experiments. Due to extreme scarcity of (b)(4) in the bone marrow (0.01-0.02%), **10 recipient mice for each genotype** (b)(4) **will be needed** to obtain sufficient cells for expression profiling in one experiment. As such, **2** (b)(4) **mice of each genotype will be needed to provide sufficient donor cells** for transplantation in each experiment. In order to achieve statistical significance, such experiment will be repeated 3 times. **Total required B6-LY5.2/NCr animals: 20 animals/experiment x 3 experiments = 60 mice. A total of 12 (2 donors/genotype x 2 genotypes x 3 experiments)** (b)(4) **mice will be needed as bone marrow donors.**

Summary of required animal numbers for Experiment 5:

Strains	Experimental groups (b)(4)		Total
B6-LY5.2/NCr	30	30	60
(b)(4) (BM donor)	6	6	12

V.2. Data Analysis: For all 7 experiments, gene expression, contribution of cell populations, colony/cell counts will be compared across genotypes using analysis of variance (ANOVA), student's t test, or nonparametric tests as appropriate. A sample size of 5 in each group will have 80% power to detect a difference of 2 standard deviations between groups using a 2-group t test with a 5%, two-sided significance level.
For aging studies in Experiment 2 and 6, log-rank tests will be used to compare time to development of sickness such as leukemia across groups. When the sample size in each group is 15, a 5% two-sided log-rank test for equality of survival curves will have

80% power to detect a difference in proportion surviving of at least 54 percentage points (e.g. 99% vs. 45% surviving)

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: Tissue culture was considered, however hematopoietic development is a complex process that requires proper interactions among different tissue and cell types within the context of a whole animal, and currently there is no tissue culture system capable of recapitulating this environment [8]. In addition, hematopoietic cell lines used in tissue culture studies are different from normal hematopoietic cells in that they have acquired mutations that allow them to replicate indefinitely in culture, and results from these studies may not apply to normal cells. Computer modeling was considered as well, but the regulation of hematopoietic development is still largely unknown and there is no system available in the hematopoiesis field that can be used to predict gene function and consequences of gene mutations.

V.3.2. Animal Model and Species Justification: I have chosen to use mice for my studies because they are genetically well-characterized mammalian organism that has been shown to have a very similar hematopoietic system to that of humans, and more and more studies have been carried out in mice to understand human hematopoiesis and leukemia development [9-11]. In addition, there are many tools available in mice but not in other mammalian models to genetically alter the germline and study the consequences of the mutation on multiple organ systems simultaneously. I also happen to have extensive experiences working with mouse hematopoietic system as a research fellow in the Mouse Cancer Genetics Program at National Cancer Institute. Lower organisms such as Zebrafish and Drosophila were also considered, however both species are very different from mammals and do not represent good models for studying human hematopoiesis.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	Mus musculus	
V.3.3.2. Strain/Stock:	(b)(4) B6-LY5.2/NCr	
V.3.3.3. Source/Vendor:	In-house (b)(4) (b)(4) (b)(4) (B6-LY5.2/NCr)	
V.3.3.4. Age:	B6-LY5.2/NCr (7-12	

weeks); (b)(4)
(E18.5) (b)(4)
mice (2-4 months)

V.3.3.5. Weight: N/A

V.3.3.6. Sex: Male: 33
Female: 258

V.3.3.7. Special Considerations: Specific pathogen free

V.3.4. Number of Animals Required (by Species): Mice: 291
(B6-LY5.2/NCr, 225; (b)(4) 28; (b)(4) (b)(4) 38)

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:
All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels. All animals subjected to irradiation will be treated with amoxicillin 3 days prior to irradiation to 10 days post irradiation to reduce distress caused by potential infection. Animals observed to be ill or showing signs of pain or distress including 30% reduction in body weight (compared to before transplantation), hunched posture, ruffled fur, and decreased activity will be evaluated by veterinary staff to be treated or be euthanized for relief of suffering.

V.3.5.2. Reduction: In order to reduce the animal numbers for this study, experiments have been designed to include tissue sharing among different experimental procedures including DNA extraction, RNA extraction, and protein extraction. Also, sample size calculations were performed to use least number of animals to achieve statistical significance.

V.3.5.3. Replacement: Tissue culture and computer models were considered but not adopted. Detailed explanations are provided in V.3.1.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	85	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	206	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: For retro-orbital bleeding, 3.5-4% Isoflurane will be applied before the procedure through induction chamber of an anesthesia machine at LAM until animals are fully anesthetized (judging by lack of movement and decrease in respiratory rate to about once every 4 seconds and a negative pedal withdrawal reflex).

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: N/A

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures: N/A

V.4.1.3.1. Sources Searched: N/A

V.4.1.3.2. Date of Search: N/A

V.4.1.3.3. Period of Search: N/A

V.4.1.3.4. Key Words of Search: N/A

V.4.1.3.5. Results of Search: N/A

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: N/A

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: Transplantation of fetal liver and bone marrow cells will be carried out by one time tail vein injection of cells resuspended in 200ul of PBS using 28 Gauge x ½ inch needles. Since transplanted cells are from mice congenic to the recipients, no immune rejection is expected after transplantation. Injection of hematopoietic cells through the tail vein is not known to cause any toxic effects to mice. All cells for injection will be obtained from mice housed in USU colony. Injection of all biological materials will be in accord with USU IACUC Policy #23 on the use of biological in experimental animals.

Transplant recipient mice in Experiment 3, 4 and 5 will also receive 5 intra-peritoneal injections with 2 day intervals of 200 ul poly I:C (a chemical used to induce Cre gene expression) at a concentration of 2 ug/ul diluted in sterile phosphate buffered saline. 28 Gauge x ½ inch needles will be used for the injections. Injection of poly I:C at this dosage is safe for mice[12].

V.4.4.2. Biosamples: Retro-orbital bleeding is chosen for the protocol based on the blood sample size required for our study and available in-house expertise. Given the relatively large sample size (200 ul of blood from each animal each time) that is needed for our study to perform FACS analysis, 2 other blood collection procedures including from the saphenous vein and jugular vein were considered as suitable alternatives.[13,14] Saphenous bleeding is the only procedure that is less painful than retro-oribal bleeding and does not require anesthesia. However, this procedure requires extensive experience and has not been performed on mice by any LAM technicians previously. In contrast, retro-orbital bleeding has been routinely carried out by staff in my laboratory under previously approved protocols and will be done under isoflurane anesthesia.

In this protocol, 200ul blood will be obtained each time by retro-orbital bleeding at one week after poly I:C injections at 1, 2 and 4 month after last 5-FU injection as described in Experiment 4. IACUC policy on retro-orbital bleeding (POLICY 001) will be strictly followed when performing this procedure. Mice will be anesthetized with Isoflurane. When the animal is no longer moving voluntarily it is placed on a paper towel. The forefinger of the operator's nondominant hand is used to pull the facial skin taut and cause the eyes to protrude slightly while the skin at the back of the neck is grasped by the thumb and remaining fingers to restrain. Breathing and color are monitored throughout the procedure to ensure that the restraint does not compromise the airway. The tip of a capillary tube is gently inserted below the eye at approximately a 45 degree angle into the space between the globe and the lower eyelid. When the tip of the pipette contacts the boney floor of the orbit it is gently twisted between thumb and forefinger to rupture the capillary sinus. Blood is allowed to flow by capillary action into the pipette. Care is taken not to take any more blood than is needed. At the conclusion of the blood withdrawal tension on the animal is released and a gauze pad is gently pressed over the eye for about 30 seconds until the bleeding has stopped. A drop of

tetracaine is added in the eye to reduce any postprocedural discomfort that may occur. Normal color and respiration are reconfirmed and the animal is returned to its cage for recovery. Alternate eyes are used for successive bleeds. A maximum of two bleeds per eye (four bleeds total) will be done.

In addition, hematopoietic tissues including bone marrow, spleen, thymus, lymph nodes and control tissue brain, lung, and kidney will be extracted only after euthanasia.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Cage cards will be used to distinguish different study groups.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: Recipient B6-LY5.2/NCr mice will be irradiated on the day of transplantation with1100 rads of total body irradiation from a ^{137}Cs source located at room (b)(6) Mice will be transported and irradiated at room temperature in irradiator disks (purchased from Braintree Scientific) that maintain a filter protected sterile environment and hold up to 20 mice at each time. The entire process will last no more than 30 minutes and during which time no food or water will be provided for the animals. Hematopoietic cells resuspended in PBS in a volume of 200 ul will be introduced by tail vein injection. Following transfer of the hematopoietic cells mice will be monitored monitored twice daily and three times between days 7-10 after irradiation for signs of distress including rough coat, listlessness, 30% reduction in body weight (compared to before transplantation), or hunched posture. Any recipients displaying any combination of two or more signs of distress above will be euthanized immediately by PI staff. The dose of irradiation used is expected to be lethal within 10 days in the absence of bone marrow reconstitution by transplanted cells. Successful recolonization of the recipient bone marrow in transplanted animals results in restoration of constitutive hematopoiesis over the normal life span of the recipient. Past experience has shown that lethally irradiated mice can suffer from infection. To prevent infection, all recipient mice will receive amoxicillin at a concentration of 0.5mg/ml in the drinking water from 3 days prior to irradiation to 10 days post irradiation, and sterile feed and autoclaved caging will be provided.

V.4.4.8. Tissue Sharing: Tissues may be shared with other investigators upon request.

V.4.5. Study Endpoint:
The study endpoint for mice is between 2 to 16 months of age as specified in the experimental design and general procedures section. Animals could be euthanized earlier due to leukemia development or engraftment failure of transplanted hematopoietic cells after irradiation. To capture and euthanize animals in distress as soon as possible, irradiated mice will be monitored at least twice daily by PI staff. Since morbidity due to bone marrow failure usually occurs between 7 and 10 days after

irradiation in our past experience, one additional inspection daily will be added during this period to catch any possible distressed animals. Signs of these conditions include rough coat, listlessness, 30% reduction in body weight (compared to before transplantation), or hunched posture. Any animals displaying any combination of two or more symptoms will be euthanized immediately.

V.4.6. Euthanasia:
Animals under this protocol will be euthanized by research personnel. Mice will be euthanized using cylinderized CO2. These animals will be placed in a cage or chamber that is not crowded and exposed to a mix of CO2 and oxygen. The CO2 flow rate will displace at least 20% of the chamber volume per minute. Flow will continue for at least one minute after visible sign of death. CO2 euthanasia will be followed by cervical dislocation to ensure death. Gestation day 18.5 fetuses will be first anesthetized by placing them in a Petri dish that is placed on top of ice for 15 minutes and then euthanized by decapitation using surgical scissors. Both procedures are consistent with the 2013 AVMA Guidelines for the Euthanasia of Animals.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:
Building(s) _____ Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:
Sterile micro-isolator cages, feed (9% fat), and bedding will be used.

Food Restriction:	Yes	_____	No	X
Fluid Restriction:	Yes		No	X

V.5.1.3. Exceptions: N/A _____ _____

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
Animals will be observed at least once daily by Center for LAM personnel. PI group will also observe the colony twice daily except during the period 7-10 days post-irradiation during which they will be monitored three times daily. Animals or pups observed to be ill or showing signs of pain or distress including weight loss, hunched posture, ruffled fur, and decreased activity will be evaluated by veterinary staff to be treated or be euthanized. PI should be contacted for potential tissue collection needs if sick animals are to be eutharized by LAM personnel.
V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health

rounds are conducted by LAM personnel once daily during weekend and holidays. Sick or moribund animal will be evaluated by veterinary staff to be treated or be euthanized. PI should be contacted for potential tissue collection needs if euthanasia is necessary. Euthanasia by LAM personnel should proceed if PI can not be reached.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Animal monitoring, euthanasia, tissue extraction, intra-peritoneal injection	(b)(6)	PI, 12 years of experience	Animal Care and Use Introductory Training Course at NCI, 2001; Animal Care and Use Training Course at NCI, 2007; Investigator training course at USUHS, 2007.
Tail vein injection, animal monitoring, Euthanasia, tissue extraction	(b)(6)	Technician, 5 years of experience	Investigator training course at USUHS, 2007; Rodent Handling Training by PI, 2007
Euthanasia, tissue extraction	(b)(6)	Technician,4 years of experience	Investigator training course at USUHS, 2007; Rodent Handling course at USUHS, 2007

VII. BIOHAZARDS/SAFETY: N/A

VIII. ENCLOSURES:

References:

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IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 5/31/13

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 5/31/13

Principal Investigator Signature Date

I. **Painful Procedure(s):**

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress (WILL) **or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature

5/11/2013

Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: Generation and characterization of genetically engineered mouse strains carrying Prdm16 mutations

C. Principal Investigator: (6)(6) Department of Pediatrics

D. Performing Organization: Uniformed Services University of the Health Sciences

E. Funding: Department of Pediatrics

F. Objective and Approach: The objective of this protocol is to characterize the function of a novel gene Prdm16 during normal mammalian development, with a focus on hematopoietic development. Different genetically engineered mutant mouse strains for Prdm16 will be generated. Developmental defects in these mutant animals will be characterized comparing to wild-type littermates. The three R's (reduction, replacement, and refinement) of animal usage will be employed.

G. Indexing Terms (Descriptors): Animals, mice, Prdm16, and knockout

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

February 19, 2014

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF PEDIATRICS

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Full Committee Review on February 19, 2014:

Animal Protocol Title: "Characterizing the Role of (b)(4) During Human Leukemia Development Using a Knock-In Mouse Model"

USUHS Protocol Number: (b)(6)

Expiration Date: February 18, 2017

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Characterizing the role of (b)(4) during human leukemia development using a knock-in mouse model

GRANT TITLE (if different from above): Characterization of molecular mechanisms underlying cancer development using mouse models.

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Pediatrics, USUHS

EARLIEST ANTICIPATED FUNDING START DATE: 1/19/2014

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6) _____ PED (b)(6) 12/11/13

Principal Investigator Signature | Department | Office/Lab Telephone | Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) _____ Chair (b)(6) 12/11/13

Research Unit Chief / Dept. Head Signature | Title | Telephone | Date

Typed Name: (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) _____ DMB (b)(6) 12/11/13

Statistician Signature | Department | Telephone | Date

Typed Name (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) _____ LAM (b)(6) Dec 12, 2013

Veterinarian Signature | Department | Telephone | Date

Typed Name: (b)(6)

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) Department of Pediatrics

ANIMAL PROTOCOL TITLE: Characterizing the role of (b)(4) during human leukemia development using a knock-in mouse model

GRANT TITLE (if different from above): Characterization of molecular mechanisms underlying cancer development using mouse models

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): N/A

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS: High levels of (b)(4) expression has been considered an important factor that contributes to the induction of leukemia development in humans. This study is designed to use mouse models to study the molecular mechanism through which (b)(4) overexpression induces human leukemia development. We will use genetically engineered mice carrying increased levels of (b)(4) to characterize the mechanisms responsible for positive effects of (b)(4) overexpression on the growth of blood cells, and to identify cooperating mutations for leukemia development. For some studies, genetically modified immature mouse blood cells will be transplanted into normal mice to better characterize their potential to induce leukemia development. Transplant recipient mice will be irradiated before transplantation to enhance the establishment of the transplant donor cells in their bone marrow. Irradiation at our proposed dosage is lethal to mice if not treated afterwards. However, these recipient mice in our study will be fully rescued by the subsequent transplantation of donor immature blood cells.

II. BACKGROUND:

II.1. Background: (b)(4) is a zinc finger transcription factor involved in chromosome translocations in human myeloid leukemias[1]. However, the mechanisms that (b)(4) overexpression causes leukemia development remain unclear. We have shown that (b)(4) alone is able to immortalize myeloid progenitor cells that normally lack the capability of self-renewal, suggesting that (b)(4) may be responsible for the self-renewal capability of leukemic stem cells[2]. In order to investigate further the mechanism that (b)(4) induces leukemia, we have generated a (b)(4) (b)(4) In this model (b)(4) (b)(4) (b)(4) Once bred with mice carrying hematopoietic-specific or inducible Cre recombinase, (b)(4) be induced tissue specifically or temporally in hematopoietic cells of the compound offspring. We plan to use this model to study the molecular mechanisms responsible for (b)(4) (b)(4) Also, in previous studies, we have found that induction of (b)(4) is not sufficient for leukemia induction in these animals, suggesting that additional

mutations may be required. Therefore, we also plan to apply retroviral insertional mutagenesis to this model to identify such cooperating mutations for (b)(4) in inducing leukemia development

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: BRD, NIH RePORTER, and PubMed

II.2.2. Date of Search: 12/4/2013 for BRD and NIH RePORTER, 1/28/2014 for PubMed

II.2.3. Period of Search:
BRD: 1998 – 2009
NIH RePORTER: 1990 – 2013
PubMed:1950-1/28/2014

II.2.4. Key Words and Search Strategy: This study is mainly designed to use an inducible knock-in mouse model and retroviral insertional mutagenesis to identify cooperating partner of (b)(4) for leukemia development. Therefore, the following combination of search terms was used: (b)(4) [text] in combination with "knock-in"[text] and "retroviral insertional mutagenesis" [text].

II.2.5. Results of Search: No projects were returned on BRD. One project entitled "Collaborative pathways that lead to leukemia" was returned on NIH RePORTER. However, this project is focused on a different oncogene CALM-AF10 and (b)(4) activation was identified as one of its cooperating mutations by retroviral insertional mutagenesis. (b)(4) is likely to have many more cooperating partners remained to be identified, which will be addressed by our proposed study. A total of 21 publications [8-28] were returned on PubMed. Vast majority of the articles concentrate either on findings that retroviral vectors or replication competent retrovirus can cause insertional activation of (b)(4) or on designing new retroviral vectors to reduce insertional mutagenesis for gene therapy purpose.[8,10,12-19,21-28] One article describes the functional regulation of (b)(4) by acetylation.[9] Only two articles describe finding of two cooperating partners for (b)(4) in leukemia development, CALM-AF10 and Trib1.[11,20] As discussed earlier, (b)(4) is likely to have more cooperating partners and our proposed insertional mutagenesis study using our unique (b)(4) knock-in mice could be the best approach for identifying them.

III. OBJECTIVE\HYPOTHESIS: The objective of this study is to understand the mechanisms that cooperate with (b)(4) to induce leukemia development using an inducible (b)(4) knock-in mouse model.

IV. MILITARY RELEVANCE: Leukemia represents a significant challenge for DoD medical care. Leukemia occurrence in the general population of U.S. is about 12 every 100,000 people according to statistical studies by National Cancer Institute. About half of these patients will die due to limited treatment options for this devastating disease. There are estimated over 5 million military personnel and their family members under DoD based on a 2004 study reported by Military Family Research Institute at Purdue

University. Therefore, more than 600 new leukemia cases and over 300 deaths due to leukemia are estimated to occur every year in the DoD health care system. These numbers could be even higher as the occurrence of leukemia could potentially further increase among military personnel due to possible exposure to toxic substances or radiation on the battlefield. Finding better treatments for leukemia patients represent an important need for the DoD health care system. Our proposed study will help gain insights into the mechanisms underlying leukemia initiation and progression, and mechanistic understanding of leukemia development should help find preventive measures and better treatments for this devastating disease.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1: To test whether leukemia development can be accelerated by retroviral insertional mutagenesis in bone marrow progenitors of (b)(4) mice and to identify cooperating mutations for (b)(4)

Our preliminary studies using (b)(4) mice have suggested that overexpression of (b)(4) is not sufficient to induce leukemia development and additional mutations may be required. To test this idea, we would like to examine whether leukemia transformation of bone marrow progenitors of (b)(4) mice can be accelerated by random mutations caused by retroviral infection and integration using a bone marrow transduction and transplantation system. (b)(4) mice of 2 genotypes (generated under breeding protocol (b)(6) : (eki/eki, tg/+) and (eki/eki, +/+), will be used as bone marrow transplantation donors for this study. While both mice are homozygous for the inducible (b)(4) allele, only (eki/eki, tg/+) mice express a Tamoxifen-inducible Cre recommbinase transgene and can be induced to overexpress (b)(4) (eki/eki, +/+) mice are included as controls. These mice (between 6-12 weeks old) will be first treated with 5-fluorouracil (5-FU). The 5-FU treatment helps deplete the number of more mature progenitor cells in the bone marrow and induce the cycling of hematopoietic stem cells which facilitate their transduction by retrovirus. Mice will be treated once with 0.2ml of 5-FU in phosphate buffered saline (PBS) via intra-peritoneal injection to achieve a dose of 150 mg/kg body weight 4 days before bone marrow harvesting. The treated mice will be euthanized by CO2 asphyxiation and bone marrow cells will be harvested from their femurs under sterile conditions. The dose of 5-FU used in these experiment is not expected to cause animal morbidity or illness. The harvested bone marrow cells will be infected with a replication incompetent retroviral vector pMYs expressing marker GFP only, and treated with Tamoxifen, and subsequently transplanted along with supporting bone marrow cells from un-irradiated B6-LY5.2/NCr mice into 2 groups of irradiated 7-12 weeks old B6-LY5.2/NCr recipient mice [one group each for the infected (eki/eki, tg/+) and (eki/eki, +/+) donor cells]. The recipient mice of both groups will be subsequently aged for 6 months for leukemia development. Any remaining transplant recipient mice 6 months after transplantation will be euthanized.

pMYs virus used for the transduction of bone marrow progenitors will be generated from sterile culture medium of PLAT-E cells, a human cell line designed to package retroviral particles in ecotropic envelops and free of other known viruses[4]. This virus is replication deficient, and therefore is no longer infectious after initial infection. Prior to and during infection, these bone marrow cells may be cultured in the presence of

recombinant murine growth factors to stimulate their proliferation (In-vitro procedures only).

Recipient B6-LY5.2/Cr mice will be irradiated on the day of transfer of hematopoietic cells with 1100 rads of total body irradiation from a ^{137}Cs source located at room (b)(6) The Infected bone marrow cells (1 x 10^6 cells/mouse) along with 5 x 10^5 supporting bone marrow cells from un-irradiated B6-LY5.2/NCr mice will be resuspended in 200ul PBS and introduced into each recipient by one time tail vein injections. Past experience has shown that lethally irradiated mice can suffer from infection during engraftment of the transplanted cells. To prevent infection, all recipient mice will receive amoxicillin at a concentration of 1mg/ml in the drinking water from 5 days prior to irradiation to 10 days post irradiation. Although occurring rarely, engraftment failure of the transplanted cells will also lead to recipient death between 7 and 10 days after injection. Therefore, to prevent any potential suffering during this critical period of time, recipient mice will be monitored twice daily by PI staff between 7 and 10 days after transplantation for signs of distress including rough coat, listlessness, or hunched posture. Any transplant recipients showing such signs will be euthanized immediately. Leukemia development and progression is a slow process; therefore, for the time periods before day 7 and after day 10 post transplantation, transplant recipient mice will be monitored once daily by PI staff during week days and by LAM personnel during weekends and holidays for signs of leukemia development, which are similar to the above-described signs of engraftment failure. Any transplant recipients showing such signs will be euthanized immediately and fresh tissues including bone marrow, spleen, thymus, liver, and kidney will be collected and examined by flow cytometry, tissue culturing and pathology. We have not observed development of any other cancers besides leukemia in B6-LY5.2/NCr mice irradiated with the same dosage (1100 rads) in our past experience.

In order to statistically establish that retroviral insertional mutagenesis can accelerate leukemia transformation of (b)(4) expressing cells, a group of 15 irradiated B6-LY5.2/NCr recipient mice will need to be transplanted with transduced bone marrow progenitors from (eki/eki, tg/+) mice and another 15 mice will receive similarly infected cells from (eki/eki, +/+) mice as the control group.

Insertional mutagenesis by retroviral vectors is a valuable tool for identifying cooperating mutations in leukemia development [3]. Taking advantage of leukemias generated in this study, we would also like to identify at least (b)(4) (b)(4) We were able to identify 2 cooperating mutations for another oncogene Sox4 from 15 leukemias using a similar insertional mutagenesis approach[3]. Therefore, in order to identify at least (b)(4) we would also like to generate a total of 30 accelerated leukemias that will be used for cloning viral insertions for the purpose of identifying cooperating partner genes for (b)(4) in leukemia induction. We estimate that 60% of the mice that receive pMYs virus transduced cells will develop leukemia within 6 months. Therefore, a total of 50 recipient mice will need to be transplanted with cells transduced by pMYs virus in order to generate 30 leukemias (50 recipients x 60% leukemia penetrance). Therefore, we will transplant 50 instead of 15 recipient mice with cells from (eki/eki, tg/+) mice and 15 recipients with cells from (eki/eki, +/+) mice.

Since 1 x10^6 infected bone marrow cells will be transplanted into each recipient mouse, a minimum of 1 x 10^6 cells x 50 recipients = 5 x 10^7 bone marrow cells will be needed from (eki/eki, tg/+) mice and another 1 x 10^6 cells x 15 recipients= 1.5 x10^7 cells from (eki/eki, +/+) mice. When mice have been treated with 5-FU, harvest of

bone marrow from one mouse is expected to yield 1 x 10^6 cells. When these cells are cultured in the presence of growth factors and in contact with retrovirus, only 50% of the cells can be recovered from culture, yielding 5 x10^5 cells. As such, each transplant recipient will require 2 mice to provide sufficient donor cells. Therefore, for 50 transplant recipients for (eki/eki, tg/+) mice as discussed above, a total of **5 x 10^7 donor cells / (5 x 10^5cells/donor) = 100 donor (eki/eki, tg/+) mice will be needed.** Similarly, for 15 transplant recipients for (eki/eki, +/+) mice, a total of **1.5 x 10^7 donor cells / (5 x 10^5cells/donor) = 30 donor (eki/eki, +/+) mice will be needed.**

We will try to accomplish this study in 8 experiments; therefore, 8 additional B6-LY5.2/NCr will be needed to provide supporting bone marrow cells. **Required B6-LY5.2/NCr recipient animals: 50 animals for (eki/eki, tg/+) + 15 animals for (eki/eki, +/+) + 8 supporting marrow donors = 73 mice.**

Summary of required animal numbers for Experiment 1:

Strains	Experimental groups		Supporting B.M.	Total
	eki/eki, tg/+	eki/eki, +/+		
B6-LY5.2/NCr	50	15	8	**73**
(b)(4) (BM donor)	100	30		**130**

Study endpoint for transplant recipient B6-LY5.2/NCr mice:
6 months after transplantation.

V.1.2. Experiment 2: To test whether (b)(4) -induced self-renewal is mediated by Wnt/β-catenin signaling.

We have found that Wnt/β-catenin pathway is activated in myeloid progenitor cells overexpressing (b)(4) suggesting that the self-renewal function of (b)(4) may be mediated by the Wnt/β-catenin pathway. To test this idea, we will test whether β-catenin deficient myeloid progenitors can be immortalized by retroviral vectors expressing (b)(4) We will breed β-catenin conditional knockout Ctnnb1-CKO mice with Cre-ERT2 mice carrying an inducible Cre transgene as described in Experiment 1 to generate Ctnnb1-ERT2 (cko/cko, tg/+) and control (cko/cko, +/+) mice (The generation of these mice is supported by the breeding protocol (b)(6) Bone marrow cells harvested from these mice will be tested for immortalization in culture by Evi1 expression after treatment with Tamoxifen which will induce deletion of β-catenin only in cells from (cko/cko, tg/+) mice but not in cells from control (cko/cko, +/+) mice.

In order to achieve statistical significance, bone marrow cells from 5 mice of each genotype will be tested. Therefore, **a total of: 5 animals/genotype x 2 genotypes = 10 mice.**

Summary of required animal numbers for Experiment 2:

Strains	Experimental groups		Total
	cko/cko, tg/+	cko/cko, +/+	
Ctnnb1-ERT2	5	5	**10**

V.2. **Data Analysis**: For Experiment 1, log-rank tests will be used to compare time to development of leukemia across groups. When the sample size in each group is 15, a 5% two-sided log-rank test for equality of survival curves will have 80% power to detect a difference in proportion surviving of at least 54 percentage points (e.g. 99% vs. 45% surviving). A sample size of 5 animals in each group (as in Experiment 2), when using t-test, will have 80% power to detect a difference of 2 standard deviations between groups.

V.3. **Laboratory Animals Required and Justification**:

V.3.1. **Non-animal Alternatives Considered**: Cell line studies was considered, however it does not allow identifying (b)(4) targets involved in immortalization as cell lines are already immortal. Also, *In vivo* study is extremely important in leukemia research as the evolvement of leukemic cells is not an isolated event and involves constant interaction with the host environment which has yet to be replicated in tissue culture settings. Computer modeling was considered as well, but the mechanisms underlying leukemia development is still largely unknown and there is no system available in the leukemia field that can be used to predict target genes involved in the immortalization of hematopoietic cells.

V.3.2. **Animal Model and Species Justification**: I have chosen to use mice for my studies because they are genetically well-characterized mammalian organism that has been shown to have a very similar hematopoietic system to that of humans, and more and more studies have been carried out in mice to understand human leukemia development and normal hematopoiesis[5-7]. In addition, there are many tools available in mice but not in other mammalian models to genetically alter the germline and study the consequences of the mutation in normal cells. I also happen to have extensive experiences (for the past 9 years) working with mouse hematopoietic system. Lower organisms such as Zebrafish and Drosophila were also considered, however both species are very different from mammals and do not represent good models for studying human hematopoiesis.

V.3.3. **Laboratory Animals**

	Species #1	Species #2
V.3.3.1. **Genus & Species**:	Mus. Musculus	
V.3.3.2. **Strain/Stock**:	B6-LY5.2/NCr (b)(4) Ctnnb1-ERT2	
V.3.3.3. **Source/Vendor**:	(b)(4) B6-LY5.2/NCr) In-house (b)(4)	

(b)(4) Ctnnb1-ERT2)

V.3.3.4. Age: 6-12 weeks

V.3.3.5. Weight: 20-25g

V.3.3.6. Sex: Male: 65 (b)(4) (b)(4) 65)
Female: 148 (b)(4) (b)(4) 65; B6-LY5.2/NCr, 73; Ctnnb1-ERT2, 10)

V.3.3.7. Special Considerations: N/A

V.3.4. Number of Animals Required (by Species): 213 mice (b)(4) (b)(4) 130; B6-LY5.2/NCr, 73; Ctnnb1-ERT2, 10)

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels. Animals observed to be ill or showing signs of pain or distress including weight loss, hunched posture, ruffled fur, and decreased activity will be evaluated by veterinary staff to be treated or be euthanized immediately for relief of suffering.

V.3.5.2. Reduction: The estimated numbers of animals for proposed studies have been computed by power analysis to ensure that the smallest numbers of animals required to evaluate the hypotheses are being used.

V.3.5.3. Replacement: Tissue culture and computer models were considered but not adopted. Detailed explanations are provided in V.3.1.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	148	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	65 (B6-LY5.2/NCr mice for receiving irradiation)	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: N/A

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: N/A

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: AWIC and Altweb

V.4.1.3.2. Date of Search: 12/9/2013

V.4.1.3.3. Period of Search: N/A

V.4.1.3.4. Key Words of Search: "Irradiation" in combination with "mouse bone marrow transplantation" was used as key words.

V.4.1.3.5. Results of Search: No relevant matches were returned in both databases.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: Whole body irradiation of recipient mice is essential for successful mouse bone marrow transplantation as it is the only way to efficiently deplete host bone marrow stem cells and to greatly increase donor cell engraftment in mice.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. **Multiple Major Survival Operative Procedures**: N/A

V.4.3.6.1. **Procedures**: N/A

V.4.3.6.2. **Scientific Justification**: N/A

V.4.4. **Animal Manipulations**:

V.4.4.1. **Injections**: 28 Gauge x ½ inch needles will be used for all injections. Some mice as specified in Experiment 1 of the experimental design and general procedures section will receive one time intra-peritoneal injection of 5-FU resuspended in phosphate buffered saline (PBS) at a dosage of 150 mg/kg body weight in 200ul volume as described in Experiment section. 5-FU treatment of mice at this dosage is toxic to proliferating cells in the hematopoietic system representing more mature progenitors, but is not expected to cause animal suffering and death.

Transplantat on of bone marrow cells will be carried out by one time tail vein njections of cells resuspended in 200ul of PBS. Recipient mice will be restrained for the injection using a Tailveiner restrainer from Braintree Scientific. Since transplanted cells are from mice congenic to the recipients, no immune rejection is expected a'ter transplantation. Injection of hematopoietic cells through the tail vein is not known to cause any toxic effects to mice All cells for injection will be obtained from mice housed in USU colony. In,ection of all biological materials will be n accord with USU IACUC Policy #23 on the use of biological in experimental animals.

V.4.4.2. **Biosamples**: Hematopoietic tissues including bone marrow, thymus, spleen, and lymph nodes will be collected at euthanasia.

V.4.4.3. **Adjuvants**: N/A

V.4.4.4. **Monoclonal Antibody (MAbs) Production**: N/A

V.4.4.5. **Animal Identification**: Cage cards will be used to distinguish different study groups.

V.4.4.6. **Behavioral Studies**: N/A

V.4.4.7. **Other Procedures**: Recipient B6-LY5.2/NCr mice will be irradiated on the day of transplantation with1100 rads of total body irradiation from a ^{137}Cs source located at room (b)(6) Mice will be transported and irradiated at room temperature in irradiator disks (purchased from Braintree Scientific) that maintain a filter protected sterile environment and hold up to 20 mice at each time. The entire process will last no more than 30 minutes and during which time no food or water will be provided for the animals. Hematopoietic cells resuspended in PBS in a volume of 200 ul will be introduced by tail vein injection. Following transfer of the hematopoietic cells mice will be monitored daily for signs of distress including rough coat listlessness, decreased body weight, or hunched posture. Any recipients displaying any combination of two or more signs of distress above will be euthanized immediately by PI staff. The dose of

irradiation used is expected to be lethal within 10 days in the absence of bone marrow reconstitution by transplanted cells. Successful recolonization of the recipient bone marrow in transplanted animals results in restoration of constitutive hematopoiesis over the normal life span of the recipient. Past experience has shown that lethally irradiated mice can suffer from infection. To prevent infection, all recipient mice will receive amoxicillin at a concentration of 1mg/ml in the drinking water from 5 days prior to irradiation to 10 days post irradiation.

V.4.4.8. Tissue Sharing: N/A

V.4.5. Study Endpoint: The study endpoint for irradiated B6-LY5.2/NCr recipient mice is 6 months after transplantation as specified in the experimental design and general procedures section. Animals could be euthanized earlier due to leukemia development or engraftment failure of transplanted hematopoietic cells after irradiation. Signs of these conditions include rough coat, listlessness, decreased body weight, or hunched posture. Any animals displaying any combination of two or more symptoms will be euthanized immediately.

V.4.6. Euthanasia: Animals under this protocol will be euthanized by PI staff or LAM personnel at study end point or when moribund due to bone marrow engraftment failure or leukemia development as described under V.1.1 Experiment 1. Mice will be euthanized using cylinderized CO2. These animals will be placed in a cage or chamber that is not crowded and exposed to a mix of CO2 and oxygen. The CO2 flow rate will displace at least 20% of the chamber volume per minute. Flow will continue for at least one minute after visible sign of death. CO2 euthanasia will be followed by cervical dislocation to ensure death. This procedure is 2013 AVMA Guidelines for the Euthanasia of Animals.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:
Building(s) _____ Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions: To prevent infection after bone marrow transplantation, all recipient mice will be provided with sterile feed and autoclaved caging will be provided.

Food Restriction:	Yes _____	No X
Fluid Restriction:	Yes _____	No X

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be observed at least once daily by Center for LAM personnel. PI group will also observe the colony once daily during week days except during the period 7-10 days post-irradiation during which they will be monitored twice daily. Animals observed to be ill or showing signs of pain or distress including weight loss, hunched posture, ruffled fur, and decreased activity will be evaluated by veterinary staff to be treated or be euthanized. PI should be contacted for potential tissue collection needs if sick animals are to be euthanized by LAM personnel.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays. Sick or moribund animal will be evaluated by veterinary staff to be treated or be euthanized. PI should be contacted for potential tissue collection needs if euthanasia is necessary. Euthanasia by LAM personnel should proceed if PI can not be reached.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Animal monitoring, euthanasia, tissue extraction, intra-peritoneal injection	(b)(6)	PI, 12 years of experience	Animal Care and Use Introductory Training Course at NCI, 2001; Animal Care and Use Training Course at NCI, 2007; Investigator training course at USUHS, 2007.
Tail vein injection, animal monitoring, Euthanasia, tissue extraction	(b)(6)	Technician, 5 years of experience	Investigator training course at USUHS, 2007; Rodent Handling Training

			by PI, 2007
Euthanasia, tissue extraction	(b)(6)	Technician, 5 years of experience	Investigator training course at USUHS, 2007; Rodent Handling course at USUHS, 2007

VII. **BIOHAZARDS/SAFETY**: Replication defective ecotropic retroviral vectors will be used to infect mouse bone marrow cells before transplantation of the infected cells into recipient mice. These viruses will be collected from culture medium of cell lines designed to package viral particles in ecotropic envelops. After initial infection these vectors are no longer infectious and are replication deficient. There is no evidence in the literature that humans can be infected by ecotropic retroviruses. Any person handling these retroviral vectors will be required to wear lab coat and gloves as precaution. Registration for the use of these viral vectors has been submitted to IBC. 5-FU and tamoxifen are harmful if ingested, injected or through skin contact. Therefore, lab coat, gloves, and safety glasses are also required when preparing the solution and carrying out animal injection using these substances.

VIII. **ENCLOSURES**:

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IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course

(b)(6) _____ 12/11/13

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 12/11/13

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) _____ 12/11/13

Principal Investigator Signature **Date**

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: Characterizing the role of (b)(4) during human leukemia development using a knock-in mouse model

C. Principal Investigator: (b)(6) Department of Pediatrics

D. Performing Organization: Uniformed Services University of the Health Sciences

E. Funding: Department of Pediatrics

F. Objective and Approach: The objective of this study is to understand the mechanisms that (b)(4) induces human leukemia development. Genetically engineered mouse strain overexpressing (b)(4) will be generated and used for studying leukemia induction. The three R's (reduction, replacement, and refinement) of animal usage will be employed.

G. Indexing Terms (Descriptors): Animals, mice, and knock-in

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

Institutional Animal Care and Use Committee
Phone: (b)(6)
Fax: (b)(6)

March 26, 2015

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF PEDIATRICS

SUBJECT: IACUC Approval of Protocol - Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on March 26, 2015:

Animal Protocol Title: "Characterization of ptpn-11 Gain of Function Mutations Using Transgenic Mouse Models"

USUHS Protocol Number: (b)(6)

Expiration Date: March 25, 2018

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc: Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Characterization of ptpn-11 and Cbl Gain of Function Mutations using Transgenic mouse models

GRANT TITLE (if different from above): Characterization of a murine model of JMML

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Department of Pediatrics

EARLIEST ANTICIPATED FUNDING START DATE: FEB 2015

PRINCIPAL INVESTIGATOR: (b)(6) MD, PhD

(b)(6)
Principal Investigator Signature

PED
Department

(b)(6)
Office/Lab Telephone

2/9/15
Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)
Research Unit Chief / Dept. Head Signature
Typed Name: (b)(6) MD

Chair
Title

(b)(6)
Telephone

2/19/15
Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)
Statistician Signature
Typed Name (b)(6) MD

PED
Department

(b)(6)
Telephone

2/23/15
Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)
Attending/Consulting Veterinarian Signature
Typed Name (b)(6) VMD

LAM
Department

(b)(6)
Telephone

2/27/15
Date

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) Pediatrics, (b)(6)

ANIMAL PROTOCOL TITLE: Characterization of ptpn-11 and Cbl Gain of Function Mutations using Transgenic Mouse Models

GRANT TITLE (if different from above): Characterization of a murine model of JMML

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): None

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS: Our laboratory is interested in studying how cancers develop so that we can ultimately help find better treatments. More specifically, we are studying how Juvenile Myelomonocytic Leukemia (JMML), an aggressive form of childhood leukemia, develops and will try to understand the different steps that are required for the development of this aggressive disease. While the cure rates for childhood leukemia has increased to above 80%, the cure rate for JMML is less than 50%, and we do not understand why JMML is a more aggressive disease or why chemotherapy is ineffective against JMML. Despite difficulty in improving the treatments, much has been learned about the genetic lesions which cause JMML. Over the past few years, mutations in ptpn-11 as well as cbl were found in patients with JMML. In fact, 90% of patients with JMML will harbor mutations in ptpn-11, k-ras, n-ras, Cbl, or Nf-1 mutations. Interestingly all of these genes are known to be involved in a common biochemical pathway called the "ras pathway" defining JMML as a disease caused by hyperactive RAS signaling.

Over the past three years, we have developed transgenic mice which carry mutant ptpn-11 transgenes as well the mutant cbl transgenes. Upon aging the ptpn-11 mutant mice, some of the mice develop a myeloproliferative disorder with an enlarged spleen. However, they do not go on to develop fulminant JMML which is likely due to several factors such as requiring additional genetic lesions as well as the fact that our transgenic mice have lower level of expression than the endogenous gene. It also is likely that the level of the normal endogenous gene affects the phenotype of these mice. Our future goals are to further characterize the Cbl transgenic mice to see if they develop JMML as well as explore the other genetic lesions which cooperate with ptpn-11 and Cbl in leading to the development of JMML.

II. BACKGROUND:

II.1. Background: Juvenile Myelomonocytic Leukemia (JMML) is a devastating childhood cancer which is rapidly fatal with infiltration of myeloid cells into multiple organs [1]. Despite remarkable advances in the cure rates of childhood leukemia, we

have made little progress with JMML with stem cell transplant providing the only hope for cure [2]. The discovery that the *ptpn-11* gene is mutated in up to 35% of JMML patient samples has provided an important clue in understanding the pathogenesis of this disease. It is now accepted that perturbations in the RAS pathway are essential in the development of JMML. Moreover, about 25% of JMML samples contain an oncogenic *ras* mutation [3, 4]. Now, with the addition of *c-cbl* mutations which is seen in about 15% of JMML patient samples, it appears that ~90% of the initiating mutations are accounted for and all of them involve genes in the RAS pathway [5, 6].

The *ptpn-11* gene encodes for SHP-2 which is a non-receptor protein tyrosine phosphatase. In order to better understand how *ptpn-11* mutations predisposed these patients to develop myeloid malignancies, we first over-expressed leukemia-associated SHP-2 protein and found that they enhanced the proliferation of murine hematopoietic cells as well as making them hypersensitive to granulocyte-macrophage colony-stimulating factor (GM-CSF). It was also found that these effects are dependent on the increased phosphatase activity of the SHP-2 protein and that these leukemia associated *ptpn-11* mutations were more potent than the Noonan syndrome mutations [7, 8]. Others have also reported similar effects of *ptpn-11* mutations on activating the RAS pathway and are summarized in the review by Mohi [9, 10].

During the past few years, new mouse models with mutant Shp-2 have been published. Chan et. al. reported the development of a knock in mouse model expressing the *ptpn-11*^{D61Y} mutation which is the second most common mutation after *ptpn-11* ^{E76K} mutation. These mice develop a fatal myeloproliferative disorder with a median survival of 45 weeks. These mice also show enhanced phosphorylation of Erk and Stat5 which is similar to *kras*^{G12D} knock in mouse model [11, 12]. Recently, Xu et. al. reported the phenotype of knock in mice which have the *ptpn-11* ^{E76K} mutation and found that these mice develop a myeloproliferative disorder as well as myeloid leukemias and lymphoid leukemias [13]. We have also generated transgenic mice carrying the *ptpn-11* ^{E76K} mutation but these mice develop only mild myeloproliferative disease with splenomegaly when aged but do not die from this disease. This is most likely due to levels of the mutant gene being expressed compared to the knock in mice which have only 50% of the wild type protein being expressed whereas the transgenic mice have normal levels of the wild type protein.

The *c-cbl* gene encodes a multifunctional adaptor protein which contains an N-terminal tyrosine-kinase binding (TKB) domain, a ring finger motif which contain E3 ligase activity, and a C-terminal ubiquitin-associated domain. It is thought that the TKB domain is involved in adaptor functions of the protein whereas the ubiquitin ligase domain results in monoubiquitylation of receptors which promotes lysosomal mediated degradation of activated receptors (reviewed in[14]). Previous mutations in myeloid malignancies have been described where mutations occur in the ring finger domain or the linker domain [15, 16]. It seems that amino acid residue 371, which resides in the linker domain and separates the TKB domain to the Ring domain, is critical in the transformation potential of *cbl* as deletion of Y371 both abolishes the E3 ligase activity and is oncogenic whereas mutation which only abolish E3 ligase activity is not oncogenic [17]. Interestingly, there was a hotspot for mutations at residue 371 in patients with JMML where 1/3 of mutations found in *c-cbl* were found to be a tyrosine to

histidine mutation, *cbl*^{Y371H} [5]. We have generated transgenic mice carrying the *cbl*^{Y371H} mutation. Because Cbl mutations require homozygosity in patients to develop disease, it is likely that we will need to inactivate the normal copy of mouse Cbl in order for the mutant over-expression to result in a disease phenotype. Our initial attempt at generating this model resulted in embryonic lethality due to the deleterious effect of having only mutant Cbl during development. We are now trying to develop a conditional deletion model so that we can bypass the embryonic lethality.

Animal models can be very useful in preclinical testing of novel therapies and especially important for rare diseases such as JMML. Genetically engineered mouse models are now being generated which accurately recapitulate human diseases [18]. Because the RAS pathway is clearly involved in the development of JMML, new drugs which target the downstream effectors of the RAS pathway are attractive candidates. For a rare disease such as JMML, clinical trials would be very time-consuming due to the lack of patient numbers and would not allow analysis of variety of agents which are now becoming available. Thus, transgenic mouse models engineered to phenocopy the biology found in JMML patients would be an attractive platform to test new therapeutic strategies with the statistical power to make an informed choice in taking new therapies to clinical trials.

Finally, In order to investigate cooperating mutations required for the development of ptpn-11 induced cancers, we plan on performing crosses with previously generated mouse models from other oncogenes and tumor suppressor genes. In this way, we will be able to test the interaction of *ptpn-11* mutations with other known oncogenic pathways.

References

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3. **Loh, M.L., et al., *Mutations in PTPN11 implicate the SHP-2 phosphatase in leukemogenesis.* Blood, 2004. 103(6): p. 2325-31.**
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17. **Thien, C.B., F. Walker, and W.Y. Langdon, *RING finger mutations that abolish c-Cbl-directed polyubiquitination and downregulation of the EGF receptor are insufficient for cell transformation.* Mol Cell, 2001. 7(2): p. 355-65.**
18. **Frese, K.K. and D.A. Tuveson, *Maximizing mouse cancer models.* Nat Rev Cancer, 2007. 7(9): p. 645-58.**

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: BRD, NIH RePORTER, PUBMED

II.2.2. Date of Search: Feb 4, 2015

II.2.3. Period of Search:
BRD: 1996-2014
NIH RePorter: 2010-2012
PUBMED: 1960-2014

II.2.4. Key Words and Search Strategy: Mouse models of JMML, Shp-2, ptpn-11, Cbl

II.2.5. Results of Search: There are no reported studies using the ptpn-11 BAC. There is a published mouse model of Noonan syndrome using the "knock in" approach where one specific mutation was tested. There is now a knock in mutation of the ptpn-

11^{E76K} mutation but it was not published when this work was started. There are no mouse models of Cbl^{Y371H} mutation. There is a mutant Cbl ringer finger knock in mice carrying the Cbl^{C379A} which is equivalent to the Cbl^{C381A} which are found in myeloid diseases.

III. OBJECTIVE\HYPOTHESIS: The objective of this protocol is to gain insight into how mutations in *ptpn-11* gene and Cbl results in the development of an aggressive form of childhood leukemia called Juvenile Myelomonocytic Leukemia. By using an animal model of JMML, we will be able to analyze the specific pathways which are perturbed by ptpn-11 mutations as well as investigating the cooperating mutations which are necessary for the development of this cancer. Finally, since the mice carry the exact mutations found in human cancers, we hope to eventually test new therapeutic strategies using this animal model to help develop better therapies for this aggressive disease.

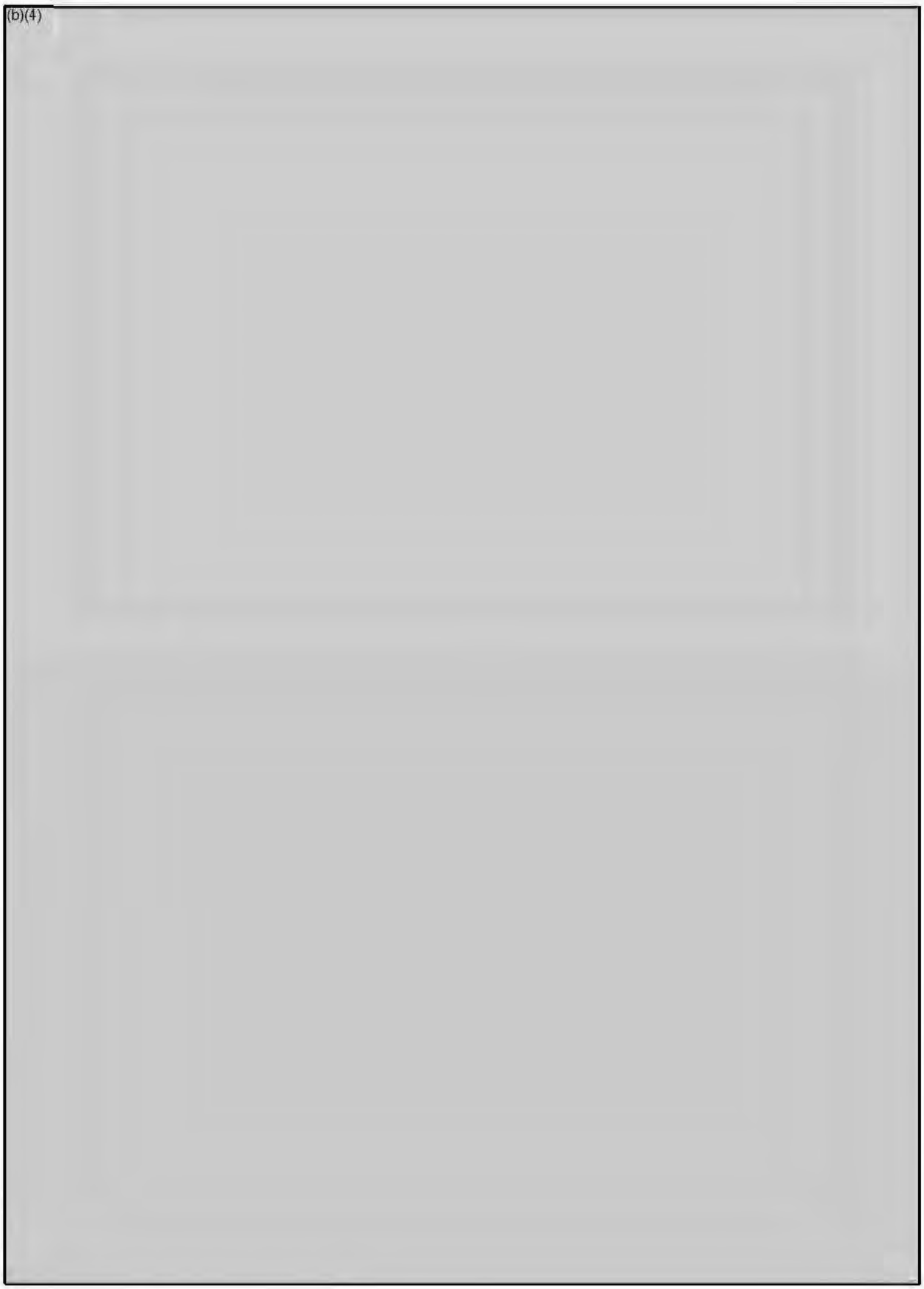
IV. MILITARY RELEVANCE: Military personnel on the battlefield may be exposed to radiation or toxic substances that could induce leukemia development later in life. Understanding the mechanisms underlying leukemia development should help find better treatments for this devastating disease. Although the focus of this study is studying the mechanisms involved in a specific form of childhood leukemia, all cancers share many similarities in pathogenesis such as activation of oncogenes and inactivation of tumor suppressor genes. Therefore, accomplishing this study has the potential to identify novel pathways that could be targeted for treating other cancers.

V. MATERIALS AND METHODS:

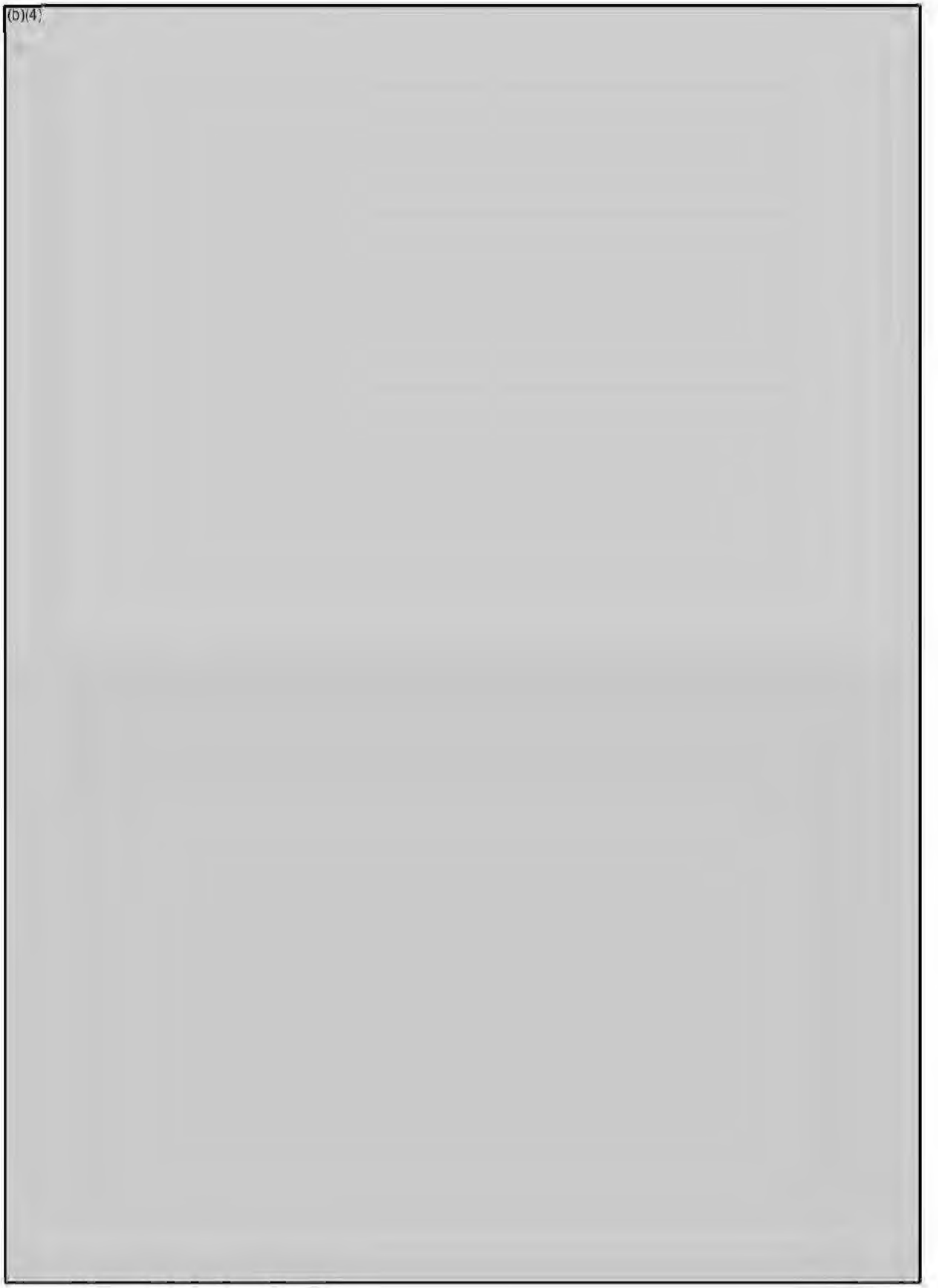
V.1. Experimental Design and General Procedures:

(b)(4)

(b)(4)



(b)(4)



(b)(4)

V.1.4. **Experiment 4: To determine the leukemia initiating population from mutant Cbl mice through bone marrow transplantation.**

Once we have mice that develop MPD and or leukemia, we will further characterize the disease phenotype by examination of the cell surface markers by flow cytometry to determine the type of leukemia. In order to determine whether the myeloproliferative disorder and or leukemia cells from the JMML mouse model is which is a hallmark of leukemia cells and its leukemia initiating cells, we will perform bone marrow transplantation experiments. Leukemic cells or pre-leukemic cells will be collected from donor transgenic mice and then transplanted into lethally radiated normal non transgenic mice. Lethally irradiated hosts will be given normal supporting cells to allow the mice to recover counts with transplanted cells and then followed for the development of disease.

In addition, in order to identify the leukemia initiating cells, different populations will be sorted by flow cytometry and then tested by bone marrow transplantation. Bone marrow will be extracted from mice at 6 to 12 weeks of age after euthanasia, and Hematopoietic Stem Cells (HSC), common myeloid progenitors (CMPs) and granulocyte macrophage progenitor (GMPs) will be purified from transgenic mice and will be transplanted into lethally irradiated B6-LY5.2/Cr mice (8-12 weeks old females, purchased from (b)(4) . The recipient mice will then be aged for up to 12 months after transplantation by monthly blood counts (see section V.4.1.2.1 for anesthesia procedures and section v.4.4.2 for biosample collection) and monitored for the development of disease in order to determine the identity of the leukemia initiating population. In addition, RNA, DNA, and protein will also be extracted from bone marrow cells for gene expression analyses such as microarray analysis and RT-PCR, and western blot analysis.

CMPs and GMPs represent very small percentage of total bone marrow cells. Around 3 mice of each genotype are required to obtain sufficient CMPs (about $3x\ 10^4$ cells) and GMPs (about 6×10^4 cells) for our proposed molecular studies in each experiment. Therefore, for 2 independent experiments, 2 x 6 animals/experiment x 5000

cells = 60000 CMP cells which can be obtained from 6 total animals with the correct genotype. In addition, purification of CMPs and GMPs is a very labor intensive procedure considering the large number of femurs that have to be processed in each setting and requires at least 2 people proficient in this procedure to work together. This procedure also contains several sensitive steps that tend to be error-prone such as 2 separate immuno-staining procedures with different cocktail of antibodies and one final 5-color cell sorting step. Therefore, animals generated in breeding protocol which don't have the correct genotype will be used for training and practice in preparation for these experiments after euthanasia. We will estimate that 20 mice are needed to learn the skills.

Recipient B6-LY5.2/Cr mice will be irradiated on the day of transfer of hematopoietic cells with 850 to 1050 rads of total body irradiation from a ^{137}Cs source located at room (b)(6) Hematopoietic cells resuspended in PBS in a volume of 200 ul will be introduced by tail vein injection. Following transfer of the hematopoietic cells, mice will be monitored for signs of distress daily such as ruffled fur, hunched back, and decreased activity. Past experience has shown that lethally irradiated mice can suffer from infection during engraftment of the transplanted cells. To prevent infection, all recipient mice will receive amoxicillin at a concentration of 1mg/ml in the drinking water from 5 days prior to irradiation to 10 days post irradiation. Although occurring rarely, engraftment failure of the transplanted cells will also lead to recipient death between 7 and 10 days after injection. Therefore, to prevent any potential suffering during this critical period of time, recipient mice will be monitored at least twice daily by PI staff between 7 and 10 days after transplantation for signs of distress including rough coat, listlessness, or hunched posture. Any transplant recipients showing such signs will be euthanized immediately. The dose of irradiation used is expected to be lethal within 10 to 14 days in the absence of bone marrow reconstitution by transplanted cells. Successful recolonization of the recipient bone marrow in transplanted animals results in restoration of constitutive hematopoiesis over the normal life span of the recipient.

Each transplantation experiment consists of 3-6 recipients and 5 experimental groups and requires a total of 25 B6-LY5.2/Cr animals as listed below: 1. PBS injection as positive control for irradiation (3 animals); 2. Supporting bone marrow (5×10^5 bone marrow cells from non-irradiated B6-LY5.2/Cr mice) as positive control for engraftment (3 animals); 3. CMPs (6 animals); 4. GMPs (6 animals); 5.HSCs (6 animals). For each transplant recipient in groups 3-5, 5×10^3 of specified myeloid progenitors will be injected via tail vein along with 5×10^5 bone marrow cells from non-irradiated B6-LY5.2/Cr mice for radioprotection. Therefore, in addition to the total of 24 recipients, one B6-LY5.2/Cr mouse is needed to provide non-irradiated bone marrow cells for each experiment. Group 1 recipients should die within 2 weeks after transplantation and will be euthanized as soon as they show sign of stress. Recipients in groups 2-5 should all survive after transplantation. While group 2 recipients will be euthanized at 4 months after transplantation, some of the recipients in group 3-5 will be euthanized at various times (2, 4, and 6 months) after transplantation for analysis of reconstitution. Bone marrow and spleen after euthanasia will be collected for FACS analysis. This experiment will be repeated 2 times to reach any conclusions.

So the minimum number of B6-LY5.2/Cr mice required for this study is: 25

animals/experiment x 2 experiments = 50.

Requested animal numbers:
Transgenic mouse donors: Need 3 per experiment X 2 experiments X 50 = 300
B6-LY5.2/Cr: 50

Total Requested; 370 (300 donor animals and 48 irradiated recipients and 2 unirradiated mice and 20 practice mice)

V.2. Data Analysis:

For all the experiments, gene expression and serial blood counts will be compared across genotypes using analysis of variance (ANOVA), student's t test, or nonparametric tests as appropriate. A sample size of 5 in each group will have 80% power to detect a difference of 2 standard deviations between groups using a 2-group t test with a 5%, two-sided significance level. This applies to experiment 4 where we are using 6 mice in order to ensure that we meet the minimum 5 needed as sometimes a mouse is lost unpredictably.

For aging studies, log-rank tests will be used to compare time to development of sickness such as cancer across genotype. When the sample size in each group is 20, a 5% two-sided log-rank test for equality of survival curves will have 80% power to detect a difference in proportion surviving to 18 months of at least 40 percentage points (e.g. 99% vs. 59% surviving). This applies to experiment 3.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: Tissue culture was considered, however cancer is a complex disease that requires proper interactions among different tissue and cell types within the context of a whole animal, and currently there is no tissue culture system capable of recapitulating this environment. In addition, cell lines used in tissue culture studies are different from normal hematopoietic cells in that they have acquired mutations that allow them to replicate indefinitely in culture, and results from these studies may not apply to normal cells. For example, leukemia associated ptpn-11 mutations introduced into 3T3 fibroblast cell line could not detect any phenotype (KL, unpublished data) while the introduction of Noonan syndrome associated mutation cleared had multiple phenotypes when introduced into the mouse germline (Araki et al, 2004).

Computer modeling was considered as well, but the regulation of hematopoietic development is still largely unknown and there is no system available in the hematopoiesis field that can be used to predict gene function and consequences of gene mutations.

V.3.2. Animal Model and Species Justification: We have chosen to use mice for our studies because they are genetically well-characterized mammalian organism that has been shown to have a very similar hematopoietic system to that of humans. In addition, there are many tools available in mice but not in other mammalian models to genetically alter the germline and study the consequences of the mutation on multiple organ systems simultaneously. We are using "knock out" mice which carry a null *ptpn-11* allele or null cbl allele as well as other transgenic mice such as the vav-icre mice to help characterize the phenotype of ptpn-11 mutant mice and Cbl mutant mice. Other organisms such as Zebrafish and Drosophila were also considered, but both species do not represent the best model for studying human hematopoiesis.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	*Mus musculus*	*Mus musculus*
V.3.3.2. Strain/Stock:	(b)(4)	
V.3.3.3. Source/Vendor:	In house	In house
V.3.3.4. Age:	6 weeks to 16 weeks	6 weeks to 12 weeks
V.3.3.5. Weight:	N/A	N/A
V.3.3.6. Sex:	~20 female	~10 females and 10 males
V.3.3.7. Special Considerations:	SPF	SPF
V.3.4. Number of Animals Required (by Species):	Mice 20	Mice 20
	Species #3	Species #4
V.3.3.1. Genus & Species:	*Mus musculus*	*Mus musculus*
V.3.3.2. Strain/Stock:	(b)(4)	
V.3.3.3. Source/Vendor:	In house	In house
V.3.3.4. Age: Age range is acceptable.	6 weeks to 12 weeks	6 weeks to 12 weeks

V.3.3.5. Weight: Weight range is acceptable.	N/A	N/A
V.3.3.6. Sex:	~10 females and 10 males	30 males and 30 females
V.3.3.7. Special Considerations:	SPF	SPF
V.3.4. Number of Animals Required (by Species):	Mice 20	Mice 60
	Species #5	Species #6
V.3.3.1. Genus & Species:	*Mus musculus*	*Mus musculus*
V.3.3.2. Strain/Stock:	C57Bl6/Ly 5.2	(b)(4)
V.3.3.3. Source/Vendor:	(b)(4)	In House
V.3.3.4. Age: Age range is acceptable.	6 weeks to 12 weeks	6 weeks to 6 months
V.3.3.5. Weight: Weight range is acceptable.	N/A	N/A
V.3.3.6. Sex:	35 males and 35 females	~30 males and 30 females
V.3.3.7. Special Considerations:	SPF	SPF
V.3.4. Number of Animals Required (by Species):	Mice 70	Mice 60
	Species #7	Species #8
V.3.3.1. Genus & Species:	*Mus musculus*	*Mus musculus*
V.3.3.2. Strain/Stock:	Cbl^{Y371H};$Cbl^{flox/flox}$	Cbl^{Y371H};$Cbl^{flox/flox}$;vav-icre
V.3.3.3. Source/Vendor:	In house	In house
V.3.3.4. Age: Age range is acceptable.	6 weeks to 12 weeks	6 weeks to 12 weeks

V.3.3.5. Weight: Weight range is acceptable.	N/A	N/A
V.3.3.6. Sex:	~30 females and 30 males	150 males and 150 females
V.3.3.7. Special Considerations:	SPF	SPF
V.3.4. Number of Animals Required (by Species):	Mice 60	Mice 300

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels. Animals observed to be ill or showing signs of pain or distress such as hunched posture, ruffled fur, and decreased activity will be evaluated by veterinary staff to be treated or be euthanized for relief of suffering.

V.3.5.2. Reduction: In order to reduce the animal numbers for this study, experiments have been designed to include tissue sharing among different experimental procedures including DNA extraction, RNA extraction, and protein extraction.

V.3.5.3. Replacement: Tissue culture and computer models were considered but not adopted. Detailed explanations are provided in V.3.1.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment: Animals observed to be ill or showing signs of pain or distress such as hunched posture, ruffled fur, and decreased activity will be evaluated by veterinary staff to be treated or be euthanized for relief of suffering.

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	(b)(4)	
V.4.1.1.1.1. Column C:	20	20
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:		
	Ptpn-11$^{WT/fl}$; phox2b-cre	(b)(4)
V.4.1.1.1.1. Column C:	20	60

V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:		
	C57Bl6/Ly 5.2	(b)(4)
V.4.1.1.1.1. Column C:	22	60
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	48	
	(b)(4)	
V.4.1.1.1.1. Column C:	60	300
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:		

V.4.1.2. Pain Relief / Prevention: Because we are trying to generate a mouse model of leukemia, some mice will become ill. Animals observed to be ill or showing signs of pain or distress such as hunched posture, ruffled fur, and decreased activity will be evaluated by veterinary staff to be treated or be euthanized for relief of suffering. All procedures except blood draw will be after euthanization. To prevent infection after irradiation, all animals will be given prophylactic antibiotics for 14 days.

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

Local anesthesia for blood draw: For superficial temporal vein sampling, we will not use local anesthesia. For lateral tain vein or ventral artery sampling, we will use lidocaine cream or ethyl chloride spray for local anesthesia. For other types of sampling, we will use either isoflurane chamber or injectable anesthesia. Isoflurane chamber is available in LAM for use by investigators and for injectable anesthesia, intraperitoneal injection with 0.1 cc of ketamine/xylazine mixture (10:1 mixture of 100mg/ml solution) will be used if needed.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: To prevent infection in irradiated mice, all recipient mice will receive amoxicillin at a concentration of 100ug/ml in the drinking water from 3 days prior to irradiation to 14 days post irradiation.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: Pubmed

V.4.1.3.2. Date of Search: 2/20/15

V.4.1.3.3. Period of Search: 1990-2014
V.4.1.3.4. Key Words of Search: animal models, animal irradiation procedure and alternatives
V.4.1.3.5. Results of Search:
Based on the search results, replacement of proposed procedure regarding irradiation of mice for bone marrow transplantation was considered but there is no viable replacement for irradiation.
V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: Irradiation is used to prepare the recipient mice for accepted donor bone marrow. Irradiation itself is not painful but results in side effects which can be painful but is needed for transplant procedures. Chemotherapy can be used but results in the same side effects or worse. There are colony formation assays which can be correlated with ability to reconstitute the hematopoietic system. However, there is no substitute for testing in an animal model. We will use the minimum amount of mice necessary for transplant studies and euthanize animals as soon as signs of distress appear.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: N/A

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: intravenous injection as described in V4.4.1 below and phlebotomy as described in V4.4.2 below.

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: Intravenous injections will be required for infusing whole bone marrow cells, sorted stem cells, and/or leukemia cells for transplant experiments. A 27 gauge needle will be used for tail vein injections with cells in phosphate buffered saline at a maximum of 10% blood volume to be delivered. For inhalation anesthesia, we will use the isoflurane chamber available in LAM or for injectable anesthesia, intraperitoneal injection with 0.1 cc of ketamine/xylazine mixture (10:1 mixture of 100mg/ml solution) will be used if needed.

V.4.4.2. Biosamples: Hematopoietic tissues including bone marrow, thymus, spleen, and lymph nodes will be collected at euthanasia. In addition, animals that are being aged will receive periodic blood counts (not more than monthly) done via tail vein phlebotomy or other IACUC accepted methods such as sub-mandibular phlebotomy. For tail vein phlebotomy, a lancet is used to make an incision at the tail vein and drops of blood are collected using a capillary collection tube. For submandibular phlebotomy, anesthesia is used for the procedure followed by an incision using a lancet and collection using capillary collection tube. Only about 50 to 100 microliter of blood will be collected and used for blood counts which are less than 5% of blood volume.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: The identification system adopted consists of two parts, a specific animal number assigned to each mouse in the colony and a specific ear punch pattern generated to distinguish individual mouse inside of a cage of 5 animals. Specifically, one animal will not be ear-punched while 4 other animals will be punched in either the top or bottom part of the left or right ear. These ear punch patterns will be assigned with specific codes: 00 for no punches, 01 for top right ear, 03 for bottom right ear, 10 for top left ear, and 30 for bottom left ear. These ear punch codes will be recorded along with the individual animal number assigned to each mouse and the genotypes on the cage card. Ear punches will be performed by me or my future staff.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: Irradiation for bone marrow reconstitution experiments: Recipient B6-LY5.2/Cr mice will be irradiated on the day of transfer of hematopoietic cells with 850 to 1050 rads of total body irradiation from a ^{137}Cs source located at room (b)(6) Hematopoietic cells resuspended in PBS in a volume of 200 ul will be introduced by tail vein injection. Following transfer of the hematopoietic cells, mice will be monitored for signs of distress daily such as ruffled fur, hunched back, and decreased activity. All irradiated mice will be monitored by the PI or research staff daily for 14 days. Any recipients found morbid will be euthanized immediately.

V.4.4.8. Tissue Sharing: Tissues may be shared with other investigators upon request.

V.4.5. Study Endpoint: The study endpoint for aged animals is up to 18 months of age or when euthanized earlier due to symptoms of leukemia such as bleeding, listlessness, and cachexia or other diseases. The criteria for early removal of aged animals will be if animals appear to be ill or in discomfort based on the evaluation by the veterinary or research staff and will be treated or euthanized as appropriate.

V.4.6. Euthanasia: Animals under this protocol will be euthanized by research personnel. In general, mice will be euthanized using cylinderized CO2. These animals will be placed in a cage or chamber that is not crowded and exposed to a mix of CO2

and oxygen. The CO2 flow rate will displace at least 20% of the chamber volume per minute. Flow will continue for at least one minute after visible sign of death. CO2 euthanasia will be followed by cervical dislocation to ensure death.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes _____	No X
Fluid Restriction:	Yes	No X

V.5.1.3. Exceptions: None _____ _____

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be observed at least once daily by Center for LAM personnel. PI will observe the colony at least once a week unless the animals have been irradiated in which case they will be observed daily for 14 days post-irradiation. Animals or pups observed to be ill or showing signs of pain or distress such as hunched posture, ruffled fur, and decreased activity will be evaluated by veterinary staff to be treated or be euthanized. PI should be contacted for potential tissue collection needs if sick animals are to be euthanized by LAM personnel.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
IP injections, tail vein injections, tail vein blood collection, and CO2 euthanasia punching	(b)(6)	14 years of experience	Animal Care and Use Introductory Training Course at Lawerence Berkeley Laboratory, 1989, at Northwestern University, 1992, at UCSF 2005, USUHS 2009) Experimental gen etics of laboratory mouse, course at Jackson laboratory, 1992.
Co2 euthanasia		5 years of experience	Animal Care and Use Introductory Training Course at USUHS April, 2009.

VII. BIOHAZARDS/SAFETY:

Personal protective equipment will be used such as gloves, lab coat and mask when handling mice and its tissues. Anesthetic agents and drugs will be handled wearing gloves with active scavenging system such as a hood or suction device to eliminate potential exposure when using inhalation anesthesia.

VIII. ENCLOSURES:

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6)

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6)

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number: (b)(6)

B. Animal Protocol Title: Characterization of ptpn-11 and Cbl Gain of Function Mutations using Transgenic mouse models

C. Principal Investigator: (b)(6) MD, PhD, Pediatrics

D. Performing Organization: Uniformed Services University of the Health Sciences

E. Funding: Department of Pediatrics

F. Objective and Approach: Juvenile Myelomonocytic Leukemia (JMML) is an aggressive disease of childhood with a very poor prognosis. Despite improvements in cure rates of childhood leukemia approaching 90%, most patients with JMML ultimately succumb to their disease. The discovery that JMML samples contained a mutated form of the *PTPN11* and Cbl gene has given us new insights into the biological differences between JMML and other leukemias. It has highlighted the importance of gain of function mutations which activate the ras pathway in the pathogenesis of JMML.

Our laboratory is interested in studying how mutations in genes involved in the ras pathway cause the development of JMML and investigating the cooperating events that make this leukemia different. Using known leukemia associated mutations identified from human JMML samples, we have generated mouse models of this disease using a bacterial artificial chromosome (BAC) containing the *PTPN11* locus. In addition, we have also incorporated the Cbl mutations to be expressed using the conditional *PTPN11* BAC as the vector to drive tissue specific expression.

We have been expressing the transgene in specific tissues by crossing the transgenic mice with different Cre expressing strains and are evaluating the mice by serial blood counts. We are planning to further investigate the cooperative events required for the development of JMML by utilizing genetic crosses to investigate the effect of two different oncogenic mutations. We hope to develop novel therapeutic strategies targeting the ras pathway using these mice as a therapeutic platform in the future.

The objective of this protocol is to characterize the role of mutant *ptpn-11* gene and Cbl during normal mammalian development, with a focus on hematopoietic development and leukemogenesis. Different genetically engineered mutant mouse strains for ptpn-11 and Cbl will be generated and characterized. Developmental defects in these mutant animals will be characterized comparing to wild-type littermates. In addition, mutant ptpn-11 mice and Cbl mice will be bred with other transgenic mice to further characterize the mutant phenotype and to study the function of ptpn-11 and Cbl. The three R's (reduction, replacement, and refinement) of animal usage will be employed.

G. Indexing Terms (Descriptors): Transgenic mice, ptpn-11, shp-2, Cbl, JMML

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Institutional Animal Care and Use Committee
Phone: (b)(6)
Fax: (b)(6)

April 29, 2016

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF PEDIATRICS

SUBJECT: IACUC Approval of Protocol - Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on April 29, 2016:

Animal Protocol Title: "Role of Setbp1 in Leukemic Stem Cell Self-Renewal in Mice"

USUHS Protocol Number: (b)(6)

Expiration Date: April 28, 2019

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a post approval meeting with LAM (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206 ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Role of Setbp1 in leukemia-initiating cell self-renewal in mice

GRANT TITLE (if different from above): Role of XPO1/CRM1 in SETBP1-induced Leukemia Development

USUHS PROJECT NUMBER/ DAI GRANT NUMBER: (b)(6)

FUNDING AGENCY: WRNMMC

EARLIEST ANTICIPATED FUNDING START DATE: 5/6/2016

PRINCIPAL INVESTIGATOR: (b)(6)

(b)(6)	PED	(b)(6)	4/6/2016
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	Chair	(b)(6)	4/7/2016
Research Unit Chief / Dept. Head Signature	Title	Telephone	Date

Typed Name: Dr (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)		(b)(6)	4/7/16
Statistician Signature	Department	Telephone	Date

Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics. All signatures are required prior to submission to the IACUC Office.

(b)(6)	LAM	(b)(6)	4/6/16
Attending/Consulting Veterinarian Signature	Department	Telephone	Date

Printed Name: (b)(6) VMD

The aims of the supporting grant must be submitted along with the 3206. If funding is from NIH please also submit the Vertebrate Animal Section (VAS).

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) Department of Pediatrics, (b)(6)

PRINCIPAL INVESTIGATOR EMAIL: (b)(6)

ANIMAL PROTOCOL TITLE: Role of Setbp1 in leukemia-initiating cell self-renewal in mice

GRANT TITLE (if different from above): Role of (b)(4) in SETBP1-induced Leukemia Development

USUHS PROJECT NUMBER: (b)(6)

DAI GRANT NUMBER: (b)(6)

CO-INVESTIGATOR(S): N/A

TECHNICIANS(S): (b)(6)
(b)(6)

I. NON-TECHNICAL SYNOPSIS: This study is designed to use mice as a model to investigate the role of a gene called Setbp1 in the development of leukemia, a deadly disease affecting many people, and also to identify better therapeutic strategies for Setbp1-induced leukemia. Mice carrying bone marrow cells that contain extra copies of the Setbp1 gene or Setbp1 mutations identified in leukemia patients will be generated in some studies by bone marrow transplantation and examined to understand the mechanisms for leukemia development. Bone marrow donor mice will be treated with a drug called 5-fluorouracil to facilitate the introduction of normal or mutant Setbp1 gene copies into their bone marrow cells. 5-fluorouracil treatment at our proposed dosage will not cause adverse symptoms in mice. Bone marrow recipient mice will be irradiated before transplantation to enhance the establishment of the transplanted cells in their bone marrow. Irradiation at our proposed dosage is lethal to mice if not treated afterwards. However, these recipient mice in our study will be fully rescued by the subsequent transplantation of donor bone marrow cells. Some recipient mice that developed leukemia will also be treated with a drug called (b)(4) to test its potential therapeutic effects against Setbp1-induced leukemia. (b)(4) treatment at our proposed dosage will not cause adverse symptoms in mice. The accomplishment of this study will help develop more effective therapies for leukemia patients in the future.

II. BACKGROUND:

II.1. Background: Targeting the unlimited self-renewal capability of leukemia-initiating cells (LICs) represents a new promising strategy for leukemia therapy [1,2]. However, only a small number of genes/mutations have been identified to confer this feature to LICs, which has significantly hampered our understanding of the underlying mechanism(s) for LIC self-renewal and our ability to design effective inhibitory strategies. We have previously found that overexpression of *Set binding protein 1*

(*Setbp1*), which encodes an AT-hook transcription factor, could confer unlimited self-renewal capability to normal mouse bone marrow progenitor cells and cause their immortalization in culture, suggesting that it could be a LIC self-renewal regulator [3]. The relevance of *SETBP1* overexpression to human leukemia development is also indicated by a previous study showing its overexpression in a significant fraction (up to 27%) of human acute myeloid leukemia cells [4]. More recently, in our collaboration with Dr. (b)(4) highly recurrent point mutations of *SETBP1* were identified in 15% of chronic myelomonocytic leukemia and 17% secondary acute myeloid leukemia patients in a sequencing screen of over 700 myelodysplastic syndrome (MDS) and myeloid leukemia patients [5]. These point mutations are missense mutations concentrated in a stretch of 15 amino acids of SETBP1 protein with D868N and I871T being two of the most prevalent changes. Given the high prevalence of these mutations in human patients, it is essential to understand the mechanisms through which they may contribute to leukemogenesis. Under previously approved protocol (b)(6) we have discovered that both *Setbp1* and *Setbp1* mutations are potent oncogenes capable of inducing myeloid leukemia development in mice. However, the molecular mechanisms responsible for their oncogenicity remain unclear. In the proposed studies, we will examine the potential complex formation and cooperation of Setbp1 and Setbp1 mutations with proteins including (b)(4) (b)(4) in inducing leukemia development.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: DTIC, NIH RePORTER and PubMed

II.2.2. Date of Search: April 4, 2016

II.2.3. Period of Search:
DTIC: 1985 – 2016
NIH RePORTER: 1988 – 2016
PubMed: 1950 – 4/4/2016

II.2.4. Key Words and Search Strategy: This study is mainly designed to use the mouse bone marrow transduction and transplantation model to characterize the function of Setbp1 in leukemia development. Therefore, "Setbp1" together with "leukemia" and "mice" were used as the search term.

II.2.5. Results of Search: Four projects were returned on DTIC. Two of them are my animal study protocols from 2008, and the other two are projects focused on prostate cancer. Three projects were returned on NIH RePORTER. One of them is my project funded by NCI. Two other projects returned are focused on cooperation of SETBP1 mutations with CSF3R mutations in chronic neutrophilic leukemia and atypical chronic myeloid leukemia. There is no overlap of these studies with my current study, which investigates cooperation between Setbp1 and (b)(4) in development of acute

myeloid leukemia. Six articles were returned on PubMed as listed below. Article #1, #5, and #6 were published from my laboratory. Article #2 studied the engraftment of human leukemia cells with a SETBP1 mutation in immune-deficient mice. Article #3 is a review article on chronic neutrophilic leukemia. Article #4 studied potential cooperation between SETBP1 and AXSL mutations, which is different from the focus of this proposal. Thus, all six articles have no overlap with my proposed studies.

1: Vishwakarma BA, Nguyen N, Makishima H, Hosono N, Gudmundsson KO, Negi V, Oakley K, Han Y, Przychodzen B, Maciejewski JP, Du Y. Runx1 repression by histone deacetylation is critical for Setbp1-induced mouse myeloid leukemia development. Leukemia. 2016 Jan;30(1):200-8. doi: 10.1038/leu.2015.200. Epub 2015 Jul 24. PubMed PMID: 26205084; PubMed Central PMCID: PMC4703539.

2: Sivagnanalingam U, Balys M, Eberhardt A, Wang N, Myers JR, Ashton JM, Becker MW, Calvi LM, Mendler JH. Residual Disease in a Novel Xenograft Model of RUNX1-Mutated, Cytogenetically Normal Acute Myeloid Leukemia. PLoS One. 2015 Jul 15;10(7):e0132375. doi: 10.1371/journal.pone.0132375. eCollection 2015. PubMed PMID: 26177509; PubMed Central PMCID: PMC4503761.

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III. **OBJECTIVE\HYPOTHESIS**: The objectives of this study are to gain novel insights into the cellular and molecular mechanisms by which Setbp1 overexpression and missense mutations induce leukemia development, and also to test new therapeutic

strategies for *SETBP1*-induced leukemias based on these insights through the use of mouse models.

IV. MILITARY RELEVANCE: Leukemia represents a significant challenge for DoD medical care. Leukemia occurrence in the general population of U.S. is about 12 every 100,000 people according to statistical studies by National Cancer Institute. About half of these patients will die due to limited treatment options for this devastating disease. There are estimated over 5 million military personnel and their family members under DoD based on a 2004 study reported by Military Family Research Institute at Purdue University. Therefore, more than 600 new leukemia cases and over 300 deaths due to leukemia are estimated to occur every year in the DoD health care system. These numbers could be even higher as the occurrence of leukemia could potentially further increase among military personnel due to their possible exposure to toxic substances or radiation on the battlefield. Finding better treatments for leukemia patients represents an important need for the DoD health care system. Our proposed study will help gain insights into the mechanisms underlying leukemia initiation and progression, and mechanistic understanding of leukemia development should help find preventive measures and better treatments for this devastating disease.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1: To determine the therapeutic effects of (b)(4) (b)(4) on *Setbp1*-induced myeloid leukemias.

Based on our finding of (b)(4) we have found that treatment with (b)(4) caused apoptosis and differentiation of Setbp1-immortalized myeloid progenitors in culture, suggesting that (b)(4) inhibitors could be used for treatment of human myeloid leukemias with SETBP1 overexpression or SETBP1 activation mutations. To further test this hypothesis, we would like to test the (b)(4) (b)(4) purity= 99.79%), an orally bioavailable (b)(4) in treating murine myeloid leukemias induced by overexpression of wild-type Setbp1 (Setbp1-WT) or Setbp1 activation mutation D868N (Setbp1-DN) *in vivo*. The use of (b)(4) is in accordance with IACUC policy 27 of guidelines for the use of non-pharmaceutical grade compounds in laboratory animals as there is no equivalent veterinary or human drug to (b)(4) that is available for experimental use.

Specifically, bone marrow or spleen cells (viably frozen) from previous Setbp1-induced leukemic mice (generated previously in B6-LY5.2/Cr mice by bone marrow transplantation under (b)(5) will be transplanted into irradiated 7-12 weeks old B6-LY5.2/Cr mice. These B6-LY5.2/Cr recipients will be irradiated on the day of transplantation with1,100 rads of total body irradiation from a ^{137}Cs source located at room (b)(6) The leukemia cells (1×10^6 cells/mouse) along with 5×10^5 supporting bone marrow cells from un-irradiated B6-LY5.2/Cr mice resuspended in PBS in a volume of 200 ul will be introduced into each recipient by tail vein injection.

Following transfer of the hematopoietic cells mice will be monitored for signs of distress daily. Any recipients found morbid will be euthanized immediately. The dose of irradiation used is expected to be lethal within 10 days in the absence of bone marrow reconstitution by transplanted cells. Successful recolonization of the recipient bone marrow in transplanted animals results in restoration of constitutive hematopoiesis over the normal life span of the recipient. Past experience has shown that lethally irradiated mice can suffer from infection. To prevent infection, all recipient mice will receive amoxicillin at a concentration of 1 mg/ml in the drinking water from 5 days prior to irradiation to 10 days post irradiation. Although occurring rarely, engraftment failure of the transplanted cells will also lead to recipient death between 7 and 10 days after injection. Therefore, to prevent any potential suffering during this critical period of time, recipient mice will be monitored twice daily by PI staff between 7 and 10 days after transplantation for signs of distress including rough coat, listlessness, or hunched posture. Any transplant recipients showing such signs will be euthanized immediately.

At one week after transplantation, the recipient mice will be divided into 2 groups, one receiving (b)(4) at 20 mg/kg of body weight (in 200ul 50%PEG400/50%ddH2O) through oral gavage once every 2 days for 22 days (total 10 doses) and the other 200ul vehicle only. (b)(4) is a new investigational compound and no chemical purity standards have been established; however, (b)(4) treatment at the proposed dosage has been shown to be safe for mice [6]. Treated mice will be monitored for signs of leukemia development including rough coat, listlessness, and hunched posture. Any recipients displaying any combination of two or more signs of distress above will be euthanized immediately. It is expected that mice receiving vehicle only will develop leukemia within 2-3 weeks after transplantation. Absence or delayed leukemia development in mice treated with (b)(4) will strongly suggest that (b)(4) can be effective in treating human myeloid leukemia patients with SETBP1 overexpression or activation mutations.

The study end point is 3 months after transplantation when all survived animals will be euthanized. A total of 6 leukemias (3 induced by Setbp1-WT and 3 induced by Setbp1-DN) will be transplanted in order to draw any significant conclusions. For each leukemia, 10 recipient mice will be transplanted (5 for (b)(4) treatment, 5 for vehicle). Therefore, **this experiment requires a total of 60 B6-LY5.2/Cr recipients (6 leukemias x 10 recipients/leukemia). 6 additional B6-LY5.2/Cr mice will be used as donors for supporting bone marrow as each transplantation experiment requires one such donor.**

Required animal numbers for Experiment 1:

Strains	Experimental groups		Supporting B.M.	Total
	(b)(4)	vehicle		
B6-LY5.2/Cr	30 (Category E)	30 (Category E)	6 (Category C)	66

V.1.2. Experiment 2: To determine the cooperation between Setbp1 and (b)(4) (b)(4) gene in leukemia development using mouse bone marrow transduction and transplantation.

We have identified [(b)(4)] as a potential cooperating partner for Setbp1 activation in acute myeloid leukemia development due to its complex formation with [(b)(4)] and Setbp1 and its overexpression in Setbp1-induced leukemia cells. To directly test this idea, we will co-transduce 5-fluorouracil (5-FU) treated mouse bone marrow progenitors with retroviral vectors expressing [(b)(4)] and Setbp1-WT/Setbp1-DN and assess their leukemogenic potential in comparison with singly transduced cells in transplantation recipient mice.

5-FU treated bone marrow progenitors from C57BL/6 mice (8 to 12 weeks old females purchased from [(b)(4)] will be transduced with retrovirus expressing Setbp1-WT/Setbp1-DN alone, [(b)(4)] alone, or the combination, and transplanted into irradiated B6-LY5.2/Cr recipients. The 5-FU treatment helps deplete the number of more mature progenitor cells in the bone marrow and induce the cycling of hematopoietic stem cells which are targets of retroviral transduction in the next step. Mice will be treated once with 0.2ml of 5-FU (pharmaceutical grade) in phosphate buffered saline (PBS) via intra-peritoneal injection to achieve a dose of 150 mg/kg body weight 4 days before bone marrow harvesting. The treated mice will be euthanized by CO2 asphyxiation and bone marrow cells will be harvested from their femurs under sterile conditions. The dose of 5-FU used in these experiment is not expected to cause any animal morbidity or illness.

Bone marrow cells extracted from 5-FU treated mice will be infected with replication incompetent retroviral vectors carrying Setbp1-WT/Setbp1-DN and/or [(b)(4)] (retroviral vector usage has been approved by IBC under project # [(b)(6)] These viruses will be collected from culture medium of cell lines designed to package viral particles in ecotropic envelops. After initial infection these viruses are no longer infectious and are replication deficient. Prior to, during, and post infection, these bone marrow progenitors may be cultured in the presence of recombinant murine growth factors including interleukin-3 (IL-3), interleukin-6 (IL-6), and stem cell factor (SCF) to stimulate their proliferation (In-vitro procedures only). The infected bone marrow progenitor cells (1 x 10^6 cells/mouse) along with 5 x 10^5 supporting bone marrow cells from un-irradiated B6-LY5.2/NCr mice resuspended in PBS in a volume of 200 ul will be introduced into each recipient by tail vein injection. Following transfer of the hematopoietic cells mice will be monitored for possible bone marrow engraftment failure and leukemia development and euthanized if necessary as described in Experiment 1. Any recipients found in distress will be euthanized immediately. At 1, 2, and 4 month after transplantation, 200ul of blood may be collected from each transplant recipient by retro-orbital bleeding to monitor the engraftment of the transduced cells by flow cytometry.

In order to draw any significant conclusions, fifteen recipient mice will be used in each group. Therefore, we will **transplant a total of 45 B6-LY5.2/Cr recipient mice for testing cooperation between Setbp1-WT and** [(b)(4)] (45 recipients for 3 groups of transduced cells). In addition, **5 un-irradiated B6-LY5.2/Cr mice will be required to provide supporting bone marrow cells** as we plan to accomplish above transplantation studies in 5 experiments, each experiment requires one supporting bone marrow donor.

Each transplant recipient will be transplanted with 1 x 10^6 transduced cells, which requires 2 C57BL/6 mice to provide sufficient donor cells. Therefore, for a total of 45 primary transplant recipients as discussed above, **a total of 90 C57BL/6 mice (45 recipients x 2) is required to provide sufficient donor cells for testing cooperation between Setbp1-WT and** (b)(4)

Same transplantation studies will be carried out separately for testing cooperation between Setbp1-DN and (b)(4) **and similarly a total of 50 B6-LY5.2/Cr recipient mice and 90 C57BL/6 mice will be required.**

Required animal numbers for Experiment 2:

For testing Setbp1-WT (b)(4) cooperation:

Strains	Experimental groups			Supporting B.M.	Total
	Setbp1-WT (b)(4)	Setbp1-WT	(b)(4)		
B6-LY5.2/Cr	15 (Category E)	15 (Category E)	15 (Category E)	5 (Category C)	50
C57BL/6 (donors)	90 for 3 groups (Category C)				90

For testing Setbp1-DN (b)(4) cooperation:

Strains	Experimental groups			Supporting B.M.	Total
	Setbp1-DN (b)(4)	Setbp1-DN	(b)(4)		
B6-LY5.2/Cr	15 (Category E)	15 (Category E)	15 (Category E)	5 (Category C)	50
C57BL/6 (donors)	90 for 3 groups (Category C)				90

V.1.3. Experiment 3: To examine the requirement for (b)(4) gene in Setbp1-induced leukemia development.

We also found (b)(4) (b)(4) suggesting that they may cooperate to induce leukemia development. In order to test this hypothesis, we would like to analyze the effects of (b)(4) on the maintenance of leukemia induced by either Setbp1-WT or Setbp1-DN. To achieve this goal, we will obtain (b)(4) mice (b)(4) and breed these mice with Cre-ER mice in which Cre expression can be induced by Tamoxifen treatment. Bone marrow progenitors from the offspring (b)(4) will be used to generate Setbp1 and Setbp1(DN)-induced leukemia cells in which (b)(4) can be deleted by Tamoxifen treatment after transplantation into secondary recipients.

A. Generation of myeloid leukemias using bone marrow progenitors from (b)(4) mice.

Age- and sex-matched littermates at 2 to 3 months of age consisting of 2 genotypes, (cko/cko,tg/+) and (cko/cko,+/+), will be treated with 5-FU as described in Experiment 2.

Bone marrow cells extracted from 5-FU treated mice will be infected with replication incompetent retroviral vectors carrying Setbp1-WT or Setbp1-DN. Since bone marrow progenitors from (b)(4) mice are positive for Ly5.2, transduced cells will be subsequently transplanted into C57BL/6 recipient mice. C57BL/6 recipient mice will be irradiated and treated with Amoxicillin as described for B6-LY5.2/Cr mice in Experiment 2. For both Setbp1-WT and Setbp1-DN, three independent (cko/cko,tg/+) leukemias and three control (cko/cko,+/+) leukemias will need to be generated for studies in B. Due to the scarcity of bone marrow progenitor cells, 2 animals of same genotype are needed for generating one leukemia. **So the minimum animal number required for (b)(4) (b)(4) mice is: 2 donor animals/leukemia x 12 leukemias = 24. In addition, 4 un-irradiated C57BL/6 mice will be required to provide supporting bone marrow cells** as we plan to accomplish above transplantation studies in 4 experiments, each experiment requires one supporting bone marrow donor.

B. Secondary transplantations will be carried out to examine the requirement for (b)(4) for the maintenance of leukemia cells. Six independently generated (cko/cko,tg/+) leukemias will be transplanted into newly irradiated C57BL/6 mice at a dosage of 1 $x10^6$cells/mouse together with 5 $x10^5$ supporting bone marrow cells from non-irradiated C57BL/6 mice. Each leukemia will be transplanted into 10 recipient mice, which will be divided into 2 groups. Starting at 7 days after transplantation, one group will be treated with Tamoxifen at 200mg/kg body weight in 200ul of corn oil while the other with corn oil only by 3 intra-peritoneal injections at 2-day intervals. Treated mice will be monitored for the development of leukemia as described in Experiment 1 for 3 months. Six (cko/cko,+/+) leukemias will also be transplanted and treated similarly to control for effects of Tamoxifen treatment. It is expected that mice receiving (cko/cko,tg/+) leukemia cells and vehicle as well as mice transplanted with (cko/cko,+/+) leukemia cells will develop leukemia within 2-3 weeks after transplantation. Absence or delayed leukemia development in mice receiving (cko/cko,tg/+) leukemia cells and Tamoxifen will strongly suggest that (b)(4) is critical for maintenance of leukemia. Therefore, **this experiment requires a total of 120 C57BL/6 recipients (12 leukemias x 10 recipients/leukemia). 12 additional C57BL/6 mice will be used as donors for supporting bone marrow as each transplantation experiment requires one such donor.**

Required animal numbers for Experiment 3A:

Strains	Experimental groups		Supporting B.M.	Total
	Setbp1-WT	Setbp1-DN		
(b)(4)	12 (Category C)	12 (Category C)		24
C57BL/6	6 (Category E)	6 (Category E)	4 (Category C)	16

Required animal numbers for Experiment 3B:

Strains	Experimental groups		Supporting B.M.	Total
	Tamoxifen	vehicle		

C57BL/6	60 (Category E)	60 (Category E)	12 (Category C)	132

V.1.4. Experiment 4: Conditional knock-in mice will be used to compare the cellular and molecular effects induced by Setbp1-WT and Setbp1-DN.

Our previous studies using mouse bone marrow transduction and transplantation system showed that Setbp1-DN induced leukemia development with much shorter latencies than Setbp1-WT, however the responsible mechanisms are difficult to study in this system due to small number of cells that engraft in the recipient mice. To circumvent this problem, (b)(4) (b)(4) (b)(4) the presence of Cre recombinase. After breeding with Mx1-cre mice in which Cre expression can be induced by poly(I:C) injections, (b)(4) (b)(4) (b)(4) will be used to study leukemia promoting effects induced by Setbp1-WT and -DN after poly(I:C) injections.

A. We will compare early cellular and molecular effects of Setbp1-WT and Setbp1-DN activation. Specifically, age- and sex-matched littermates consisting of 2 genotypes, (ki/+,tg/+) and (ki/+,+/+), will receive 3 intra-peritoneal injections of poly(I:C) at 2-day intervals at 2 to 3 months of age to induce the expression of Setbp1-WT or Setbp1-DN. The injections will be carried out using 28 Gauge x ½ inch needles and 200ul of poly(I:C) at a concentration of 2 ug/ul diluted in sterile phosphate buffered saline will be injected each time. Injection of poly I:C at this dosage is safe for mice. The injected animals will be euthanized at approximately 1 month after the final injection and tissues including hematopoietic tissues such as bone marrow, thymus, and spleen will be used for 2 types of experiments including the preparation of RNA, DNA, and protein for gene expression analysis and fluorescence activated cell sorting (FACS) for analysis of cell number, cell cycle and apoptosis status of hematopoietic stem and progenitor populations. Each type of experiment will need to be successfully repeated 5 times to reach any conclusions. So the minimum animal number for both (b)(4) and (b)(4) mice is: 2 experiment types x 2 genotypes x 5 animals/genotype x 2 strains = 40.

B. Another 30 animals of each strain consisting of 2 genotypes, (ki/+,tg/+) and (ki/+,+/+) will be aged for one year after poly(I:C) injections to test whether leukemias can be eventually induced by activation of Setbp1-WT or Setbp1-DN in these animals. Injected mice will be monitored for signs of leukemia development including rough coat, listlessness, and hunched posture. Any recipients displaying any combination of two or more signs of distress above will be euthanized immediately and fresh tissues including bone marrow, spleen, thymus, liver, and kidney will be collected and examined by flow cytometry, tissue culturing and pathology. Any remaining mice 1 year after poly(I:C) injections will be euthanized

Required animal numbers for Experiment 4A:

Strains	Experimental groups	Total

	ki/+,tg/+	ki/+,+/+	
(b)(4)	10 (Category C)	10 (Category C)	20
(b)(4)	10 (Category C)	10 (Category C)	20

Required animal numbers for Experiment 4B:

Strains	Experimental groups		Total
	ki/+,tg/+	ki/+,+/+	
(b)(4)	15 (Category C)	15 (Category C)	30
(b)(4)	15 (Category C)	15 (Category C)	30

V.2. Data Analysis: For all molecular experiments, engraftment/contribution of cell populations, cell cycle and apoptosis status, and colony/cell counts will be compared across groups using analysis of variance (ANOVA), student's t test, or nonparametric tests as appropriate. A sample size of 5 in each group will have 80% power to detect a difference of 2 standard deviations between groups using a 2-group t test with a 5%, two-sided significance level.
For aging studies, log-rank tests will be used to compare time to development of leukemia across groups. When the sample size in each group is 15, a 5% two-sided log-rank test for equality of survival curves will have 80% power to detect a difference in proportion surviving of at least 54 percentage points (e.g. 99% vs. 45% surviving)

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: Tissue culture study was considered, however it does not allow identifying and characterizing the effects of mutations within the context of the whole animal. *in vivo* study is extremely important in leukemia research as the evolvement of leukemic cells is not an isolated event and involves constant interaction with the host environment which has yet to be replicated in tissue culture settings. In addition, hematopoietic cell lines used in tissue culture studies are different from normal hematopoietic cells in that they have acquired mutations that allow them to replicate indefinitely in culture, and results from these studies may not apply to normal cells. Computer modeling was considered as well, but the mechanisms underlying leukemia development is still largely unknown and there is no system available in the leukemia field that can be used to predict target genes involved in the transformation of hematopoietic cells.

V.3.2. Animal Model and Species Justification: I have chosen to use mice for my studies because they are a genetically well-characterized mammalian organism that has been shown to have a very similar hematopoietic system to that of humans, and more and more studies have been carried out in mice to understand human leukemia development and normal hematopoiesis [7-9]. In addition, there are many tools available in mice but not in other mammalian models to genetically alter the germline and study

the consequences of the mutation in normal cells. I also happen to have extensive experiences (for the past 13 years) working with mouse hematopoietic system. Lower organisms such as Zebrafish and Drosophila were also considered, however both species are very different from mammals and do not represent good models for studying human hematopoiesis.

V.3.3. Laboratory Animals

ALTERNATIVES CONSIDERATIONS: Does the protocol have any provisions that would qualify it to be identified as one that Refines, Reduces, or Replaces (3R's) the use of animals in relation to other protocols or procedures performed in the past?

Y/N (circle) **SECTION V.3.5.**

Exceptions to the *Guide for the Care and Use of Laboratory Animals* (Please check all applicable):

[ ] Use of Paralytics (V.4.1.2.3.)
[ ] Prolonged Restraint (V.4.2.)
[ ] Multiple Major Survival Surgery (V.4.3.6.)
X Use of Non-pharmaceutical grade chemicals (V.4.4.1.)
[ ] Use of Complete Freund's Adjuvant (V.4.4.3.)
[ ] Death as an endpoint (V.4.5.)
[ ] Food/Water Restriction (V.5.1.2.
[ ] Single Housing of Social Species (V.5.1.3)
[ ] Restriction of Environmental Enrichment (V.5.3.2.)
[ ] Drug Use/Controlled Substances (Appendix A)

IDENTIFICATION OF SPECIES AND STRAIN: In accounting for animal numbers, please ensure that the strain of animal as well as the species is identified. If more than one strain of any species will be used, please list each proposed strain in a separate column. If more than two species/strains are to be used, duplicate Sections V.3.3.1 – V.3.4 , and Section V.4.1.1.1, on subsequent pages to cover all requested strains.

		Species/Strain #1	Species/Strain #2
V.3.3.1.	Genus & Species:	*Mus. musculus*	*Mus. musculus*
V.3.3.2.	Strain/Stock:	C57BL/6	B6-LY5.2/Cr
V.3.3.3.	Source/Vendor:	(b)(4)	
V.3.3.4.	Age:	6-12 weeks	6-12 weeks
V.3.3.5.	Weight:	20-25g	20-25g

V.3.3.6. **Sex**:	Female	Female
V.3.3.7. **Special Considerations**:	N/A	N/A
V.3.4. **Number of Animals Required (by Species/Strain)**:	328 mice	166 mice

	Species/Strain #3	Species/Strain #4
V.3.3.1. **Genus & Species**:	*Mus. musculus*	*Mus. musculus*
V.3.3.2. **Strain/Stock**:	(b)(4)	
V.3.3.3. **Source/Vendor**:	In-house	In-house
V.3.3.4. **Age**:	6-12 weeks	6-12 weeks
V.3.3.5. **Weight**:	20-25g	20-25g
V.3.3.6. **Sex**:	Female	Female
V.3.3.7. **Special Considerations**:	N/A	N/A
V.3.4. **Number of Animals Required (by Species/Strain)**:	24 mice	50 mice

	Species/Strain #5	Species/Strain #
V.3.3.1. **Genus & Species**:	*Mus. musculus*	
V.3.3.2. **Strain/Stock**:	(b)(4)	
V.3.3.3. **Source/Vendor**:	In-house	
V.3.3.4. **Age**:	6-12 weeks	
V.3.3.5. **Weight**:	20-25g	
V.3.3.6. **Sex**:	Female	
V.3.3.7. **Special Considerations**:	N/A	
V.3.4. **Number of Animals Required (by Species/Strain)**:	50 mice	

V.3.5. **Refinement, Reduction, Replacement (3 Rs)**:

V.3.5.1. Refinement: All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels. Animals observed to be ill or showing signs of pain or distress including weight loss, hunched posture, ruffled fur, and decreased activity will be evaluated by veterinary staff to be treated or be euthanized immediately for relief of suffering. Anesthesia will be used for retro-orbital bleeding.

V.3.5.2. Reduction: In order to reduce the animal numbers in this study, experiments have been designed to include tissue sharing among different experimental procedures including DNA, RNA, and protein extractions, and also animal sharing for purification of different hematopoietic progenitor populations. The estimated number of animals for proposed studies have been computed by power analysis to ensure that the smallest numbers of animals required to evaluate the hypotheses are being used.

V.3.5.3. Replacement: Tissue culture and computer models were considered but not adopted. Detailed explanations are provided in V.3.1.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species/Strain #1	Species/Strain #2
V.4.1.1.1.1. Column C:	196	16
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:	132 (for receiving irradiation)	150 (for receiving irradiation)
	Species/Strain #3	**Species/Strain #4**
V.4.1.1.1.1. Column C:	24	50
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:		
	Species/Strain #5	**Species/Strain #**
V.4.1.1.1.1. Column C:	50	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:		

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: For retro-orbital bleeding, 3.5-4% Isoflurane will be applied before the procedure through induction chamber of an anesthesia machine at LAM until animals are fully anesthetized (judging by lack of movement and decrease in respiratory rate to about once every 4 seconds and a negative pedal withdrawal reflex).

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: N/A

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: AWIC and Altweb

V.4.1.3.2. Date of Search: 3/30/2016

V.4.1.3.3. Period of Search: N/A

V.4.1.3.4. Key Words of Search: "Irradiation" in combination with "mouse bone marrow transplantation" was used as key words.

V.4.1.3.5. Results of Search: No relevant matches were returned in both databases.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: Whole body irradiation of recipient mice is essential for successful mouse bone marrow transplantation as it is the only way to efficiently deplete host bone marrow stem cells and to greatly increase donor cell engraftment in mice.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: 28 Gauge x ½ inch needles will be used for all injections.

Some mice as specified the experimental design and general procedures section will receive one time intra-peritoneal injection of 5-FU (15 mg/ml) resuspended in phosphate buffered saline (PBS) at a dosage of 150 mg/kg body weight in 200ul volume as described in Experiment section. 5-FU treatment of mice at this dosage is toxic to proliferating cells in the hematopoietic system representing more mature progenitors, but is not expected to cause animal suffering and death.

Some mice will receive 3 intra-peritoneal injections of Tamoxifen at 200mg/kg body weight at 2-day intervals dissolved in corn oil or corn oil only at 200ul volume per animal each time. Injection of Tamoxifen at this dosage is safe for mice.

Some mice will receive 3 intra-peritoneal injections of poly(I:C) at 2-day intervals at 2 to 3 months of age to induce the expression of Setbp1-WT or Setbp1-DN. 200ul of poly(I:C) at a concentration of 2 ug/ul diluted in sterile phosphate buffered saline will be injected each time. Injection of poly I:C at this dosage is safe for mice.

Transplantation of bone marrow or spleen cells will be carried out by one time tail vein injections of cells resuspended in 200ul of PBS. Since transplanted cells are from mice congenic to the recipients, no immune rejection is expected after transplantation. Injection of hematopoietic cells through the tail vein is not known to cause any toxic effects to mice. All cells for injection will be obtained from mice housed in USU colony. Injection of all biological materials will be in accord with USU IACUC Policy #23 on the use of biological materials in experimental animals.

V.4.4.2. Biosamples: Retro-orbital bleeding is chosen for the protocol based on the blood sample size required for our study and available in-house expertise. Given the relatively large sample size (200 ul of blood from each animal each time) that is needed for our study to perform FACS analysis, 2 other blood collection procedures including from the saphenous vein and jugular vein were considered as suitable alternatives. Saphenous bleeding is the only procedure that is less painful than retro-orbital bleeding and does not require anesthesia. However, this procedure requires extensive experience and has not been performed on mice by staff in my laboratory. In contrast, retro-orbital bleeding has been routinely carried out by staff in my laboratory under previously approved protocols and will be done under isoflurane anesthesia.
In this protocol, 200ul blood will be obtained each time by retro-orbital bleeding at 1 and 4 month after transplantation for certain animals as described in Experiment 1A. Using the same procedure, same volume of bleeds will also be obtained 3 times after transplantation as described in Experiment 2. IACUC policy on retro-orbital bleeding (POLICY 001) will be strictly followed when performing this procedure. Mice will be anesthetized with Isoflurane. When the animal is no longer moving voluntarily it is placed on a paper towel. The forefinger of the operator's non-dominant hand is used to pull the facial skin taut and cause the eyes to protrude slightly while the skin at the back

of the neck is grasped by the thumb and remaining fingers to restrain. Breathing and color are monitored throughout the procedure to ensure that the restraint does not compromise the airway. The tip of a capillary tube is gently inserted below the eye at approximately a 45 degree angle into the space between the globe and the lower eyelid. When the tip of the pipette contacts the boney floor of the orbit it is gently twisted between thumb and forefinger to rupture the capillary plexus/sinus. Blood is allowed to flow by capillary action into the pipette. Care is taken not to take any more blood than is needed. At the conclusion of the blood withdrawal tension on the animal is released and a gauze pad is gently pressed over the eye for a few seconds until the bleeding has stopped. A drop of tetracaine is added in the eye to reduce any post-procedural discomfort that may occur. Normal color and respiration are reconfirmed and the animal is returned to its cage for recovery. Alternate eyes are used for successive bleeds. A maximum of two bleeds per eye will be done. In addition, hematopoietic tissues including bone marrow, spleen, thymus, lymph nodes and control tissue brain, lung, and kidney will be extracted after euthanasia.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Cage cards will be used to distinguish different study groups.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures:

Oral gavage through 20 Gauge x1.5 inch gavage needles will be used to administer mice with (b)(4) at 20 mg/kg of body weight (in 200ul 50%PEG400/50%ddH2O) once every 2 days for 22 days (total 10 doses). This dosage of (b)(4) when used in mice was shown to be safe and not to cause any significant side effects [6].

Recipient B6-LY5.2/Cr or C57BL/6 mice will be irradiated on the day of transplantation with1100 rads of total body irradiation from a ^{137}Cs source located at room (b)(6) Mice will be transported and irradiated at room temperature in irradiator disks (purchased from Braintree Scientific) that maintain a filter protected sterile environment and hold up to 20 mice at each time. The entire process will last no more than 30 minutes and during which time no food or water will be provided for the animals. Hematopoietic cells resuspended in PBS in a volume of 200 ul will be introduced by tail vein injection. The dose of irradiation used is expected to be lethal within 10 days in the absence of bone marrow reconstitution by transplanted cells. Successful recolonization of the recipient bone marrow in transplanted animals results in restoration of constitutive hematopoiesis over the normal life span of the recipient. Past experience has shown that lethally irradiated mice can suffer from infection. To prevent infection, all recipient mice will receive amoxicillin at a concentration of 1 mg/ml in the drinking water from 5 days prior to irradiation to 10 days post irradiation, and sterile feed and autoclaved caging will be provided. Although occurring rarely, engraftment failure of the transplanted cells will also lead to recipient death between 7 and 10 days after injection.

Therefore, to prevent any potential suffering during this critical period of time, recipient mice will be monitored twice daily by PI staff between 7 and 10 days after transplantation for signs of distress including rough coat, listlessness, or hunched posture. Any transplant recipients showing such signs will be euthanized immediately. Leukemia development and progression is a slow process; therefore, for the time periods before day 7 and after day 10 post transplantation, transplant recipient mice will be monitored once daily by PI staff during week days and by LAM personnel during weekends and holidays for signs of leukemia development, which are similar to the above-described signs of engraftment failure. Any recipients displaying any combination of two or more signs of distress above will be euthanized immediately by PI staff.

V.4.4.8. Tissue Sharing: Tissues may be shared with other investigators upon request.

V.4.5. Study Endpoint: The study endpoint for transplant donor mice is between 6 to 12 weeks of age. The study endpoint for transplant recipient mice is between 4 to 15 months of age as specified in the experimental design and general procedures section. Animals could be euthanized earlier due to engraftment failure of transplanted hematopoietic cells after irradiation or leukemia development. Although occurring rarely, engraftment failure of the transplanted cells will lead to recipient death between 7 and 10 days after injection. Therefore, to prevent any potential suffering during this critical period of time, recipient mice will be monitored twice daily by PI staff between 7 and 10 days after transplantation for signs of distress including rough coat, listlessness, or hunched posture. Any transplant recipients showing any combination of two or more signs of distress above will be euthanized immediately. Leukemia development and progression is a slow process; therefore, for the time periods after day 10 post transplantation, transplant recipient mice will be monitored once daily by PI staff during week days and by LAM personnel during weekends and holidays for signs of leukemia development, which are similar to the above-described signs of engraftment failure. Any recipients displaying any combination of two or more signs of distress above will be euthanized immediately by PI staff.

V.4.6. Euthanasia: Animals under this protocol will be euthanized by research personnel. Mice will be euthanized using cylinderized CO2. These animals will be placed in a cage or chamber that is not crowded and exposed to a mix of CO2 and oxygen. The CO2 flow rate will displace at least 20% of the chamber volume per minute. Flow will continue for at least one minute after visible sign of death. CO2 euthanasia will be followed by cervical dislocation to ensure death. This is consistent with the 2013 AVMA Guidelines for the Euthanasia of Animals.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. **Study Room**:

Building(s) _____ Room Number(s) (b)(6)

V.5.1.2. **Special Husbandry Provisions**:

Food Restriction: Yes _____ No x

Fluid Restriction: Yes _____ No x

V.5.1.3. **Exceptions**: N/A

V.5.2. **Veterinary Medical Care**:

V.5.2.1. **Routine Veterinary Medical Care**: LAM animal care technicians will observe all animals at least once daily during rounds. The clinical veterinarian will check the rounds sheets daily and evaluate overall animal health IAW LAM. Bone marrow transplant recipient mice will be monitored twice daily by PI staff between 7 and 10 days after transplantation. For the time periods before day 7 and after day 10 post transplantation, transplant recipient mice will be monitored once daily by PI staff during week days.

V.5.2.2. **Emergency Veterinary Medical Care**: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. **Environmental Enrichment**:

V.5.3.1. **Enrichment Strategy**: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. **Enrichment Restrictions**: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Experience of person performing activity (species specific) (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)	Dates of the USU Investigator Training course and required IACUC CITI Training
CO2 euthanasia, intra-peritoneal injection	(b)(6)	PI, 15 years of experience on mouse CO2 euthanasia and intra-peritoneal injection	Rodent Handling Training at NCI, 2001	Investigator training course at USUHS (2007) CITI training (7/22/2014)

Tail vein injection, CO2 euthanasia	(b)(6)	Technician, 8 years of experience on mouse tail vein injection and CO2 euthanasia	Rodent Handling Training by PI, 2007	Investigator training course at USUHS (2007) CITI training (9/30/2014)
CO2 Euthanasia, intra-peritoneal injection		Technician, 8 years of experience on mouse CO2 euthanasia and intra-peritoneal injection	Rodent Handling course at USUHS, 2007	Investigator training course at USUHS (2007) CITI training (6/27/2014)
Oral gavage, Retro-orbital bleeding, CO2 euthanasia		1 year of experience on mouse oral gavage, 5 years experience on retro-orbital bleeding and CO2 euthanasia	Rodent Handling course at USUHS, 2011	Investigator training course at USUHS (2011) CITI training (7/1/2015)
Retro-orbital bleeding, CO2 euthanasia		5 years of experience on mouse retro-orbital bleeding and CO2 euthanasia	Rodent Handling course at USUHS, 2011	Investigator training course at USUHS (2011) CITI training (7/15/2014)
Retro-orbital bleeding, CO2 euthanasia		5 years of experience on mouse retro-orbital bleeding and CO2 euthanasia	Rodent Handling course at USUHS, 2010	Investigator training course at USUHS (2010) CITI training (7/20/2015)
Retro-orbital bleeding, CO2 euthanasia		3 years of experience mouse retro-orbital bleeding and CO2 euthanasia	Rodent Handling course at USUHS, 2012	Investigator training course at USUHS (2012) CITI training (7/2/2014)

VII. BIOHAZARDS/SAFETY:

A. Zoonotic Disease: N/A

B. Safety Hazards: Replication defective ecotropic retroviral vectors will be used to infect mouse bone marrow cells before transplantation of the infected cells into recipient mice. These viruses will be collected from culture medium of cell lines designed to package viral particles in ecotropic envelops. After initial infection these vectors are no longer infectious and are replication deficient. There is no evidence in the literature that humans can be infected by ecotropic retroviruses. Any person handling these retroviral vectors will be required to wear lab coat and gloves as precaution. 5-FU, Tamoxifen, and (b)(4) are inhibitors of Thymidine Synthase, Estrogen Receptor, and (b)(4) respectively. They do not evaporate at room temperature, but are potentially harmful if ingested. Lab coat gloves, and mask are required when preparing the solutions and carrying out animal injection and oral gavage using these substances. Lab coat, gloves, and mask are also required at any time when handling animals to minimize exposure to allergens from animals. For animal irradiation, mice will be transported and irradiated in irradiator disks (purchased from Braintree Scientific) that maintain a filter protected sterile environment. Personnel performing irradiation will be required to wear lab coat and gloves and carry a thermoluminescent dosimeter issued by Radiation Safety Office for monitoring potential radiation exposure during the procedure.

C. Isoflurane: Isoflurane will be used for retro-orbital bleeding.

D. Isoflurane Exposure: To reduce isoflurane exposure, an isoflurane vaporizer with pass-through charcoal canister filter in the exhaust gas line will be used to anesthetize mice for retro-orbital bleeding.

E. Sharp Instruments: Needles will not be re-capped and will be disposed in sharp containers after usage.

F. Infectious Agents that do not cause Zoonoses: N/A

VIII. ENCLOSURES:

1. Dick, J.E. Acute myeloid leukemia stem cells. *Annals of the New York Academy of Sciences* **1044**, 1-5 (2005).
2. Lobo, N.A., Shimono, Y., Qian, D. & Clarke, M.F. The biology of cancer stem cells. *Annual review of cell and developmental biology* **23**, 675-699 (2007).
3. Oakley, K., *et al.* Setbp1 promotes the self-renewal of murine myeloid progenitors via activation of Hoxa9 and Hoxa10. *Blood* **119**, 6099-6108 (2012).
4. Cristobal, I., *et al.* SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. *Blood* **115**, 615-625 (2010).
5. Makishima, H., *et al.* Somatic SETBP1 mutations in myeloid malignancies. *Nature genetics* **45**, 942-946 (2013).
6. (b)(4)

7. Maeda, T., *et al.* Regulation of B versus T lymphoid lineage fate decision by the proto-oncogene LRF. *Science* **316**, 860-866 (2007).
8. Rossi, D.J., *et al.* Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature* **447**, 725-729 (2007).
9. Hock, H., *et al.* Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. *Nature* **431**, 1002-1007 (2004).

IX. EXTRAMUAL COLLABORATION: N/A

If there are any collaborations with outside entities, please explain here.

X. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 4/6/2016

Principal Investigator Signature — Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely,

"Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) [redacted]

Principal Investigator Signature

4/6/2016
Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL** or **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) [redacted]

Principal Investigator Signature

4/6/2016
Date

XI. PROTOCOL ABSTRACT:

A. **Animal Protocol Number:**

B. **Animal Protocol Title:** Role of Setbp1 in leukemia-initiating cell self-renewal in mice

C. **Principal Investigator:** (b)(6) Department of Pediatrics

D. **Performing Organization:** Uniformed Services University of the Health Sciences

E. **Funding:** WRNMMC

F. **Objective and Approach:** The objectives of this study are to gain novel insights into the cellular and molecular mechanisms by which Setbp1 overexpression and missense mutations induce leukemia development, and also to test new therapeutic strategies for *SETBP1*-induced leukemias based on these insights through the use of mouse models. The three R's (reduction, replacement, and refinement) of animal usage will be employed.

G. **Indexing Terms (Descriptors):** Animals, mice, Setbp1, and leukemia

APPENDIX A.

PI NAME: (b)(6)
PROTCOL NUMBER:
DRUGS AND CONTROLLED SUBSTANCES

Please list ALL drugs and controlled substances that will be used under this protocol, indicating the DEA Schedule if known. Provide both the Approved Name and the Proprietary Name of each drug if known.

This list, once approved, will be used by the IACUC and the USU Pharmacy to determine which drugs and controlled substances can be supplied to the Principal Investigator. The Pharmacy will not dispense to the PI any drug that is not included in this list.

Drug Name (Please give both approved and Proprietary Names if possible)	DEA Schedule (I, II, III, or IV) (if known)
1.5-fluorouracil (5-FU)	
2. Tamoxifen	
3. (b)(4)	

Date of Protocol Expiration Date:

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4712
http://www.usuhs.mil

January 9, 2013

MEMORANDUM FOR DR. (b)(6)

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on January 9, 2013:

Animal Protocol Title: "Skin Cancer Biomarkers for Exposure to (b)(4) Ionizing Radiation (Mus Musculus)"

USUHS Protocol Number: (b)(6)

Expiration Date: January 8, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

Revised

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Skin cancer biomarkers for exposure to (b)(4) ionizing radiation (*Mus musculus*)

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)
FUNDING AGENCY: US Military Cancer Institute

EARLIEST ANTICIPATED FUNDING START DATE: November 15, 2012

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6)
Principal Investigator Signature

(b)(6)
Department Office/Lab Telephone

11/12/2012
Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)
Dept. Head Signature

Chair
Title

(b)(6)
Telephone

11/12/2012
Date

Typed Name: (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)
Statistician Signature

(b)(6)
Department Telephone

11/12/2012
Date

Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)
Attending/Consulting Veterinarian Signature

LAM
Department

(b)(6)
Telephone

11/21/12
Date

Typed Name: LTC (b)(6)

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D., (b)(6)

ANIMAL PROTOCOL TITLE: Skin cancer biomarkers for exposure to (b)(4) ionizing radiation (*Mus musculus*)

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6) M.D./Ph.D., Collaborator

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

There is increased risk to US military personnel for unintentional exposure to radiation, either due to a nuclear accident or as the result of terrorist activity. Although significant research has been conducted on the effects of high dose radiation, the true risk of exposure to (b)(4) radiation is not well understood. One theory regarding risk from (b)(4) radiation exposures is the linear no threshold (LNT) model, which states that any level of radiation exposure can result in health risks. Some data indicate that there are significant differences (qualitative and quantitative) between the effects of (b)(4) high dose radiation on specific biomolecular targets including DNA, transcription factors, and molecules involved in initiation of (b)(4) Cellular changes leading to the generation of cancer in response to (b)(4) gamma irradiation have not been well defined, and biomarkers to predict these changes have not been identified. The proposed research will develop a murine model for (b)(4) radiation-induced skin cancer and identify cutaneous biomarker (b)(4)

(b)(4)

II. BACKGROUND:

II.1. Background:

(b)(4)

(b)(4) The biochemical consequences of radiation exposure are pronounced and include: radiation-induced gene expression changes, DNA and protein modifications, and metabolomic, lipidomic, immunomodulatory, cytogenetic and inflammatory mediator changes. (b)(4)

(b)(4)

(b)(4) Examination of transcriptional responses to (b)(4) radiation demonstrated the altered expression of

up to 1,400 genes of which a significant number are correlated with specific cancers. At present, however, much less is known about how (b)(4) radiation effects (b)(4) (b)(4) leading to cancer development.

Findings by several laboratories have provided substantial evidence that some (but not all) changes that occur at high dose radiation (b)(4) For instance, unrepaired DNA damage, as detected by micronuclei, can be reliably observed in murine skin but only following a (b)(4) irradiation. Changes in gene expression can be detected after exposure of (b)(4) but it is unknown whether these early changes in gene expression lead to malignant transformation in response to (b)(4) radiation.

Mitochondrial dysfunction caused by ionizing radiation is thought to be an underlying mechanism driving protein oxidation, where the increased production of reactive oxygen species (ROS) occurs due to a disruption in the electron transport chain. The resulting rise in ROS catalyzes the modification of cellular proteins. Covalent modifications are hallmarks of oxidative stress and are well recognized for their contribution to resulting pathologies. Protein oxidation by oxidative stress and free radical damage, such as that induced by radiation, is a hallmark mechanism underlying the pathology of radiation injury. Protein modification can be utilized as a sensitive system for the detection of radiation-induced cell damage for the following reasons. First, radiation-induced damage of proteins is dose- and time-dependent and thus reflects biologic consequence. Second, data from our laboratory and others indicates that damaged can proteins reflect organ-specific injury. And third, the scientific tools required for analyzing proteins damaged by radiation are generally well established. Importantly, these modifications are chemically stable, protein specific and easily detected using highly sensitive antibody-based procedures.

(b)(4)

(b)(4)

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:
Biomedical Research Database (BRD)NIH RePORTER (replacement database for CRISP) *http://projectreporter.nih.gov/reporter.cfm*
PubMed

II.2.2. Date of Search: November 2, 2012

II.2.3. Period of Search: all dates searched for each database: for BRD, 1998-2012; for RePorter, 1972-2012; for PubMed, 1970-2012.

II.2.4. Key Words and Search Strategy: (b)(4) ionizing radiation, skin, cancer

II.2.5. Results of Search:
Results from NIH RePORTER:
5 grants were found that had the key words.

1. NIH R01 EB008713 by J.P. Desai "Robotic haptic feedback system for BX/RFA of breast tumor under MRI". This grant investigates the use of radiofrequency ablation (RFA) for early stage breast cancer. The grant also proposes the use of MRI instead of CT scans for localizing breast tumors to avoid exposure of patients to ionizing radiation.
2. (b)(4)
3. NIH Z1A CP01033 by M.S. Linet "Studies of populations exposed to occupational sources of radiation". This grant proposes to examine cancer incidence and mortality among US radiologic technologists, in Chornobyl accident survivors and clean-up workers, and in female flight attendants. Skin cancer rates will be examined as results of UV exposures No animal studies are proposed.
4. R21 CA161670 by K.Sheng "A mechanistic model to derive lung tumor motion from the whole surface tracking". This project will investigate technological improvements to the calculations of clinical radiologic instrumentation to allow the delivery of synchronized radiation to tumors, taking into consideration internal tumor motion. The experiments proposed are geared toward tracking lung tumors.

Results from BRD: No matches

Results from PubMed:
There were 271 results from the key words, however, most of these were not related to the induction of cancer by (b)(4) ionizing radiation. Many manuscripts were related to cancer

treatment and not to the induction of cancer in normal cells. A large number of the manuscripts examined UV radiation effects.

Epidemiological studies of exposures to (b)(4) ionizing radiation:

1) (b)(4)

2)

3)

4)

5)

6)

In vivo studies:

1) (b)(4)

2)

3)

Non-in vivo model systems for examining ionizing radiation effects

1) (b)(4)

2)

3)

4)

5)

6)

7)

8) (b)(4)

III. OBJECTIVE\HYPOTHESIS:

(b)(4)

IV. MILITARY RELEVANCE:

The Department of Defense is prepared to execute military missions within radiation environments, to manage radiation crises associated with terrorist activities, and to manage consequences in the event of nuclear weapons detonation. Recent studies indicate that there is an increasing risk of exposure to ionizing radiation (IR) to military personnel due to increasing terrorist activities, including potential use of stolen nuclear weapons or dispersal of radioactive material in a "dirty bomb" (b)(4) (b)(4) Because of potential exposure of military personnel to radiation, the DoD is committed to protect members of the Armed Services from the adverse health effects of ionizing radiation. (b)(4) IR causes short term, acute injuries including hematopoetic and GI syndromes. However (b)(4) IR (as may result from a radiation dispersal device) can cause cancer including skin cancer.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

The threshold dose of radiation for induction of skin cancers is not known.

V.1.1. Experiment 1: Determination of the threshold doses and time courses of gamma radiation required for the induction of skin cancer in a susceptible murine model.

(b)(4)

Note: female mice will be used for these studies because of reduced aggressive behavior in females during long housing times. Also, homozygous (b)(4) mice will not be used as these mice develop spontaneous tumors in ~2 months.

Non-irradiated (b)(4) mice = (b)(4) mice

(b)(4) animals/group) x (4 radiation doses) x (1 time point) = (b)(4) mice

Control (nonirradiated) (b)(4) mice = (b)(4) mice

(b)(4) animals/group) x (1 radiation doses) x (1 time point) = (b)(4) mice

(b)(4) Endpoint: Animals will be euthanized at months postirradiation. At earlier time points, mice will be euthanized if skin tumors (or any other tumors resulting from radiation exposure) are equal to 20 mm in diameter, or if multiple tumors appear that together equal 20 mm diameter. This is the recommended maximal tumor size for mice according to NCI Frederick ACUC Guidelines Involving Experimental Neoplasia Proposals in Mice and Rats, 2006.

V.1.2. Experiment 2: Identification of skin proteins that undergo carbonylation and the formation of hydroxynonenal (HNE) adducts and the expression of specific markers for (b)(4) gamma radiation in a murine model of radiation-induced skin cancer.

(b)(4)

(b)(4)

Preliminary findings in our laboratory indicate that N = is sufficient for the detection of (b)(4) (b)(4) and signal transduction pathway activation in response to radiation. For these experiments, both male and female mice can be utilized. Also, each animal will provide its own control tissue outside of the field of radiation.

(b)(4) mice

(b)(4) animals/group) x (4 radiation doses) x (5 time points) = (b)(4) mice

(b)(4) mice

(b)(4) animals/group) x (1 radiation doses) x (5 time point) = (b)(4) mice

Study endpoints: Animals will be euthanized at (b)(4) days postirradiation. At earlier time points, mice will be euthanized if skin tumors (or any other tumors resulting from radiation exposure) are equal to 20 mm in diameter, or if multiple tumors appear that together equal 20 mm diameter. This is the recommended maximal tumor size for mice according to NCI Frederick ACUC Guidelines Involving Experimental Neoplasia Proposals in Mice and Rats, 2006.

V.2. Data Analysis:

Mean with standard error, or percentage will be reported if applicable. A significance level is set at 5% for each test. All statistical tests are two-sided. A statistical software, SAS will be used for statistical analyses.

Experiment 1: Log-Rank test will be used for comparison among groups. If significant, Fisher's exact test will be used to detect significant differences across the groups in (b)(4) tumor development rates at months. Kaplan-Meier curves will be displayed. N = (b)(4) mice per group will have 80% power in detecting a significant difference between two groups if any, given type I error of 5% where the treatment group survival rate is at least 68%, compared to a control group survival rate of 20%. Similar statements would apply, if the treatment group survival rates are at least 83%, 78%, or 73%, compared to vehicle group survival rates of 35%, 30%, and 25%, respectively (b)(4)

Experiment 2: Analysis of variance (ANOVA) will be used to detect significant difference among the groups. If significant, then the pair-wise comparison by Tukey-Kramer will be used to identify which group is different from the other. For experiment 2, N = mice per (b)(4) group could have 80% power to detect any significant difference between groups if any, given type I error of 5% and delta/sigma has at least 1.4, where the delta is the estimated mean differences between the two groups and sigma is the pooled standard deviation of the two groups (b)(4)

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

For the last 4 years, we have utilized primary cell culture systems to examine radiation effects on cells; however, we must determine whether our culture findings are relevant in whole animals. We will continue to use primary cell culture whenever possible to avoid unnecessary use of animals. We have also considered computer models as alternatives to animals. However, this alternative also lacks the knowledge base and complexity needed to evaluate radiation-induced cancer risks.

V.3.2. Animal Model and Species Justification:

Tissue culture conditions cannot fully substitute for the complexities of whole organisms in response to radiation injury. Such injury involves the interactions between the hematopoietic system and other organ systems. In order to develop viable treatments for humans, we find that our research must be conducted in whole animals.

The mouse as the best species to use for radiation protocols: 1) mice provide the least sentient species available whose response to radiation has been clearly identified, with physiological responses to radiation and cancer development that are similar to human physiology, 2) extensive literature is available on responses of mice to radiation and cancer, 3) more reagents are available to study the relevant cell types and molecules in mice than in any other species except humans, and the availability of transgenic and knockout mice make this model extremely attractive for further research into molecular mechanisms, and 4) our research group have extensive experience with radiation effects in mice, including radiation injury and radioprotection research.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	Mus musculus	Mus musculus
V.3.3.2. Strain/Stock:	(b)(4)	
V.3.3.3. Source/Vendor:		
V.3.3.4. Age:	8-12 weeks	8-12 weeks
V.3.3.5. Weight:	Normal adult weight range for this strain: 17-21 g	Normal adult weight range for this strain: 17-21 g
V.3.3.6. Sex:	(b)(4)	
V.3.3.7. Special Considerations:	None	

V.3.4. Number of Animals Required (by Species): mice

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:
Animals demonstrating morbidity due to radiation exposure (b)(4) will be considered to have arrived at the study endpoint and will be immediately euthanized (See sections V.4.5 Study Endpoint and V.4.6 Euthanasia).

Ketamine/xylazine anesthesia will be used during X-ray irradiation to prevent stress from the time the mice are placed in restraint. Under the supervision of AFFRI veterinary staff, we found that in female mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia with no morbidity or mortality (b)(4) (b)(4) We propose to use this range of concentration of anesthetics, together with a heating pad to keep mice warm during the period of recovery from anesthesia. Standard intraperitoneal injection methods are used for injection of the anesthesia, for which all laboratory personnel have received training.

V.3.5.2. Reduction:

Whenever possible, control groups will be shared between experimental groups for our experiments. Tissues will be collected for multiple assays from the same animals further reducing the total number of animals needed. At every stage of the research, planned experiments will be reevaluated, in an attempt to reduce the numbers of experiments and eliminate repeats of experiments where statistical significance has already been obtained.

V.3.5.3. Replacement:
At this time, it is not feasible to use non-animal systems in place of animal models to address the research questions in this protocol.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals: (b)(4)

(b)(4)

V.4.1.1.1.1. Column C:
V.4.1.1.1.2. Column D:
V.4.1.1.1.3. Column E:

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:
Ketamine/xylazine anesthesia will be used during X-ray irradiation to prevent stress from the time the mice are placed in restraint. Under the supervision of AFFRI veterinary staff, we found that in female mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia with no morbidity or mortality (b)(4) (b)(4) We propose to use this range of concentration of anesthetics, together with a heating pad to keep mice warm during the period of recovery from anesthesia. Standard intraperitoneal injection methods are used for injection of the anesthesia, for which all laboratory personnel have received training.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Not applicable

V.4.1.2.3. Paralytics: Not applicable

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched:
Sources searched: DoD Bioedical Research Data Base (BRD), CRISP, PubMED

V.4.1.3.2. Date of Search:

V.4.1.3.3. Period of Search:
Search covered published manuscripts from ~1965-present. (BRD search included all available years 1998 -2012).

V.4.1.3.4. Key Words of Search:
Pain, analgesia, anesthesia, mice, radiation, ionizing radiation, humane

V.4.1.3.5. Results of Search:
The literature search revealed that, at present, there are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. In some cases radiation has been demonstrated to reduce pain in laboratory mice (b)(4) and has been in humans reported to alleviate cancer related bone pain (b)(4) (b)(4) A recent report showed that administration of botulinum toxin A was effective for reduction of pain and muscle spasms after clinical radiation for treatment of tumors (b)(4) Application of natural honey 20 min prior to radiation exposure was also shown to reduce mucositis with associated pain in humans following head/neck radiotherapy (b)(4) (b)(4) it has not been determined whether honey acts as a local radioprotectant. Spinal cord stimulation has also been reported in humans to alleviate pain resulting from radiotherapy (b)(4) however, this method would not be practical for use in mice because of the extensive spinal cord manipulation required. Human administration of morphine also relieves pain (b)(4) but again this would not be practical for mice in our experiments because of side effects of the drug. In humans, exposure to total body irradiation may induce vomiting and diarrhea, causing pain and distress. However, mice are not susceptible to vomiting.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
Anesthesia will be used before (b)(4) irradiation to avoid stress from restraint. Radiation (b)(4) used for these experiments is not expected to induce pain in any animals, and most time points will be within 30 days postirradiation, prior to the development of cancers. However, animals that are observed to be in pain will be euthanized. Pain will be evaluated using several standard indications including: grimacing, abnormal breathing, hunched or fluffed fur and inactivity, or ataxia. In experiments where tissue collection is needed, animals will be euthanized with pentobarbitol prior to sample collection as described elsewhere. We will utilize the attached score sheets to provide a scale for measurement of criteria for euthanasia. Any animal scoring 12 or more total on the score sheet will be euthanized.

V.4.2. Prolonged Restraint:
(b)(4) irradiation will require restraint in designed jigs for ~20 min to receive X-ray exposure (b)(4) animals will be anesthetized during this period (see Anesthesia above) and tails will be taped to one side with surgical tape. The jigs for use during (b)(4) irradiation were examined and approved by the AFRRI Veterinary staff. An image of the jigs is attached. Under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff, we found that in female CBA mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia (25 G needle) with no morbidity or mortality (b)(4) The length of time for anesthesia is needed to ensure that animals do not regain

consciousness while being positioned in the lead shield for irradiation. Positioning of the mice must be done carefully to ensure that all animals receive the same exposures. We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. Standard i.p. injection methods are used for injection of the anesthesia.

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: Not applicable

V.4.3.2. Procedure: Not applicable

V.4.3.3. Post-surgical Provisions: Not applicable

V.4.3.4. Location: Not applicable

V.4.3.5. Surgeon: Not applicable

V.4.3.6. Multiple Major Survival Operative Procedures: Not applicable

V.4.3.6.1. Procedures: Not applicable

V.4.3.6.2. Scientific Justification: Not applicable

V.4.4. Animal Manipulations:

V.4.4.1. Injections:
Intra-peritoneal injections of pentobarbital for euthanasia 0.1-0.2 ml/mouse Fatal Plus (39-78 mg pentobarbital/mouse), will be performed with a 25 G needle. I.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine will be done using a 25 G needle. This has been extensively examined by the AFRRI Veterinary staff and shown to effectively produce non-lethal anesthesia (b)(4)

V.4.4.2. Biosamples:
Ear punch tissue will be used both for animal identification and for genotyping. Ear punches will be performed without anesthesia.

Sections of skin tissue or skin tumor tissue will be taken in from the backs of euthanized mice. All other tissues will be available to other researchers for tissue sharing.

V.4.4.3. Adjuvants: Not applicable

V.4.4.4. Monoclonal Antibody (MAbs) Production: Not applicable

V.4.4.5. Animal Identification:
All cages will have cage cards identifying the treatment groups for each experiment. For experiments 2 and 3, each animal within a cage will receive ear punches for individual

identification (1 = no cut; 2 = right ear upper corner; 3 = left ear upper corner; 4 = both ears).

V.4.4.6. Behavioral Studies: Not applicable

V.4.4.7. Other Procedures:

Ear punch

The ear punch will be soaked in alcohol to disinfect it before use and between animals. The device will be placed on the pinna of the ear (external ear) in a location where the mouse will be marked for identification. The punch will be pressed firmly to punch a circular hole through the ear, being careful not to rip the delicate membrane of the pinna. The ear will then be gently separated from the device. The ear punch will be cleaned and disinfected in alcohol before use in another mouse.

(b)(4) **irradiation**

The methodology for (b)(4) irradiation of mice including approval of holding jigs and development of anesthesia, was developed in conjunction with the AFRRI Veterinary Staff.

(b)(4) irradiation will be performed using the RS2000 irradiator (b)(6)
Thirty minutes prior to irradiation, the RS2000 irradiator and lead shield will be decontaminated thoroughly with MB10.

Mice will be anesthetized by injection in the USUHS LAM facility. Anesthetized mice will be placed in ventilated Lucite jigs, which restrain the animals to prevent lateral or vertical movement. Tails will be taped to prevent any forward motion of animals. Mice will be irradiated at (b)(4) irradiation. Following radiation exposure, mice will be immediately returned to the LAM and allowed to recover from anesthesia under supervision with heating pads. Immediately upon recovery, animals will be returned to standard cages that house 4 animals per cage. Lucite jigs will be sanitized after each use.

V.4.4.8. Tissue Sharing:

For the proposed studies (b)(4) tissue will be obtained. All other tissues will be available for tissue sharing with other investigators.

V.4.5. Study Endpoint:

(b)(4) The endpoint for Experiment 1 is months. The endpoints for Experiment 2 are (b)(4) (b)(4) days postirradiation. Morbid animals will be monitored at least twice daily, morning and afternoon. The experimental design does not contemplate supportive therapy. Criteria to be used for health evaluation while the animals are on study include: 20% weight loss (mice will be weighed every other day—based on our experience with total body irradiation in which animals can recover from ~25% weight loss (b)(4) ruffled fur, dehydration, decreased activity, and hunched body position, dyspnea, or panting. Animals exhibiting these signs, and scoring above 11 on the pain score sheet may be euthanized, and considered to be at the study endpoint. Also, at earlier time points, mice will be euthanized if skin tumors (or any other tumors

resulting from radiation exposure) are equal to 20 mm in diameter, or if multiple tumors appear that together equal 20 mm diameter. This is the recommended maximal tumor size for mice according to NCI Frederick ACUC Guidelines Involving Experimental Neoplasia Proposals in Mice and Rats, 2006.

V.4.6. Euthanasia:
Animals will be euthanized at the stated endpoints of the experiments by injection of 0.1-0.2 ml/mouse Fatal Plus (39-78 mg pentobarbital/mouse), IP using a 25 G needle. Euthanasia will be confirmed by cervical dislocation. Animals which appear to be experiencing pain or distress as a result of radiation exposure or are found moribund will also be euthanized by pentobarbitol and necropsied. At the end of experiments, unused animals will be transferred to other protocols or euthanized by exposure to 100% CO_2 from a compressed gas cylinder in accordance with the current American Veterinary Medical Association (AVMA) Guidelines for Euthanasia. Exsanguination, cervical dislocation, and/or pneumothorax will be used to ensure euthanasia.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. Food and water and Rodent Diet will be available ad libitum. The 12:12 hr (lights off at 6 pm) room light cycle is fine for all the studies.

V.5.1.1. Study Room: will be performed in the
Radiation Building (b)(6) Room Number (b)(6)

Euthanasia Building (b)(6) Room number (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No x

Fluid Restriction: Yes _____ No x

V.5.1.3. Exceptions: None
V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
Experimental animals will be observed at least twice a day by one of the study investigators or technicians. VSD personnel will also monitor the animals twice a day during their rounds. As described in another section (study endpoint) morbid animals will be monitored at least twice daily, morning and afternoon. The experimental design does not contemplate supportive therapy. The justification for not providing supportive care has been described in the study end point section as well as in the pain category section. Criteria to be used for health evaluation while the animals are on study include: 20% weight loss (mice will be weighed every other day—based on our experience with total body irradiation in which animals can recover from ~25% weight loss (b)(4)

ruffled fur, dehydration, decreased activity, and hunched body position, dyspnea, or panting. Animals exhibiting these signs may be euthanized, and considered to be at the study endpoint.

V.5.2.2. Emergency Veterinary Medical Care:
All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.
V.5.3.2. Enrichment Restrictions: None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Ear punch, irradiation, anesthesia, euthanasia	(b)(6)	8+ years	(b)(6)
Ear punch, irradiation, anesthesia, euthanasia		8 years	
		7 years	

VII. BIOHAZARDS/SAFETY: None

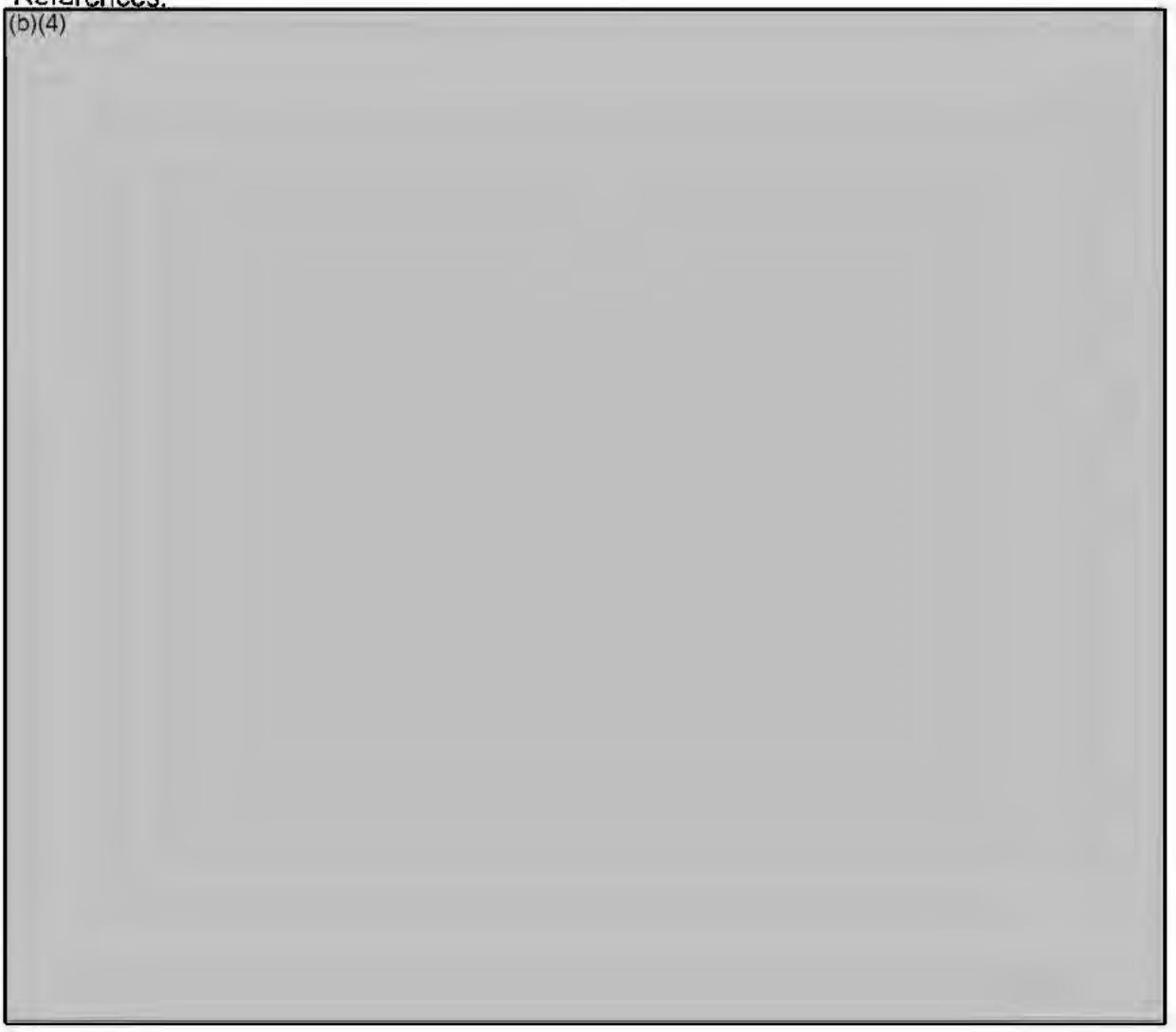
VIII. ENCLOSURES:

1) Images of Lucite jigs for (b)(4) irradiation.

2) Critieria for euthanasia
3) Pain score sheet
4) (b)(4) MSDS

References:

(b)(4)



IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ Nov 12, 2012

Prin ature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ Nov 12, 2012

Princi Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL** ~~or **WILL NOT**~~ be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

P re

Nov 20, 2012

Date

X. PROTOCOL ABSTRACT:

There is increased risk to US military personnel for unintentional exposure to radiation, either due to a nuclear accident or as the result of terrorist activity. Although significant research has been conducted on the effects of high dose radiation, the true risk of exposure to (b)(4) radiation is not well understood. One theory regarding risk from (b)(4) radiation exposures is the linear no threshold (LNT) model, which states that any level of radiation exposure can result in health risks. Some data indicate that there are significant differences (qualitative and quantitative) between the effects of (b)(4) high dose radiation on specific biomolecular targets including DNA, transcription factors, and molecules involved in initiation of (b)(4) Cellular changes leading to the generation of cancer in response to (b)(4) gamma irradiation have not been well defined, and biomarkers to predict these changes have not been identified. The proposed research will develop a murine model for (b)(4) radiation-induced skin cancer and identify cutaneous biomarker (b)(4)
(b)(4)

A. Animal Protocol Number:

B. Animal Protocol Title:

Skin cancer biomarkers for exposure to (b)(4) ionizing radiation (*Mus musculus*)

C. Principal Investigator:

(b)(6)

D. Performing Organization:

USUHS

E. Funding: US Military Cancer Institute

F. Objective and Approach:

(b)(4)

G. Indexing Terms (Descriptors): pulmonary injury, radiation, (b)(4) (b)(4) mice, animals

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4712
http://www.usuhs.mil

January 3, 2013

MEMORANDUM FOR DR. (b)(6)

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on January 3, 2013:

Animal Protocol Title: (b)(4)
(b)(4)

USUHS Protocol Number: (b)(6)

Expiration Date: January 2, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER S(b)(6)
PROTOCOL NUMBER:

Revised

IACUC Date Stamp

PROTOCOL TITLE: (b)(4)
(b)(4)

GRANT TITLE (if different from above): (b)(4)
(b)(4)

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: DTRA

EARLIEST ANTICIPATED FUNDING START DATE: Current

PRINCIPAL INVESTIGATOR:
(b)(6) 11/26/2012
Date

Associate Professor, (b)(6)
(b)(6) office: (b)(6) fax: (b)(6)

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) Chair (b)(6) 11/29/12
Dept. Head Signature Title Telephone Date
Typed Name: (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the st

(b)(6) (b)(6) 11/26/2012
Statistician Signature Department Telephone Date
Typed Name (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) LAM (b)(6) 11/29/12
Attending/Consulting Veterinarian Signature Department Telephone Date
Typed Name: LTC (b)(6)

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D. (b)(6)

ANIMAL PROTOCOL TITLE: (b)(4)
(b)(4)

GRANT TITLE (if different from above): (b)(4)
(b)(4)

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S):
TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

The terrorist attack of September 11, 2001 against the United States has increased the concern that terrorists may acquire and use radioactive sources (e.g. dirty radioactive bombs) to expose military and civilian populations to ionizing radiation. However, despite this concern, there are currently no FDA approved drugs that can be used for the general population to prevent death that is likely to result from exposure to ionizing radiation. (b)(4) irradiation in humans can result in (b)(4) injuries. However, because rodents are more resistant to (b)(4) injuries than humans, there is currently no murine model that has been developed to study both (b)(4) radiation injuries in the same animal.

(b)(4)

II. BACKGROUND:

II.1. Background:

The acute toxic effects of exposure to high doses of ionizing radiation in humans have been shown to occur due to two sequential effects: first the ablation of immune cells, resulting in loss of immune function and increasing likelihood of pathogenic infection (b)(4) and subsequently the loss of epithelial progenitor cells of the gastrointestinal (GI) crypt (b)(4) (b)(4) Both of these events have been shown to be due to the

loss of fast-growing pluripotent and/or progenitor cell types. The time course of the acute injuries correlates with the life span of the mature cell types of the tissue, which are not replaced because of radiation damage to the adult pluripotent cells and adult stem cells. There is currently no FDA approved prophylactic or treatment for prevention of acute radiation injuries following high-dose (b)(4) adiation exposure.

In some cases supportive measures can be used to treat acute radiation syndrome, and survival from (b)(4) adiation exposure is now possible (b)(4) This results in current efforts to mitigate or treat later stage effects of ionizing radiation exposure. (b)(4)

(b)(4)

In humans, death from radiation (b)(4) can occur following survival from (b)(4) Cumulative evidence from a number of laboratories, including our own, shows that there is a threshold dose of radiation of (b)(4) o induce sufficient (b)(4) for the (b)(4) phases to occur in mice; below these doses, no permanent injury manifests, although some short-term effects are observed (b)(4) However, in humans, there is currently no accepted protocol for preventative treatment of radiation-induced (b)(4) and morbidity as high as 50% is associated with each phase (b)(4) (b)(4) injuries from radiation is considered to be a serious problem in the field for the development of agents to prevent these radiation injuries.

(b)(4) **as a radioprotectant:** The biochemical properties of the isoflavones and the investigation of (b)(4) as a protectant against gamma radiation-induced lethality have been investigated by (b)(4), (b)(6) (b)(4) A single i.p. dose of (b)(4) administered hr prior to a lethal dose of (b)(4) (b)(4) radiation resulted in day survival rates of 65%, 91%, and 85% respectively. Mice receiving saline or PEG vehicle alone survived at 8% and 15% respectively. All doses of (b)(4) protected mice significantly ($p < 0.001$) better than vehicle or saline control groups.

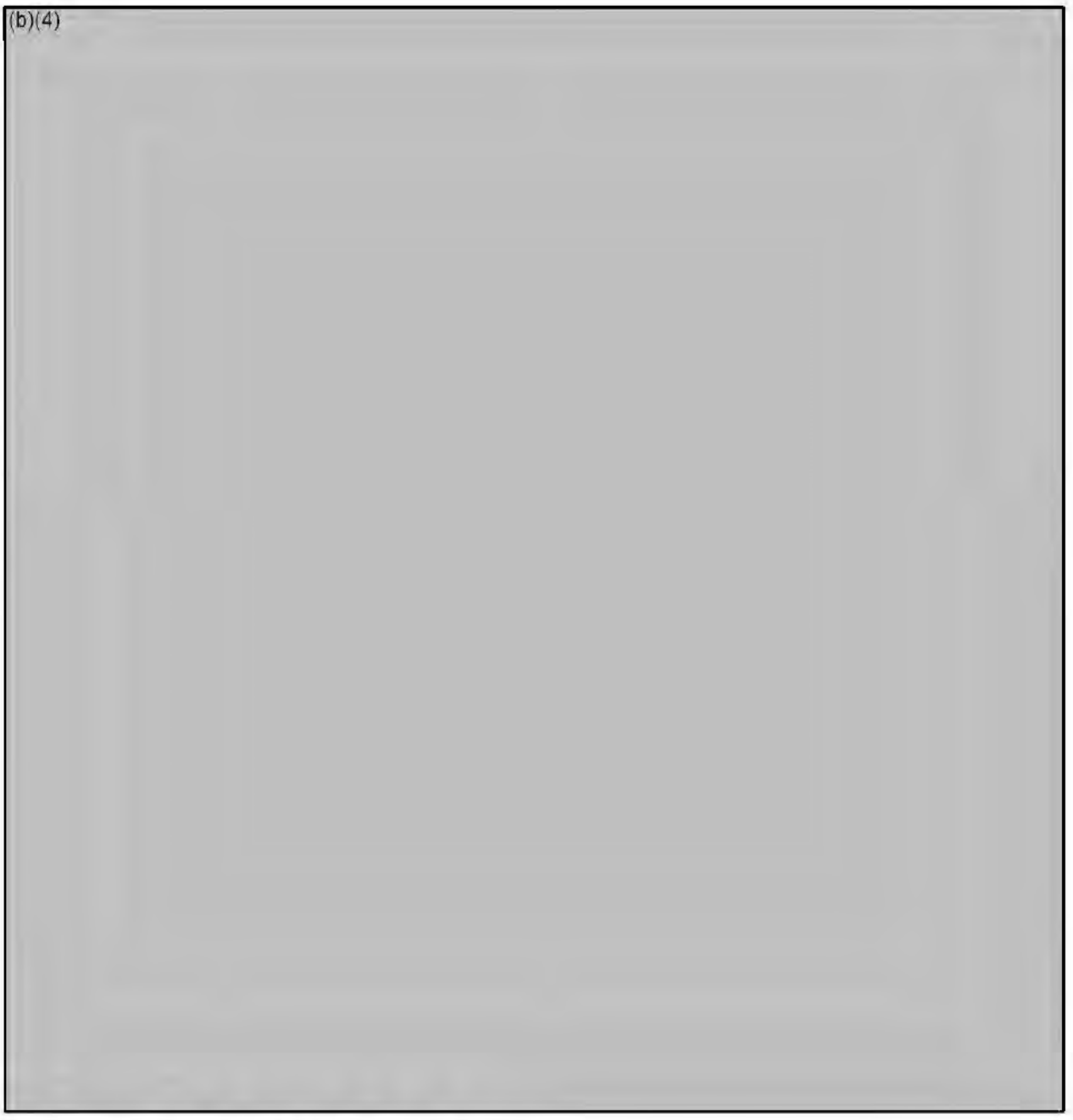
The behavioral toxicity was investigated for male mice receiving a single i.p. injection (0.1 ml) of saline, PEG vehicle, or (b)(4) Locomotor activity (total distance traveled), grip strength and motor coordination were evaluated on days (b)(4) post-injection (b)(4) and body weight was measured over days. On day (b)(4) (b)(4) tissues from the testes, liver, adrenal gland, mesenteric lymph node, spleen, and bone marrow of the femur and sternum were collected, fixed in buffered formalin, paraffin embedded, sectioned, and stained by hematoxylin and eosin. The results indicated that there were no significant effects of (b)(4) or vehicle on locomotor activity, grip strength, motor coordination, or body weight, compared to the saline-treated control group. In addition, no gross morphological changes or histopathological alterations were observed (b)(4)

(b)(4) **as a radioprotectant and an antifibrotic agent:** (b)(4) (b)(4) and its proteolytic product (b)(4) are believed to have their primary biological

functions in blood pressure homeostasis. However (b)(4) have been demonstrated to be upregulated in fibrotic tissues. Inhibition of (b)(4) or blockade of the (b)(4) receptors on cells has been shown to ameliorate drug-induced (b)(4) in murine and rat models for (b)(4) (b)(4) receptor antagonists also prevent cardiac remodeling following ischaemia/reperfusion in rat models of cardiac remodeling diseases (b)(4) Several studies have also shown that inhibition of (b)(4) or (b)(4) signaling mitigates radiation-induced injuries, including to the heart and (b)(4) (b)(4) One study shows that the (b)(4) reduces acute damage to the jejunal mucosa in mice following exposure to (b)(4) This work examined only initial tissue injury and did not extend findings past 5 days, and did not examine mortality. A second study found a reduction in (b)(4) in rats exposed to (b)(4) (b)(4) radiation (b)(4)

Preliminary Studies:

(b)(4)



II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:
Biomedical Research Database (BRD)NIH RePORTER (replacement database for CRISP) *http://projectreporter.nih.gov/reporter.cfm*
PubMed **II.2.2. Date of Search:**
Data base searches conducted on 11/06/12.

II.2.3. Period of Search:
Searches conducted for the entire time periods available in each data base. For BRD, 1998-2012; for RePorter, 1972-2012. For PubMed, 1970-2012.

II.2.4. Key Words and Search Strategy:

Searches were performed using the following key word combinations:

(b)(4)

II.2.5. Results of Search:

1) BRD: (b)(4) returned 0 results.

One grant was funded to examine the activity of (b)(4)

(b)(4)

2) A RePORTER search revealed 8 current grants studying the effects of radiation on

(b)(4)

None of the funded studies duplicate our research of (b)(4)

(b)(4)

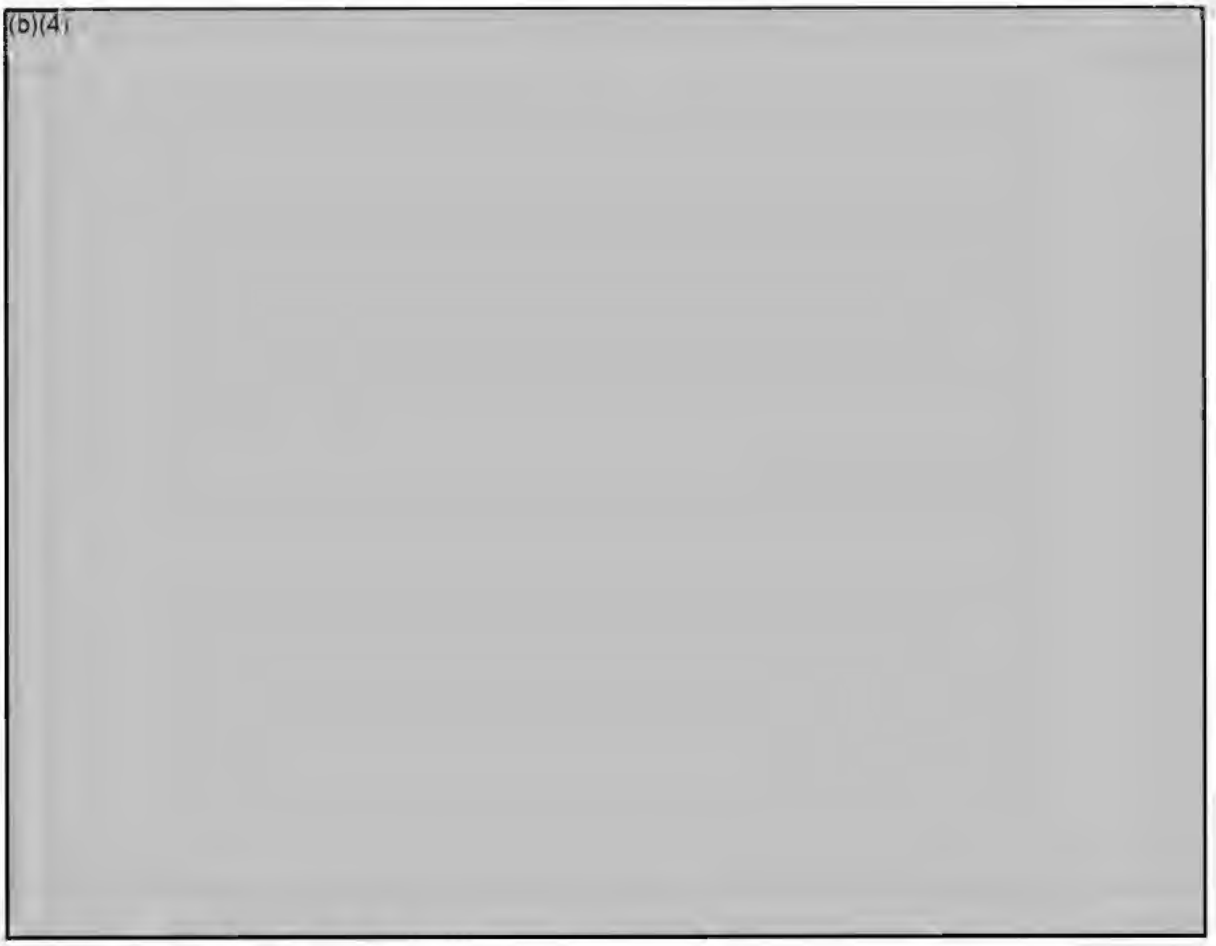
3) PUBMED Search *8/3/09*

None of the publications available described (b)(4)

(b)(4)

(b)(4)

(b)(4)



Summary of other publications:

(b)(4)

(b)(4)

III. OBJECTIVE\HYPOTHESIS:

Our objective is to: 1) continue development of a murine model for both the (b)(4) and (b)(4) injuries; and 2) examine protective effects of (b)(4) against these two injuries in the developed model. (b)(4)

(b)(4)

IV. MILITARY RELEVANCE:

The Department of Defense is prepared to execute military missions within radiation environments, to manage radiation crises associated with terrorist activities, and to manage consequences in the event of nuclear weapons detonation. Recent studies indicate that there is an increasing risk of exposure to ionizing radiation (IR) to military personnel due to increasing terrorist activities, including potential use of stolen nuclear weapons or dispersal of radioactive material in a "dirty bomb" (b)(4) Because of potential exposure of military personnel to radiation, the DoD is committed to protect members of the Armed Services from the adverse health effects of ionizing radiation. High dose IR causes short term, acute injuries including hematopoetic and GI syndromes. However, both high and low dose IR (as may result from a radiation dispersal device) can cause (b)(4) as demonstrated by Japanese atomic bomb survivor data as well as accidental exposures to Russian populations (b)(4) (b)(4) While early effects of radiation injury now have treatment/ prophylactic strategies, the delayed effects currently have no treatment. Lack of treatment for late effects of radiation, including (b)(4) will likely lead to delayed morbidity and mortality for personnel exposed to IR.

The Department of Defense (DoD) Chemical and Biological Defense Program 2007 Annual Report to Congress, Chapter 2.7.6 Medical Radiological Defense, addresses the requirements for the development of radioprotectants. This priority is outlined for the development of

protectants for both acute and delayed radiation injuries, with an emphasis on (b)(4) (b)(4) According to this report, the mitigation of (b)(4) is a major research thrust area. (b)(4)

(b)(4)

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

(b)(4)

(b)(4) irradiation will be performed using the RS2000 irradiator (b)(6) Thirty minutes prior to irradiation, the RS2000 irradiator and lead shield will be decontaminated thoroughly with MB10. Sham irradiation will be performed by placing the mice in jigs for equal lengths of time with no irradiation. As measurements of (b)(4) from radiation, five criteria will be examined:

(b)(4)

(b)(4)

V.1.1. Experiment 1:

Experiment 1 Determination of field of exposure for (b)(4) irradiation (b)(4)

This pilot experiment will be performed to identify the correct shield cut out size required for exposure of the mouse (b)(4) with minimal exposure to the brain or intestinal tract. Mice will be exposed to (b)(4) with x-ray film in a cassette beneath the shield to determine the correct placement of lead strips to close the size of the opening in the lead shield. Animals will be euthanized once the correct opening size is determined.

V.1.2. Experiment 2:

(b)(4)

PEG-400 (0.1 ml/mouse) vehicle only or (b)(4) will be administered by a single (b)(4) i.p. injection h before radiation exposure. (b)(4) will be provided in the drinking water at (b)(4) to give a dosage of (b)(4) per day for the duration of the experiment starting on day 0. The intake and dosage of drug has been previously studied in our laboratory. Amounts of water with and without (b)(4) were measured daily in sham irradiated and (b)(4) irradiated mice to verify that (b)(4) has no effect on water consumption and the dosage of (b)(4) remains in the effective dose range after radiation (b)(4). Mice will be anesthetized and exposed to (b)(4) x-ray irradiation to give exposures equal to (b)(4) irradiation (b)(4) dose rate. Treatment groups will be: 1) PEG; 2) PEG + (b)(4) 3) (b)(4) 4) (b)(4) (b)(4) water bottles will be prepared by the (b)(6 aboratory, and will be replaced 2X per week. Extra water bottles will be prepared in advance in case they are needed to replace a leaking water bottle. (b)(4) is stable in (b)(4) water for several weeks; bottles will be prepared and available for use over weekends and holidays.

The dose rate of (b)(4) to achieve the total (b)(4) exposure desired will be determined by the Radiation Department, which will perform dosimetry using phantoms in the RS2000 irradiator.

Mice will be monitored monthly using non-invasive, unrestrained (b)(4) to determine (b)(4) For these studies (b)(4) mice are required per radiation dose. The time point for (b)(4) is days, based on data (b)(4) from previous studies by others (b)(4) The time point fo (b)(4) is (b)(4) days, based on previous studies by others (b)(4) The experiment will be repeated

because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) [redacted] mice per group) x (4 treatment groups) x (1 time point) x (3 (b)(4) [redacted] radiation levels) = (b)(4) [redacted]
(b)(4) [redacted] mice/experiment) x (2 experiments) = [redacted] mice

Endpoints: The final endpoint for the experiment is [redacted] days. Mice displaying obvious dyspnea, (b)(4) lethargy, or other markers of morbundity and which appear to be in distress will be euthanized. (b)(4) [redacted] will be histologically examined for (b)(4) [redacted] remodeling. The scale for pain or lethargy is attached. Mice scoring a total of 12 under any measurement will be euthanized.

V.1.3. Experiment 3

Experiment 3: Determination of the mechanism of reduction of (b) [redacted] injury by (b)(4) [redacted]
(b)(4) [redacted]

We have determined that between (b)(4) [redacted] days (b)(4) [redacted] cells exhibit markers of (b)(4) [redacted] and (b)(4) [redacted] following (b)(4) [redacted] irradiation. We will investigate the mechanism of action of (b)(4) [redacted] examining these markers of cell injury.

We will use 4 time points: (b)(4) [redacted] days postirradiation, using (b)(4) [redacted] (b)(4) [redacted] irradiation. Treatment groups will be: 1) PEG; 2) PEG + (b)(4) [redacted] 3) (b)(4) [redacted] 4) (b)(4) [redacted] (b)(4) [redacted] The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) [redacted] mice per group) x (4 treatment groups) x (4 time points) x (3 (b)(4) [redacted] radiation levels) = (b)(4) [redacted]
(b)(4) (b)(4) [redacted] mice/experiment) x (2 experiments) = [redacted] mice
(b)(4) [redacted] sham-irradiated mice will be required as controls.

Endpoint: Endpoints for the experiments will be (b)(4) [redacted] days. Early time points will be taken if any animal exhibits signs or symptoms of morbidity or moribundity. Such animals will be euthanized at the time of observation.

V.2. Data Analysis:

Mean with standard error, or percentage will be reported if applicable. A significance level is set at 5% for each test. All statistical tests are two-sided. A statistical software, SAS will be used for statistical analyses.

Experiments 2 and 3: Analysis of variance (ANOVA) will be used to detect significant difference among the groups. If significant, then the pair-wise comparison by Tukey-Kramer will be used to identify which group is different from the other. For experiment 2, N = [redacted] mice per group could (b)(4) have 80% power to detect any significant difference between groups if any, given type I error of 5% and delta/sigma has at least 1.4, where the delta is the estimated mean differences between the two groups and sigma is the pooled standard deviation of the two groups (b)(4) [redacted] For experiment 3, previous data from this laboratory indicate that the coefficient of variation (CV) of the data is about 0.5, and that differences of between 2- and 30- fold are likely to be
(b)(4) observed. A sample size of [redacted] per group will have 80% power to detect 3-fold increases in biomarkers based on a lognormal t test with a 5% two-sided significance level and a CV of 0.5.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

For the last 8 years, we have utilized primary cell culture systems to examine anti-apoptotic mechanisms; however, we must determine whether our culture findings are relevant in whole animals. We will continue to use primary cell culture whenever possible to avoid unnecessary use of animals. We have also considered computer models as alternatives to animals. However, this alternative also lacks the knowledge base and complexity needed to evaluate radioprotective and radiotherapeutic agents.

V.3.2. Animal Model and Species Justification:

Tissue culture conditions cannot fully substitute for the complexities of whole organisms in response to radiation injury. Such injury involves the interactions between the hematopoietic system and other organ systems. In order to develop viable treatments for humans, we find that our research must be conducted in whole animals.

(b)(4),(b)(6) the mouse as the best species to use for radiation protection and radiotherapy protocols: 1) mice provide the least sentient species available whose response to radiation has been clearly identified, with physiological responses to radiation and radioprotectants that are similar to human physiology, 2) extensive literature is available on responses of mice to radiation and countermeasures, 3) more reagents are available to study the relevant cell types and molecules in mice than in any other species except humans, and the availability of transgenic and knockout mice *make* this model extremely attractive for further research into molecular mechanisms, and 4) (b)(6) (b)(6) have extensive experience with this model in radiation injury and radioprotection research (b)(6)

V.3.3. Laboratory Animals

V.3.3.1. Genus & Species: *Mus musculus*

V.3.3.2. Strain/Stock: CBA

V.3.3.3. Source/Vendor: (b)(4)

V.3.3.4. Age: 10-14 weeks

V.3.3.5. Weight: Normal adult weight range for this strain: 17-21 g.

V.3.3.6. Sex: Female. Only females will be used in this study for the following reasons: 1) we have so far utilized female mice to lay the foundation of our studies for radiation-induced (b)(4) injuries; 2) because of long housing periods, it is advantageous to use female mice that are less aggressive toward each other.

V.3.3.7. Special Considerations: Commercially procured animals must be adventitious disease free. Mice- Pathogen-free, including *Pseudomonas aeruginosa*, and Pasteurella: All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus,

Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus; *Helicobacter* spp. *Mycoplasma pulmonis*, and *Clostridium piliforme*. Endoparasite- and ectoparasite-free.

V.3.4. Number of Animals Required (by Species):

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: Animals demonstrating moribundity due to radiation exposure (b)(4) will be considered to have arrived at the study endpoint (b)(4) (b)(4) will likely minimize pain and distress, while (b)(4) has been shown in studies of chemotherapy agents to function as a repair and (b)(4) factor.

Ketamine/xylazine anesthesia will be used during X-ray irradiation to prevent stress from the time the mice are placed in restraint. Under the training protocol at AFRRI inconjunction with the AFFRI veterinary staff, we found that in female C57BL/6J mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia with no morbidity or mortality (b)(4) We propose to use this range of concentration of anesthetics, together with a heating pad to keep mice warm during the period of recovery from anesthesia. The temperature in room where the mice will be irradiated will be measured, and if necessary a space heater will be used to increase the temperature. Standard intraperitoneal injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which all laboratory personnel have received training at AFRRI.

V.3.5.2. Reduction: Whenever possible, control groups will be shared between experimental groups for our experiments. Tissues will be collected for multiple assays from the same animals further reducing the total number of animals needed. At every stage of the research, planned experiments will be reevaluated, in an attempt to reduce the numbers of experiments and eliminate repeats of experiments where statistical significance has already been obtained. We also plan to take as many tissues as we can reasonably analyze for experiments examining protein oxidation.

V.3.5.3. Replacement: At this time, it is not feasible to use non-animal systems in place of animal models to address the research questions in this protocol.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals: (b)(4)

V.4.1.1.1.1. Column C: (b)(4)

V.4.1.1.1.2. Column D:

V.4.1.1.1.3. Column E: (b)(4)

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:
We will be unable to use analgesics since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4) (b)(4) Some sedatives have been reported to contribute to postoperative (b)(4) which likely reduce some functions of radiation. Additionally, it has been shown that the biometabolism of analgesics can induce protein oxidation, which would interfere with sample analysis (b)(4)

An anesthesia protocol has been developed for use during the X-ray irradiation. Ketamine will be used with xylazine for longer anesthesia in mice to be place in restraints for (b)(4) irradiation. Under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff, we found that in female CBA mice, ~12 weeks old,i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia (25 G needle) with no morbidity or mortality (b)(4) We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. Standard i.p. injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which all laboratory personnel have received training at AFRRI.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:
Animals will be housed and cared for as described in Section V.5. Veterinary Care both before and after dose administrations and irradiation procedures.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: DoD Bioedical Research Data Base (BRD), CRISP, PubMED

V.4.1.3.2. Date of Search: 11/7/12

V.4.1.3.3. Period of Search: Search covered published manuscripts from ~1965-present. (BRD search included all available years 1998 -2012).

V.4.1.3.4. Key Words of Search: Pain, analgesia, anesthesia, mice, radiation, ionizing radiation, humane, (b)(4)

V.4.1.3.5. Results of Search:
The literature search revealed that, at present, there are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. In some cases radiation has been demonstrated to reduce pain in laboratory mice (b)(4) and has been in humans reported to alleviate cancer related bone pain (b)(4) (b)(4) A recent report showed that administration of botulinum toxin A was effective for reduction of pain and muscle spasms after clinical radiation for treatment of tumors (b)(4) (b)(4) Application of natural honey 20 min prior to radiation exposure was also shown to reduce mucositis with associated pain in humans following head/neck radiotherapy (b)(4) it has not been determined whether honey acts as a local radioprotectant. Spinal cord stimulation has also been reported in humans to alleviate pain resulting from radiotherapy (b)(4) however, this method would not be

practical for use in mice because of the extensive spinal cord manipulation required. Human administration of morphine also relieves pain (b)(4) but again this would not be practical for mice in our experiments because of side effects of the drug. In humans, exposure to (b)(4) irradiation may induce vomiting and diarrhea, causing pain and distress. However, mice are not susceptible to vomiting. The animals that will be administered (b)(4) will likely receive pain relief as phytoestrogens have been reported to reduce neuropathic pain in rodents (b)(4) preliminary results also indicate that (b)(4) ameliorates acute radiation-induced injuries (b)(4)

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
Anesthesia will be used before (b)(4) irradiation to avoid stress from restraint. (b)(4)
(b)(4)
(b)(4) we will be unable to use analgesics after irradiation since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4)
(b)(4)
(b)(4) In experiments where tissue collection is needed, animals will be euthanized with pentobarbitol prior to sample collection as described elsewhere.

V.4.2. Prolonged Restraint:
No prolonged restraint is required for (b)(4) irradiation. Whole body (b)(4) is non-invasive and unrestrained. (b)(4) irradiation will require restraint in designed jigs for 20-40 min to receive X-ray exposure (b)(4) animals will be anesthetized during this period (see Anesthesia above) and tails will be taped to one side with surgical tape. The jigs for use during (b)(4) irradiation were examined and approved by the AFRRI Veterinary staff. An image of the jig is now provided (see attached). Under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff, we found that in female CBA mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia (25 G needle) with no morbidity or mortality (b)(4) We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. Standard i.p. injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which all laboratory personnel have received training at AFRRI. AFFRI Veterinary staff have provided training and supervision for the placement of anesthetized animals in the approved jigs for irradiation.

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations: N/A

V.4.4.1. Injections:
Intra-peritoneal injections of pentobarbital for euthanasia 0.1-0.2 ml/mouse Fatal Plus (39-78 mg pentobarbital/mouse), will be performed with a 25 G needle. I.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine will be done using a 25 G needle. This has been extensively examined by the AFRRI Veterinary staff and shown to effectively produce non-lethal anesthesia (b)(4)

V.4.4.1.1. Pharmaceutical Grade Drugs
(b)(4)

V.4.4.1.2. Non-Pharmaceutical Grade Drugs
(b)(4)

Endotoxin in the drugs will be measured by the PI, or information from the company will be provided prior to administration of drugs to the animals.

V.4.4.2. Biosamples:
All biosamples will be taken after euthanasia. (b)(4) (b)(4) or analysis will removed *en bloc*. Intestinal tissue will be taken in smaller amounts (the first segment of the small intestine). All other tissues will be available to other researchers for tissue sharing.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification:
All cages will have cage cards identifying the treatment groups for each experiment. For experiments 2 and 3, each animal within a cage will receive ear punches for individual identification (1 = no cut; 2 = right ear upper corner; 3 = left ear upper corner; 4 = both ears).

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures:

(b)(4) **Irradiation**

The methodology for (b)(4) irradiation of mice including approval of holding jigs and development of anesthesia was developed in conjunction with the AFRRI Veterinary Staff. (b)(4) irradiation will be performed using the RS2000 irradiator (b)(6) Thirty minutes prior to irradiation, the RS2000 irradiator and lead shield will be decontaminated thoroughly with MB10.

Mice will be anesthetized by injection in the USUHS LAM facility. Anesthetized mice will be placed in ventilated Lucite jigs, which restrain the animals to prevent lateral or vertical movement. Tails will be taped to prevent any forward motion of animals. Mice will be irradiated at (b)(4) irradiation. The temperature in room where the mice will be irradiated will be measured, and if necessary a space heater will be used to increase the temperature. Following radiation exposure, mice will be immediately returned to the LAM and allowed to recover from anesthesia under supervision with heating pads. Immediately upon recovery, animals will be returned to standard cages that house 4 animals per cage. Lucite jigs will be sanitized after each use.

(b)(4)

(b)(4) The animals will experience no restraint, no anesthesia, no drugs, or other manipulation during this time. Following the measurement, to be performed in the room in which they are housed, the animals will be returned to their original cages. Chambers will be sanitized after each use.

Ear punch
The ear punch will be soaked in alcohol to disinfect it before use and between animals. The device will be placed on the pinna of the ear (external ear) in a location where the mouse will be marked for identification. The punch will be pressed firmly to punch a circular hole through the ear, being careful not to rip the delicate membrane of the pinna. The ear will then be gently separated from the device. The ear punch will be cleaned and disinfected in alcohol before use in another mouse.

V.4.4.8. Tissue Sharing:

For most studies (b)(4) will be obtained. All other tissues will be available for tissue sharing with other investigators. Our laboratory has made extensive use of shared tissues for training and technique development for tissue. (b)(4)

V.4.5. Study Endpoint:

(b)(4)

(b)(4) Early endpoints will be used in the case of morbidity and morbundity. Specific health assessment criteria including rapid weight loss, ruffled fur, dried lacrimal fluid accumulated in the cul-de-sac of their eyes, are cool to the touch, decreased appetite, diarrhea, difficulty breathing or panting (b)(4) (b)(4) will used to determine early study endpoints for euthanasia. Following (b)(4) irradiation, mice may exhibit injuries from the (b)(4) irradiation during the first week postirradiation. Also, mice are expected to develop (b)(4) injury on days (b)(4) postirradiation. During the critical time periods (the first week postirradiation and (b)(4) days postirradiation), mice will be monitored by the PI or other members of the investigative team at least twice daily, early morning and late afternoon including weekends or holidays. Mice will be considered moribund when, in addition to showing some or all of the signs of morbidity, they exhibit labored

breathing plus either an inability to remain upright or are unmotivated to move. Morbid or moribund mice will be euthanized.

V.4.6. Euthanasia:
Animals will be euthanized at the stated endpoints of the experiments by i.p. injection of ml/mouse Fatal Plus. PHS policy is intraperitoneal injection of at least 200 mg/kg sodium pentobarbital euthanasia solution; Fatal Plus: 390 mg pentobarbitol/1 ml
1 ml/390 ml x 0.02 kg/mouse x 200 mg/1 kg = 0.01-0.02
IP will be performed using a 25 G needle. (Please note that Euthasol cannot be used as additives have effects on lung tissues. We do have Fatal Plus for these experiments.)
Euthanasia will be confirmed by cervical dislocation. Animals which appear to be experiencing pain or distress as a result of radiation exposure or are found moribund will also be euthanized by pentobarbitol and necropsied. At the end of experiments, unused animals will be transferred to other protocols or euthanized by exposure to 100% CO_2 from a compressed gas cylinder in accordance with the current American Veterinary Medical Association (AVMA) Guidelines for Euthanasia. Exsanguination, cervical dislocation, and/or pneumothorax will be used to ensure euthanasia.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. Food and water and Rodent Diet will be available ad libitum. The 12:12 hr (lights off at 6 pm) room light cycle is fine for all the studies.

V.5.1.1. Study Room: will be performed in the
Radiation Building (b)(6) Room Number (b)(6)

Euthanasia Building (b)(6) Room number (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No x

Fluid Restriction: NOTE: (b)(4) **treated water will be provided.** No x

V.5.1.3. Exceptions: None

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
Experimental animals will be observed at least twice a day by one of the study investigators or technicians. VSD personnel will also monitor the animals twice a day during their rounds. As described in another section (study endpoint) morbid animals will be monitored at least twice daily, morning and afternoon. The experimental design does not contemplate supportive therapy. The justification for not providing supportive

care has been described in the study end point section as well as in the pain category section. Criteria to be used for health evaluation while the animals are on study include: 20% weight loss (mice will be weighed every other day—based on (b)(0) with (b)(4) irradiation in which animals can recover from ~25% weight loss (b)(4) (b)(4) ruffled fur, dehydration, decreased activity, and hunched body position, dyspnea, or panting. Animals exhibiting these signs may be euthanized, and considered to be at the study endpoint. Increased monitoring of animals will begin on day (b)(4) through the end of the experiment, based on our previous data that indicated that mortality from (b)(4) irradiation occurs between days (b)(4) postirradiation.

V.5.2.2. Emergency Veterinary Medical Care:
All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.
V.5.3.2. Enrichment Restrictions: none

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

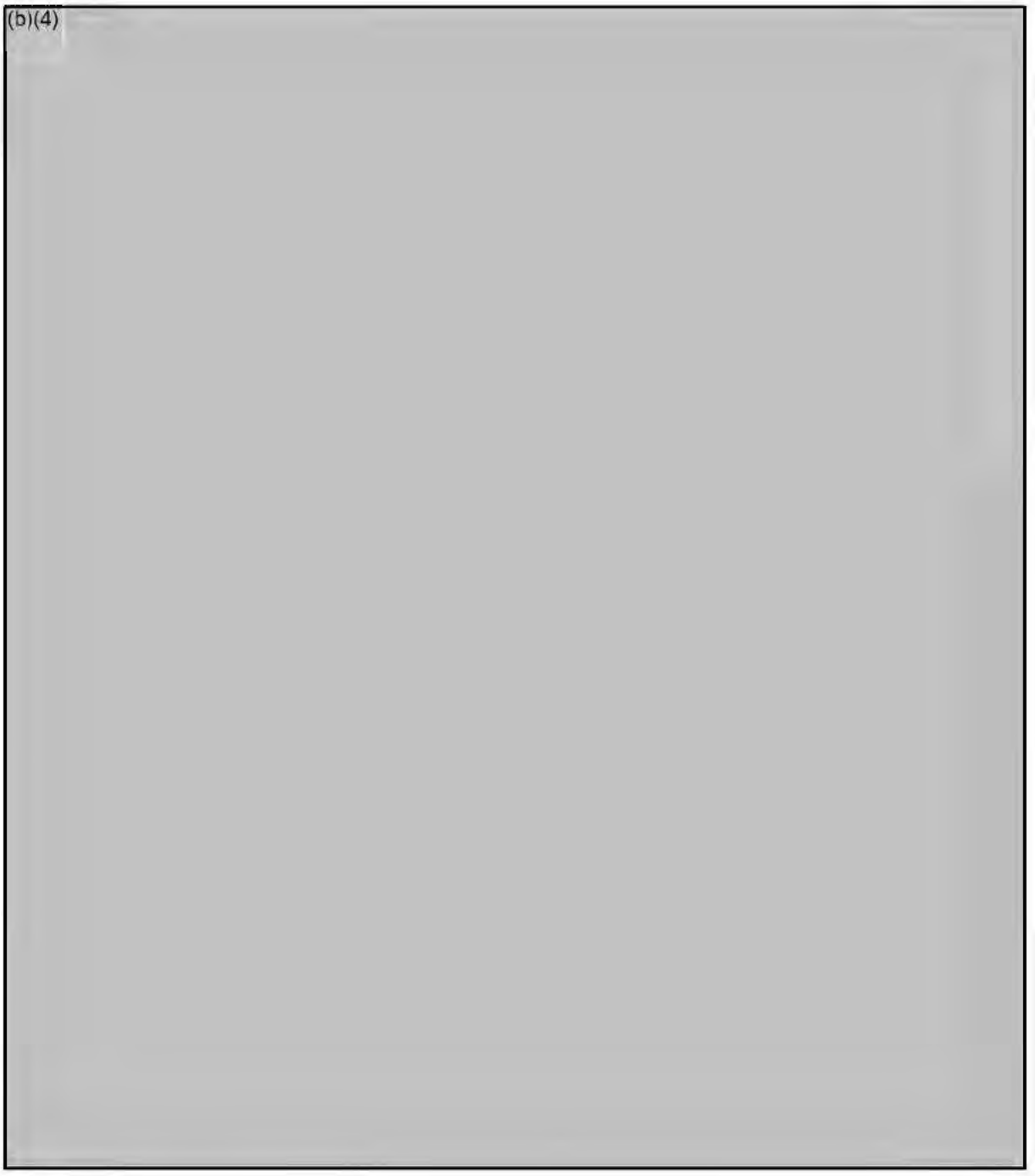
STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Ear punch, irradiation, anesthesia, euthanasia	(b)(6)	8+ years	(b)(6)
Ear punch, irradiation, anesthesia, euthanasia		6 years	

VII. BIOHAZARDS/SAFETY: None

VIII. ENCLOSURES: Images of jigs, (b)(4) MSDS, manuscripts (b)(4) (b)(4)

References:


(b)(4)

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ 11/26/12

P ature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ 11/26/12

Pr Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Princi ure

11/26/12

Date

X. PROTOCOL ABSTRACT:

The terrorist attack of September 11, 2001 against the United States has increased the concern that terrorists may acquire and use radioactive sources (e.g. dirty radioactive bombs) to expose military and civilian populations to ionizing radiation. However, despite this concern, there are currently no FDA approved drugs that can be used for the general population to prevent death that is likely to result from exposure to ionizing radiation. (b)(4) irradiation in humans can result in both (b)(4) injuries. However, because rodents are more resistant to (b)(4) injuries than humans, there is currently no murine model that has been developed to study both acute and delayed radiation injuries in the same animal.

(b)(4)

A. Animal Protocol Number:

B. Animal Protocol Title: (b)(4)

(b)(4)

C. Principal Investigator: (b)(6)

D. Performing Organization: Uniformed Services University of the Health Sciences

E. Funding: DTRA

F. Objective and Approach:

(b)(4)

G. Indexing Terms (Descriptors): (b)(4)

animals, mice

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4712
http://www.usuhs.mil

February 25, 2013

MEMORANDUM FOR DR. (b)(6)

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on February 25, 2013:

Animal Protocol Title: "Murine Model for (b)(4) Radiation Injuries (Mus Musculus)"

USUHS Protocol Number: (b)(6)

Expiration Date: February 24, 2016

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

Learning to Care for Those in Harm's Way

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

Revised

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Murine model for (b)(4) radiation injuries (*Mus musculus*)

GRANT TITLE (if different from above): (b)(4)
(b)(4)

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: DTRA

EARLIEST ANTICIPATED FUNDING START DATE: Current

PRINCIPAL INVESTIGATOR:

(b)(6) Feb 4, 2013
Date

Associate Professor (b)(6)
(b)(6) office: (b)(6) fax: (b)(6)

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific re(b)(6) ood scientific research practice.

Dept. Head Signature — Chair (Title) — (b)(6) (Telephone) — 2/4/13 (Date)
Typed Name: (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) — (b)(6) (Department Telephone) — Feb 4, 2013 (Date)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) — LAM (Department) — (b)(6) (Telephone) — 2/4/2013 (Date)
Attending/Consulting Veterinarian Signature
Typed Name: LTC (b)(6)

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D., (b)(6)

ANIMAL PROTOCOL TITLE: Murine model for (b)(4) radiation injuries (*Mus musculus*)

GRANT TITLE (if different from above): (b)(4)
(b)(4)

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S):

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

The terrorist attack of September 11, 2001 against the United States has increased the concern that terrorists may acquire and use radioactive sources (e.g. dirty radioactive bombs) to expose military and civilian populations to ionizing radiation. However, despite this concern, there are currently no FDA approved drugs that can be used for the general population to prevent death that is likely to result from exposure to ionizing radiation. (b)(4) irradiation in humans can result in (b)(4) injuries. However, because rodents are more resistant to (b)(4) injuries than humans, there is currently no murine model that has been developed to study both (b)(4) radiation injuries in the same animal.

(b)(4),(b)(6)

II. BACKGROUND:

II.1. Background:

The acute toxic effects of exposure to high doses of ionizing radiation in humans have been shown to occur due to two sequential effects: first the ablation of immune cells, resulting in loss of immune function and increasing likelihood of pathogenic infection (b)(4) and subsequently the loss of epithelial progenitor cells of the gastrointestinal (GI) crypt (b)(4) (b)(4) Both of these events have been shown to be due to the loss of fast-growing pluripotent and/or progenitor cell types. The time course of the acute injuries correlates with the life span of the mature cell types of the tissue, which are not replaced because of radiation damage to the adult pluripotent cells and adult stem cells. There is currently no FDA approved prophylactic or treatment for prevention of acute radiation injuries following high-dose (b)(4) radiation exposure.

In some cases supportive measures can be used to treat acute radiation syndrome, and survival from (b)(4) radiation exposure is now possible (b)(4) This results in current efforts to mitigate or treat later stage effects of ionizing radiation exposure. (b)(4)

(b)(4)

In humans, death from radiation (b)(4) can occur following survival from (b)(4) Cumulative evidence from a number of laboratories, including our own, shows that there is a threshold dose of radiation of (b)(4) to induce sufficient (b)(4) for the (b)(4) phases to occur in mice; below these doses, no permanent injury manifests, although some short-term effects are observed (b)(4) However, in humans, there is currently no accepted protocol for preventative treatment of radiation-induced (b)(4) and morbiditiy as high as 50% is associated with each phase (b)(4) (b)(4) injuries from radiation is considered to be a serious problem in the field for the development of agents to prevent these radiation injuries.

(b)(4) **as a radioprotectant:** The biochemical properties of the isoflavones and the investigation of (b)(4) as a protectant against gamma radiation-induced lethality have been investigated by (b)(4),(b)(6) (b)(4) A single i.p. dose of (b)(4) administered - hr prior to a lethal dose of (b)(4) (b)(4) radiation resulted in -day survival rates of 65%, 91%, and 85% respectively. Mice receiving saline or PEG vehicle alone survived at 8% and 15% respectively. All doses of (b)(4) protected mice significantly ($p < 0.001$) better than vehicle or saline control groups.

The behavioral toxicity was investigated for male mice receiving a single i.p. injection (0.1 ml) of saline, PEG vehicle, or (b)(4) Locomotor activity (total distance traveled), grip strength and motor coordination were evaluated on days (b)(4) post-injection (b)(4) and body weight was measured over days. On day (b)(4)

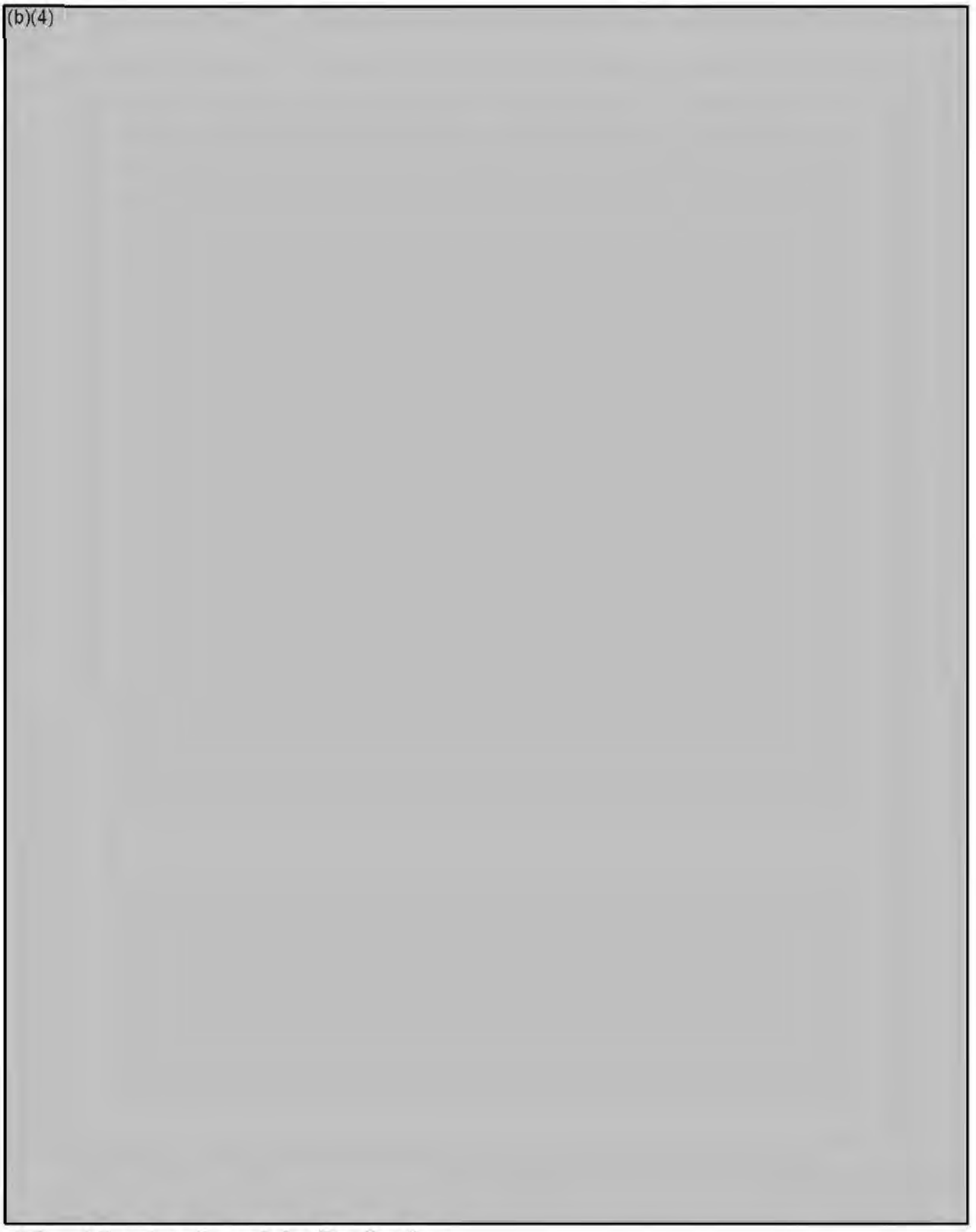
(b)(4) tissues from the testes, liver, adrenal gland, mesenteric lymph node, spleen, and bone marrow of the femur and sternum were collected, fixed in buffered formalin, paraffin embedded, sectioned, and stained by hematoxylin and eosin. The results indicated that there were no significant effects of (b)(4) or vehicle on locomotor activity, grip strength, motor coordination, or body weight, compared to the saline-treated control group. In addition, no gross morphological changes or histopathological alterations were observed (b)(4)

(b)(4) **as a radioprotectant and an antifibrotic agent:** (b)(4) (b)(4) and its proteolytic product (b)(4) are believed to have their primary biological functions in blood pressure homeostasis. However, (b)(4) have been demonstrated to be upregulated in fibrotic tissues. Inhibition of (b)(4) or blockade of the (b)(4) receptors on cells has been shown to ameliorate drug-induced (b)(4) in murine and rat models for (b)(4) (b)(4) receptor antagonists also prevent cardiac remodeling following ischaemia/reperfusion in rat models of cardiac remodeling diseases (b)(4) Several studies have also shown that inhibition of (b)(4) (b)(4) signaling mitigates radiation-induced injuries, including to the heart and (b)(4) (b)(4) One study shows that the (b)(4) reduces acute damage to the jejunal mucosa in mice following exposure to (b)(4) This work examined only initial tissue injury and did not extend findings past (b)(4) days, and did not examine mortality. A second study found a reduction in (b)(4) in rats exposed to (b)(4) (b)(4) radiation (b)(4)

Preliminary Studies:

(b)(4)

(b)(4)



II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:

Biomedical Research Database (BRD)NIH RePORTER (replacement database for CRISP) *http://projectreporter.nih.gov/reporter.cfm*
PubMed **II.2.2. Date of Search:**
Data base searches conducted on 1/06/13.

II.2.3. Period of Search:
Searches conducted for the entire time periods available in each data base. For BRD, 1998-2013; for RePorter, 1972-2013. For PubMed, 1970-2013.

II.2.4. Key Words and Search Strategy:
Searches were performed using the following key word combinations:
(b)(4)

II.2.5. Results of Search:
1) BRD (b)(4) returned 0 results.
One grant was funded to examine the activity of (b)(4)
(b)(4)

2) A RePORTER search revealed 8 current grants studying the effects of radiation on (b)(4)
(b)(4)

None of the funded studies duplicate our research of (b)(4)
(b)(4)

3) PUBMED Search

None of the publications available described (b)(4)

(b)(4)

(b)(4)

Summary of other publications:

(b)(4)

(b)(4)

III. OBJECTIVE\HYPOTHESIS:

Our objective is to: 1) continue development of a murine model for both the (b)(4) (b)(4) injuries; and 2) examine protective effects of (b)(4) against these two injuries in the developed mode (b)(4)

(b)(4)

IV. MILITARY RELEVANCE:

The Department of Defense is prepared to execute military missions within radiation environments, to manage radiation crises associated with terrorist activities, and to manage consequences in the event of nuclear weapons detonation. Recent studies indicate that there is an increasing risk of exposure to ionizing radiation (IR) to military personnel due to increasing terrorist activities, including potential use of stolen nuclear weapons or dispersal of radioactive material in a "dirty bomb" (b)(4) Because of potential exposure of military personnel

to radiation, the DoD is committed to protect members of the Armed Services from the adverse health effects of ionizing radiation. High dose IR causes short term, acute injuries including hematopoetic and GI syndromes. However, both high and low dose IR (as may result from a radiation dispersal device) can cause (b)(4) as demonstrated by Japanese atomic bomb survivor data as well as accidental exposures to Russian populations (b)(4) (b)(4) While early effects of radiation injury now have treatment/ prophylactic strategies, the delayed effects currently have no treatment. Lack of treatment for late effects of radiation, including (b)(4) will likely lead to delayed morbidity and mortality for personnel exposed to IR.

The Department of Defense (DoD) Chemical and Biological Defense Program 2007 Annual Report to Congress, Chapter 2.7.6 Medical Radiological Defense, addresses the requirements for the development of radioprotectants. This priority is outlined for the development of protectants for both acute and delayed radiation injuries, with an emphasis on (b)(4) (b)(4) According to this report, the mitigation of (b)(4) is a major research thrust area. (b)(4)

(b)(4)

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

The goal of this project is to investigate the protective effects and mechanisms of (b)(4) (b)(4) treatment for radiation-induced (b)(4) injuries.

(b)(4)

(b)(4) Mice will be exposed to both (b)(4) (b)(4) irradiation to induce the total radiation doses required to induce both

(b)(4) injuries, to simulate the injuries that occur in humans as a result of (b)(4) irradiation.

(b)(4) irradiation will be performed in the (b)(6) using the standard Lucite holders for irradiation. Sham irradiation will be performed by placing the mice in Lucite holders for equal time periods without irradiation. These experiments will involve short end points (less than (b)(4) days) for examination of five criteria. Survival at (b)(4) days will be used to evaluate the (b)(4) **A protocol for (b)(4) irradiation of mice for the proposed experiments in this protocol was approved by the AFRRI IACUC (b)(6) We are awaiting approval for a modification of this protocol to move the mice to USUHS for (b)(4) irradiation and housing thereafter.**

(b)(4) radiation used to induced (b)(4) irradiation will be performed using the RS2000 irradiator (b)(6) Thirty minutes prior to irradiation, the RS2000 irradiator and lead shield will be decontaminated thoroughly with MB10. Sham irradiation will be performed by placing the mice in jigs for equal lengths of time with no irradiation.

V.1.1. Experiment 1:

Experiment 1 Final development of a murine model that incorporates both (b)(4) (b)(4) radiation injuries. (b)(4)

Rationale: (b)(4)

(b)(4)

This experiment, combining (b)(4) doses to achieve (b)(4) dose, will also be used to determine that we do not encounter a significant effect from (b)(4) the dose. To minimize a possible (b)(4) effect, we will keep the interval between radiation exposures as low as possible, (b)(4) min.

To complete our development of the model, we will use five doses of (b)(4) irradiation between (b)(4) Groups will be: 1) (b)(4) irradiation only; and 2) (b)(4) irradiation + (b)(4) irradiation. Time points will be: survival at (b)(4) days postirradiation (b)(4) (b)(4) irradiation will be given to equal a total exposure of (b)(4) (b)(4) Mice will first be exposed to (b)(4) irradiation in the (b)(6) facility (b)(4) then anesthetized and exposed to X-ray irradiation of the (b)(4) in the RS2000 at USU, dose rate (b)(4)

Control mice will be anesthetized and placed in holders to provide a sham for (b)(4) irradiation. The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) Mice needed for experiment: group to reach statistical significance for survival.
(b)(4) mice/group) x (6 conditions) x (1 time point) = animals.
The experiment will be repeated 1 time to confirm findings (total animals). (b)(4)

(b)(4) Endpoint: The endpoint will be days. We will measure survival at both (b)(4) days for the study. Early time points will be taken if any animal exhibits signs or symptoms of morbidity or moribundity (score of >11 as described in the pain score sheet). Specific health assessment criteria including rapid weight loss, ruffled fur, dried lacrimal fluid accumulated in the cul-de-sac of their eyes, are cool to the touch, decreased appetite, diarrhea, difficulty breathing or panting will used to determine early study endpoints for euthanasia (See section V.4.5). Such animals will be euthanized at the time of observation.

Table 1: Schematic for Experiment 1

(b)(4)

V.1.2. Experiment 2

Experiment 2 Determine the effect of (b)(4) on survival following (b)(4) (b)(4) irradiation. (b)(4)

Rationale: Experiment 1 will be used to determine overlapping effects of (b)(4) irradiation on (b)(4) injury by (b)(4) irradiation. This section objective will be to determine the effects and the mechanisms of (b)(4) for the prevention of (b)(4) induced by exposure to (b)(4) irradiation. The approach will be to use murine model of both (b)(4) radiation injuries developed in Experiment 1. We will assess the mechanisms of protection using (b)(4) cellular, histological, molecular, and biochemical markers. The rationale for undertaking this research is that once the mechanisms of (b)(4) prevention of (b)(4) are known, these may help designing therapeutic strategies against radiation-induced (b)(4) injury.

PEG-400 (0.1 ml/mouse) vehicle only or (b)(4) will be administered by a single (b)(4)
(b)(4) (b)(4) i.p. injection h before radiation exposure (b)(4) will be provided in the drinking water at (b)(4) to give a dosage of (b)(4) per day for the duration of the experiment starting on day 0. Our preliminary findings indicate that there are no adverse effects due to long term use of (b)(4) We have used this drug in the water to treat mice for up to days with no findings of (b)(4) adverse effects. Mice will be exposed to (b)(4) using (b)(4) dose rate. Subsequently, mice will be anesthetized and exposed to (b)(4) x-ray irradiation to give exposures equal to (b)(4) irradiation, (b)(4) dose rate. Treatment groups will be: 1) PEG; 2) PEG + (b)(4) 3) (b)(4) and 4) (b)(4) water will be provided for all

mice to reduce opportunistic infections that occur following (b)(4) irradiation. For mice to be treated with (b)(4) will be provided in (b)(4) water. (b)(4) water bottles and (b)(4) water bottles will be prepared by the (b)(6) laboratory, and will be replaced 2X per week in accordance with LAM protocols. Extra water bottles will be prepared in advance and they will be available in the room where the animals are housed in case they are needed to replace a leaking water bottle.

Mice will be monitored monthly using non-invasive, unrestrained (b)(4) to determine (b)(4) For these studies (b)(4) mice are required per radiation dose. The time point for (b)(4) is (b)(4) days, based on data from previous studies by others (b)(4) The time point for (b)(4) is (b)(4) days, based on previous studies by others (b)(4) Although breathing will be monitored, (b)(4) results will not be used as a sole indicator for euthanasia. (See section V.4.6).

The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) (b)(4) mice per group) x (4 treatment groups) x (1 time point) x (3 radiation levels) = (b)(4) (b)(4)
(b)(4) mice/experiment) x (2 experiments) = mice

Endpoints. The final endpoint for the experiment is days. Mice displaying obvious dyspnea, (b)(4) extreme lethargy, or other markers of morbundity as described in the pain score sheet (score of >11), and which appear to be in distress will be euthanized. Specific health assessment criteria including rapid weight loss, ruffled fur, dried lacrimal fluid accumulated in the cul-de-sac of their eyes, are cool to the touch, decreased appetite, diarrhea, difficulty breathing or panting will used to determine early study endpoints for euthanasia (See section V.4.5). (b)(4)
(b)(4)

Table 2. Schematic for Experiments 2 and 3.

(b)(4)

V.2. Data Analysis:

Mean with standard error, or percentage will be reported if applicable. A significance level is set at 5% for each test. All statistical tests are two-sided. A statistical software, SAS will be used for statistical analyses.

Experiments 1 and 2. Log-Rank test will be used for comparison among groups. If significant, Fisher's exact test will be used to detect significant differences across the groups in survival (b)(4) rates at day Kaplan Meier curves will be displayed. N = mice per group will have 80% (b)(4) power in detecting a significant difference between two groups if any, given type I error of 5% where the treatment group survival rate is at least 68%, compared to a control group survival rate of 20%. Similar statements would apply, if the treatment group survival rates are at least 83%, 78%, or 73%, compared to vehicle group survival rates of 35%, 30%, and 25%, respectively (b)(4)

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

For the last 8 years, we have utilized primary cell culture systems to examine anti-apoptotic mechanisms; however, we must determine whether our culture findings are relevant in whole animals. We will continue to use primary cell culture whenever possible to avoid unnecessary use of animals. We have also considered computer models as alternatives to animals. However, this alternative also lacks the knowledge base and complexity needed to evaluate radioprotective and radiotherapeutic agents.

V.3.2. Animal Model and Species Justification:

Tissue culture conditions cannot fully substitute for the complexities of whole organisms in response to radiation injury. Such injury involves the interactions between the hematopoietic system and other organ systems. In order to develop viable treatments for humans, we find that our research must be conducted in whole animals.

(b)(4),(b)(6) the mouse as the best species to use for radiation protection and radiotherapy protocols. 1) mice provide the least sentient species available whose response to radiation has been clearly identified, with physiological responses to radiation and radioprotectants that are similar to human physiology, 2) extensive literature is available on responses of mice to radiation and countermeasures, 3) more reagents are available to study the relevant cell types and molecules in mice than in any other species except humans, and the availability of transgenic and knockout mice make this model extremely attractive for further research into molecular mechanisms, and 4) (b)(6) (b)(6) have extensive experience with this model in radiation injury and radioprotection research (b)(6)

V.3.3. Laboratory Animals

V.3.3.1.	**Genus & Species:**	*Mus musculus*
V.3.3.2.	**Strain/Stock:**	CBA
V.3.3.3.	**Source/Vendor:**	(b)(4)
V.3.3.4.	**Age:**	10-14 weeks
V.3.3.5.	**Weight:**	Normal adult weight range for this strain: 17-21 g.
V.3.3.6.	**Sex:**	Female. Only females will be used in this study for the following reasons: 1) we have so far utilized female mice to lay the foundation of our studies for radiation-induced (b)(4) injuries; 2) because of long housing periods, it is advantageous to use female mice that are less aggressive toward each other.
V.3.3.7.	**Special Considerations:**	Commercially procured animals must be adventitious disease free. Mice- Pathogen-free, including *Pseudomonas aeruginosa*, and Pasteurella. All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus,

Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus, *Helicobacter* spp. *Mycoplasma pulmonis*, and *Clostridium piliforme*. Endoparasite- and ectoparasite-free

V.3.4. Number of Animals Required (by Species):

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: Animals demonstrating moribundity due to radiation exposure (b)(4) will be considered to have arrived at the study endpoint and will be immediately euthanized (See sections V.4.5 Study Endpoint and V.4.6 Euthanasia) (b)(4) will likely minimize pain and distress, while (b)(4) has been shown in studies of chemotherapy agents to function as a repair and (b)(4) factor.

Ketamine/xylazine anesthesia will be used during X-ray irradiation to prevent stress from the time the mice are placed in restraint. Under the training protocol at AFRRI inconjunction with the AFFRI veterinary staff, we found that in female C57BL/6J mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia with no morbidity or mortality (b)(4) We propose to use this range of concentration of anesthetics, together with a heating pad to keep mice warm during the period of recovery from anesthesia. The temperature in room where the mice will be irradiated will be measured, and if necessary a space heater will be used to increase the temperature. Standard intraperitoneal injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which all laboratory personnel have received training at AFRRI.

V.3.5.2. Reduction: Whenever possible, control groups will be shared between experimental groups for our experiments. Tissues will be collected for multiple assays from the same animals further reducing the total number of animals needed. At every stage of the research, planned experiments will be reevaluated, in an attempt to reduce the numbers of experiments and eliminate repeats of experiments where statistical significance has already been obtained. We also plan to take as many tissues as we can reasonably analyze for experiments examining (b)(4)

V.3.5.3. Replacement: At this time, it is not feasible to use non-animal systems in place of animal models to address the research questions in this protocol.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals: (b)(4)

V.4.1.1.1.1. Column C: (b)(4)

V.4.1.1.1.2. Column D: (b)(4)

V.4.1.1.1.3. Column E:

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:
We will be unable to use analgesics since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4) (b)(4) Some sedatives have been reported to contribute to postoperative (b)(4) which likely reduce some functions of radiation. Additionally, it has been shown that the biometabolism of analgesics can induce protein oxidation, which would interfere with sample analysis (b)(4)

An anesthesia protocol has been developed for use during the X-ray irradiation. Ketamine will be used with xylazine for longer anesthesia in mice to be place in restraints for (b)(4) irradiation. Under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff, we found that in female CBA mice, ~12 weeks old,i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia (25 G needle) with no morbidity or mortality (b)(4) We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. Standard i.p. injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which all laboratory personnel have received training at AFRRI.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:
Animals will be housed and cared for as described in Section V.5. Veterinary Care both before and after dose administrations and irradiation procedures.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: DoD Bioedical Research Data Base (BRD), REPORTER, PubMED

V.4.1.3.2. Date of Search: 1/7/13

V.4.1.3.3. Period of Search: Search covered published manuscripts from ~1965-present. (BRD search included all available years 1998 -2012).

V.4.1.3.4. Key Words of Search: Pain, analgesia, anesthesia, mice, radiation, ionizing radiation, humane, (b)(4)

V.4.1.3.5. Results of Search:
The literature search revealed that, at present, there are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. In some cases radiation has been demonstrated to reduce pain in laboratory mice (b)(4) and has been in humans reported to alleviate cancer related bone pain (b)(4) (b)(4) A recent report showed that administration of botulinum toxin A was effective for reduction of pain and muscle spasms after clinical radiation for treatment of tumors (b)(4) (b)(4) Application of natural honey 20 min prior to radiation exposure was also shown to reduce mucositis with associated pain in humans following head/neck

radiotherapy (b)(4) it has not been determined whether honey acts as a local radioprotectant. Spinal cord stimulation has also been reported in humans to alleviate pain resulting from radiotherapy (b)(4) however, this method would not be practical for use in mice because of the extensive spinal cord manipulation required. Human administration of morphine also relieves pain (b)(4) but again this would not be practical for mice in our experiments because of side effects of the drug. In humans, exposure to (b)(4) irradiation may induce vomiting and diarrhea, causing pain and distress. However, mice are not susceptible to vomiting. The animals that will be administered (b)(4) will likely receive pain relief as phytoestrogens have been reported to reduce neuropathic pain in rodents (b)(4) preliminary results also indicate that (b)(4) ameliorates acute radiation-induced injuries (b)(4)

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

Anesthesia will be used before (b)(4) irradiation to avoid stress from restraint. (b)(4) (b)(4) (b)(4) we will be unable to use analgesics after irradiation since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4) (b)(4) (b)(4) In experiments where tissue collection is needed, animals will be euthanized with pentobarbitol prior to sample collection as described elsewhere.

V.4.2. Prolonged Restraint:

(b)(4) irradiation will require restraint in designed jigs for 20-40 min to receive X-ray exposure (b)(4) animals will be anesthetized during this period (see Anesthesia above) and tails will be taped to one side with surgical tape. The jigs for use during (b)(4) irradiation were examined and approved by the AFRRI Veterinary staff. An image of the jig is now provided (see attached). Under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff, we found that in female CBA mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia (25 G needle) with no morbidity or mortality (b)(4) We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. Standard i.p. injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which all laboratory personnel have received training at AFRRI. AFFRI Veterinary staff have provided training and supervision for the placement of anesthetized animals in the approved jigs for irradiation.

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations: N/A

V.4.4.1. Injections:
Intra-peritoneal injections of pentobarbital for euthanasia 0.1-0.2 ml/mouse Fatal Plus (39-78 mg pentobarbital/mouse), will be performed with a 25 G needle. I.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine will be done using a 25 G needle. This has been extensively examined by the AFRRI Veterinary staff and shown to effectively produce non-lethal anesthesia (b)(4) PEG-400 (0.1 ml/mouse) vehicle only or (b)(4) will be
(b)(4) administered by a single (b)(4) i.p. injection [redacted] before radiation exposure also with 25 G needles.

V.4.4.1.1. Pharmaceutical Grade Drugs

(b)(4)

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

(b)(4)

Endotoxin in the drugs will be measured by the PI, or information from the company will be provided prior to administration of drugs to the animals. Concentrated PEG 400 has antibacterial activities, and solutions of PEG do not have bacterial contamination. (b)(4) will be handled under sterile conditions.

V.4.4.2. Biosamples:
All biosamples will be taken after euthanasia. (b)(4)
(b)(4) or analysis will removed *en bloc*. Intestinal tissue will be taken in smaller amounts (the first segment of the small intestine). All other tissues will be available to other researchers for tissue sharing.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification:
All cages will have cage cards identifying the treatment groups for each experiment. For experiments 2 and 3, each animal within a cage will receive ear punches for individual identification (1 = no cut; 2 = right ear upper corner; 3 = left ear upper corner; 4 = both ears).

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures:

(b)(4),(b)(6)

(b)(4) irradiation

The methodology for (b)(4) irradiation of mice including approval of holding jigs and development of anesthesia was developed in conjunction with the AFRRI Veterinary Staff. (b)(4) irradiation will be performed using the RS2000 irradiator (b)(6) Thirty minutes prior to irradiation, the RS2000 irradiator and lead shield will be decontaminated thoroughly with MB10.

Mice will be anesthetized by injection in the USUHS LAM facility. Anesthetized mice will be placed in ventilated Lucite jigs, which restrain the animals to prevent lateral or vertical movement. Tails will be taped to prevent any forward motion of animals. Mice will be irradiated at (b)(4) irradiation. The temperature in room where the mice will be irradiated will be measured, and if necessary a space heater will be used to increase the temperature. Following radiation exposure, mice will be immediately returned to the LAM and allowed to recover from anesthesia under supervision with heating pads. Immediately upon recovery, animals will be returned to standard cages that house 4 animals per cage. Lucite jigs will be sanitized after each use.

(b)(4)

(b)(4) The animals will experience no restraint, no anesthesia, no drugs, or other manipulation during this time. Following the measurement, to be performed in the room in which they are housed, the animals will be returned to their original cages. Chambers will be sanitized after each use.

Ear punch

The ear punch will be soaked in alcohol to disinfect it before use and between animals. The device will be placed on the pinna of the ear (external ear) in a location where the mouse will be marked for identification. The punch will be pressed firmly to punch a circular hole through the ear, being careful not to rip the delicate membrane of the pinna. The ear will then be gently separated from the device. The ear punch will be cleaned and disinfected in alcohol before use in another mouse.

V.4.4.8. Tissue Sharing:

For most studies (b)(4) will be obtained. All other tissues will be available for tissue sharing with other investigators. Our laboratory has made extensive use of shared tissues for training and technique development for tissue. (b)(4)

V.4.5. Study Endpoint:

(b)(4)

(b)(4) Early endpoints will be

used in the case of morbidity and morbundity. Specific health assessment criteria including rapid weight loss, ruffled fur, dried lacrimal fluid accumulated in the cul-de-sac of their eyes, are cool to the touch, decreased appetite, diarrhea, difficulty breathing or panting will used to determine early study endpoints for euthanasia. Following (b)(4) irradiation, mice may exhibit radiation injuries during the first week postirradiation. Also, mice are expected to develop (b)(4) postirradiation. During these critical time periods (b)(4) (b)(4) mice will be monitored by the PI or other members of the investigative team at least twice daily, early morning and late afternoon including weekends or holidays. Mice will be considered moribund when, in addition to showing some or all of the signs of morbidity, they exhibit labored breathing plus either an inability to remain upright or are unmotivated to move. Morbid or moribund mice will be euthanized.

V.4.6. Euthanasia:

Animals will be euthanized at the stated endpoints of the experiments by i.p. injection of ml/mouse Fatal Plus. PHS policy is intraperitoneal injection of at least 200 mg/kg sodium pentobarbital euthanasia solution; Fatal Plus: 390 mg pentobarbitol/1 ml

1 ml/390 ml x 0.02 kg/mouse x 200 mg/1 kg = 0.01-0.02

IP will be performed using a 25 G needle. (Please note that Euthasol cannot be used as additives have effects (b)(4) We do have Fatal Plus for these experiments.) Euthanasia will be confirmed by cervical dislocation. Animals which appear to be experiencing pain or distress as a result of radiation exposure or are found moribund will also be euthanized by pentobarbitol and necropsied. At the end of experiments, unused animals will be transferred to other protocols or euthanized by exposure to 100% CO_2 from a compressed gas cylinder in accordance with the current American Veterinary Medical Association (AVMA) Guidelines for Euthanasia. Exsanguination, cervical dislocation, and/or pneumothorax will be used to ensure euthanasia.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: After animals receive (b)(4) irradiation, they will remain at USUHS in a designated quarantine room, as specified by LAM veterinarians. Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. Food and acidified water and Rodent Diet will be available ad libitum. The 12:12 hr (lights off at 6 pm) room light cycle is fine for all the studies. Special caging is not needed; standard shoebox cages will be used. Acidified water to reduce infection will be provided for all cages. Autoclaved bedding and food will be used for all cages.

V.5.1.1. Study Room: will be performed in the

Radiation Building (b)(6) Room Number (b)(6)

Euthanasia Building (b)(6) Room number (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No x
Not sterilized

Fluid Restriction: NOTE: (b)(4) No x
water and (b)(4)
(b)(4) treated
water will be
provided.

V.5.1.3. **Exceptions**: None

V.5.2. **Veterinary Medical Care**:

V.5.2.1. **Routine Veterinary Medical Care**:
Experimental animals will be observed at least twice a day by one of the study investigators or technicians. VSD personnel will also monitor the animals twice a day during their rounds. As described in another section (study endpoint) morbid animals will be monitored at least twice daily, morning and afternoon. The experimental design does not contemplate supportive therapy. The justification for not providing supportive care has been described in the study end point section as well as in the pain category section. Criteria to be used for health evaluation while the animals are on study include: 20% weight loss (mice will be weighed every other day—based on our experience with (b)(4) irradiation in which animals can recover from ~25% weight loss (b)(4) (b)(4) ruffled fur, dehydration, decreased activity, and hunched body position, dyspnea, or panting. Animals exhibiting these signs may be euthanized, and considered to be at the study endpoint. Increased monitoring of animals will begin on (b)(4) through the end of the experiment, based on our previous data that indicated that mortality from (b)(4) injury occurs mostly between days (b)(4) postirradiation and from (b)(4) irradiation occurs between days (b)(4) postirradiation.

V.5.2.2. **Emergency Veterinary Medical Care**:
All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. **Environmental Enrichment**:

V.5.3.1. **Enrichment Strategy**: All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.
V.5.3.2. **Enrichment Restrictions**: none

VI. **STUDY PERSONNEL QUALIFICATIONS AND TRAINING**:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or	Name of person	Qualifications of person	Specific training in this

procedure (e.g., tail vein injections, euthanasia)	performing activity	performing activity (e.g., research technician, 2 yrs experience)	activity or procedure (e.g., rodent handling class, 1999)
Ear punch, irradiation, anesthesia, euthanasia	(b)(6)	8+ years	(b)(6)
Ear punch, irradiation, anesthesia, euthanasia		6 years	

VII. BIOHAZARDS/SAFETY: None

VIII. ENCLOSURES: Images of jigs, (b)(4) MSDS

References:

(b)(4)

(b)(4)

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User

(b)(6) _____ Feb 5 2013
Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____
_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ Feb 5 2013
Prin Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature

Date

X. PROTOCOL ABSTRACT:

The terrorist attack of September 11, 2001 against the United States has increased the concern that terrorists may acquire and use radioactive sources (e.g. dirty radioactive bombs) to expose military and civilian populations to ionizing radiation. However, despite this concern, there are currently no FDA approved drugs that can be used for the general population to prevent death that is likely to result from exposure to ionizing radiation. (b)(4) irradiation in humans can result in (b)(4) injuries. However, because rodents are more resistant to (b)(4) injuries than humans, there is currently no murine model that has been developed to study both (b)(4) radiation injuries in the same animal.

(b)(4)

A. **Animal Protocol Number:**

B. **Animal Protocol Title:** (b)(4)

(b)(4)

C. **Principal Investigator:** (b)(6)

D. **Performing Organization:** Uniformed Services University of the Health Sciences

E. **Funding:** DTRA

F. **Objective and Approach:**

(b)(4)

G. **Indexing Terms (Descriptors):** (b)(4)

(b)(4) animals, mice

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

February 21, 2014

MEMORANDUM FOR DR. (b)(6)

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on February 21, 2014:

Animal Protocol Title: (b)(4) Protection from Radiation-Induced (b)(4) (b)(4) Injuries in a Murine Model (Mus Musculus)"

USUHS Protocol Number: (b)(6)

Expiration Date: February 20, 2017

Supporting Grant(s) Number: TBD

Name of Principal Investigator: Dr (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

Revised

IACUC Date Stamp

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: (b)(4) Protection from Radiation-Induced (b)(4) Injuries in a Murine Model (*Mus musculus*)

GRANT TITLE (if different from above): Enhancing cancer treatment by normal tissue protection

USUHS PROJECT NUMBER: To be assigned

FUNDING AGENCY: NIH NIAID

EARLIEST ANTICIPATED FUNDING START DATE: April 2014

(b)(6) **ATOR:**

Date

Associate Professor (b)(6)
301-295-3236 office; 301-295-3220 fax; regina.day@usuhs.edu

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)

Dept. Head Signature
Typed Name: (b)(6)

Chair — Title
(b)(6) — Telephone
2/3/14 — Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)

(b)(6) — Department Telephone

Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)

Attending/Consulting Veterinarian Signature
Typed Name: ~~LTC~~ MAJ (b)(6)

LAM — Department
(b)(6) — Telephone
2/10/2014 — Date

USUHS Form 3206- Animal Study Proposal Form Instructions

USUHS / DOD – SPONSORED ANIMAL RESEARCH PROPOSALS MUST USE THIS STANDARDIZED FORMAT

Reference DOD Directive 3216.1 & USUHS Instruction 3203

****

Specific information requested in the following animal-use protocol template is a result of requirements of the Animal Welfare Act regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD directives.

****

This document is intended to be an aid in the preparation of a USUHS DOD – sponsored animal use proposal. The instructions and written explanations provided for individual paragraphs (ref. animal-use protocol template in AR 40-33 / USUHSINST 3203, Appendix C) are coded as hidden text. To see the instructions and examples for each section, select the "**Show/Hide ¶**" button on your tool bar. To print the hidden text, select "Print" on the drop down file menu. Under the "Options" button, select "Hidden text" under the "Include with document" section. Use of a word processor makes completion of this template a "fill-in-the-blanks" exercise. Please provide all response entries in the following font: Arial, Regular, 12, Black. Please do NOT submit this page of instructions with your animal protocol submission.

With the exception of title headings, each paragraph and subparagraph in the following template must have a response. Portions of the template that are not applicable to your particular protocol, (i.e. no surgery or no prolonged restraint) should be marked "**N/A**". There are no space limitations for the responses.

Pertinent standing operating procedures or similar documents that are readily available to your IACUC may be referenced to assist in the description of specific procedures. It is critical that only animal studies or procedures documented in an IACUC – approved protocol be performed at your facility. Additionally, Principal Investigators, or other delegated research personnel, should keep accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates to their approved protocol.

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D. (b)(6)

ANIMAL PROTOCOL TITLE: (b)(4) Protection from Radiation-Induced (b)(4) Injuries in a Murine Model (*Mus musculus*)

GRANT TITLE (if different from above): Enhancing cancer treatment by normal tissue protection

USUHS PROJECT NUMBER: To be determined

CO-INVESTIGATOR(S): Dr. (b)(6)

POSTDOCS, STUDENTS, TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

Despite advances in precision of targeting tumors with radiation therapy (RT) (eg., intensity-modulated RT and stereotactic radiosurgery), loco-regional recurrence is still a major problem due to the inability to deliver a sufficiently high dose to achieve tumor control without unacceptable late normal tissue and organ toxicity. Tumors in which loco-regional control is problematic include locally advanced breast, lung, head and neck, brain, cervical cancer, and others. Two other situations where radiation protection may be useful are: 1) in children, where RT can impair normal growth and development; and 2) in patients receiving combined RT and chemotherapy, where the acute effects of treatment may be severe, particularly when RT and chemotherapy are given concurrently. One method to improve the therapeutic index is by use of agents that differentially protect normal tissues relative to tumors.

The only FDA-approved radioprotector is amifostine, a pro-drug that is converted to an active thiol-type free-radical scavenger. Amifostine is approved to reduce xerostomia in patients receiving head and neck irradiation, but its use is limited because it must be given intravenously shortly before RT and it has systemic toxicity (b)(4)

(b)(4)

(b)(4) Our aim is to develop an effective, non-toxic radioprotectant/ radiotherapeutic treatment strategy that can be used for protection against radiation injury to normal tissues during clinical radiation for cancer treatment.

II. BACKGROUND:

II.1. Background:

The acute toxic effects of exposure to high doses of ionizing radiation in humans have been shown to occur due to two sequential effects: first the ablation of immune cells, resulting in loss of immune function and increasing likelihood of pathogenic infection (b)(4) ; and subsequently the loss of epithelial progenitor cells of the gastrointestinal (GI) crypt (b)(4)

(b)(4) Both of these events have been shown to be due to the loss of

fast-growing pluripotent and/or progenitor cell types. The time course of the acute injuries correlates with the life span of the mature cell types of the tissue, which are not replaced because of radiation damage to the adult pluripotent cells and adult stem cells. There is currently no FDA approved prophylactic or treatment for prevention of acute radiation injuries following high-dose (b)(4) radiation exposure.

(b)(4)

In humans, severe radiation (b)(4) can result in death (b)(4) (b)(4) Cumulative evidence from a number of laboratories, including our own, shows that there is a threshold dose of radiation of (b)(4) to induce sufficient (b)(4) for the (b)(4) phases to occur in mice; below these doses, no permanent injury manifests, although some short-term effects are observed (b)(4) However, in humans, there is currently no accepted protocol for preventative treatment of radiation-induced (b)(4) and morbiditiy as high as 50% is associated with each phase (b)(4) (b)(4) (b)(4) injuries from radiation is considered to be a serious problem in the field for the development of agents to prevent these radiation injuries.

(b)(4) as a radioprotectant:

Preliminary Studies:

(b)(4),(b)(6)

(b)(4)

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:

Biomedical Research Database (BRD),
NIH RePORTER (replacement database for CRISP) http://projectreporter.nih.gov/reporter.cfm,
and PubMed

PubMed

II.2.2. Date of Search:

Data base searches conducted on 1/30/14.

II.2.3. Period of Search:

Searches conducted for the entire time periods available in each data base. For BRD, 1998-2014; for RePorter, 1972-2012. For PubMed, 1970-2014.

II.2.4. Key Words and Search Strategy:

Searches were performed using the following key word combinations: (b)(4)

(b)(4)

II.2.5. Results of Search:

1) BRD: Search resulted in 0 projects with these key words

2) A RePORTER search: Search resulted in 0 projects with these key words

3) PUBMED Search *8/3/09*

(b)(4)

(b)(4)

Summary of other publications:

(b)(4)

III. OBJECTIVE\HYPOTHESIS:

(b)(4)

IV. MILITARY RELEVANCE:

Military personnel and their families receive medical treatment for cancer, including clinical radiation. The development of agents for the protection of normal tissues during cancer radiotherapy would allow the use of higher doses of radiation which has been shown to improve the level of tumor control and cancer irradiation. The goals of this research are consistent with improved cancer treatment in military

personnel and their families.

Additionally, the Department of Defense is prepared to execute military missions within radiation environments, to manage radiation crises associated with terrorist activities, and to manage consequences in the event of nuclear weapons detonation. Recent studies indicate that there is an increasing risk of exposure to ionizing radiation (IR) to military personnel due to increasing terrorist activities, including potential use of stolen nuclear weapons or dispersal of radioactive material in a "dirty bomb" (b)(4) Because of potential exposure of military personnel to radiation, the DoD is committed to protect members of the Armed Services from the adverse health effects of ionizing radiation. High dose IR causes short term, acute injuries including hematopoetic and GI syndromes. However, both high and low dose IR (as may result from a radiation dispersal device) can cause (b)(4) as demonstrated by Japanese atomic bomb survivor data as well as accidental exposures to Russian populations (b)(4) (b)(4) While early effects of radiation injury now have treatment/ prophylactic strategies, the delayed effects currently have no treatment. Lack of treatment for late effects of radiation, including (b)(4) (b)(4) will likely lead to delayed morbidity and mortality for personnel exposed to IR.

The Department of Defense (DoD) Chemical and Biological Defense Program 2007 Annual Report to Congress, Chapter 2.7.6 Medical Radiological Defense, addresses the requirements for the development of radioprotectants. This priority is outlined for the development of protectants for both acute and delayed radiation injuries, with an emphasis on (b)(4) According to this report, the mitigation of (b)(4) is a major research thrust area. This project is also designed to investigate mechanism(s) of protection to aid in the development of such agents.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

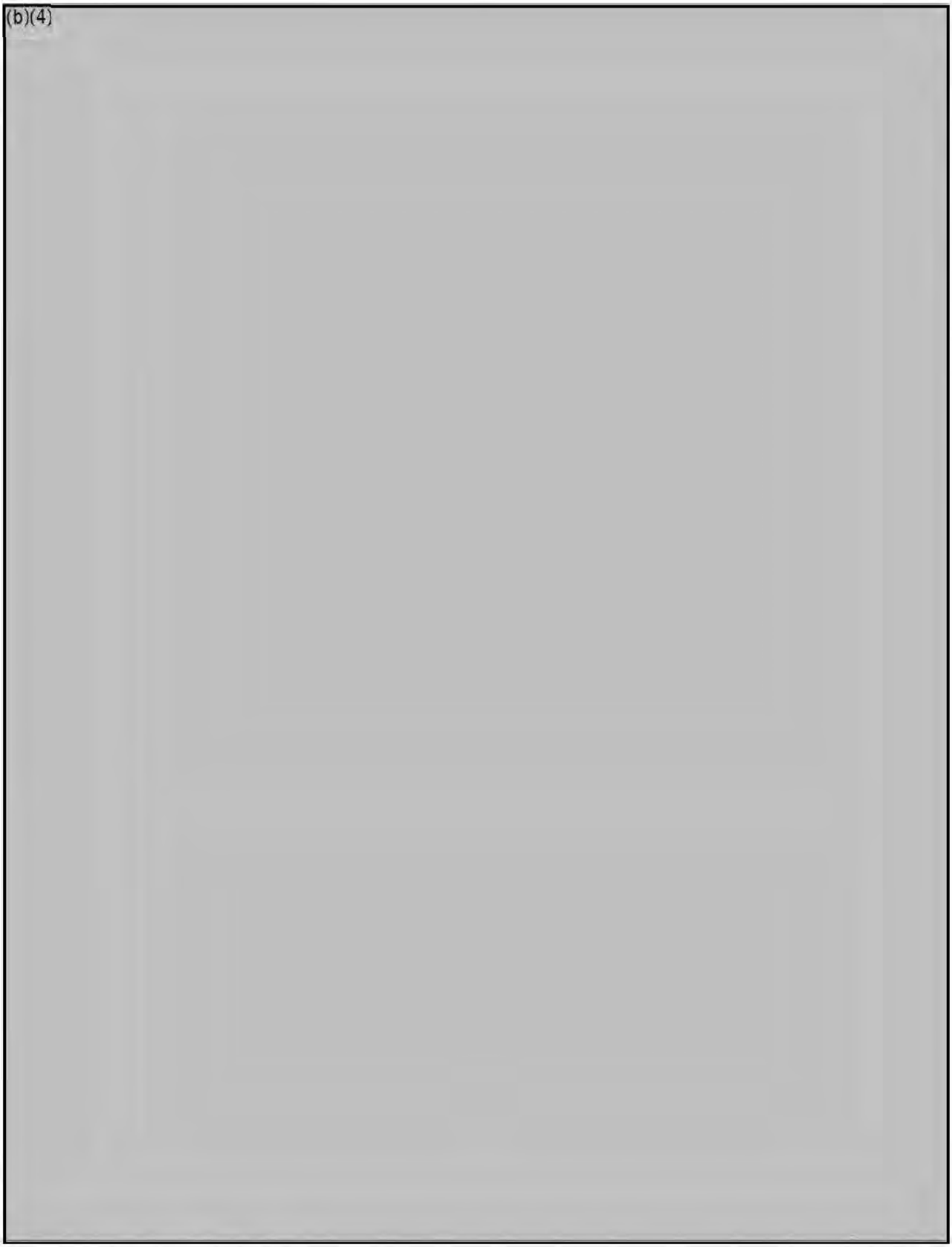
The goal of this project is to investigate the protective effects and mechanisms of (b)(4) for radiation-induced (b)(4) injuries.

(b)(4) Rationale for Doses and Times of Administration:

(b)(4)

Radiation Rationale for Doses and Times of Administration

(b)(4)


(b)(4)

(b)(4)

Experiments 1-5: Effect of (b) on radiation-induced (b)(4) in C57L mice.

V.1.1. Pilot study for (b)(4) protection (b)(4)
This pilot experiment will be performed to survival from (b)(4)
Two groups will be studied: (b)(4)
(b)(4)

(b)(4) mice per group) x (2 treatment groups) x (1 time point) = mice (b)(4)

Endpoint: The final endpoint for the experiment is days. Mice displaying obvious dyspnea, (b)(4)
lethargy, or other markers of morbundity and which appear to be in distress will be euthanized.
(b)(4) The scale for pain or
lethargy is attached. Mice scoring a total of 12 under any measurement will be euthanized.

Note: If the Pilot Study for survival does not support the hypothesis (b)(4)
(b)(4) Experiments 2-4 will not be conducted. We will consult with the USU IACUC for
the possibility of conducting Experiment 5 to determine the mechanism for the lack of protection of the
(b)(4)

V.1.2. Experiment 2: Dose-modifying effect of (b)(4)
(b)(4)
Once we have established the efficacy of (b)(for protecting mice from (b)(4) irradiation at the
(b)(4) we will determine the dose modifying effect using three doses of (b)(4) irradiation (b)(4)
(b)(4)
(b)(4) Two groups
of mice will be studied: 1) IR +vehicle (control) and 2) IR + (experimental). For these studies, (b)(4)
(b)(4) we will require mice per group in the radiation groups. We will include controls with animals (b)(4)
per group: 1) Sham IR + vehicle and 2) Sham IR.

Radiation groups
(b)(4) mice per group) x (2 treatment groups) x (1 time point) x (3 (b)(4) radiation levels) = (b)(4)
(b)(4) (b)(4) mice/experiment) x (2 experiments) = mice

Control groups
(b)(4) mice per group) x (2 treatment groups) x (1 time point) = mice (b)(4)

Endpoint: The final endpoint for the experiment is days. Mice displaying obvious dyspnea, (b)(4)
lethargy, or other markers of morbundity and which appear to be in distress will be euthanized. (b)(4)
(b)(4) The scale for pain or lethargy is
attached. Mice scoring a total of 12 under any measurement will be euthanized.

V.1.3. Experiment 3 Survival from fractionated-dose irradiation (b)(4)
Humans receive radiation for cancer treatment in fractionated doses. Our preliminary studies will
utilize single doses of radiation for the induction of (b)(4) injuries. Once we have established
the efficacy of (b)(4 for the protection of (b)(4 tissue against radiation injury from single high doses of

radiation, we will investigate the use of (b)(4) for protection against fractionated radiation. A schedule
(b)(4) of fractionated radiation, using (b)(4) fractions of (b)(4) given equally spaced (b)(4) over (b)(4) days, has been demonstrated in a murine model to be effective for the induction of radiation (b)(4) injuries (b)(4) We will utilize two treatment groups: 1) IR + vehicle; 2) IR + (b)(4)

(b)(4) (b)(4) mice per group) x (2 treatment groups) x (1 time point) = (b)(4)
(b)(4) (b)(4) mice/experiment) x (2 experiments) = (b)(4) mice

Endpoint: The final endpoint for the experiment is (b)(4) days. Mice displaying obvious dyspnea, (b)(4) lethargy, or other markers of morbundity and which appear to be in distress will be euthanized. (b)(4) (b)(4) The scale for pain or lethargy is attached. Mice scoring a total of 12 under any measurement will be euthanized.

V.1.4. Experiment 4 (b)(4) protection against (b)(4)
(b)(4)
We will investigate the degree of (b)(4) protection against radiation-induced (b)(4) We will determine levels of (b)(4) For
(b)(4) this experiment we will require only (b)(4) mice/group, and we will examine 4 groups: 1) sham + vehicle; 2)
(b)(4) sham + (b)(4) 3) IR + vehicle; 4) IR + (b)(4)

(b)(4) (b)(4) mice per group) x (4 treatment groups) x (1 time point) = (b)(4)
(b)(4) (b)(4) mice/experiment) x (2 experiments) = (b)(4) mice

Endpoint: The final endpoint for the experiment is (b)(4) days. Mice displaying obvious dyspnea, (b)(4) lethargy, or other markers of morbundity and which appear to be in distress will be euthanized. (b)(4) The scale for pain or lethargy is attached. Mice scoring a total of 12 under any measurement will be euthanized.

V.1.5. Experiment 5 (b)(4) protection against (b)(4)
(b)(4)
(b)(4) For this experiment we will require only
(b)(4) (b)(4) mice/group, and we will examine 4 groups: 1) sham + vehicle; 2) sham + (b)(4) 3) IR + vehicle; 4) IR + (b)(4) The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) (b)(4) mice per group) x (4 treatment groups) x (4 time points) = (b)(4)
(b)(4) (b)(4) mice/experiment) x (2 experiments) = (b)(4) mice

Endpoints: Endpoints for the experiments will be (b)(4) days. Early time points will be taken if any animal exhibits signs or symptoms of morbidity or moribundity. Such animals will be euthanized at the time of observation.

Experiments 6-7: Effect of (b)(4) on radiation-induced (b)(4)

V.1.6. Experiment 6 (b)(4) protection against (b)(4)
(b)(4)

irradiation. We will use two groups: 1) IR + vehicle; 2) IR + (b)(4) For this experiment we will use (b)(4) animals per group.

(b)(4) mice per group) x (2 treatment groups) x (2 time points) = (b)(4)
(b)(4) mice/experiment) x (2 experiments) = mice

Endpoints: Endpoints for the experiments will be (b)(4) days. Early time points will be taken if any animal exhibits signs or symptoms of morbidity or moribundity. Such animals will be euthanized at the time of observation.

V.1.7. Experiment 7 (b)(4) protection against (b)(4)

(b)(4)

(b)(4) For this experiment we will require only mice/ (b)(4) group, and we will examine 4 groups: 1) sham + vehicle; 2) sham + (b)(4) 3) IR + vehicle; 4) IR + (b)(4) The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) mice per group) x (4 treatment groups) x (4 time points) = (b)(4)
(b)(4) mice/experiment) x (2 experiments) = mice

Endpoints: Endpoints for the experiments will be (b)(4) days. Early time points will be taken if any animal exhibits signs or symptoms of morbidity or moribundity. Such animals will be euthanized at the time of observation.

V.2. Data Analysis:

Mean with standard error, or percentage will be reported if applicable. A significance level is set at 5% for each test. All statistical tests are two-sided. A statistical software, SAS will be used for statistical analyses.

Experiments 1-3: Analysis of variance (ANOVA) will be used to detect significant difference among the groups. If significant, then the pair-wise comparison by Tukey-Kramer will be used to identify which group is different from the other. For experiment 2, N = mice per group could (b)(4) have 80% power to detect any significant difference between groups if any, given type I error of 5% and delta/sigma has at least 1.4, where the delta is the estimated mean differences between the two groups and sigma is the pooled standard deviation of the two groups (b)(4)

Experiments 4,5,7: Previous data from this laboratory indicate that the coefficient of variation (CV) of the data is about 0.5, and that differences of between 2- and 30- fold are likely to be observed.
(b)(4) A sample size of per group will have 80% power to detect 3-fold increases in biomarkers based on a lognormal t test with a 5% two-sided significance level and a CV of 0.5.

Experiment 6: Previous data from this laboratory indicate that the coefficient of variation (CV) of the data is about 0.5, and that differences of between 2- and 30- fold are likely to be observed. A
(b)(4) sample size of per group will have 80% power to detect 3-fold increases in biomarkers based on a lognormal t test with a 5% two-sided significance level and a CV of 0.5.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

For the last 9 years, we have utilized primary cell culture systems to examine anti-apoptotic mechanisms; however, we must determine whether our culture findings are relevant in whole animals. We will continue to use primary cell culture whenever possible to avoid unnecessary use of animals. We have also considered computer models as alternatives to animals. However, this alternative also lacks the knowledge base and complexity needed to evaluate radioprotective and radiotherapeutic agents.

V.3.2. Animal Model and Species Justification:

Tissue culture conditions cannot fully substitute for the complexities of whole organisms in response to radiation injury. Such injury involves the interactions between the hematopoietic system and other organ systems. In order to develop viable treatments for humans, we find that our research must be conducted in whole animals.

Work by (b)(6) also advocates the mouse as the best species to use for radiation protection and radiotherapy protocols: 1) mice provide the least sentient species available whose response to radiation has been clearly identified, with physiological responses to radiation and radioprotectants that are similar to human physiology, 2) extensive literature is available on responses of mice to radiation and countermeasures, 3) more reagents are available to study the relevant cell types and molecules in mice than in any other species except humans, and the availability of transgenic and knockout mice make this model extremely attractive for further research into molecular mechanisms, and 4) (b)(6) have extensive experience with this model in radiation injury and radioprotection research (b)(6) (b)(6)

The strains of mice to be utilized for each portion of the study are based on the literature and on findings from our own laboratory. Strain C57L mice have been demonstrated to have patterns of radiation-induced (b)(4) injury similar to that observed in humans (b)(4) Our laboratory has found that C57BL/6 mice have radiation-induced (b)(4) damage similar to that reported in the literature for humans.

V.3.3. Laboratory Animals

V.3.3.1.	**Genus & Species:**	*Mus musculus*	*Mus musculus*
V.3.3.2.	**Strain/Stock:**	C57L	C57BL/6
V.3.3.3.	**Source/Vendor:**	(b)(4)	
V.3.3.4.	**Age:**	10-14 weeks	10-14 weeks
V.3.3.5.	**Weight:**	Normal adult weight range for this strain: 17-21 g	Normal adult weight range for this strain: 17-21 g

V.3.3.6. **Sex**:	Female. Only females will be used in this study for the following reasons: 1) we have so far utilized female mice to lay the foundation of our studies for radiation-induced (b)(4) (b)(4) injuries; 2) because of long housing periods, it is advantageous to use female mice that are less aggressive toward each other.	Female. Only females will be used in this study for the following reasons: 1) we have so far utilized female mice to lay the foundation of our studies for radiation-induced (b)(4) (b)(4) injuries; 2) because of long housing periods, it is advantageous to use female mice that are less aggressive toward each other.
V.3.3.7. **Special Considerations**:	Commercially procured animals must be adventitious disease free. Mice- Pathogen-free, including *Pseudomonas aeruginosa*, and Pasteurella: All mice need to be free of the following agents: Sendai Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus; *Helicobacter* spp. *Mycoplasma pulmonis* and *Clostridium piliforme*. Endoparasite- and ectoparasite-free.	

V.3.4. **Number of Animals Required (by Species)**: C57L C57BL/6 (b)(4)

V.3.5. **Refinement, Reduction, Replacement (3 Rs)**:

V.3.5.1. **Refinement**: Animals demonstrating moribundity due to radiation exposure (b)(4) (b)(4) will be considered to have arrived at the study endpoint and will be immediately euthanized (See sections V.4.5 Study Endpoint and V.4.6 Euthanasia).

Ketamine/xylazine anesthesia will be used during X-ray irradiation to prevent stress from the time the mice are placed in restraint. Under the training protocol at AFRRI inconjunction with the AFFRI veterinary staff, we found that in female CBA or C57BL/6J mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in a minimum of 40 min anesthesia with no morbidity or mortality (b)(4) We will use a heating pad to keep mice warm during the period of recovery from anesthesia. The temperature in room where the mice will be irradiated will be measured, and if necessary a space heater will be used to increase the temperature. Standard intraperitoneal injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which all laboratory personnel have received training at AFRRI.

V.3.5.2. **Reduction**:

Whenever possible, control groups will be shared between experimental groups for our experiments. Tissues will be collected for multiple assays from the same animals further reducing the total number of animals needed. At every stage of the research, planned experiments will be reevaluated, in an attempt to reduce the numbers of experiments and eliminate repeats of experiments where statistical significance has already been obtained. For (b)(4) irradiation studies, each animal will be able to provide its own control tissue from the non-irradiated area. Preliminary results from our laboratory demonstrate that tissue from the abdominal region is normal under our experimental conditions.

V.3.5.3. Replacement: At this time, it is not feasible to use non-animal systems in place of animal models to address the research questions in this protocol.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals: (b)(4)

V.4.1.1.1.1. Column C: (b)(4)

V.4.1.1.1.2. Column D:

V.4.1.1.1.3. Column E:

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:
We will be unable to use general analgesics since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4) (b)(4) Some sedatives have been reported to contribute to postoperative (b)(4) which likely reduce some functions of radiation. For C57BL/6 mice exposed to (b)(4) irradiation to induce radiation (b)(4) daily on animals displaying radiation injury to alleviate discomfort and to prevent infection. This agent is also used for humans.

An anesthesia protocol has been developed for use during the X-ray irradiation. Ketamine will be used with xylazine for longer anesthesia in mice to be place in restraints for (b)(4) irradiation. Under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff, we found that in female C57BL/6 and CBA mice, ~12 weeks old,i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in in a minimum of 40 min anesthesia (25 G needle) with no morbidity or mortality (b)(4) We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. Standard i.p. injection methods are used for injection of the anesthesia, as recommended and approved by the AFRRI veterinary staff and for which all laboratory personnel have received training at AFRRI.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:
Animals will be housed and cared for as described in Section V.5. Veterinary Care both before and after dose administrations and irradiation procedures. The PI and research staff will monitor animals after radiation or sham irradiation during anesthesia recovery. The PI and research staff will also monitor animals daily following radiation exposure, and twice daily during critical periods. Otherwise, the animals will receive standard veterinary care in the USU vivarium.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: DoD Bioedical Research Data Base (BRD), NIH Reporter, PubMED

V.4.1.3.2. Date of Search: 1/30/14

V.4.1.3.3. Period of Search: Search covered published manuscripts from ~1965-present. (BRD search included all available years 1998 -2014).

V.4.1.3.4. Key Words of Search: Pain, analgesia, anesthesia, mice, radiation, ionizing radiation, humane, (b)(4)

V.4.1.3.5. Results of Search:
The literature search revealed that, at present, there are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. In some cases radiation has been demonstrated to reduce pain in laboratory mice (b)(4) and has been in humans reported to alleviate cancer related bone pain (b)(4) (b)(4) A recent report showed that administration of botulinum toxin A was effective for reduction of pain and muscle spasms after clinical radiation for treatment of tumors (b)(4) (b)(4) Application of natural honey 20 min prior to radiation exposure was also shown to reduce mucositis with associated pain in humans following head/neck radiotherapy (b)(4) it has not been determined whether honey acts as a local radioprotectant. Spinal cord stimulation has also been reported in humans to alleviate pain resulting from radiotherapy (b)(4) however, this method would not be practical for use in mice because of the extensive spinal cord manipulation required. Human administration of morphine also relieves pain (b)(4) but again this would not be practical for mice in our experiments because of side effects of the drug. In humans, exposure to (b)(4) irradiation may induce vomiting and diarrhea, causing pain and distress. However, mice are not susceptible to vomiting. There are no reports regarding the effects of on pain in general or (b)(4) radiation-induced pain.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
Anesthesia will be used before (b)(4) irradiation to avoid stress from restraint. (b)(4) (b)(4) (b)(4) we will be unable to use analgesics after irradiation since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4) (b)(4) (b)(4) Pain will be evaluated using several standard indications including: grimacing, abnormal breathing, hunched or fluffed fur and inactivity, or ataxia. In experiments where tissue collection is needed, animals will be euthanized with pentobarbitol prior to sample collection as described in Section V.4.6. We will utilize the attached score sheets to provide a scale for measurement of criteria for euthanasia. Score sheet will be used daily after irradiation. During critical periods, animals will be monitored twice daily, and during the second monitoring score sheets will be updated if necessary for that day. Any animal scoring 12 or more total on the score sheet will be euthanized. In experiments where tissue collection is needed, animals will be euthanized with pentobarbitol prior to sample collection as described elsewhere.

V.4.2. **Prolonged Restraint**:
(b)(4) irradiation will require restraint in designed jigs for ~20 min to receive X-ray exposure (b)(4) animals will be anesthetized during this period (see Anesthesia above) and tails will be taped to one side with surgical tape. The jigs for use during (b)(4) irradiation were examined and approved by the AFRRI Veterinary staff. An image of the jigs is attached. Under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff, we found that in female CBA and C57BL/6 mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in a minimum of 40 min anesthesia (25 G needle) with no morbidity or mortality (b)(4) The length of time for anesthesia is needed to ensure that animals do not regain consciousness while being positioned in the lead shield for irradiation. Positioning of the mice must be done carefully to ensure that all animals receive the same exposures. We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. Standard i.p. injection methods are used for injection of the anesthesia. We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. AFFRI Veterinary staff provided training and supervision for the placement of anesthetized animals in the approved jigs for irradiation.

V.4.3. **Surgery**:
V.4.3.1. **Pre-surgical Provisions**: N/A

V.4.3.2. **Procedure**: N/A

V.4.3.3. **Post-surgical Provisions**: N/A

V.4.3.4. **Location**: N/A

V.4.3.5. **Surgeon**: N/A

V.4.3.6. **Multiple Major Survival Operative Procedures**:
V.4.3.6.1. **Procedures**: N/A

V.4.3.6.2. **Scientific Justification**: N/A

V.4.4. **Animal Manipulations**: N/A

V.4.4.1. **Injections**:
Intra-peritoneal injections of pentobarbital for euthanasia 0.01-0.02 ml/mouse Fatal Plus (39-78 mg pentobarbital/mouse), will be performed with a 25 G needle. I.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine will be done using a 25 G needle. This level of anesthesia was first developed for our use in collaboration with the AFRRI Veterinary staff and shown to effectively produce non-lethal anesthesia for in a minimum of 40 min (b)(4)

(b)(4)

V.4.4.1.1. Pharmaceutical Grade Drugs

(b)(4)

V.4.4.1.2. Non-Pharmaceutical Grade Drugs
None.

V.4.4.2. Biosamples:
All biosamples will be taken after euthanasia. (b)(4) will removed *en bloc*. (b)(4) will be removed from the (b)(4) as control tissue. All other tissues will be available to other researchers for tissue sharing.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification:
All cages will have cage cards identifying the treatment groups for each experiment. For experiments 2 and 3, each animal within a cage will receive ear punches for individual identification (1 = no cut; 2 = right ear upper corner; 3 = left ear upper corner; 4 = both ears).

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures:

(b)(4) irradiation
The methodology for (b)(4) irradiation of mice including approval of holding jigs and development of anesthesia was developed in conjunction with the AFRRI Veterinary Staff. (b)(4) irradiation will be performed using the RS2000 irradiator in (b)(6)
Thirty minutes prior to irradiation, the RS2000 irradiator and lead shield will be decontaminated thoroughly with MB10.

Mice will be anesthetized by injection in the USUHS LAM facility. Anesthetized mice will be placed in ventilated Lucite jigs, which restrain the animals to prevent lateral or vertical movement. Tails will be taped to prevent any forward motion of animals. Mice will be irradiated at (b)(4) to deliver (b)(4) irradiation. The temperature in room where the mice will be irradiated will be measured, and if necessary a space heater will be used to increase the temperature. Following radiation exposure, mice will be immediately returned to the LAM and allowed to recover from anesthesia under supervision with heating pads. Immediately upon recovery, animals will be returned to standard cages that house 4 animals per cage. Lucite jigs will be sanitized after each use.

Ear punch
The ear punch will be soaked in alcohol to disinfect it before use and between animals. The device will be placed on the pinna of the ear (external ear) in a location where the mouse will be marked for identification. The punch will be pressed firmly to punch a circular hole through the ear, being careful not to rip the delicate membrane of the pinna. The ear will then be gently separated from the device. The ear punch will be cleaned and disinfected in alcohol before use in another mouse.

V.4.4.8. Tissue Sharing:

For most studies, (b)(4) tissue will be obtained. All other tissues will be available for tissue sharing with other investigators. Our laboratory has made extensive use of shared tissues for training and technique development for (b)(4) tissue.

V.4.5. Study Endpoint:

(b)(4)

(b)(4) Early endpoints will be used in the case of morbidity and morbundity. Specific health assessment criteria including rapid weight loss, ruffled fur, dried lacrimal fluid accumulated in the cul-de-sac of their eyes, are cool to the touch, decreased appetite, dehydration, diarrhea, difficulty breathing or panting (b)(4) (b)(4) or hunched body position will used to determine early study endpoints for euthanasia. The experimental design does not contemplate supportive therapy, and animals exhibiting these signs may be euthanized, and considered to be at the study endpoint.

Following (b)(4) irradiation, mice may exhibit injuries from the (b)(4) irradiation *during* the first week postirradiation. Also, mice are expected to develop (b)(4) injury on days (b)(4) postirradiation. During the critical time periods (b)(4) days postirradiation), mice will be monitored by the PI or other members of the investigative team at least twice daily, early morning and late afternoon including weekends or holidays.

V.4.6. Euthanasia:

Animals will be euthanized at the stated endpoints of the experiments by i.p. injection of ml/mouse Fatal Plus. PHS policy is intraperitoneal injection of at least 200 mg/kg sodium pentobarbital euthanasia solution; Fatal Plus: 390 mg pentobarbitol/1 ml.

1 ml/390 ml x 0.02 kg/mouse x 200 mg/1 kg = 0.01-0.02

IP will be performed using a 25 G needle. (Please note that Euthasol cannot be used as additives have effects on (b)(4) We do have Fatal Plus for these experiments.) Euthanasia will be confirmed by cervical dislocation. Animals which appear to be experiencing pain or distress as a result of radiation exposure or are found moribund will also be euthanized by pentobarbitol and necropsied. At the end of experiments, unused animals will be transferred to other protocols or euthanized by exposure to 100% CO_2 from a compressed gas cylinder in accordance with the current American Veterinary Medical Association (AVMA) Guidelines for Euthanasia. Exsanguination, cervical dislocation, and/or pneumothorax will be used to ensure euthanasia.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. Mice will be housed 4 per cage. Food and water and Rodent Diet will be available ad libitum. The 12:12 hr (lights off at 6 pm) room light cycle is fine for all the studies.

V.5.1.1. Study Room:

Radiation Building (b)(6) Room Number (b)(6)

Euthanasia Building (b)(6) Room number (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes _____	No	x
Fluid Restriction:	**NOTE: Acidified water will be provided to reduce infections after radiation exposure.**	No	x

Acidified water (pH 2.5 – 3.0) is produced at AFRRI for their vivarium using 2.0 N HCl.

V.5.1.3. Exceptions: None

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
Experimental animals will be observed at least twice a day by one of the study investigators or technicians. LAM personnel will also monitor the animals twice a day during their rounds. As described in another section (study endpoint) morbid animals will be monitored at least twice daily, morning and afternoon. The experimental design does not contemplate supportive therapy. The justification for not providing supportive care has been described in the study end point section as well as in the pain category section. Criteria to be used for health evaluation while the animals are on study include: 20% weight loss compared with average weights of sham irradiated animals (mice will be weighed every other day between days (b)(4) —based on our experience with (b)(4) irradiation in which animals can recover from ~25% weight loss (b)(4) ruffled fur, dehydration, decreased activity, and hunched body position, dyspnea, or panting. Animals exhibiting these signs may be euthanized, and considered to be at the study endpoint. Increased monitoring of C57L animals will begin on day (b)(through the end of the experiment, based on our previous data that indicated that mortality from (b)(4) irradiation occurs between days (b)(4) postirradiation. Increased monitoring of C57BL/6 animals exposed to (b)(4) irradiation for (b)(4) will be monitored between days (b)(4) when the most prominent (b)(4) (b)(4) is expected. In the event of debilitating illness the decision to euthanize will be made by the veterinarian and/or PI.

V.5.2.2. Emergency Veterinary Medical Care:
All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays. In the case of an emergency health problem, if the responsible person (e.g. investigator) is not available or if the investigator and veterinary staff cannot reach consensus on treatment, the veterinarian has the authority to treat the animal, remove it from the experiment, institute appropriate measures to relieve severe pain or distress, or perform euthanasia if necessary.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions: none

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

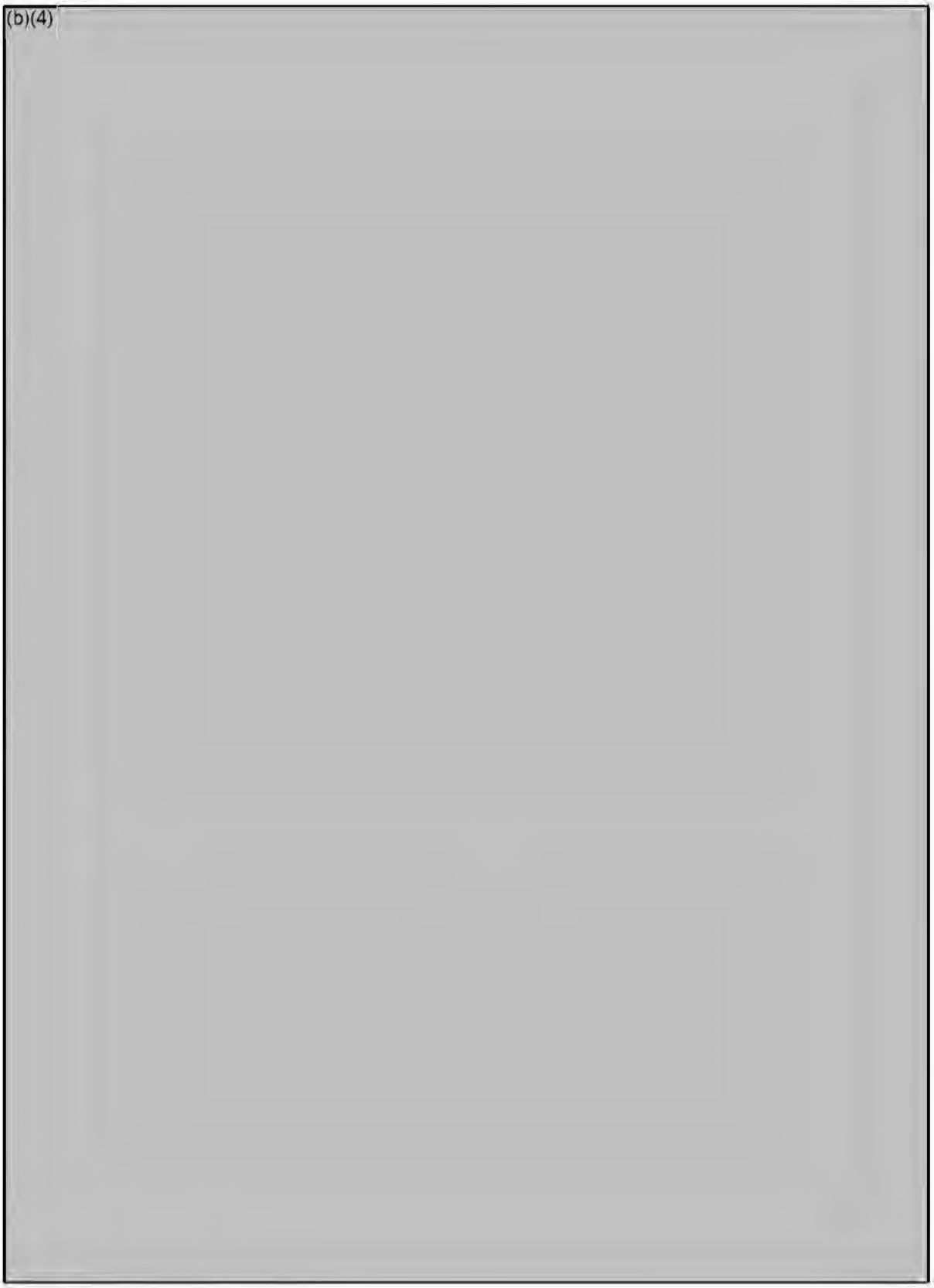
Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Ear punch, irradiation, anesthesia, euthanasia	(b)(6)	8+ years	(b)(6)
Ear punch, irradiation, anesthesia, euthanasia		6 years	
Rodent handling		10+ years	
Anesthesia, euthanasia, irradiation		5+ years	

VII. BIOHAZARDS/SAFETY: None

VIII. ENCLOSURES: Image of jigs for (b)(4) irradiation; pain score sheet; criteria for euthanasia

References:

(b)(4)


(b)(4)

(b)(4)

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ Feb 10, 2014

Princi ure Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

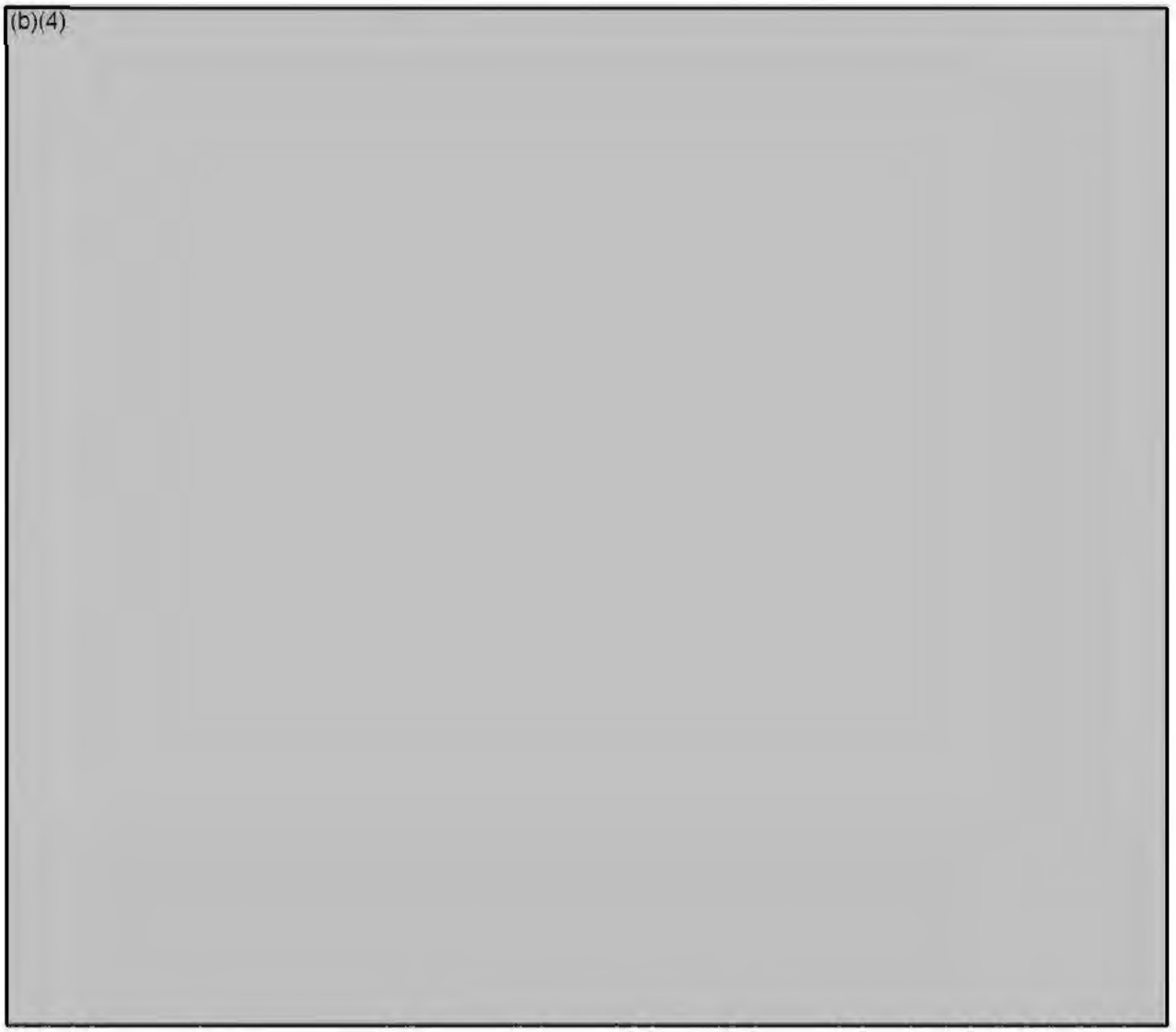
_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ Feb 10, 2014

Prin gnature Date


(b)(4)

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Inv

Feb 10 2014

Date

X. PROTOCOL ABSTRACT:

A. **Animal Protocol Number:**

B. **Animal Protocol Title:** (b)(4) Protection from Radiation-Induced (b)(4) (b) njuries in a Murine Model (*Mus musculus*)

C. **Principal Investigator:** (b)(6)

D. **Performing Organization:** Uniformed Services University of the Health Sciences

E. **Funding:** NIH/NIAID

F. **Objective and Approach:**

(b)(4)

G. **Indexing Terms (Descriptors):** (b)(4) (b)(4) animals, mice

X. PROTOCOL ABSTRACT:

A. **Animal Protocol Number**: (b)(6)

B. **Animal Protocol Title**: (b)(4) Protection from Radiation-Induced (b)(4) (b)(4) Injuries in a Murine Model (*Mus musculus*)

C. **Principal Investigator**: (b)(6)

D. **Performing Organization**: Uniformed Services University of the Health Sciences

E. **Funding**: NIH/NIAID

F. **Objective and Approach:**

(b)(4)

G. **Indexing Terms (Descriptors)**: (b)(4) (b)(4) animals, mice

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

June 2, 2014

MEMORANDUM FOR DR. (b)(6)

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on June 2, 2014:

Animal Protocol Title: (b)(4) Inhibition to Mitigate Radiation-Induced (b)(4) in the (b)(4) Mus Musculus)"

USUHS Protocol Number: (b)(6)

Expiration Date: June 1, 2017

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: (b)(4) Inhibition to Mitigate Radiation-Induced (b)(4) in the (b)(4) (Mus musculus)

GRANT TITLE (if different from above): (b)(4) Inhibition to Mitigate Radiation-Induced (b)(4) in Vivo.

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: USUHS Standard Grant

EARLIEST ANTICIPATED FUNDING START DATE: Dec 2014

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6) April 14, 2014
Date

Associate Professor (b)(6)
(b)(6) office: (b)(6) fax: (b)(6)

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) Chair (b)(6) 4/16/14
Dept. Head Signature Title Telephone Date
Typed Name: (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) (b)(6) April 14 2014
Signature Department Telephone Date
Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) LAM (b)(6) 20 April 2014
Attending/Consulting Veterinarian Signature Department Telephone Date
Typed Name: MAJ (b)(6)

USUHS Form 3206- Animal Study Proposal Form Instructions

USUHS / DOD – SPONSORED ANIMAL RESEARCH PROPOSALS MUST USE THIS STANDARDIZED FORMAT

Reference DOD Directive 3216.1 & USUHS Instruction 3203

****

Specific information requested in the following animal-use protocol template is a result of requirements of the Animal Welfare Act regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD directives.

****

This document is intended to be an aid in the preparation of a USUHS DOD – sponsored animal use proposal. The instructions and written explanations provided for individual paragraphs (ref. animal-use protocol template in AR 40-33 / USUHSINST 3203, Appendix C) are coded as hidden text. To see the instructions and examples for each section, select the "**Show/Hide ¶**" button on your tool bar. To print the hidden text, select "Print" on the drop down file menu. Under the "Options" button, select "Hidden text" under the "Include with document" section. Use of a word processor makes completion of this template a "fill-in-the-blanks" exercise. Please provide all response entries in the following font: Arial, Regular, 12, Black. Please do NOT submit this page of instructions with your animal protocol submission.

With the exception of title headings, each paragraph and subparagraph in the following template must have a response. Portions of the template that are not applicable to your particular protocol, (i.e. no surgery or no prolonged restraint) should be marked "**N/A**". There are no space limitations for the responses.

Pertinent standing operating procedures or similar documents that are readily available to your IACUC may be referenced to assist in the description of specific procedures. It is critical that only animal studies or procedures documented in an IACUC – approved protocol be performed at your facility. Additionally, Principal Investigators, or other delegated research personnel, should keep accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates to their approved protocol.

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR (b)(6) Ph.D., (b)(6)

ANIMAL PROTOCOL TITLE: (b)(4) Inhibition to Mitigate Radiation-Induced (b)(4) in the (b)(4) (Mus musculus)

GRANT TITLE (if different from above): (b)(4) Inhibition to Mitigate Radiation-Induced (b)(4) in Vivo.

USUHS PROJECT NUMBER: (b)(6) (USUHS Standard Grant)

CO-INVESTIGATOR(S): (b)(6) M.D., Ph.D.
POSTDOCS, STUDENTS, TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

Exposure to high dose radiation can occur as the result of a nuclear accident or terrorist event. Exposure to ionizing radiation in humans results in severe and usually fatal illnesses due to multi-organ damage. The effects of radiation on the lung are delayed, but result in high mortality. Despite a large amount of research into the effects of high dose radiation exposure, there has been a delay in the development of drugs or treatments to prevent radiation injury in individuals exposed to radiation.

Our long term goal is to develop agents to prevent immediate and delayed effects of ionizing radiation in multiple organs. Our aim is to investigate drugs targeting the (b)(4) (b)(4) for the prevention of radiation-induced cell damage to the (b)(4) Ionizing radiation has detrimental effects on (b)(4) and leading to increased (b)(4) that persists for long periods of time following the initial damage by radiation exposure.

Previous studies from our lab demonstrated that the principle effect of radiation on normal cells is loss of ability of cells to grow normally and instead to become (b)(4) in a process termed (b)(4) Our data further indicate that radiation-induced (b)(4) (b)(4) The activation of (b)(4) in vivo correlates with (b)(4) upregulation in (b)(4) tissue in a murine model of radiation-induce multi-organ injuries. Therefore, we hypothesize that inhibition the (b)(4) prevent (b)(4) (b)(4) resulting in the mitigation of radiation injuries.

Our proposed studies here will examine the effects of (b)(4) inhibition mitigating (b)(4) radiation injuries in murine models for these injuries. We will utilize C57L mice exposed to (b)(4) X-ray irradiation at (b)(4) to determine the efficacy and mechanisms of (b)(4) for the protection of the (b)(4) against radiation injury. We will utilize C57BL/6 mice exposed to (b)(4) radiation in the (b)(4) region to determine the efficacy and mechanisms of protection of the (b)(4) against radiation injury. Our aim is to develop an effective, non-toxic radioprotectant/ radiotherapeutic treatment strategy that can be used for protection against radiation injury to normal tissues during clinical radiation for cancer treatment.

II. BACKGROUND:

II.1. Background:

Ionizing radiation has been demonstrated to ablate normal vascular function, compromising the endothelial barrier, leading to tissue edema and inflammation in cycles that occur long after the initial radiation insult (b)(4) The cellular injuries from radiation occur in several stages. Early damage occurs in minutes to several hours after radiation exposure and is believed to lead to early inflammatory reactions in response to the activation of proteolytic enzymes (b)(4) (b)(4) Subsequent cycles of cell damage (days to months after radiation exposure) are believed to be due to another mechanism, potentially cellular senescence or apoptosis, possibly associated with inflammation.

The acute toxic effects of exposure to high doses of ionizing radiation in humans have been shown to occur due to two sequential effects: first the ablation of immune cells, resulting in loss of immune function and increasing likelihood of pathogenic infection (b)(4) and subsequently the loss of epithelial progenitor cells of the gastrointestinal (GI) crypt (b)(4) (b)(4) Both of these events have been shown to be due to the loss of fast-growing pluripotent and/or progenitor cell types. The time course of the acute injuries correlates with the life span of the mature cell types of the tissue, which are not replaced because of radiation damage to the adult pluripotent cells and adult stem cells. There is currently no FDA approved prophylactic or treatment for prevention of acute radiation injuries following high-dose (b)(4) radiation exposure.

(b)(4)

In humans, severe radiation (b)(4) can result in death (b)(4) (b)(4) Cumulative evidence from a number of laboratories, including our own, shows that there is a threshold dose of radiation of (b)(4) to induce sufficient (b)(4) for the (b)(4) phases to occur in mice; below these doses, no permanent injury manifests, although some short-term effects are observed (b)(4) However, in humans, there is currently no accepted protocol for preventative treatment of radiation-induced (b)(4) and morbidity as high as 50% is associated with each phase (b)(4) (b)(4) injuries from radiation is considered to be a serious problem in the field for the development of agents to prevent these radiation injuries.

(b)(4) **as a radioprotectant:** Inhibitors for (b)(4) signaling have been tested in animal models for cancer and brain injury, and a number of these agents are currently being used in clinical trials.

(b)(4)

(b)(4) inhibitors are oral drugs with accepted safety profiles.

Preliminary Studies:

Model system for radiation-induced (b)(4) We determined that although (b)(4) irradiation at (b)(4) can induce some early alterations of gene expression in the (b)(4) (b)(4) this level of radiation does not induce observable radiation-induced (b)(4) in C57BL/6J mice (b)(4) For this reason, we have decided to perform studies of radiation-induced (b)(4) using higher doses of (b)(4) irradiation. Using X-ray radiation sources at AFRRI and USUHS, we have determined that (b)(4) irradiation is a threshold dose of radiation exposure for the induction of radiation (b)(4) in several strains of mice including CBA and C57BL/6. Published findings indicate that C57L mice respond to radiation in a manner similar to these mice (b)(4)

Model system for radiation-induced (b)(4) We previously determined that (b)(4) irradiation of C57BL/6 mice induces (b)(4) (b)(4) there is some tissue repair—(b)(4) (b)(4) However, histological evaluation revealed continued (b)(4) underlying structural remodeling of the (b)(4) Our studies suggest that (b)(4) is a threshold dose of radiation for unrepaired (b)(4) in these mice. These findings from our laboratory have not yet been published.

(b)(4) **as a radiation countermeasure:** Our preliminary studies indicate that in primary cultures of human (b)(4) are upregulated within several hours of radiation exposure (b)(4) Additionally the (b)(4) (b)(4) receptor is activated rapidly in response to radiation and remains activated for up to h (b)(4) postirradiation (b)(4) We determined that the inhibition of (b)(4) blocked radiation-induced (b)(4) in both human primary cell cultures (b)(4) (b)(4) Cells maintained normal morphology and proliferative capacity. This finding suggested that radiation-induced (b)(4) in primary cells requires the (b)(4) pathway. We have also observed the activation of (b)(4) from irradiated mice in a time course that correlates with the onset of cellular (b)(4) We hypothesize that the inhibition of radiation-induced cellular (b)(4) in vivo may also be blocked by (b)(4) leading to the preservation of normal cellular function after radiation exposure and preventing delayed tissue damage.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:

Biomedical Research Database (BRD), NIH RePORTER (replacement database for CRISP) *http://projectreporter.nih.gov/reporter.cfm*, and PubMed

II.2.2. Date of Search:

Data base searches conducted on 04/02/14.

II.2.3. Period of Search:

Searches conducted for the entire time periods available in each data base. For BRD, 1998-2014; for RePorter, 1972-2013. For PubMed, 1970-2014.

II.2.4. Key Words and Search Strategy:

Searches were performed using the following key word combinations: (b)(4) (b)(4)

II.2.5. Results of Search:

1) BRD: Search resulted in 0 projects with these key words.

2) A RePORTER search: Search resulted in 0 projects with these key words

3) PUBMED Search

(b)(4)

Summary of other publications:

(b)(4)

(b)(4)

III. OBJECTIVE\HYPOTHESIS:

(b)(4)

IV. MILITARY RELEVANCE:

Military personnel and their families receive medical treatment for cancer, including clinical radiation. The development of agents for the protection of normal tissues during cancer radiotherapy would allow the use of higher doses of radiation which has been shown to improve the level of tumor control and cancer irradiation. The goals of this research are consistent with improved cancer treatment in military personnel and their families.

Additionally, the Department of Defense is prepared to execute military missions within radiation environments, to manage radiation crises associated with terrorist activities, and to manage consequences in the event of nuclear weapons detonation. Recent studies indicate that there is an increasing risk of exposure to ionizing radiation (IR) to military personnel due to increasing terrorist activities, including potential use of stolen nuclear weapons or dispersal of radioactive material in a "dirty bomb" (b)(4) . Because of potential

exposure of military personnel to radiation, the DoD is committed to protect members of the Armed Services from the adverse health effects of ionizing radiation. High dose IR causes short term, acute injuries including hematopoetic and GI syndromes. However, both high and low dose IR (as may result from a radiation dispersal device) can cause (b)(4) as demonstrated by Japanese atomic bomb survivor data as well as accidental exposures to Russian populations (b)(4) (b)(4) While early effects of radiation injury now have treatment/ prophylactic strategies, the delayed effects currently have no treatment. Lack of treatment for late effects of radiation, including (b)(4) will likely lead to delayed morbidity and mortality for personnel exposed to IR.

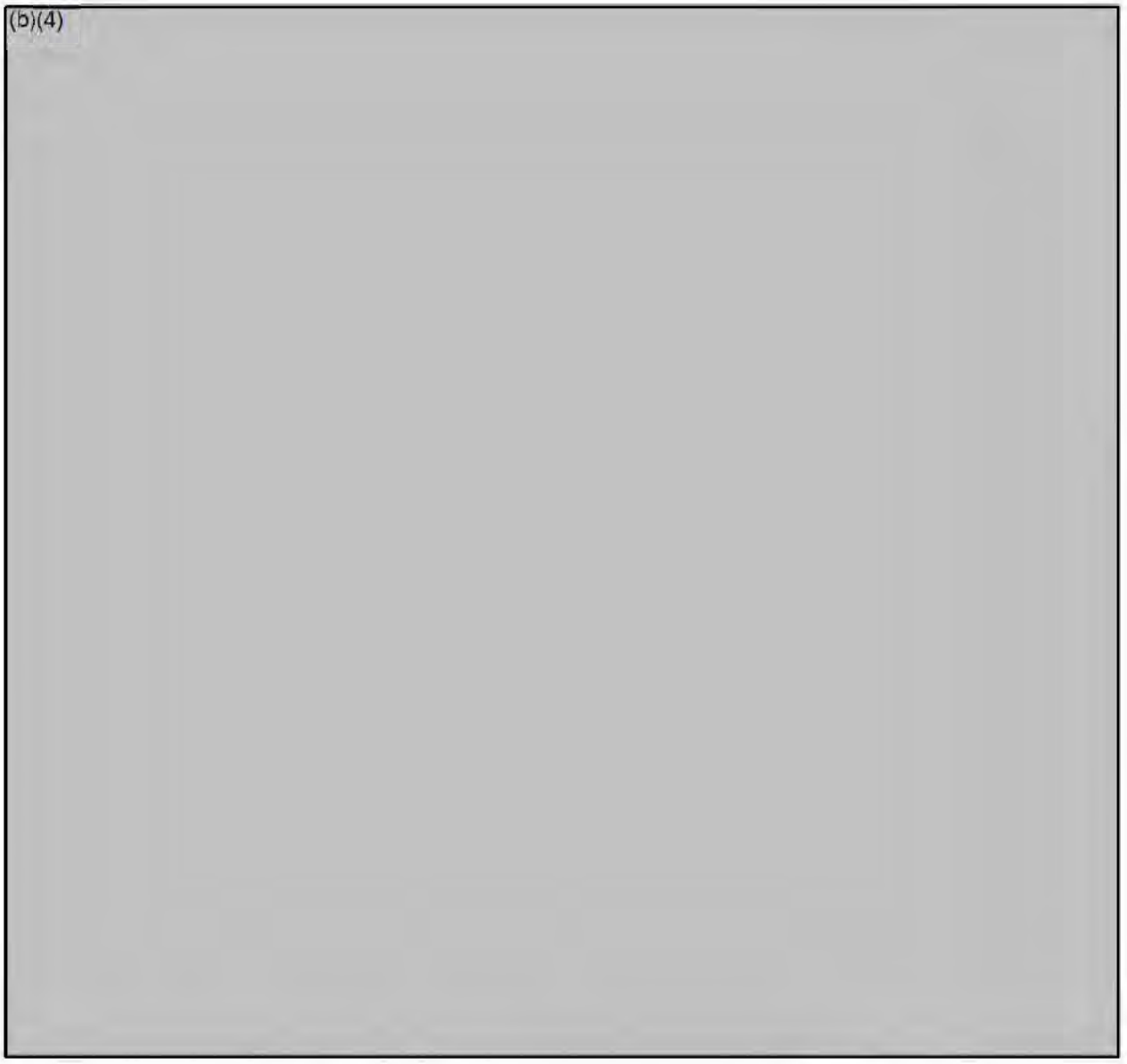
The Department of Defense (DoD) Chemical and Biological Defense Program 2007 Annual Report to Congress, Chapter 2.7.6 Medical Radiological Defense, addresses the requirements for the development of radioprotectants. This priority is outlined for the development of protectants for both acute and delayed radiation injuries, with an emphasis on (b)(4) According to this report, the mitigation of (b)(4) is a major research thrust area. This project is also designed to investigate mechanism(s) of protection to aid in the development of such agents.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

The goal of this project is to investigate the protective effects and mechanisms of (b)(4) treatment for radiation-induced (b)(4)

(b)(4)



Experiments 1-2: Effect of (b)(4) inhibition on radiation-induced (b)(4) injury following (b)(4) ionizing radiation in C57L mice and C57BL/6 mice, respectively.

V.1.1. Effects of (b)(4) administration for (b)(4) injuries (b)(4) (b)(4)

This experiment will determine whether (b)(4) inhibition prevents radiation-induced (b)(4) and improves (b)(4) or vehicle will be administered h postirradiation (b)(4) with administration daily for (b)(4) after radiation exposure (b)(4) Two groups will be studied: (b)(4) + vehicle and (b)(4)

(b)(4) mice per group) x (2 treatment groups) x (1 time point) = mice (b)(4)

Endpoint: The final endpoint for the experiment is days. Mice displaying obvious dyspnea, (b)(4) lethargy, or other markers of morbundity and which appear to be in distress will be euthanized.

(b)(4) The scale for pain or lethargy is attached. Mice scoring a total of 12 under any measurement will be euthanized.

V.1.2. Experiment 2: Effects of (b)(4) inhibition for the mitigation of (b)(4) radiation injury (b)(4)

We will determine the effect of (b)(4) inhibition on radiation (b)(4) The effect of (b)(4) inhibition on (b)(4) will be scored at (b)(4) days postirradiation. (b)(4) or vehicle will be administered (b)(4) h postirradiation, with administration daily for (b)(4) after radiation exposure (b)(4) region irradiation will be used. Groups will be: 1) radiation + no treatment; 2) radiation +vehicle (control); and 3) irradiation + (b)(4) (b)(4) For these studies, we will require (b)(4) mice per group in the radiation groups. We will obtain control tissue outside of the field of radiation, which previous experiments in our laboratory have demonstrated are appropriate as controls.

The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

Radiation groups

(b)(4) (b)(4) mice per group) x (3 treatment groups) x (2 time points) = (b)(4)

(b)(4) (b)(4) mice/experiment) x (2 experiments) = (b)(4) mice

Endpoint: The final endpoint for the experiment is (b)(4) days. Mice displaying obvious lethargy, or other markers of morbundity and which appear to be in distress will be euthanized. The scale for pain or lethargy is attached. Mice scoring a total of 12 under any measurement will be euthanized.

Experiments 3-5: Effect of (b)(4) inhibition for mitigation of radiation-induced cellular (b)(4) These experiments will be performed only if positive results are obtained from experiments 1 or 2.

V.1.3. Experiment 3 Effect of (b)(4) inhibition on (b)(4) (b)(4)

If we find that (b)(4) inhibition results in reduction of radiation-induced (b)(4) injury at (b)(4) days postirradiation, we will further investigate the mechanism of the improvement. We will determine whether (b)(4) reduces (b)(4) and improves cell survival following radiation exposure in the (b)(4) We will use 3 time point groups (b)(4) days postirradiation to examine markers for (b)(4) by western blotting and immunohistochemistry (b)(4) (b)(4) will be assessed by immunohistochemistry and by qRT-PCR for immune cell (b)(4) markers (b)(4) will also be determined using the tissue from Experiment 2, with the time points (b)(4) days postirradiation. Groups will be: 1) radiation + no treatment; 2) radiation + vehicle; 3) radiation + (b)(4) Control tissue will be obtained from outside of the radiation field.

The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) (b)(4) mice per group) x (3 treatment groups) x (3 time points) = (b)(4)

(b)(4) (b)(4) mice/experiment) x (2 experiments) = (b)(4)

Endpoint: The endpoints for the experiment are days (b)(4) postirradiation. Mice displaying obvious dyspnea, lethargy, or other markers of morbundity and which appear to be in distress will be

euthanized. The scale for pain or lethargy is attached. Mice scoring a total of 12 under any measurement will be euthanized.

V.1.4. Experiment 4 (b)(4) **inhibition on** (b)(4)
(b)(4)
We will investigate the effecs of (b)(4) inhibition of (b)(4) We will determine levels of (b)(4) using histological methods at (b)(4) days after (b)(4) irradiation, as well as (b)(4) For this
(b)(4) experiment we will require only mice/group, and we will examine 6 groups: 1) sham; 2) sham + vehicle 3) sham + (b)(4) 4) IR 5) IR + vehicle; 6) IR + (b)(4)

The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) mice per group) x (6 treatment groups) x (1 time point) = (b)(4)
(b)(4) mice/experiment) x (2 experiments) = mice

Endpoint: The final endpoint for the experiment is days. Mice displaying obvious dyspnea, (b)(4) lethargy, or other markers of morbundity and which appear to be in distress will be euthanized. (b)(4) The scale for pain or lethargy is attached. Mice scoring a total of 12 under any measurement will be euthanized.

V.1.5. Experiment 5 (b)(4) **inhibition on** (b)(4)
(b)(4) **following irradiation** (b)(4)
We will investigate the activation of (b)(4) pathway and monitor (b)(4) We will examine these aspects through immunohistochemistry and determine if the (b)(4) treatment was sufficient to inhibit the feed-forward (b)(4) Four time points will be examined, correlating with the times we have observed for (b)(4) (b)(4) following radiation exposure: (b)(4) weeks postirradiation. We will analyze (b)(4) and the expression of (b)(4)
(b)(4) For this experiment we will require only mice/group, and we will examine 6 groups: 1) sham irradiation + no treatment; 2) sham + vehicle 3) sham + (b)(4) 4) IR; 5) IR + vehicle; 6) IR + (b)(4) The experiment will be repeated because it is a generally accepted principle of the scientific method that experiments must be repeated to evaluate a hypothesis.

(b)(4) mice per group) x (6 treatment groups) x (4 time points) = (b)(4)
(b)(4) mice/experiment) x (2 experiments) = mice

Endpoints: Endpoints for the experiments will (b)(4) weeks postirradiation. Early time points will be taken if any animal exhibits signs or symptoms of morbidity or moribundity. Such animals will be euthanized at the time of observation.

V.2. Data Analysis:

Mean with standard error, or percentage will be reported if applicable. A significance level is set at 5% for each test. All statistical tests are two-sided. A statistical software, SAS will be used for statistical analyses.

Experiment 1: Analysis of variance (ANOVA) will be used to detect significant difference among the groups. If significant, then the pair-wise comparison by Tukey-Kramer will be used to identify which group is different from the other. For experiment 1, N = mice per group could have 80%(b)(4) power to detect any significant difference between groups if any, given type I error of 5% and delta/sigma has at least 1.4, where the delta is the estimated mean differences between the two groups and sigma is the pooled standard deviation of the two groups (b)(4)

Experiments 2-5: Previous data from this laboratory indicate that the coefficient of variation (CV) of the data is about 0.5, and that differences of between 2- and 30- fold are likely to be observed. A (b)(4) sample size of [redacted] per group will have 80% power to detect 3-fold increases in biomarkers based on a lognormal t test with a 5% two-sided significance level and a CV of 0.5.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

For the last 10 years, we have utilized primary cell culture systems to examine anti-apoptotic mechanisms; however, we must determine whether our culture findings are relevant in whole animals. We will continue to use primary cell culture whenever possible to avoid unnecessary use of animals. We have also considered computer models as alternatives to animals. However, this alternative also lacks the knowledge base and complexity needed to evaluate radioprotective and radiotherapeutic agents.

V.3.2. Animal Model and Species Justification:

Tissue culture conditions cannot fully substitute for the complexities of whole organisms in response to radiation injury. Such injury involves the interactions between the hematopoietic system and other organ systems. In order to develop viable treatments for humans, we find that our research must be conducted in whole animals.

Work by (b)(6) [redacted] also advocates the mouse as the best species to use for radiation protection and radiotherapy protocols: 1) mice provide the least sentient species available whose response to radiation has been clearly identified, with physiological responses to radiation and radioprotectants that are similar to human physiology, 2) extensive literature is available on responses of mice to radiation and countermeasures, 3) more reagents are available to study the relevant cell types and molecules in mice than in any other species except humans, and the availability of transgenic and knockout mice make this model extremely attractive for further research into molecular mechanisms, and 4) our research group has extensive experience with this model in radiation injury and radioprotection research.

The strains of mice to be utilized for each portion of the study are based on the literature and on findings from our own laboratory. (b)(4) [redacted] (b)(4) [redacted] Our laboratory has found that C57BL/6 mice have radiation-induced (b)(4) damage similar to that reported in the literature for humans.

V.3.3. Laboratory Animals

V.3.3.1. Genus & Species:	*Mus musculus*	*Mus musculus*
V.3.3.2. Strain/Stock:	C57L	C57BL/6
V.3.3.3. Source/Vendor:	(b)(4) [redacted]	
V.3.3.4. Age:	10-14 weeks	10-14 weeks
V.3.3.5. Weight:	Normal adult weight range for this strain: 17-21 g.	Normal adult weight range for this strain: 17-21 g.

V.3.3.6. Sex:	Female. Only females will be used in this study for the following reasons: 1) we have so far utilized female mice to lay the foundation of our studies for radiation-induced (b)(4) (b)(4) injuries; 2) because of long housing periods, it is advantageous to use female mice that are less aggressive toward each other.	Female. Only females will be used in this study for the following reasons: 1) we have so far utilized female mice to lay the foundation of our studies for radiation-induced (b)(4) (b)(4) injuries; 2) because of long housing periods, it is advantageous to use female mice that are less aggressive toward each other.
V.3.3.7. Special Considerations:	Commercially procured animals must be adventitious disease free. Mice- Pathogen-free, including *Pseudomonas aeruginosa*, and Pasteurella. All mice need to be free of the following agents: Sendai, Pneumonia Virus of Mice (PVM), Reovirus-3 (Reo 3), Mouse Adenovirus (MAD-1, MAD-2), Mouse Cytomegalovirus Virus (MCMV), Ectromelia, K virus, Lymphocytic Choriomeningitis Virus (LCM), Epidemic Diarrhea of Infant Mice (EDIM), Hantaan virus, Rotavirus, Mouse Parvovirus (MPV), Polyoma Virus, Mouse Minute Virus (MMV), Mouse Thymic Virus (MTV), Theiler's Mouse Encephalomyelitis Virus (TMEV/GDVII), *Encephalitozoon cuniculi*, CAR bacillus, *Helicobacter* spp. *Mycoplasma pulmonis*, and *Clostridium piliforme*. Endoparasite- and ectoparasite-free.	

V.3.4. Number of Animals Required (by Species): C57L C57BL/6 (b)(4)

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: Animals demonstrating moribundity due to radiation exposure will be considered to have arrived at the study endpoint and will be immediately euthanized (See sections V.4.5 Study Endpoint and V.4.6 Euthanasia).

Ketamine/xylazine anesthesia will be used during X-ray irradiation to prevent stress from the time the mice are placed in restraint. Under the training protocol at AFRRI in conjunction with the AFFRI veterinary staff, we found that in female CBA or C57BL/6J mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in a minimum of 40 min anesthesia with no morbidity or mortality (b)(4) We will use a heating pad to keep mice warm during the period of recovery from anesthesia. The temperature in room where the mice will be irradiated will be measured, and if necessary a space heater will be used to increase the temperature. Standard intraperitoneal injection methods are used for injection of the anesthesia, as recommended and approved by the USUHS veterinary staff and for which all laboratory personnel have received training at USUHS.

V.3.5.2. Reduction: Whenever possible, control groups will be shared between experimental groups for our experiments. Tissues will be collected for multiple assays from the same animals

further reducing the total number of animals needed. At every stage of the research, planned experiments will be reevaluated, in an attempt to reduce the numbers of experiments and eliminate repeats of experiments where statistical significance has already been obtained. For (b)(4) irradiation studies, each animal will be able to provide its own control tissue from the non-irradiated area. Preliminary results from our laboratory demonstrate that tissue from the abdominal region is normal under our experimental conditions.

V.3.5.3. Replacement: At this time, it is not feasible to use non-animal systems in place of animal models to address the research questions in this protocol.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	C57L	C57BL/6
V.4.1.1.1.1. Column C:	(b)(4)	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:		

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:
We will be unable to use general analgesics since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4) (b)(4) Some sedatives have been reported to contribute to postoperative (b)(4) which likely reduce some functions of radiation. For C57BL/6 mice exposed to (b)(4) irradiation to induce radiation (b)(4) daily on animals displaying radiation injury to alleviate discomfort and to prevent infection. This agent is also used for humans.

An anesthesia protocol has been developed for use during the X-ray irradiation. Ketamine will be used with xylazine for longer anesthesia in mice to be place in restraints for (b)(4) irradiation. Under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff, we found that in female C57BL/6 and CBA mice, ~12 weeks old,i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in a minimum of 40 min anesthesia (25 G needle) with no morbidity or mortality (b)(4)

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:
Animals will be housed and cared for as described in Section V.5. Veterinary Care both before and after dose administrations and irradiation procedures. The PI and research staff will monitor animals after radiation or sham irradiation during anesthesia recovery. The PI and research staff will also monitor animals daily following radiation exposure, and twice daily during critical periods. Otherwise, the animals will receive standard veterinary care in the USU vivarium. Animals displaying radiation-induced (b)(4) will receive (b)(4) (b)(4) is approved for human use.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: DoD Bioedical Research Data Base (BRD), NIH Reporter, PubMED

V.4.1.3.2. Date of Search: 3/31/14

V.4.1.3.3. Period of Search: Search covered published manuscripts from ~1965-present (BRD search included all available years 1998 -2014).

V.4.1.3.4. Key Words of Search: Pain, analgesia, anesthesia, mice, radiation, ionizing radiation, humane (b)(4)

V.4.1.3.5. Results of Search:
The literature search revealed that, at present, there are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. In some cases radiation has been demonstrated to reduce pain in laboratory mice (b)(4) and has been in humans reported to alleviate cancer related bone pain (b)(4) (b)(4) A recent report showed that administration of botulinum toxin A was effective for reduction of pain and muscle spasms after clinical radiation for treatment of tumors (b)(4) (b)(4) Application of natural honey 20 min prior to radiation exposure was also shown to reduce mucositis with associated pain in humans following head/neck radiotherapy (b)(4) it has not been determined whether honey acts as a local radioprotectant. Human administration of morphine also relieves pain (b)(4) but again this would not be practical for mice in our experiments because of side effects of the drug. In humans, exposure to (b)(4) irradiation may induce vomiting and diarrhea, causing pain and distress. However, mice are not susceptible to vomiting.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
Anesthesia will be used before (b)(4) irradiation to avoid stress from restraint (b)(4) (b)(4) (b)(4) we will be unable to use analgesics after irradiation since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4) (b)(4) Pain will be evaluated using several standard indications including: grimacing, abnormal breathing, hunched or fluffed fur and inactivity, or ataxia. In experiments where tissue collection is needed, animals will be euthanized with pentobarbitol prior to sample collection as described in Section V.4.6. We will utilize the attached score sheets to provide a scale for measurement of criteria for euthanasia. Score sheet will be used daily after irradiation. During critical periods, animals will be monitored twice daily, and during the second monitoring score sheets will be updated if necessary for that day. Any animal scoring 12 or more total on the score sheet will be euthanized. In experiments where tissue collection is needed, animals will be euthanized with pentobarbitol prior to sample collection as described elsewhere.

V.4.2. Prolonged Restraint:
(b)(4) irradiation will require restraint in designed jigs for ~30 min to receive X-ray exposure (b)(4) (b)(4) animals will be anesthetized during this period (see Anesthesia above) and tails will be taped to one side with surgical tape. The jigs for use during (b)(4) irradiation were examined and approved by the AFRRI Veterinary staff. An image of the jigs is attached. Under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff, we found that in female CBA and C57BL/6 mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in a minimum of 40 min anesthesia (25 G needle) with no morbidity or mortality (b)(4) The length of time

for anesthesia is needed to ensure that animals do not regain consciousness while being positioned in the lead shield for irradiation. Positioning of the mice must be done carefully to ensure that all animals receive the same exposures. We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. Standard i.p. injection methods are used for injection of the anesthesia. We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. AFFRI Veterinary staff provided training and supervision for the placement of anesthetized animals in the approved jigs for irradiation.

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations: N/A

V.4.4.1. Injections:

Intra-peritoneal injections of pentobarbital for euthanasia 0.01-0.02 ml/mouse Fatal Plus (39-78 mg pentobarbital/mouse), will be performed with a 23 G needle. I.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine will be done using a 25 G needle. This level of anesthesia was first developed for our use in collaboration with the AFRRI Veterinary staff and shown to effectively produce non-lethal anesthesia for a minimum of 40 min (b)(4)

(b)(4)

(b)(4) Animals will be monitored using the "Pain Score Sheet".

V.4.4.1.1. Pharmaceutical Grade Drugs

(b)(4)

V.4.4.1.2. Non-Pharmaceutical Grade Drugs

(b)(4)

(b)(4)

V.4.4.2. Biosamples:
All biosamples will be taken after euthanasia. (b)(4) will removed *en bloc*. (b)(4 will be removed from the (b)(4) as experimental tissue and from the (b)(4) as control tissue. All other tissues will be available to other researchers for tissue sharing.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification:
All cages will have cage cards identifying the treatment groups for each experiment.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures:

(b)(4) **irradiation**
The methodology for (b)(4) irradiation of mice including approval of holding jigs and development of anesthesia was developed in conjunction with the AFRRI Veterinary Staff. (b)(4) irradiation will be performed using the RS2000 irradiator in (b)(6) Thirty minutes prior to irradiation, the RS2000 irradiator and lead shield will be decontaminated thoroughly with MB10.

Mice will be anesthetized by injection in the USUHS LAM facility. Anesthetized mice will be placed in ventilated Lucite jigs, which restrain the animals to prevent lateral or vertical movement. Tails will be taped to prevent any forward motion of animals. Mice will be irradiated at (b)(4) (b)(4) irradiation. The temperature in room where the mice will be irradiated will be measured, and if necessary a space heater will be used to increase the temperature. Following radiation exposure, mice will be immediately returned to the LAM and allowed to recover from anesthesia under supervision with heating pads. Immediately upon recovery, animals will be returned to standard cages that house 4 animals per cage. Lucite jigs will be sanitized after each use.

V.4.4.8. Tissue Sharing:
For most studies (b)(4) will be removed *en bloc* or (b)(4 tissue with substructure tissues will be removed. All other tissues will be available for tissue sharing with other investigators. Our laboratory has made extensive use of shared tissues for training and technique development for (b)(4) tissue.

V.4.5. Study Endpoint:
(b)(4)
(b)(4) Early endpoints will be used in the case of morbidity and morbundity. Specific health assessment criteria including rapid weight loss, ruffled fur, dried lacrimal fluid accumulated in the cul-de-sac of their eyes, are cool to the touch, decreased appetite, dehydration, diarrhea, difficulty breathing or panting (b)(4) (b)(4) or hunched body position will used to determine early study

endpoints for euthanasia. The experimental design does not contemplate supportive therapy, and animals exhibiting these signs may be euthanized, and considered to be at the study endpoint.

Following (b)(4) irradiation, mice may exhibit injuries from the (b)(4) irradiation during the (b)(4) (b)(4) postirradiation. Also, mice are expected to develop (b)(4) postirradiation. During the critical time periods (b)(4) postirradiation), mice will be monitored by the PI or other members of the investigative team at least twice daily, early morning and late afternoon including weekends or holidays.

V.4.6. Euthanasia:

Animals will be euthanized at the stated endpoints of the experiments by i.p. injection of ml/mouse Fatal Plus. PHS policy is intraperitoneal injection of at least 200 mg/kg sodium pentobarbital euthanasia solution; Fatal Plus: 390 mg pentobarbitol/1 ml.

1 ml/390 ml x 0.02 kg/mouse x 200 mg/1 kg = 0.01-0.02

IP injection will be performed using a 23 G needle. (Please note that Euthasol cannot be used as additives have effects on (b)(4) We do have Fatal Plus for these experiments.) Euthanasia will be confirmed by cervical dislocation. Animals which appear to be experiencing pain or distress as a result of radiation exposure or are found moribund will also be euthanized by pentobarbitol and necropsied. At the end of experiments, unused animals will be transferred to other protocols or euthanized by exposure to 100% CO_2 from a compressed gas cylinder in accordance with the current American Veterinary Medical Association (AVMA) Guidelines for Euthanasia. Exsanguination, cervical dislocation, and/or pneumothorax will be used to ensure euthanasia.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. Mice will be housed 4 per cage. Food and water and Rodent Diet will be available ad libitum. The 12:12 hr (lights off at 6 pm) room light cycle is fine for all the studies.

V.5.1.1. Study Room:

Radiation Building (b)(6) Room Number (b)(6)

Euthanasia Building (b)(6) Room number (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes _____	No	x
Fluid Restriction:	**NOTE: Acidified water will be provided to reduce infections after radiation exposure.**	No	x

Acidified water (pH 2.5 – 3.0) is produced at AFRRI for their vivarium using 2.0 N HCl. The water will be obtained from USUHS LAM. Unused ~~water will~~ be neutralized with sodium bicarbonate and the pH will be verified before disposal.

V.5.1.3. Exceptions: None

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care:
Experimental animals will be observed at least twice a day by one of the study investigators or technicians. LAM personnel will also monitor the animals twice a day during their rounds. As described in another section (study endpoint) morbid animals will be monitored at least twice daily, morning and afternoon. The experimental design does not contemplate supportive therapy. The justification for not providing supportive care has been described in the study end point section as well as in the pain category section. Criteria to be used for health evaluation while the animals are on study include: 20% weight loss compared with average weights of sham irradiated animals (mice will be weighed every other day between days (b)(4) (b)(4) ruffled fur, dehydration, decreased activity, and hunched body position, dyspnea, or panting. Animals exhibiting these signs may be euthanized, and considered to be at the study endpoint. Increased
(b)(4) monitoring of C57L animals will begin on day through the end of the experiment, *based on our* previous data that indicated that mortality from (b)(4) irradiation occurs between days (b)(4) postirradiation in CBA mice. Increased monitoring of C57BL/6 animals exposed to (b)(4) irradiation for (b)(4) will be monitored between days (b)(4) when the most prominent (b)(4) (b)(4) is expected. In the event of debilitating illness the decision to euthanize will be made by the veterinarian and/or PI.

V.5.2.2. Emergency Veterinary Medical Care:
All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays. In the case of an emergency health problem, if the responsible person (e.g. investigator) is not available or if the investigator and veterinary staff cannot reach consensus on treatment, the veterinarian has the authority to treat the animal, remove it from the experiment, institute appropriate measures to relieve severe pain or distress, or perform euthanasia if necessary.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions: none

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Ear punch, irradiation, anesthesia, euthanasia	(b)(6)	9+ years	(b)(6)
Irradiation, anesthesia, euthanasia		6 years	
Rodent handling		10+ years	
Anesthesia, euthanasia, irradiation		5+ years	

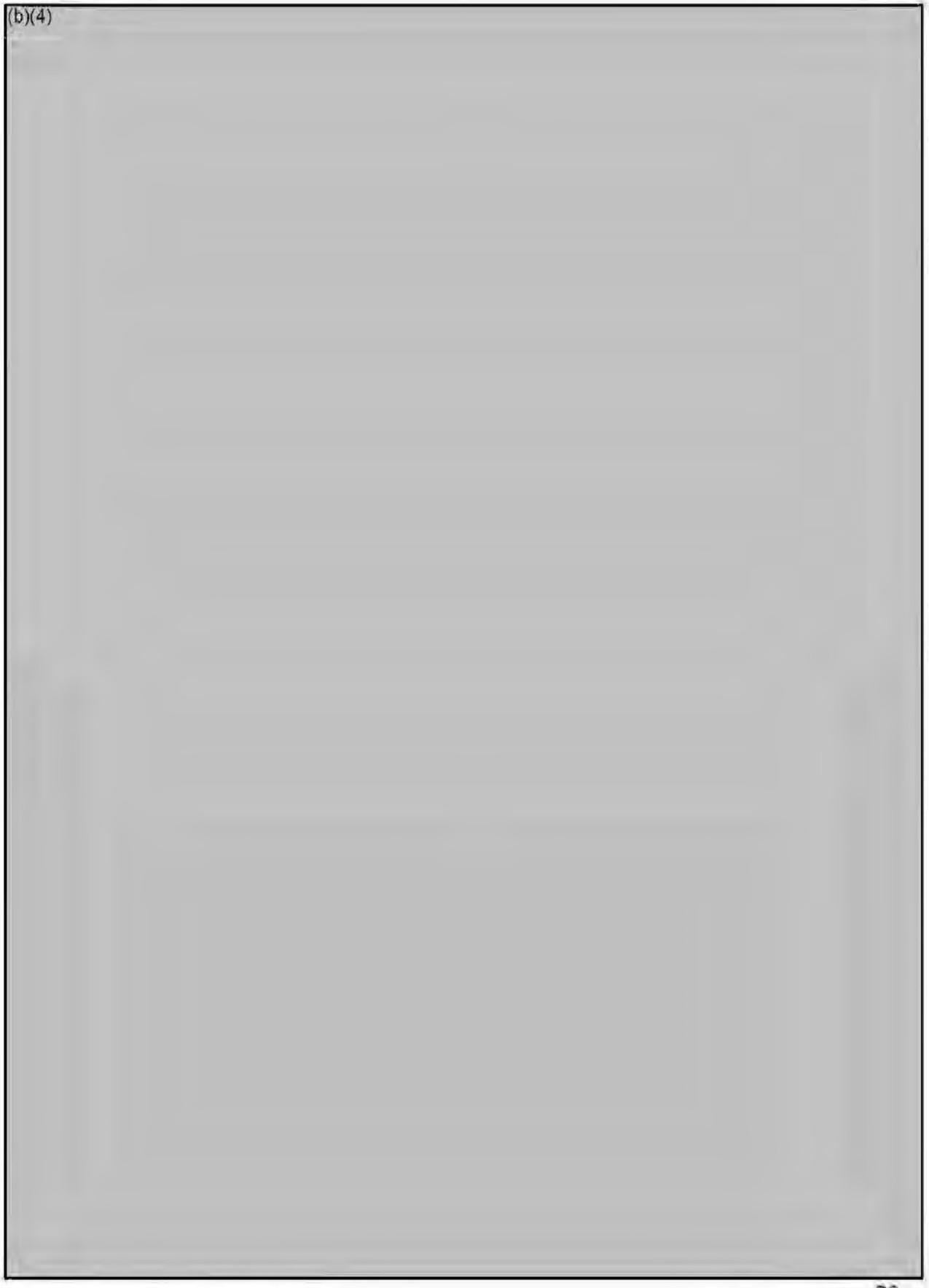
VII. BIOHAZARDS/SAFETY: Laboratory personnel working with animals will be wearing standard personal protective equipment (PPE), including dust masks, gloves, and lab coats, as required by USUHS LAM. No additional protection is required for handling irradiated animals, since the animals will be exposed to X-ray irradiation and are not themselves radioactive or otherwise infectious. The RS2000 X-ray irradiator utilizes a fully shielded cabinet for animal irradiation that has been tested and approved by the USUHS Radiation Safety Office. Personal dosimeters are worn by laboratory personnel during X-ray irradiation of the animals, as designated by the Radiation Safety Office.

VIII. ENCLOSURES: Image of jigs for (b)(4) irradiation; pain score sheet; criteria for euthanasia

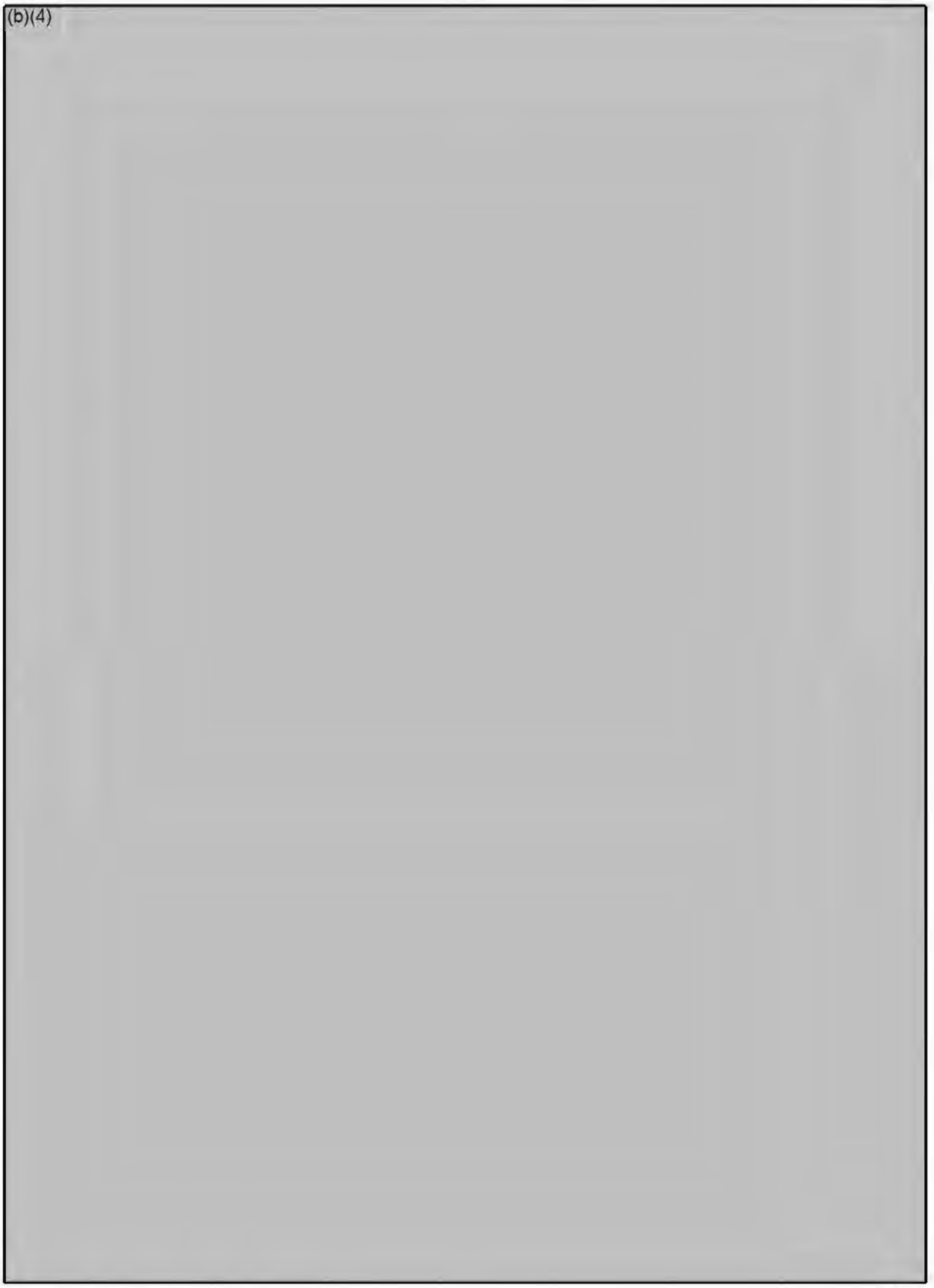
References:

(b)(4)

(b)(4)



(b)(4)



IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6)

P[redacted]gnature April 14, 2014
Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6)

Princ[redacted]ature April 14, 2014
Date

I.

Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL or WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Prin[redacted]nature

April 14, 2014
Date

X. **PROTOCOL ABSTRACT**:

A. **Animal Protocol Number**:

B. **Animal Protocol Title**: (b)(4) Inhibition to Mitigate Radiation-Induced (b)(4) (b)(4) (Murine)

C. **Principal Investigator**: (b)(6)

D. **Performing Organization**: Uniformed Services University of the Health Sciences

E. **Funding**: USU standard grant

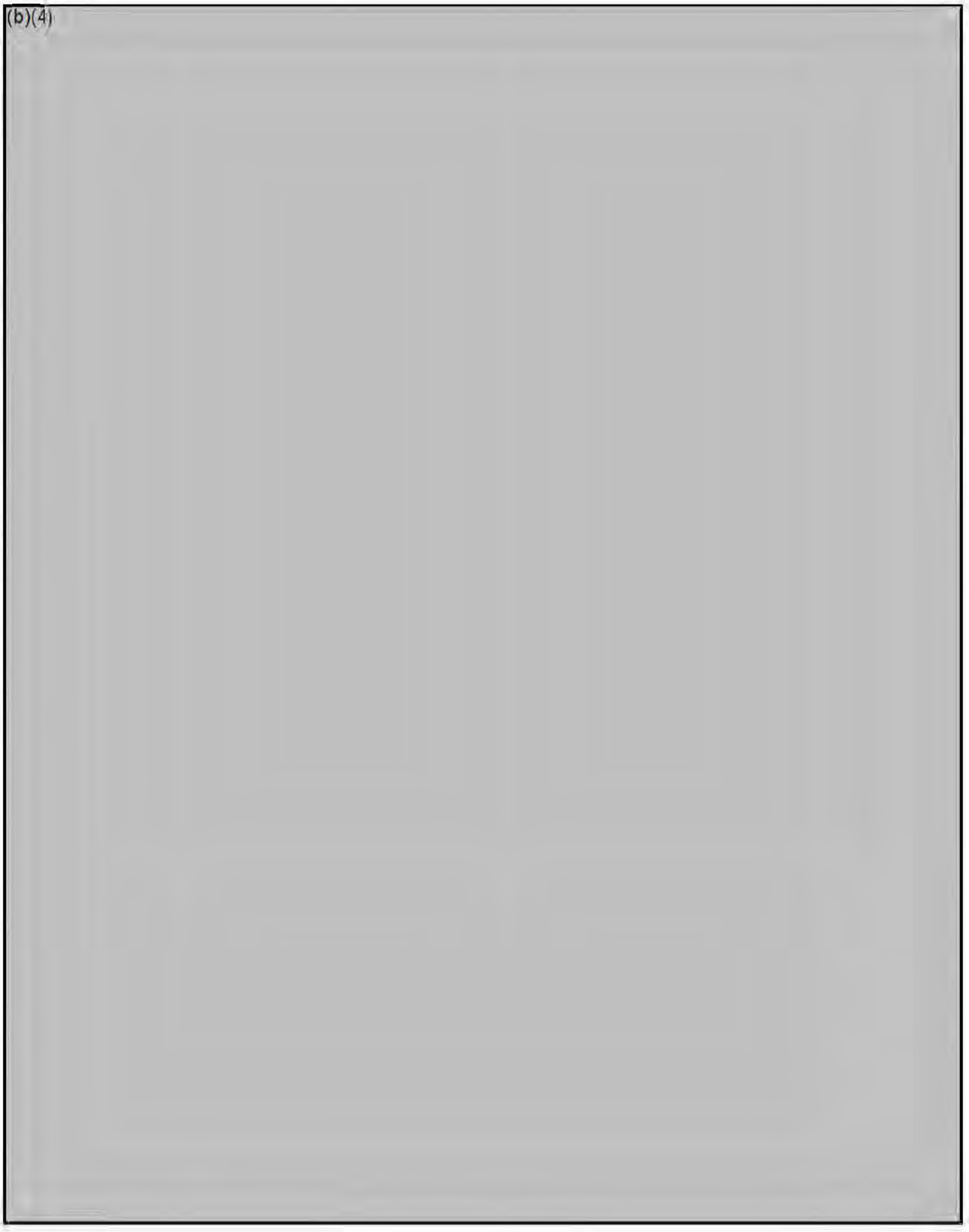
F. **Objective and Approach**:

(b)(4)

G. **Indexing Terms (Descriptors)**: (b)(4) (b)(4) animals, mice

November 20, 2012 (b)(6)

(b)(4)



Date:

Pain Score Sheet

date	Cage #				Cage #				Cage #			
animal	1	2	3	4	1	2	3	4	1	2	3	4
appearance												
respiratory rate												
behavior												
provoked behavior												
total												

date	Cage #				Cage #				Cage #			
animal	1	2	3	4	1	2	3	4	1	2	3	4
appearance												
respiratory rate												
behavior												
provoked behavior												
total												

date	Cage #				Cage #				Cage #			
animal	1	2	3	4	1	2	3	4	1	2	3	4
appearance												
respiratory rate												
behavior												
provoked behavior												
total												

date	Cage #				Cage #				Cage #			
animal	1	2	3	4	1	2	3	4	1	2	3	4
appearance												
respiratory rate												
behavior												
provoked behavior												
total												

VIII. Appendix A: Clinical Observations with Criteria for Euthanasia (Rodent)

DATE:	Time:	Animal ID#	Score
Appearance:			
	Normal (smooth coat, clear eyes/nose)		0
	Hunched and/or fluffed		1
	Ocular discharge, and/or edema		3
	Emaciated, dehydrated (skin tent) **		5
	Soft stools (fecal matter around anus)		5
	Bloody diarrhea		9
Respiratory rate:			
	Normal breathing		0
	Increased breathing (double normal rate, rapid, shallow)		6
	Abdominal breathing (gasping +/- open mouth breathing)*		12
General Behavior:			
	Normal (based on baseline observations)		0
	Stretching of hind limbs with abdominal motion (writhe) or grimace (App. B and C)		1
	Decreased mobility		2
	Ataxia, wobbly, weak**		6
	Inability to stand*		12
Provoked Behavior:			
	Normal (moves when cage is disturbed, runs from hand (mice) or investigates (rat))		0
	Subdued; responds to stimulation (moves away briskly)		1
	Subdued even to stimulation (moves away slowly)		3
	Unresponsive to gentle prodding **		6
	Does not right when placed gently on side within 5 seconds*		12
TOTAL			_____

Definitive criteria for morbidity:
Weight Loss up to 10% baseline (if available)**
Pale, white mucous membranes/ skin * *
Fever >104F/40C (if available)**

Definitive criteria for moribundity:
Weight Loss > 20% OR <15g (adult mouse)*
Blue mucous membranes/ skin (cyanosis)*
Cool to the touch or <86F/30C (if available)*

**** Regardless of score, notify appropriate person immediately.**

*** Regardless of score, immediately euthanize (death is imminent)**

Score:
< 6 Normal
6 - 9 Morbid: Monitor at least 3 times per day; notify appropriate personnel immediately
> 10 Moribund: Notify responsible personnel immediately for euthanasia (if no single criteria is 12*)
Any single criteria of 12* euthanize immediately; consider as 'found dead'

(Note: This form does not have to filled out for every individual at every observation, so long as the criteria are used in informing decisions on increased monitoring and/or euthanasia. This form may be used to make a spreadsheet for greater ease of multiple animal observations, as long as the criteria and numbering system are exactly copied.)

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

June 9, 2015

MEMORANDUM FOR DR. (b)(6)

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on June 8, 2014:

Animal Protocol Title: "Development of (b)(4) for the Mitigation of Acute Injuries from Ionizing Radiation in Gottingen Minipigs (Sus scrofa domestica)"

USUHS Protocol Number: (b)(6)

Expiration Date: June 7, 2018

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6) Ph.D.
for (b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Development of (b)(4) for the mitigation of acute injuries from ionizing radiation in Gottingen minipigs (*Sus scrofa domestica*)

GRANT TITLE (if different from above): Advanced Development of (b)(4) as a Mitigator for Acute and Delayed Ionizing Radiation Injuries

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: DMRDP

EARLIEST ANTICIPATED FUNDING START DATE: May 19, 2014

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6) _____ (b)(6) 23 March 2015

Signature Department Office/Lab Telephone Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) Chairman (b)(6) 3/26/15

Re ept. Head Signature Title Telephone Date

Typed Name: Dr. (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the st

(b)(6) (b)(6) March 23 2015

Department Telephone Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) LAM (b)(6) 24 March 2015

Attending/Consulting Veterinarian Signature Department Telephone Date

Typed Name: (b)(6) DVM, MPH

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D., (b)(6)
(b)(6)

ANIMAL PROTOCOL TITLE: Development of (b)(4) or the mitigation of acute injuries from ionizing radiation in Gottingen minipigs (*Sus scrofa domestica*)

GRANT TITLE (if different from above): Advanced Development of (b)(4) as a Mitigator for Acute and Delayed Ionizing Radiation Injuries

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6) Ph.D., AFRRI

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS: The (b)(4) system is sensitive to injury by ionizing radiation. (b)(4) Syndrome, a part of Acute Radiation Syndrome (ARS), occurs in response to exposure of significant portions of the active (b)(4) to ionizing radiation, above (b)(4) A very limited number of large animal models are available for the testing of radiation countermeasures, including non-human primates, dogs and swine. An improved understanding of the natural history of radiation sickness and development of multi-organ failure is still largely incomplete.

Dr (b)(6) has been developing a minipig model of ARS. Dr. (b)(6) has characterized (b)(4) ARS and has tested the efficacy of the radiation countermeasure G-CSF on survival and (b)(4) recovery in the irradiated Gottingen minipig. Signs and symptoms, kinetics of blood cell depletion and recovery, multi-organ dysfunction and histological findings resembled closely what observed in human victims of radiation accidents and in other large animal models (b)(4)

Our laboratory has characterized the use of (b)(4) as a radiation countermeasure for (b)(4) syndrome in mice. We have demonstrated in mice the required time course for administration of (b)(4) and dosages of the drug required for preventing mortality at (b)(4) (b)(4) levels of radiation exposure. Our research has also demonstrated that (b)(4) suppresses radiation-induced (b)(4) in the (b)(4) and other tissues, mitigates radiation-induced hypoxia, and reduces the induction of cytokines induced in response to radiation damage (b)(4)
(b)(4)

Advanced development of a radiation countermeasure requires the testing of the countermeasure in two animal species as well as demonstration of the mechanism of action in order to fulfill the FDA Animal Rule. The purpose of the current study is the testing of (b)(4) as a countermeasure in the minipig model developed by Dr (b)(6) to fulfill the requirement of a second species for radiation testing according to FDA requirements.

II. BACKGROUND:

II.1. Background:

High dose total body irradiation (TBI) in humans can result in both acute and delayed injuries. The Acute Radiation Syndrome (ARS) is characterized by the (b)(4) (b)(4) syndromes. The mechanisms leading to (b)(4) ARS remain largely unknown, but current information indicate that (b)(4) may play a major role in the loss of (b)(4) (b)(4) leading to (b)(4) There are currently no approved countermeasures for radiation-induced (b)(4) injuries, and it is our long range goal to develop effective agents for this condition. The objective of this application is to evaluate the effects of an FDA-approved drug (b)(4) for mitigating (b)(4) radiation injuries. (b)(4) as a mitigator of radiation-induced (b)(4) injury using a murine model for (b)(4) injuries, and (b)(6) have conducted significant experiments to elucidate its mechanism of action in mice and in ex vivo experiments. Radiation countermeasures must be approved for human use under the FDA Animal Rule, which requires demonstration of efficacy in two species. One species may be a rodent, and is fulfilled by our murine model experiments, however the other species must be non-rodent. Our central hypothesis is that (b)(4) administration will have significant combined effects in mitigating radiation (b)(4) radiation injuries in the Gottingen minipig model of (b)(4) ARS. (b)(4) administered daily (b)(4) was demonstrated to mitigate radiation-induced damage to the kidney in swine (b)(4) (b)(4) was also demonstrated in swine to reduce cardiac remodeling after experimentally-induced myocardial infarction (b)(4) With Dr. (b)(6) we will assess in vivo protection and potential mechanisms of action of (b)(4) against radiation-induced (b)(4) injury in the minipig model of (b)(4) ARS. This research supports DoD goals of identifying novel therapeutic strategies to mitigate/treat the health consequences of ionizing radiation exposure that exist as a threat under military operations, humanitarian, and counter terrorism environments.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: BRD, PubMed, NIH RePORTER

II.2.2. Date of Search: 1/06/2015

II.2.3. Period of Search: BRD: 1998-2014; PubMed: 1985-present; NIH RePORTER: 2006-present

II.2.4. Key Words and Search Strategy:
Searches were performed using the following key word combinations:
(b)(4) + minipig + (b)(4) + radiation, or (b)(4) + minipig + radiation, or (b)(4) + minipig + radiation, or swine + radiation

II.2.5. Results of Search:

The NIH RePORTER search of all funded grants since 2006 revealed no related grants. Swine + radiation provided 123 matches, but none related to ARS. Swine + radiation + (b)(4) (b)(4) provided no matches.

The PubMed search of the list of key words minipig + radiation + (b)(4) and swine + radiation + (b)(4) provided 1 result.

1) (b)(4)

The BRD search (1998-2007): no grants were identified for minipig + radiation searches. For swine + radiation there were 4 results:

1) Implantation Techniques and Specific Absorption Rates for Swine (*Sus scrofa*) in Directed Energy Health and Safety Studies
2) Pilot study: Development of Gottingen minipig (*Sus scrofa domestica*) as radiation injury model
3) NIRVANA: Non-Ionizing Radiation Vision for a New Army
4) Extended Electro Magnetic Medical Advisor: A Diagnostic Tool for Casualty Triage by Combat Medics

III. OBJECTIVE\HYPOTHESIS:

Data from our laboratory and the literature indicate that the inhibition of (b)(4) activation is protective against (b)(4) injuries from radiation in the murine model of radiation-induced (b)(4) syndrome. We hypothesize that inhibition of (b)(4) to inhibit (b)(4) activation may mitigate radiation induced (b)(4) injury by reducing the loss of mature blood cells, sparing (b)(4) cells, and enhancing (b)(4) (b)(4) recovery in the Gottingen minipig model of (b)(4) syndrome. We anticipate an improvement in survival of at least 30%.

IV. MILITARY RELEVANCE:

The Department of Defense (DoD) is prepared to execute military missions within radiation environments, to manage radiation crises associated with terrorist activities, and to manage consequences in the event of nuclear weapons detonation. Recent studies indicate that there is an increasing risk of exposure to ionizing radiation to military personnel due to increasing terrorist activities, including potential use of stolen nuclear weapons, the detonation of an improvised nuclear device (IND) or a large radiological dispersal device (RDD) (b)(4) (b)(4) High dose ionizing radiation results in short term, acute injuries including (b)(4) syndromes. However, both high and low dose radiation (as may result from a radiation dispersal device) can cause delayed (b)(4) as demonstrated by previous radiation accidental exposures. For example, individuals accidentally exposed to 8-24 Gy radiation in the Tokai-mura, Japan radiation accident were successfully treated for acute radiation injuries but succumbed to delayed respiratory failure between 82-210 days

postirradiation (b)(4) A radiation accident victim in Belarus also recovered from acute injuries but died from radiation-induced (b)(4) 130 days following a 10 Gy exposure (b)(4)

At present, there are no FDA approved medical countermeasures for mitigation or treatment of acute or delayed radiation injuries. The development of new medical radiological countermeasures will significantly enhance the warfighting mission by sustaining unit effectiveness and are critical for operational preparedness in nuclear/radiological threat environments. These agents are essential for the prevention of radiation casualties and preserving the Warfighter's effectiveness. One of the primary focuses of the DoD medical radiological defense research area is to develop broad-spectrum postirradiation therapeutics effective against acute radiation syndrome (April, 2009).

The DoD is committed to protecting the Warfighter from the adverse health effects of ionizing radiation. Our research is aimed at developing an effective postirradiation countermeasure to: 1) reduce the casualty load at medical treatment facilities, 2) sustain a more effective operational force after a radiation exposure event, 3) allow commanders to conduct operations in radiation field environments without undue risk of decremented performance of personnel due to acute tissue injury, and to 4) reduce the negative psychological impact on personnel tasked to operate in contaminated environments.

The research outlined in this proposal is aimed at developing effective therapeutic strategies for the combined treatment of acute and late radiation-induced injuries for the Warfighter. Results from these studies will additionally have application for civilian use and the medical community at large.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

Male Gottingen minipigs will be obtained from (b)(4) at age of approximately 4 months, and at a weight appropriate for healthy animals of that age range. Minipigs will be housed singly to avoid injuries, and fed (b)(4) according to their weight, following recommendations from the vendor (b)(4) and IAW USUHS-LAM SOP 22-205 Routine Husbandry of Pigs. Animal care and use procedures will only be performed by trained and certified personnel.

Animals will have 1-2 weeks to acclimate to the USUHS LAM (see time line, below). A blood draw will be made from each animal to establish baseline blood cell levels.

(b)(6) Animals will be transported under anesthesia to (b)(6) for (b)(4) irradiation in the (b)(6) facility (see V.4.1.2.2.). Sham irradiated animals will be anesthetized, and placed in transport and moved to (b)(6) but not irradiated. (b)(4) (b)(4) will be freshly injected into a grape or marshmallow for the animal to eat (b)(4)

Health of animals will be monitored twice-daily, and temperatures will be taken for each animal *once* a day in the morning. CBC/differential will be monitored on the days (b)(4) (b)(4) except in emergency cases, where blood for (b)(4) will be taken by LAM request. These experiments will utilize the husbandry, irradiation, and blood draw procedures perfected in the studies performed under previously approved protocols at (b)(6)

Animals will be euthanized on day (b)(4) Tissues and blood samples may be taken at the time of euthanasia and shared with other investigators for biodosimetric and mechanistic studies.

Secondary data-points will be standard hematology measurements in peripheral blood (b)(4) etc.), body temperature, body weight, and occurrence of bloody stools. On day (b)(4) each surviving minipig will be euthanized, blood taken for (b)(4) and tissues collected for histopathology and tissue repository.

Time Line for experiment:

(b)(4)

V.1.1. Experiment 1: Effects of (b)(4) treatment on (b)(4) (b)(4) in Gottingen minipigs.

Our experiments will be conducted with Gottingen minipigs. Animals will be either sham irradiated or receive (b)(4) irradiation in the (b)(6) facility. We will determine the effect of (b)(4) on normal (b)(4) after (b)(4) of treatment: 1) (b)(4) cell analysis; 2) histological analysis of (b)(4) (b)(4) 3) the levels of (b)(4) cytokines in the blood; and 4) extramedulary (b)(4) will be determined by cellular analysis of the spleen and liver. (b)(4) analysis, and spleen and liver analysis can be performed using the same animals.

Treatment groups will be: 1) sham irradiated (b)(4) 2) sham irradiated + (b)(4) 3) (b)(4) irradiation (b)(4) 4) (b)(4) irradiation + (b)(4) (b)(4) Survival will be determined at (b)(4) Samples of blood will be obtained on days - (b)(4) and at the time of euthanasia (Time 0 is the time of (b)(4) irradiation). The (b)(4) and livers will be removed for analysis. We (b)(4) require a total of N = animals per group, and 4 iterations total for this pilot experiment, (b)(4) giving a total of N = per group. The iterations would be performed over a period of (b)(4) (b)(4) will be required to complete the experiments since animals will be singly housed, which means that experiments can only be conducted when there is sufficient space in LAM for the experiment. Also (b)(6) will be shutting down their (b)(6) facility for (b)(6) meaning that there will be significant delays for the experiments to be performed. We want to ensure that we can complete this pilot to obtain clear data to determine whether a larger scale experiment should be performed. This will be sufficient to provide proof of concept for protection by (b)(4) prior to initiation of a larger study.

(b)(4) animals/group x 4 treatment groups = animals
(b)(4) One experimental repeated for 4: total = animals
Groups with no pain (Category C): (b)(4)
Groups with alleviated pain (Category D): (b)(4)
Groups with non-alleviated pain (Category E): (b)(4)

V.2. Data Analysis:

(b)(4) histology will be scored by a blinded veterinary pathologist. Experiments investigating the effects of (b)(4) (b)(4) will be done for each endpoint using ANOVA in 2^2, 2^3, and 2^4 designs. These analyses may also include replication. Other statistical analyses will be used where found appropriate. Based on previous experience in our laboratory an N of s enough to obtain statistical (b)(4) significance for survival in swine and to obtain statistical significance for other (b)(4) (b)(4) data (b)(4) Although space
(b)(4) requirements only allow N = group per iteration, we will obtain sufficient N for (b)(4) analysis after 2 iterations. For survival, a higher N is required. We believe that N = (b)(4) will be sufficient for proof of principle under the current funding.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:
For the last 9 years, we have performed extensive experiments in primary cell culture systems to investigate the effects of radiation on normal cells. However, our culture findings have not yet been shown to completely recapitulate the complexity of the whole animal system. We will continue to use primary cell culture whenever possible to avoid unnecessary use of animals. We have also considered computer models as alternatives to animals. However, this alternative also lacks the knowledge base and complexity needed to evaluate fibrosis and preventative agents.

Because we do not know all the cell types and tissues involved in radiation injury, and how these systems interact with one another for repair or for extension of initial radiation damage, there is vital information that can be gained only from the use of a whole animal system. Up until now, we have utilized mice as the least sentient whole animal system for our research of (b)(4) as a radiation countermeasure. However, in order to advance our research to FDA requirements under the Animal Rule, we must investigate the efficacy of (b)(4) in another, non-rodent species.

V.3.2. Animal Model and Species Justification:
Tissue culture conditions cannot fully substitute for the complexities of whole organisms in response to radiation injuries. Such injury involves the interactions between the (b)(4) system and other organ systems. In order to develop viable treatments for humans, we find that our research must be conducted in whole animals.

Dr. (b)(6) have investigated the Gottingen minipig as an additional large animal model for the study of (b)(4) radiation injury and

for the development of radiation countermeasures (b)(4) (b)(4) Large, long-lived, non-rodent animal models are required for drug studies submitted to the FDA for licensure applications. Our previous studies have utilized mice for the investigation of (b)(4) timing of administration, and mechanism(s) of action as a radiation countermeasure.

V.3.3. Laboratory Animals

		Strain #1	Strain #2
V.3.3.1.	**Genus & Species:**	*Sus scrofa domestica*	
V.3.3.2.	**Strain/Stock:**	Gottingen	
V.3.3.3.	**Source/Vendor:**	(b)(4)	
V.3.3.4.	**Age:**	4-6 months	
V.3.3.5.	**Weight:**	Standard (7-13 kg)	
V.3.3.6.	**Sex:**	Male	
V.3.3.7.	**Special Considerations:**	None	

V.3.4. Number of Animals Required (by Strain): *Sus scrofa domestica*
Gottingen = (b)(4)

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:
The study data-points currently mandated by the FDA for approval of radiation countermeasures under the Animal Efficacy Rule is mortality. Hence the primary study data-point in our development of Gottingen minipigs as a model for ARS is survival. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress, using an extensive set of criteria (see animal endpoints, paragraph V.4.5 – Lethargy, weight loss, temperature, respiratory distress, food intake, bleeding etc). Animal care and use procedures will only be performed by trained personnel. Extensive human interaction every weekday will reduce stress in the animals. Use of slings and anesthesia will minimize distress during (b)(4) collections and the animals will be gradually acclimated to the slings for these procedures.

V.3.5.2. Reduction:
Blood samples obtained from minipigs before irradiation will serve as controls, thus reducing the number of animals required for this study. Use of historical controls will reduce the number of animals needed for this study.

V.3.5.3. Replacement:

Our laboratory has made extensive use of primary cell culture systems for all preceding experiments. Every effort will be made to continue to use primary cell culture whenever possible in place of animals.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Strain #1	Strain #2
V.4.1.1.1.1. Column C:	(b)(4)	
V.4.1.1.1.2. Column D:		
V.4.1.1.1.3. Column E:		

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

BLOOD DRAWS
For blood draws, animals will be placed in a sling and anesthetized with inhaled isoflorane via a nose cone delivered from a precision vaporizer. If necessary, animals will be injected with Telazol (1-2 mg/kg i.m.), acepromazine (1.1-2.2mg/kg im,iv,sc), or midazolam (0.1-0.5mg/kg im or sc) in order to place the mask on the animals . (b)(4) (b)(4) after disinfection of the area. Isoflorane will be administered using a mask (1-4%), and passive scavenging will be done by F/air charcoal canister.

IRRADIATION
Prior to transportation to (b)(6) for irradiation, animals will be anesthetized at (b)(6) with Telazol® 4.4 mg/kg i.min combination with xylazine 2mg/kg im. Alternatively, ketamine (5-20 mg/kg i.m.)/xylazine (1-2 mg/kg i.m.) can also be used for anesthesia. Atropine (0.05 mg/kg s.c., i.m. or i.v.; atropine sulfate, Butler, Columbus, OH) or glycopyrrolate (0.01 mg/kg) i.m. or i.v. may be given to reduce mucosal secretions.

BONE MARROW ASPIRATION
A bone marrow aspiration will be performed immediately prior to euthanasia. Samples will be collected prior to death to obtain the best quality sample. Animals will be anesthetized with Telazol® (4.4 mg/kg i.m) or Ketamine (5-20mg/kg i.m.) in combination with xylazine 2mg/kg im.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

Body weights will be determined at least once prior to treatment, on the first day of treatment, and on days of blood collection.

TRANSPORTATION OF ANIMALS TO (b)(6) FOR IRRADIATON

Pre-Irradiation at USUHS and Transport to (b)(6) Animals will be fasted overnight (approximately 16 – 18 h). Animals will be anesthetized with injectable drugs, placed in a large animal kennel on a wheeled cart and taken to the LAM van. The anesthetized animals will be driven to (b)(6) and monitored en route. Animals will be provided with additional anesthesia if needed prior to irradiation. The kennel containing the swine will be handed over to the (b)(6) staff.

IRRADIATION

Pre-Irradiation at (b)(6) Anesthetized at USUHS as described above. Upon arrival to (b)(6) animals will be under the care of the (b)(6) veterinary staff. At (b)(6) minipigs will be transported to the (b)(6) in an approved, covered transport cage. Heart rate and temperature will be measured prior to irradiation. At least one vet tech or one veterinarian will be present at the time of irradiation. Immediately before irradiation, the vet or vet tech will determine whether or not to administer additional sedative to keep the animal anesthetized during irradiation.

Irradiation procedure: Animals will be strapped in place into slings in polyvinylchloride PVC frames for irradiation. Effect of anesthesia will be monitored in real time through video cameras positioned in the (b)(6) waiting area. Minipigs will be removed from the slings once irradiation is completed. Temperature and heart rate will be taken before returning the minipigs to their home cages.

Post-Irradiation: After irradiation, animals will be transported back to USUHS. Animals will be returned to their cages, where they will be allowed to recover from anesthesia under the careful surveillance of LAM and/or research staff who will monitor vital signs and provide thermal support (e.g. Bair Hugger blanket) if necessary (b)(4)

Preparation of the animals: Animals will be fasted overnight prior to blood draws. Blood draws will be performed by trained research and veterinary staff. For blood draws, animals will be placed in slings and sedated using inhaled isoflorane via a mask. If necessary, Telazol (1-2 mg/kg i.m.), acepromazine (1.1-2.2mg/kg im,iv.sc), or midazolam (0.1-0.5mg/kg im or sc).will be injected prior to isoflorane. 2-6 ml of blood will be withdrawn from the cephalic, saphenous, ear vein or other peripheral vein after the area has been disinfected. Blood draws will occur on days (b)(4) (see time line for experiment).

After blood draw, animals will be returned to their cages and checked to ensure that the needle puncture wound is closed and the blood has clotted.

BONE MARROW ASPIRATION

A bone marrow aspiration will be performed immediately prior to euthanasia. Samples will be collected prior to death to obtain the best quality sample. Animals will be anesthetized with Telazol® (4.4 mg/kg i.m) or Ketamine (5-20mg/ kg im) in combination with xylazine (2mg/kg im). Bone marrow espiration will be performed with a standard bone marrow aspiration needle (Jamshidi). The skin will be disinfected using isopropyl alcohol. Samples will be collected from the medial aspect of the proximal tibia, approximately at the level of the tibial crest. Alternatively, bone marrow may be aspirated from the dorsal aspect of the tuber coxae or midsternum.

V.4.1.2.3. Paralytics: Not applicable

V.4.1.3. **Literature Search for Alternatives to Painful or Distressful Procedures:**

V.4.1.3.1. **Sources Searched:** Agricola, Pubmed

V.4.1.3.2. **Date of Search:** January 8, 2015

V.4.1.3.3. **Period of Search:** All available

V.4.1.3.4. **Key Words of Search:**
The aspects of the study relevant to alternatives to painful or distressful procedures are the sequelae to exposure to [redacted] at doses causing ARS. We used the following key words in the search for alternatives: swine, radiation/ionizing radiation/gamma radiation, ARS/hematology, sequelae, pain, distress, alternatives.

(b)(4)

V.4.1.3.5. **Results of Search:**
PubMed search: There were a number of reports for the searches that were relevant for UV radiation, but these were not included in our analysis.

Swine + ionizing radiation + pain: 2 reports

Riccobono D et al. 2014 Transient gene therapy to treat cutaneous radiation syndrome development in a minipig model. Health Phys 106:713-9.

Agay E et al. 2010 Multipotent mesenchymal stem cell grafting to treat cutaneous radiation syndrome: development of a new minipig model. Exp Hematol 38: 945-56.

The two published manuscripts were investigations of mesenchymal stem cells for the treatment of cutaneous radiation syndrome in the minipig model. Although pain is mentioned in these manuscripts, management of pain is not discussed.

Swine + radiation/ARS + distress: 0 12 reports (not relevant)

Advances in Swine Biomedical Model Genomics. Lunney JK
Int J Biol Sci. 2007; 3: 179–184.

Pacing-Induced Regional Differences in Adenosine Receptors mRNA Expression in a Swine Model of Dilated Cardiomyopathy. Del Ray S, et al.
PLoS One. 2012; 7

Experimental Infection of Pigs with the Human 1918 Pandemic Influenza Virus
Weingartl HM, et al. J Virol. 2009; 83: 4287–4296.

A defect in dystrophin causes a novel porcine stress syndrome. DJ Nonneman, er al. BMC Genomics. 2012; 13: 233.

Guidelines on Management of Human Infection with the Novel Virus Influenza A (H1N1) – A Report from the Hospital das Clinicas of the University of São Paulo. Hajjar LA, et al. Clinics (Sao Paulo) 2009; 64: 1015–1024.

A Reassortment-Incompetent Live Attenuated Influenza Virus Vaccine for Protection against Pandemic Virus Strains. Hai R, et al. J Virol. 2011; 85: 6832–6843.

Deletions in the Neuraminidase Stalk Region of H2N2 and H9N2 Avian Influenza Virus Subtypes Do Not Affect Postinfluenza Secondary Bacterial Pneumonia. Chockalingam AK, et al. J Virol. 2012 April; 86: 3564

Safety assessment of probiotics for human use. Sanders ME, e. Gut Microbes. 2010; 1: 164–185.

Epidemiology of Human Infection with the Novel Virus Influenza A (H1H1) in the Hospital das Clínicas, São Paulo, Brazil 2009. Schout D, et al. Clinics (Sao Paulo) 2009; 64: 1025–1030.

After the bomb drops: A new look at radiation-induced multiple organ dysfunction syndrome (MODS) JP Williams, WH McBride. Int J Radiat Biol 2011; 87: 851–868.

Safety, Immunogencity and Efficacy of a Cold-Adapted A/Ann Arbor/6/60 (H2N2) Vaccine in Mice and Ferrets. Chen GLet al. Virology. 2010 1; 398: 109–114.

Why is the doctor in the Merry Wives of Windsor called Caius? McNair Med Hist. 1969; 13: 311–339.

Swine + radiation + alternatives: 10 reports (not relevant)

Advances in Swine Biomedical Model Genomics Lunney JK. Int J Biol Sci. 2007; 3:179–184.

A Comparison of the Growth Responses Following Intramuscular GHRH Plasmid Administration Versus Daily Growth Hormone Injections in Young Pigs. Khan AS, Draghia-Akli R, Shypailo RJ, Ellis KI, Mersmann H, Fiorotto ML. Mol Ther. 2010; 18: 327–333.

Comparison of dkgB-linked intergenic sequence ribotyping to DNA microarray hybridization for assigning serotype to Salmonella enterica. Jean Guard, Roxana Sanchez-Ingunza, Cesar Morales, Tod Stewart, Karen Liljebjelke, JoAnn Kessel, Kim Ingram, Deana Jones, Charlene Jackson, Paula Fedorka-Cray, Jonathan Frye, Richard Gast, Arthur Hinton, Jr. FEMS Microbiol Lett. 2012 December; 337(1): 61–72.

Society of Nematologists 2011 Meeting: Abstracts: Alphabetically by first author. J Nematol. 2011 Sep-Dec; 43(3-4): 223–297.

Antimicrobial Susceptibility to Azithromycin among Salmonella enterica Isolates from the United States. Sjölund-Karlsson M, et al. Antimicrob Agents Chemother. 2011; 55: 3985–3989.

A critical analysis of disease-associated DNA polymorphisms in the genes of cattle, goat, sheep, and pig. Ibeagha-Awemu EM, et al. Genome. 2008; 19: 226–245.

Competitive exclusion treatment reduces the mortality and fecal shedding associated with enterotoxigenic Escherichia coli infection in nursery-raised neonatal pigs. Genovese KJ, et al. Can J Vet Res. 2000; 64: 204–207.

How sustainable agriculture can address the environmental and human health harms of industrial agriculture. Horrigan L, Lawrence RS, Walker P. Environ Health Perspect. 2002; 110: 445–456.

Swine + radiation + ARS/hematology: 0 reports

Swine + radiation + sequelae: 2 reports

Temporal modulation of TGF-beta1 and beta actin gene expression in pig skin and muscular fibrosis after ionizing radiation. Martin M et al. 1993 Radiat Res 134:63-70.

Mechanisms of action for an anti-radiation vaccine in reducing the biological impact of high dose and dose-rate, low-linear energy transfer radiation exposure.Maliev V et al. 2007 Radiats Biol Radioecol 47:286-91.

The reports examined alterations in swine tissue following radiation exposure, but pain was not discussed.

AGRICOLA

Swine + radiation/ARS + pain: 0 reports

Swine + radiation/ARS + distress: 0 reports

Swine + radiation/ARS + alternatives: 7 reports (not relevant)

Evaluation of a porcine lens and fluorescence assay approach for in vitro ocular toxicological investigations. Oriowo, Om NAL Catalog (AGRICOLA)

Energy for swine facilities II Alternative sources of energy Fehr, RI NAL Catalog (AGRICOLA)

Molecular characterization, chromosomal location, alternative splicing and polymorphism of porcine GFAT1 gene Liu, K. NAL Catalog (AGRICOLA)

Evaluation of alternative techniques to determine pork carcass value Akridge, Jt NAL Catalog (AGRICOLA)

Genomic structure, chromosomal localization and expression profile of a porcine long non-coding RNA isolated from long SAGE libraries Ren, H. NAL Catalog (AGRICOLA)

Environmental Prevalence and Persistence of Salmonella spp in Outdoor SwineWallows Callaway, Tr NAL Catalog (AGRICOLA)

Factors affecting cellular outgrowth from porcine inner cell masses in vitro Schilperoort-Haun, Kr NAL Catalog (AGRICOLA)

Swine + radiation/ARS + hematology: 0 reports

Swine + radiation/ARS + sequelae: 0 reports

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

Due to the nature of ionizing radiation and the intended treatment given, animals are expected to die from a compromised immune system, opportunistic infections and acute radiation syndrome. The remaining surviving animals are dependent on the countermeasure given. Administering systemic anesthetic agents to animals after irradiation are known to interact with the immune system (see references in (b)(4) (b)(4) (b)(4) As a result, this will skew our results and would not be productive. Alternatively, we are providing supportive care in terms of fluids/

nutritional support to increase survival. Use of supportive care is expected to mitigate the pain associated to the sequelae of irradiation. Although we have utilized mice for most of our studies as the least sentient animals to provide preclinical information about countermeasures against the toxic effects of radiation, in order to develop (b)(4) as a countermeasure for future use for humans, efficacy of the drug must be demonstrated in an additional non-rodent species, in compliance with the Food and Drug Administration (FDA) Animal Rule. We have selected swine as a species currently acceptable by the FDA for acute radiation syndrome drug testing.

V.4.2. Prolonged Restraint:

Only short periods of restraint in the panepinto sling (<30 minutes) will be necessary for blood collections and the irradiation procedure. To minimize discomfort, a new type of sling that uses softer, elastic material to support the animal, and utilizes a more efficient restraint design that maintains the animal in place while reducing manipulation by technicians and consequently stress to the animals.

After the acclimation period, animals are acclimated to the sling for 5-10 minutes, 2-3 times before initiating blood draws. Acclimation to the sling is very rapid and non-stressful; animals are petted and gently spoken to while restrained. It has been the experience of Dr. (b)(6) group that resting on the sling does not agitate the animal at all.

Animals are anesthetized at USUHS, transported to (b)(6) (b)(6) holding area before being put into slings for irradiation (<20 minutes). Animals will be kept under continuous observation while in the sling. Slings will be cleaned with a towel after each use, and washed weekly and sanitized.

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: Not applicable

V.4.3.2. Procedure: Not applicable

V.4.3.3. Post-surgical Provisions: Not applicable

V.4.3.4. Location: Not applicable

V.4.3.5. Surgeon: Not applicable

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: Not applicable

V.4.3.6.2. Scientific Justification: Not applicable

V.4.4. Animal Manipulations:

V.4.4.1. Injections:
All injections will be administered i.m., i.v., i.c., or s.c. using sterile needles (23 – 21 gauge) and syringes. Specific drugs for injection are defined below:

Drugs for injection:
Atropine/Atropine sulfate 0.04 mg/kg. s.c., i.v., or i.m.
Euthasol® (sodium pentobarbital, 1 ml/4.5 kg i.v. or i.c.
Glycopyrrolate 0.01 mg/kg i.m. or i.v.
Ketamine/xylazine 5 - 20 mg/kg i.m. /xylazine 1 -2 mg/kg i.m.
Telazol/xylazine 4.4 mg/kg i.m./2 mg/kg i.m
Telazol 1-2mg/kg i.m.
Acepromazine 1.1-2.2mg/kg im,iv.sc
Midazolam 0.1-0.5mg/kg im or sc

V.4.4.2. Biosamples:
Blood samples will be collected from the cephalic, saphenous, ear vein, or other peripheral vein. Animals will be anesthetized with inhaled isoflorane with injected Telazol (1-2 mg/kg i.m.), Acepromazine (1.1-2.2mg/kg im,iv.sc), or Midazolam (0.1-0.5mg/kg im or sc), if necessary prior to isoflurane delivery. Blood will be drawn after disinfection of the area with 70% ethanol using a 21-25 gauge needle. Blood draws will be made on days (b)(4) (b)(4) see experimental time line); day 0 is the day of irradiation. This will be performed as it is done routinely in the previous minipig protocols by Dr (b)(6) Each sample will be a maximum of 6 ml in volume or less than 1% total blood volume (whichever is less) and will be collected from the ear vein, saphenous vein, or leg vein.

Tissue and histological samples:
Tissue samples, to include organs, (b)(4) and hairs, may be taken at the time of euthanasia for slide preparation as well as for frozen tissue repository, and may be shared with other investigators.

V.4.4.3. Adjuvants: None

V.4.4.4. Monoclonal Antibody (MAbs) Production: None

V.4.4.5. Animal Identification: Tattoos or ear tags and cage cards/tags will be used.

V.4.4.6. Behavioral Studies: None

V.4.4.7. Other Procedures:

Treatment with (b)(4)
(b)(4) will
(b)(4) given twice daily orally, mg/kg. The (b)(4) will be made up in sterile saline and injected into a grape or a marshmallow and given to the animals to be eaten immediately after injection. Animals will be observed for a few minutes after the food item is given to ensure that the entire item is eaten. This measurement is based on published reports of efficacy of (b)(4) for mitigation of radiation injuries to the (b)(4) (b)(4)

V.4.4.8. Tissue Sharing:
All tissues are available for tissue sharing since our research will utilize small quantities of tissues only.

V.4.5. Study Endpoint:

The data-point currently mandated by the FDA for approval of radiation countermeasures under the Animal Efficacy Rule is moribundity or mortality. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress. Euthanasia will be carried out when any one or combination of the following signs of moribundity are observed and in consultation with USUHS veterinarians.

Absolute

1. Non responsive, assuming the animal has recovered from anesthesia.
2. Dyspnea
3. Loss of 20% of expected weight (based on Marshall Bio-resources growth chart –weight vs age- and day 0 baseline percentile).

Non-absolute

1. Hyper/hypothermia
2. Anorexia (skip 3 consecutive BID meals)
3. Anemia/pallor, CRT >2 seconds. CRT will be performed behind the ears.
4. Petechiae/ecchymosis
5. Vomiting/diarrhea
6. Lethargy
7. Seizures or vestibular signs (falling, circling or head tilt)
8. Uncontrollable hemorrhage

V.4.6. Euthanasia:

Animals will be euthanized according to current American Veterinary Medical Association (AVMA) guidelines. Animals will be anesthetized with Telazol® (4.4 mg/kg i.m.) or ketamine (5-20 mg/kg i.m.)/xylazine (2 mg/kg i.m.). A bone marrow aspiration will be performed after the animal is anesthetized. Animals will then be euthanized with Euthasol® (sodium pentobarbital, (1 ml/4.5 kg i.v. or i.c.) or another commercial euthanasia solution. Death will be confirmed by USUHS veterinarian or veterinary technician via auscultation of the heart, with cessation of sounds for at least 2 minutes or longer as per current SOP. See item V.4.5 for euthanasia criteria before the end of the study.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations:

Animals will be singly housed. This is necessary since irradiated animals will have reduced immune function and can develop infections from biting or wounds from rough play which can be common in these animals. Rooms will be maintained on a 12:12 h light (0600 to 1800) dark cycle with the temperature set to 27 to 29°C and 30% to 70% relative humidity. Commercial diet (Mini-Swine Diet 8753, Harlan Teklad Diets, Madison, WI) will be provided. Water will be provided ad libitum. Animals will be fed twice a day to control weight gain. Animals will also need to be fasted (b)(4) h prior to anesthetizing for blood draws and for transport for irradiation at (b)(6)

V.5.1.1. Study Room:

Euthanasia will be performed in LAM.

Building(s) (b)(6) Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes X No _____

Fluid Restriction: Yes _____ No X

V.5.1.3. Exceptions:
None

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be monitored for health, humane treatment, and husbandry considerations, twice daily by LAM personnel during routine weekday rounds and at least once daily on weekends/holidays. This is in addition to, and not in place of, the monitoring that is done by the PI and their staff. In the event of a debilitating illness or adverse reaction, the decision to treat or euthanize an animal will be made by either the veterinarian and/or PI.

V.5.2.2. Emergency Veterinary Medical Care:
All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy:
All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Standard enrichment for swine will be included.

V.5.3.2. Enrichment Restrictions: None. Animals will be singly housed. This is necessary since irradiated animals will have reduced immune function and can develop serious infections from biting or wounds from rough play which can be common in these animals.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Euthanasia	(b)(6)	3 yrs as researcher 6 yrs as PI 8+yrs as researcher	(b)(6)

			(b)(6)
Blood draws from ear vein or saphenous vein	Training required		
Swine handling	Training required		
Swine irradiation and monitoring	Training required		
Swine necropsy	Training required		
Anesthesia	Training required		

(b)(6) Personnel who will be involved in handling animals at (b)(6)

Name	Degree	Training	Date	Specific training	Years
(b)(6)	DVM, ACLAM, MPH	(b)(6)	2011; 2007-2010; 2007-8	All veterinary decisions and technical procedures	14 years
	DVM, MPH, ACVPM		1999-2014	All veterinary decisions, protocol decisions and technical procedures.	16 years
	DVM, MPH, ACVPM		2007-2014	All veterinary decisions and technical procedures.	8 years
	DVM, PhD, MS, ACLAM		09/2009 04/2012	All veterinary decisions and technical procedures	7 years
	DVM, PhD		Jan-May 2014	All veterinary decisions and technical training	2.5 years
	DVM		Feb 2012	All veterinary decisions and technical training	6 years
	68T		Nov. 2011	All veterinary technical procedures	5 years
	A.A.S.		March 2013	All veterinary technical procedures	4 years
	High School		July 2013	All veterinary technical procedures	1.5 years
	RVT, RALAT		June 2014	All veterinary technical procedures	11 years
	BS		July 2014	All veterinary technical procedures	10 months

VII. BIOHAZARDS/SAFETY: PPE will be used to minimize allergens produced by swine. Standard PPE include dust mask, gloves, shoe covers, and lab coats.

VIII. **ENCLOSURES**: (b)(4) data sheets

References:

(b)(4)

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) _____ March 23 2015

Prin ure Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) _____ March 23 2015

Prin e Date

I. Painful Procedure(s):

I am **NOT** conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(4)

Principal Investigator

March 23 2015

Date

X. PROTOCOL ABSTRACT:

High dose total body irradiation (TBI) in humans can result in both acute and delayed injuries. The Acute Radiation Syndrome (ARS) is characterized by the (b)(4) (b)(4) syndromes. The mechanisms leading to (b)(4) ARS remain largely unknown, but current information indicate that (b)(4) may play a major role in (b)(4) There are currently no approved countermeasures for radiation-induced (b)(4) injuries, and it is our long range goal to develop effective agents for this condition. The objective of this application is to evaluate the effects of an Food and Drug Administration (FDA)-approved drug, (b)(4) for mitigating (b)(4) radiation injuries. (b)(4) (b)(4) as a mitigator of radiation-induced (b)(4) injury using a murine model for (b)(4) injuries, and we have conducted significant experiments to elucidate its mechanism of action in mice and in ex vivo experiments. Radiation countermeasures must be approved for human use under the FDA Animal Rule, which requires demonstration of efficacy in two species. One species may be a rodent, and is fulfilled by our murine model experiments, however the other species must be non-rodent. Our central hypothesis is that (b)(4) administration will have significant combined effects in mitigating radiation (b)(4) radiation injuries in the Gottingen minipig model of (b)(4) ARS. With Dr. (b)(6) we will assess in vivo protection and potential mechanisms of action of (b)(4) against radiation-induced (b)(4) injury in the minipig model of (b)(4) ARS. This research supports DoD goals of identifying novel therapeutic strategies to mitigate/treat the health consequences of ionizing radiation exposure that exist as a threat under military operations, humanitarian, and counter terrorism environments. We anticipate an improvement in survival of at least 30%.

A. **Animal Protocol Number**: To be assigned

B. **Animal Protocol Title**: Development of (b)(4) for the mitigation of acute injuries from ionizing radiation in Gottingen minipigs (*Sus scrofa domestica*)

C. **Principal Investigator**: (b)(6) Ph.D.

D. **Performing Organization**: USUHS/AFRRI

E. **Funding**: DMRDP

Objective and Approach

We will assess in vivo protection and potential mechanisms of action of (b)(4) against radiation-induced (b)(4) injury in the minipig model of (b)(4) acute radiation syndrome. We will administer (b)(4) orally to Gottingen minipigs following (b)(4) (b)(4) irradiation (b)(4) recovery and survival will be monitored as well as indicators of radiation-induced (b)(4)

Indexing Terms (Descriptors): radiation, acute (b)(4) injury, radiation countermeasure, swine, minipig (b)(4) irradiation

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

February 1, 2016

MEMORANDUM FOR DR. (b)(6)

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on February 1, 2016:

Animal Protocol Title: "Development of (b)(4) for the Mitigation of (b)(4) Injuries from Ionizing Radiation in Mice (Mus Musculus)"

USUHS Protocol Number: (b)(6)

Expiration Date: January 31, 2019

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MA (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Development of (b)(4) for the mitigation of (b)(4) injuries from ionizing radiation in mice (*Mus musculus*)

GRANT TITLE (if different from above): Advanced Development of (b)(4) as a Mitigator for (b)(4) Ionizing Radiation Injuries

DH **USUHS PROJECT NUMBER:** ~~new protocol number~~ (b)(6)

FUNDING AGENCY: DMRDP

EARLIEST ANTICIPATED FUNDING START DATE: May 19, 2014

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6) — Signature | (b)(6) — Department, Office/Lab Telephone | Dec 9, 2015 — Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is cons(b)(6)ntific research practice.

(b)(6) — Research Unit Chief / Dept. Head Signature | Chairman — Title | (b)(6) — Telephone | 12/9/15 — Date

Typed Name: Dr. (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the stu(b)(6)

Sta(b)(6) — Department, Telephone | Dec. 9, 2015 — Date

Typ

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) — Attending/Consulting Veterinarian Signature | LAM — Department | (b)(6) — Telephone | Dec 9, 2015 — Date

Typed Name: MAJ (b)(6)

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D., (b)(6)
(b)(6)

ANIMAL PROTOCOL TITLE: Development of (b)(4) for the mitigation of (b)(4) injuries from ionizing radiation in mice (*Mus musculus*)

GRANT TITLE (if different from above): Development of (b)(4) for the mitigation of (b)(4) from ionizing radiation in mice (*Mus musculus*)

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): none

TECHNICIANS(S): (b)(6)

I. **NON-TECHNICAL SYNOPSIS:** The terrorist attack of September 11, 2001 against the United States has increased the concern that terrorists may acquire and use radioactive sources (e.g. dirty radioactive bombs) to expose military and civilian populations to ionizing radiation. However, despite this concern, there are currently no FDA approved drugs that can be used for the general population to prevent death that is likely to result from exposure to ionizing radiation. (b)(4) irradiation in humans can result in both (b)(4) (b)(4) injuries. However, because rodents are more resistant to pulmonary injuries than humans, there is currently no murine model that has been developed to study (b)(4) (b)(4) radiation injuries in the same animal.

(b)(4)

(b)(4) In fact, we have recently found that (b)(4) can be administered h postirradiation up to h postirradiation (b)(4) to provide statistically similar levels of mitigation. Our proposed studies here will expand our understanding of the mechanism for the effects of (b)(4) for protection against (b)(4) (b)(4) radiation injuries in animal models for each of these injuries.

Our overall aim is to develop an effective, non-toxic radioprotectant/ radiotherapeutic treatment strategy that can be used for protection against both (b)(4) effects of radiation exposure in a murine model for both types of radiation injury. Our laboratory has characterized the use of (b)(4) as a radiation countermeasure for (b)(4) syndrome in mice. We have demonstrated in mice the required time course for administration of (b)(4) and dosages of the drug required for preventing mortality at the 50% lethal dose at (b)(4) (b)(4) levels of radiation exposure. Our research has also demonstrated that (b)(4) suppresses radiation-induced (b)(4) and other tissues, mitigates radiation-induced (b)(4) and reduces the induction of (b)(4) induced in response to radiation damage (b)(4)

(b)(4) We have also found that (b)(4) reduces radiation-induced (b)(4) (b)(4) when administered from h postirradiation through days (b)(4) postirradiation. We wish to determine whether a shorter time course of (b)(4) administration would have similar effects.

II. BACKGROUND:

II.1. Background:

The acute toxic effects of exposure to high doses of ionizing radiation in humans have been shown to occur due to two sequential effects: first the ablation of immune cells, resulting in loss of immune function and increasing likelihood of pathogenic infection [1]; and subsequently the loss of epithelial progenitor cells of the gastrointestinal (GI) crypt [2, 3]. Both of these events have been shown to be due to the loss of fast-growing pluripotent and/or progenitor cell types. The time course of the acute injuries correlates with the life span of the mature cell types of the tissue, which are not replaced because of radiation damage to the adult pluripotent cells and adult stem cells. There is currently no FDA approved prophylactic or treatment for prevention of acute radiation injuries following high-dose (b)(4) radiation exposure.

In some cases supportive measures can be used to treat acute radiation syndrome, and survival from (b)(4) radiation exposure is now possible [4, 5]. This results in current efforts to mitigate or treat later stage effects of ionizing radiation exposure (b)(4)

(b)(4)

In humans, death from (b)(4) can occur following survival from (b)(4) syndrome (b)(4) Cumulative evidence from a number of laboratories, including our own, shows that there is a threshold dose of radiation of (b)(4) to induce sufficient (b)(4) for the (b)(4) and (b)(4) phases to occur in mice; below these doses, no permanent injury manifests, although some short-term effects are observed (b)(4) However, in humans, there is currently no accepted protocol for preventative treatment of radiation-induced (b)(4) and morbiditiy as high as 50% is associated with each phase [6] (b)(4) (b)(4) injuries from radiation is considered to be a serious problem in the field for the development of agents to prevent these radiation injuries.

(b)(4) **as a radioprotectant and an antifibrotic agent:** (b)(4) (b)(4) and its proteolytic product (b)(4) are believed to have their primary biological functions in blood pressure homeostasis. However (b)(4) have been demonstrated to be upregulated in fibrotic tissues. Inhibition of (b)(4) or blockade of the (b)(4) receptors on cells has been shown to ameliorate drug-induced (b)(4) in murine and rat models for (b)(4) (b)(4) receptor antagonists also prevent (b)(4) following ischaemia/reperfusion in rat models of (b)(4) diseases (b)(4) Several studies have also shown that inhibition of (b)(4) signaling mitigates radiation-induced injuries, including to the (b)(4) One study shows that the (b)(4) reduces acute damage to the (b)(4) in mice following exposure to (b)(4) This work examined only initial tissue injury and

(b)(4) did not extend findings past [redacted] days, and did not examine mortality. A second study found a reduction in (b)(4) in rats exposed to (b)(4) radiation (b)(4)

Preliminary Studies:

(b)(4)

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: DTIC, PubMed, NIH RePORTER

II.2.2. Date of Search: 11/19/2015

II.2.3. Period of Search: DTIC: 1998-2014; PubMed: 1985-present; NIH RePORTER: 2006-present

II.2.4. Key Words and Search Strategy:

Searches were performed using the following key word combinations:
(b)(4) + radiation + murine/mouse (b)(4) + radiation + (b)(4) injury, + radiation + lung

II.2.5. Results of Search:

1) BRD: (b)(4) + radiation or (b)(4) + radiation
Two grants were funded to examine the activity of (b)(4)

(b)(4)

2) A RePORTER search revealed 2 current grants studying the effects of (b)(4) on radiation induced injuries for (b)(4)

(b)(4)

(b)(4)

None of the funded studies duplicate our research of (b)(4) and radiation-induced (b)(4) injuries.

3) PUBMED Search *6/3/15*
None of the publications available described a murine model for both (b)(4) (b)(4) injuries from radiation.

(b)(4)

(b)(4)

Summary of other publications

(b)(4)

III. OBJECTIVE\HYPOTHESIS:

Our objective is to: 1) examine the effects of (b)(4) administration on radiation-induced (b)(4) using the (b)(6) designed for the irradiation of small animals that we have utilized for the past 4 years for studying (b)(4) ; 2) to examine the effects of (b)(4) administration and (b)(4) of administration on radiation-induced (b)(4) using the (b)(6) and 3) examine the mechanism of delayed and limited time course of administration of (b)(4) for prevention of (b)(4)

IV. MILITARY RELEVANCE:

The Department of Defense (DoD) is prepared to execute military missions within radiation environments, to manage radiation crises associated with terrorist activities, and to manage consequences in the event of nuclear weapons detonation. Recent studies indicate that there is an increasing risk of exposure to ionizing radiation to military personnel due to increasing terrorist activities, including potential use of stolen nuclear weapons, the detonation of an improvised nuclear device (IND) or a large radiological dispersal device (RDD) (b)(4) (b)(4) High dose ionizing radiation results in short term, acute injuries including hematopoietic and GI syndromes. However, both high and low dose radiation (as may result from a radiation dispersal device) can cause (b)(4) as demonstrated by previous radiation accidental exposures. For example, individuals accidentally exposed to (b)(4) radiation in the Tokai-mura, Japan radiation accident were successfully treated for acute radiation injuries but succumbed to (b)(4) between 82-210 days postirradiation (b)(4) A radiation accident victim in

Belarus also recovered from acute injuries but died from radiation-induced (b)(4) 130 days following a (b)(4) exposure (b)(4)

At present, there are no FDA approved medical countermeasures for mitigation or treatment of acute or delayed radiation injuries. The development of new medical radiological countermeasures will significantly enhance the warfighting mission by sustaining unit effectiveness and are critical for operational preparedness in nuclear/radiological threat environments. These agents are essential for the prevention of radiation casualties and preserving the Warfighter's effectiveness. One of the primary focuses of the DoD medical radiological defense research area is to develop broad-spectrum postirradiation therapeutics effective against acute radiation syndrome (b)(4) (b)(4)

The DoD is committed to protecting the Warfighter from the adverse health effects of ionizing radiation. Our research is aimed at developing an effective postirradiation countermeasure to: 1) reduce the casualty load at medical treatment facilities, 2) sustain a more effective operational force after a radiation exposure event, 3) allow commanders to conduct operations in radiation field environments without undue risk of decremented performance of personnel due to acute tissue injury, and to 4) reduce the negative psychological impact on personnel tasked to operate in contaminated environments.

The research outlined in this proposal is aimed at developing effective therapeutic strategies for the combined treatment of acute and late radiation-induced injuries for the Warfighter. Results from these studies will additionally have application for civilian use and the medical community at large.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

(b)(4) radiation used to induced (b)(4) irradiation will be performed using the (b)(6) irradiator in (b)(6) Thirty minutes prior to irradiation, the (b)(6) irradiator and lead shield will be decontaminated thoroughly with MB10. Sham irradiation, where indicated, will be performed by placing the mice in jigs for equal lengths of time with no irradiation.

V.1.1. Experiment 1 Determine the effect of delayed administration of (b)(4) on survival following (b)(4) irradiation. mice: in category E)

(b)(4) (b)(4)

Rationale: Experiment 1 will be used to determine whether (b)(4) can be administered in a delayed time course following radiation exposure, and to determine the shortest time course of administration. Our experiment will be based on findings by (b)(4) showing that another (b)(4) can mitigate radiation-induced (b)(4) when administered starting days postirradiation continued through days postirradiation. Our laboratory also found that (b)(4) could mitigate radiation-induce (b)(4) injuries when initiated h postirradiation through weeks postirradiation; survival was not statistically different from administration of (b)(4) from h postirradiation through weeks postirradiation.

(b)(4) (b)(4) (b)(4) (b)(4)

(b)(4) will be provided in the drinking water at (b)(4) to give a dosage of (b)(4) per day for the duration of the experiment starting on day 0. Our preliminary findings indicate that there are no adverse effects due to long term use of (b)(4) We have used this drug in the water to treat mice for up to days with no findings of adverse effects. Mice will be exposed to (b)(4) (b)(4) x-ray irradiation (b)(4) dose rate, in the (b)(6)

(b)(4)

Acidified water will be provided for all mice to reduce opportunistic infections that occur following (b)(4) irradiation. For mice to be treated with (b)(4) will be provided in acidified water. Acidified water bottles and (b)(4) water bottles will be prepared by the (b)(4) and will be replaced 2× per week in accordance with LAM protocols. Extra water bottles will be prepared in advance and they will be available in the room where the animals are housed in case they are needed to replace a leaking water bottle.

Mice will be monitored monthly using non-invasive, unrestrained (b)(4) to determine (b)(4) For these studies (b)(4) mice are required per radiation dose to provide statistical significance; the experiment will not need to be repeated with this number of animals per group. The time point for (b)(4) is (b)(4) days, based on data from previous studies by others (b)(4) The time point for (b)(4) is (b)(4) days, based on previous studies by others (b)(4) Although breathing will be monitored, (b)(4) results will not be used as a sole indicator for euthanasia. (See section V.4.6).

Experiment 1a: To determine the optimal time of initiation of (b)(4) Treatment groups will be: 1) no treatment; 2) (b)(4) days postirradiation; 3) (b)(4) days postirradiation (b)(4) days postirradiation; 4) (b)(4) days postirradiation (b)(4) days postirradiation; 5) (b)(4) days postirradiation (b)(4) days postirradiation.

(b)(4) mice/group × 5 groups = mice, all category E

Experiment 1b: To determine the optimal time for ending (b)(4) administration for mitigation of (b)(4) The optimal time point for initiation of (b)(4) administration will be obtained in experiment 1a. In this experiment (b)(4) will be initiated at that optimal time point and continued for (b)(4) days. There will also be a no treatment group control,that will receive acidified water with (b)(4)

(b)(4) mice/group × 5 groups = mice, all category E

Endpoint: Endpoint for the experiment will be survival at days. Animals surviving to (b)(4) (b)(4) days will be euthanized and tissue will be obtained. Early time points will be taken if any animal exhibits signs or symptoms of morbidity or moribundity. Such animals will be euthanized at the time of observation.

V.1.2. Experiment 2 Determine the optimal dose of (b)(4) for improved survival following (b)(4) irradiation. (b)(4) mice: (b)(4) in category E)

Rationale: Experiment 2 will be used to determine whether (b)(4) dosage can be reduced for mitigation of delayed radiation-induced (b)(4) injuries. Our data from (b)(4) irradiation and the use of (b)(4) for protection against (b)(4) injury indicated that (b)(4) can be reduced to as little as (b)(4) in mice to obtain survival equivalent to (b)(4) We will use the optimal time course for (b)(4) administration, as identified in Experiments 1a and 1b (b)(4) will be administered at (b)(4) in acidified water). Control group will have acidified water with no drug administered. For these studies (b)(4) mice are required per radiation dose to provide statistical significance; the experiment will not need to be repeated with this number of animals per group.

(b)(4) mice/group × 3 groups = mice, all category E

V.1.2. Experiment 3 Determine the mechanism of action of (b)(4) for mitigation of (b)(4) injuries. [redacted] mice [redacted] in category E, [redacted] category C)

Rationale: Experiment 3 will be used to examine the mechanism of protection for (b)(4) against (b)(4) injuries using the newly identified optimized times of administration of (b)(4) from Experiments 1 and 2. Our data from our previous experiments indicates that (b)(4) reduces (b)(4) that can be detected at [redacted] days postirradiation. We will examine the optimal time course and optimal dosage of (b)(4) compared with our previous findings (b)(4) postirradiation). A control group will receive acidified water but no (b)(4) For this experiment, a sham irradiated group will also be required. Only [redacted] animals per group are needed. Tissues will be obtained from animals at the [redacted] days postirradiation (b)(4) tissue will be obtained for (b)(4) (b)(4) analysis. This experiment will need to be repeated once.

[redacted] mice/group x 4 groups = [redacted] mice, [redacted] category E, [redacted] category C
1 repeat, total animals (b)(4) category E, [redacted] category C

V.2. Data Analysis:

Experiments 1 and 2: Survival: Log-Rank test will be used for comparison among groups. If significant, Fisher's exact test will be used to detect significant differences across the groups in survival rates at day [redacted] Kaplan Meier curves will be displayed. N = [redacted] mice per group will have 80% power in detecting a significant difference between two groups if any, given type I error of 5% where the treatment group survival rate is at least 68%, compared to a control group survival rate of 20%. Similar statements would apply, if the treatment group survival rates are at least 83%, 78%, or 73%, compared to vehicle group survival rates of 35%, 30%, and 25%, respectively (b)(4)

Experiment 3: Analysis of variance (ANOVA) will be used to detect significant difference among the groups. If significant, then the pair-wise comparison by Tukey-Kramer will be used to identify which group is different from the other. Based on our current data from other radiation experiments for the induction of apoptotic and senescence markers and specific inflammatory cytokines, N = [redacted] mice per group could have 80% power to detect any significant difference between groups if any, given type I error of 5% and delta/sigma has at least 1.4, where the delta is the estimated mean differences between the two groups and sigma is the pooled standard deviation of the two groups (b)(4)

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:

For the last 9 years, we have performed extensive experiments in primary cell culture systems to investigate the effects of radiation on normal cells. However, our culture findings have not yet been shown to completely recapitulate the complexity of the whole animal system. We will continue to use primary cell culture whenever possible to avoid unnecessary use of animals. We have also considered computer models as alternatives to animals. However, this alternative also lacks the knowledge base and complexity needed to evaluate (b)(4) and preventative agents. Because we do not know all the cell types and tissues involved in radiation injury, and how these systems

interact with one another for repair or for extension of initial radiation damage, there is vital information that can be gained only from the use of a whole animal system.

V.3.2. Animal Model and Species Justification:

Tissue culture conditions cannot fully substitute for the complexities of whole organisms in response to radiation injury. Such injury involves the interactions between the hematopoietic system and other organ systems. In order to develop viable treatments for humans, we find that our research must be conducted in whole animals.

Research in the field of radiation countermeasures also advocates the mouse as the best species to use for radiation protection and radiotherapy protocols: 1) mice provide the least sentient species available whose response to radiation has been clearly identified, with physiological responses to radiation and radioprotectants that are similar to human physiology, 2) extensive literature is available on responses of mice to radiation and countermeasures, 3) more reagents are available to study the relevant cell types and molecules in mice than in any other species except humans, and the availability of transgenic and knockout mice make this model extremely attractive for further research into molecular mechanisms, and 4) our research group have extensive experience with this model in radiation injury and radioprotection research.

V.3.3. Laboratory Animals

		Strain #1	Strain #2
V.3.3.1.	**Genus & Species:**	*Mus musculus*	
V.3.3.2.	**Strain/Stock:**	CBA, female. CBA mice have previously been demonstrated to be sensitive to radiation-induced (b)(4) (b)(4) in a shorter time course than C57BL/6 mice, and in a pattern that more closely resembles (b)(4) in humans (b)(4)	
V.3.3.3.	**Source/Vendor:**	(b)(4)	
V.3.3.4.	**Age:**	6-10 weeks	
V.3.3.5.	**Weight:**	Standard (17-21 g)	
V.3.3.6.	**Sex:**	Female Only females will be used in this study for the following reasons: 1) we have so far utilized female mice to lay the foundation of our studies for radiation-induced	

(b)(4)
lung injuries; 2) because of long housing periods, it is advantageous to use female mice that are less aggressive toward each other

V.3.3.7. Special Considerations: None

V.3.4. Number of Animals Required (by Strain): *Mus musculus* =(b)(4)

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:
Animals demonstrating moribundity due to radiation exposure (b)(4) (b)(4) will be considered to have arrived at the study endpoint and will be immediately euthanized (See sections V.4.5 Study Endpoint and V.4.6 Euthanasia). The literature search revealed that, at present, there are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. In some cases radiation has been demonstrated to reduce pain in laboratory mice (b)(4) and has been in humans reported to alleviate cancer related bone pain (b)(4) (b)(4) A recent report showed that administration of botulinum toxin A was effective for reduction of pain and muscle spasms after clinical radiation for treatment of tumors (b)(4) Application of natural honey 20 min prior to radiation exposure was also shown to reduce mucositis with associated pain in humans following head/neck radiotherapy (b)(4) (b)(4) it has not been determined whether honey acts as a local radioprotectant. Spinal cord stimulation has also been reported in humans to alleviate pain resulting from radiotherapy (b)(4) however, this method would not be practical for use in mice because of the extensive spinal cord manipulation required. Human administration of morphine also relieves pain (b)(4) but again this would not be practical for mice in our experiments because of side effects of the drug. In humans, exposure to total body irradiation may induce vomiting and diarrhea, causing pain and distress. However, mice are not susceptible to vomiting.

V.3.5.2. Reduction:
Whenever possible our laboratory used shared control groups between experiments. Tissues will be collected for multiple assays from the same animals further reducing the total number of animals needed. At every stage of the research, planned experiments will be reevaluated, in an attempt to reduce the numbers of experiments and eliminate repeats of experiments where statistical significance has already been obtained. We also plan to take as many tissues as we can reasonably analyze for experiments.

V.3.5.3. Replacement:
Our laboratory has made extensive use of tissue culture techniques to study the effects of (b)(4) and radiation on cells. While these studies have provided valuable

data, it is not feasible to use non-animal systems in place of animal models to address the research questions in this protocol.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Strain #1
V.4.1.1.1.1. Column C:	(b)(4)
V.4.1.1.1.2. Column D:	
V.4.1.1.1.3. Column E:	

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

We will be unable to use analgesics since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4) (b)(4) Some sedatives have been reported to contribute to postoperative (b)(4) which likely reduce some functions of radiation. Additionally, it has been shown that the biometabolism of analgesics can induce protein oxidation, which would interfere with sample analysis (b)(4)

An anesthesia protocol has been developed for use during the X-ray irradiation. Ketamine will be used with xylazine for longer anesthesia in mice to be place in restraints for (b)(4) irradiation. Under the training protocol at AFRRI in conjunction with the AFRRI veterinary staff, we found that in female CBA mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia (25 G needle) with no morbidity or mortality (b)(4) We propose to use this concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. Standard i.p. injection methods are used for injection of the anesthesia

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions:

Animals will be housed and cared for as described in Section V.5. Veterinary Care both before and after dose administrations and irradiation procedures.

V.4.1.2.3. Paralytics: Not applicable

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: DoD Bioedical Research Data Base (BRD), REPORTER, PubMED

V.4.1.3.2. Date of Search: November 19, 2015

V.4.1.3.3. Period of Search: All available

V.4.1.3.4. Key Words of Search:
Pain, analgesia, anesthesia, mice, radiation, ionizing radiation, humane, (b)(4)

V.4.1.3.5. Results of Search:
The literature search revealed that, at present, there are no alternative procedures for irradiation because its effects cannot be otherwise duplicated. In some cases radiation has been demonstrated to reduce pain in laboratory mice (b)(4) and has been in humans reported to alleviate cancer related bone pain (b)(4) (b)(4) A recent report showed that administration of botulinum toxin A was effective for reduction of pain and muscle spasms after clinical radiation for treatment of tumors (b)(4) Application of natural honey 20 min prior to radiation exposure was also shown to reduce mucositis with associated pain in humans following head/neck radiotherapy (b)(4) (b)(4) it has not been determined whether honey acts as a local radioprotectant. Spinal cord stimulation has also been reported in humans to alleviate pain resulting from radiotherapy (b)(4) however, this method would not be practical for use in mice because of the extensive spinal cord manipulation required. Human administration of morphine also relieves pain (b)(4) but again this would not be practical for mice in our experiments because of side effects of the drug. In humans, exposure to total body irradiation may induce vomiting and diarrhea, causing pain and distress. However, mice are not susceptible to vomiting.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
Anesthesia will be used before (b)(4) irradiation to avoid stress from restraint. We will be unable to use analgesics after irradiation since they have effects on (b)(4) function that would interfere with the interpretation of our results (b)(4) (b)(4) (b)(4) In experiments where tissue collection is needed, animals will be euthanized with pentobarbitol prior to sample collection as described elsewhere.

V.4.2. Prolonged Restraint:

(b)(4) irradiation will require restraint in designed jigs for ~30 min to receive X-ray exposure (b)(4) animals will be anesthetized during this period (see Anesthesia above) and tails will be taped to one side with surgical tape. The jigs for use during (b)(4) irradiation were examined and approved by the Veterinary staff. An image of the jig is now provided (see attached). We found that in female CBA mice, ~12 weeks old, i.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine results in 60-80 min anesthesia (25 G needle) with no morbidity or mortality (b)(4) (b)(4) We propose to use this range of concentration of anesthesia, together with a heating pad to keep mice warm during the period of anesthesia. Standard i.p. injection methods are used for injection of the anesthesia, as recommended.

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: Not applicable

V.4.3.2. Procedure: Not applicable

V.4.3.3. Post-surgical Provisions: Not applicable

V.4.3.4. Location: Not applicable

V.4.3.5. Surgeon: Not applicable

V.4.3.6. Multiple Major Survival Operative Procedures:

V.4.3.6.1. Procedures: Not applicable

V.4.3.6.2. Scientific Justification: Not applicable

V.4.4. Animal Manipulations:

V.4.4.1. Injections:

Intra-peritoneal injections of pentobarbital for euthanasia 0.1-0.2 ml/mouse Fatal Plus (39-78 mg pentobarbital/mouse), will be performed with a 25 G needle. A single I.p. injection of 150 mg/kg ketamine plus 18 mg/kg xylazine will be done using a 25 G needle. This has been extensively used by our laboratory and shown to effectively produce non-lethal anesthesia. (b)(4) (b)(4) vehicle only or (b)(4) will be administered by a single (b)(4) i.p. injection h before radiation exposure also with 25 G needles.

(b)(4)

V.4.4.2. Biosamples:

All biosamples will be taken after euthanasia. (b)(4) will removed *en bloc*. All other tissues will be available to other researchers for tissue sharing.

V.4.4.3. Adjuvants: None

V.4.4.4. Monoclonal Antibody (MAbs) Production: None

V.4.4.5. Animal Identification: Ear punch and cage cards/tags will be used.

V.4.4.6. Behavioral Studies: None

V.4.4.7. Other Procedures:

Treatment with (b)(4) (b)(4) Our preliminary findings indicate that there are no adverse effects due to long term use of (b)(4) We have used this drug in the water to treat mice for up to days with no findings of adverse effects.

(b)(4)

Thoracic irradiation

The methodology for (b)(4) irradiation of mice including approval of holding jigs and development of anesthesia was developed in conjunction with the AFRRI Veterinary Staff. (b)(4) irradiation will be performed using the (b)(6) irradiator in (b)(6) inner room. Thirty minutes prior to irradiation, the (b)(6) irradiator and lead shield will be decontaminated thoroughly with MB10.

Mice will be anesthetized by IP injection in the USUHS LAM facility. Anesthetized mice will be placed in ventilated Lucite jigs, which restrain the animals to prevent lateral or vertical movement. Tails will be taped to prevent any forward motion of animals. Mice will be irradiated at (b)(4) irradiationFollowing radiation exposure, mice will be immediately returned to the LAM and allowed to recover from anesthesia under supervision with heating pads. Immediately upon recovery, animals will be returned to standard cages that house 4 animals per cage. Lucite jigs will be sanitized with soap and water after each use.

Whole body, non-invasive plethysmography
Non-anesthetized, unrestrained (b)(4) will require individual animals to be placed in a clean plexiglass chamber (~ 12 cm in diameter, ~15 cm high) for 45-60 min. Animals will not have food or water during this time, but they will have a continuous flow of fresh room air. Their (b)(4) is measured based on warming and humidifying of the air in the chamber. The animals will experience no restraint, no anesthesia, no drugs, or other manipulation during this time. Following the measurement, to be performed in the room in which they are housed, the animals will be returned to their original cages. Chambers will be sanitized after each use.

V.4.4.8. Tissue Sharing:
All tissues are available for tissue sharing since our research will utilize small quantities of tissues only.

V.4.5. Study Endpoint:
For experiments 1 and 2, survival will be the endpoint. For experiment 3, an early time point will
(b)(4) be taken, [redacted] days postirradiation. Moribundity will be used as a surrogate for mortality, and euthanasia will be used in order to minimize pain and distress. Mice will be monitored following signs of moribundity. Specific health assessment criteria including rapid weight loss (10–15%), ruffled fur, dried lacrimal fluid accumulated in the cul-de-sac of their eyes, are cool to the touch, decreased appetite, diarrhea, difficulty breathing or panting (b)(4) (b)(4) Mice exhibiting these signs will be considered morbid and will be monitored by the PI or other members of the investigative team at least twice daily, early morning and late afternoon. Mice will be considered moribund when, in addition to showing some or all of the signs of morbidity, they exhibit labored breathing plus either an inability to remain upright or are unmotivated to move.

V.4.6. Euthanasia:
Animals will be euthanized according to current American Veterinary Medical Association (AVMA) guidelines. Animals will be anesthetized with Telazol® (4.4 mg/kg i.m.) or ketamine (5-20 mg/kg i.m.)/xylazine (2 mg/kg i.m.). Animals will then be euthanized with Euthasol® (sodium pentobarbital, (1 ml/4.5 kg i.p) or another commercial euthanasia solution. A physical method of euthanasia (cervical dislocation of bilateral thoracotomy) will be used to ensure that no animals recover. See item V.4.5 for euthanasia criteria before the end of the study.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations:

Animals will be group housed. No food or liquid restrictions are required. Water containing (b)(4) will be provided and will be labeled and present in the room in case water bottles need to be refilled. Laboratory staff will be responsible for refilling water bottles.

V.5.1.1. Study Room:
Euthanasia will be performed in (b)(6)
Building(s) (b)(6) Room Number(s) (b)(6)

V.5.1.2. Special Husbandry Provisions:

Food Restriction: Yes _____ No X

Fluid Restriction: Yes _____ No X

V.5.1.3.Exceptions:
(b)(4) water with or without (b)(4) will be provided for all animals by the PI, but no restriction in fluid will be needed.

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be monitored for health, humane treatment, and husbandry considerations, twice daily by (b)(6) personnel during routine weekday rounds and at least once daily on weekends/holidays. This is in addition to, and not in place of, the monitoring that is done by the PI and their staff. In the event of a debilitating illness or adverse reaction, the decision to treat or euthanize an animal will be made by either the veterinarian and/or PI.

V.5.2.2. Emergency Veterinary Medical Care:
All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by (b)(6) personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy:
All animals on this protocol will be provided with routine environmental enrichment in accordance with (b)(6) SOPs and IACUC Policies. Standard enrichment for mice will be included, such as nestlets.

V.5.3.2. Enrichment Restrictions: No restrictions

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Euthanasia	(b)(6)	9 yrs experience for euthanasia	(b)(6)
Euthanasia		10 yrs for euthanasia	
Euthanasia		12 yrs for euthanasia	
Thoracic irradiation		4 yrs experience for thoracic irradiation	
Thoracic irradiation		4 yrs experience for thoracic irradiation	
Thoracic irradiation		4 yrs experience for thoracic irradiation	

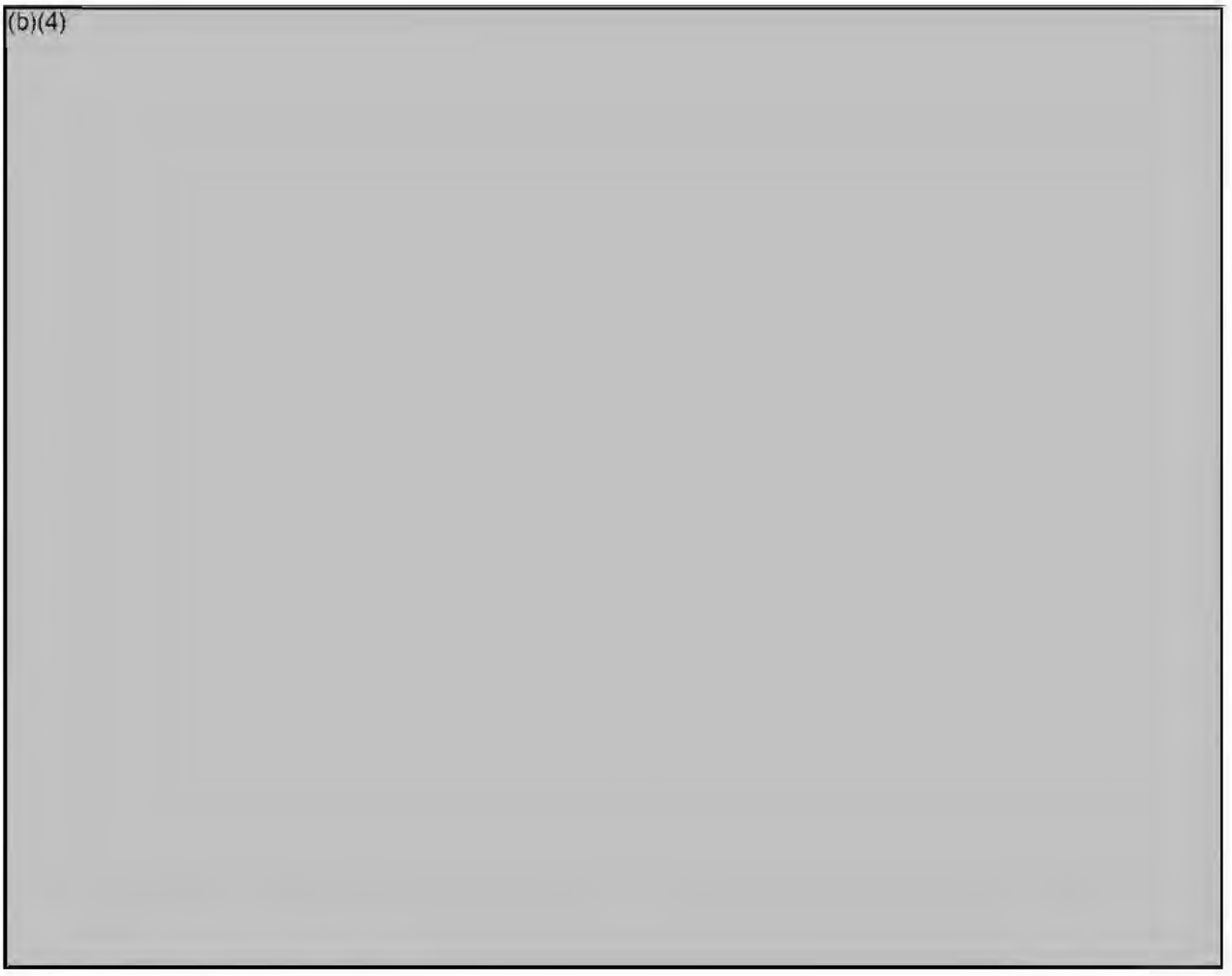
VII. BIOHAZARDS/SAFETY: PPE will be used to minimize allergens produced by mice. Standard PPE include dust mask, gloves, shoe covers, and lab coats.

VIII. ENCLOSURES: (b)(4) data sheets

References:

(b)(4)

(b)(4)



IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation

(b)(6) [redacted]

Dec 9 2015
Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) [redacted]

Dec 9 2015
Date

I. Painful Procedure(s):

I am **NOT** conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) [redacted]

Dec 9 2015
Date

X. PROTOCOL ABSTRACT:

The terrorist attack of September 11, 2001 against the United States has increased the concern that terrorists may acquire and use radioactive sources (e.g. dirty radioactive bombs) to expose military and civilian populations to ionizing radiation. However, despite this concern, there are currently no FDA approved drugs that can be used for the general population to prevent death that is likely to result from exposure to ionizing radiation. (b)(4) irradiation in humans can result in both (b)(4) injuries. However, because rodents are more resistant to (b)(4) injuries than humans, there is currently no murine model that has been developed to study both (b)(4) radiation injuries in the same animal.

Our studies have shown that administration of the (b)(4) (b)(4) can protect laboratory mice from the acute lethal effects of ionizing irradiation. We have tested (b)(4) in a high-dose (b)(4) only irradiation model for the generation of (b)(4) in mice. Our preliminary data indicate that (b)(4) protects (b)(4) from radiation-induced and increases the mean survival time from (b)(4) irradiation by ~50-75%. Our proposed studies here will examine the optimal time course for (b)(4) administration and the optimal dosage of (b)(4) in mice. Our aim is to develop an effective, non-toxic radioprotectant/ radiotherapeutic treatment strategy that can be used for protection against both (b)(4) effects of radiation exposure in a murine model for both types of radiation injury.

A. Animal Protocol Number: To be assigned

B. Animal Protocol Title: Development of (b)(4) for the mitigation of (b)(4) (b)(4) injuries from ionizing radiation in mice (*Mus musculus*)

C. Principal Investigator: (b)(6) Ph.D.

D. Performing Organization: USUHS

E. Funding: DMRDP

Objective and Approach

We will assess *in vivo* protection of the (b)(4) from radiation injuries using (b)(4) administration and short time courses of administration of (b)(4) in mice.

Indexing Terms (Descriptors): radiation, (b)(4) injury, radiation countermeasure, mouse, (b)(4) irradiation

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

May 16, 2016

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF PHARMACOLOGY

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on May 16, 2016:

Animal Protocol Title: "Kappa Receptor Antagonists as Rapid Acting Antidepressants in Mice (mus musculus)"

USUHS Protocol Number: (b)(6)

Expiration Date: May 15, 2019

Supporting Grant(s) Number: USU TBD

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the RATS system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

4/5/16
IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Kappa Receptor Antagonists as Rapid Acting Antidepressants in Mice (*Mus musculus*)

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: USU TBD NIH NIMH (b)(6)

FUNDING AGENCY: NIMH

EARLIEST ANTICIPATED FUNDING START DATE: April 1, 2016

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

(b)(6)		(b)(6)	
Principal Investigator Signature	Pharmacology Department	Office/Lab Telephone	March 21, 2016 Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)		(b)(6)	
Research Unit Chief / Dept. Head Signature	Chair PHA Title	Telephone	March 31, 2016 Date

Typed Name: (b)(6)

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)		(b)(6)	
Statistician Signature	PHA Department	Telephone	March 31, 2016 Date

Typed Name: (b)(6)

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if

(b)(6)

Typed Name (b)(6) VMD

Attending/Consulting Veterinarian Signature	LAM Department	(b)(6) Telephone	March 28, 2016 Date

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) Ph.D.

ANIMAL PROTOCOL TITLE: Kappa Receptor Antagonists as Rapid Acting Antidepressants in Mice (*Mus musculus*)

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER:

CO-INVESTIGATOR(S): (b)(6) Ph.D.

TECHNICIANS(S): To be hired (2)

I. NON-TECHNICAL SYNOPSIS:

The purpose of studies in this protocol is to use mouse models to determine whether kappa opioid receptor antagonists are likely to be effective for the clinical treatment of depression and anxiety disorders and should be advanced to the next stage of investigation. Current antidepressant medications have a slow onset of action and many patients (20-40%) fail to respond to multiple trials and are treatment resistant. Recently, ketamine has been shown to produce rapid antidepressant effects that are sustained for about a week in treatment-resistant human patients and in animal models, but hallucinogenic and amnesic effects limit its widespread application. This proposal will compare the effects of ketamine with two novel kappa opioid antagonists (LY2456302 and LY2444296) because preliminary data showed that other kappa opioid antagonists can produce rapid and sustained antidepressant and anxiolytic effects in animal models. The results of this project will establish whether these two novel kappa opioid receptor antagonists have the ability to produce rapid antidepressant and anxiolytic responses in mice that are similar to those of ketamine and to determine whether they share a common molecular mechanism of action.

II. BACKGROUND:

II.1. Background:

There is a clear medical need for new drugs that would expand options for treating comorbid depression and anxiety, especially for treatment-resistant patients. This is especially true recently since an increasing number of patients (30-40%) are refractory to current antidepressant medications. In the past few years, low dose ketamine has been shown to produce antidepressant effects in treatment-resistant patients, showing that novel antidepressants can be developed. The effects of ketamine are a medical breakthrough because they occur immediately and last for about a week, unlike most antidepressants that require a month or longer before they even begin to work. The overall goal of this research program is to use established rodent models for antidepressant and anxiolytic drug effects to provide evidence that kappa opioid

receptor (KOR) antagonists have the potential to be developed as rapid and sustained antidepressants.

KOR antagonists are another unique class of drugs that may have rapid antidepressant effects. KORs and their endogenous ligand dynorphin are established to underlie dysphoria and depression accompanying exposure to stress. Some experimental KOR antagonists have been shown to produce antidepressant and anxiolytic effects in animal models, but pharmacological characteristics (irreversible blockade) of these compounds have made them unsuitable for clinical development in humans.

The proposed studies will evaluate the effects of two novel KOR antagonists, LY2456302 and LY2444295, as potential antidepressant and anxiolytic drugs using animal behavior tests. These are new compounds that can be developed in humans. Studies will determine whether the KOR antagonists produce their behavioral effects in mice more rapidly than established antidepressants and are sustained with a similar time course to ketamine. The behavioral tests used will be: the forced swim test and novelty-induced hypophagia, tests that are sensitive to traditional antidepressant drugs. The experiments focus on comparing the time of onset or duration of activity for ketamine, LY2456302 and LY2444295 with the known delayed action for SSRI antidepressants. Other experiments will establish the mechanism of action of the KOR antagonists using mice with constitutive deletion of kappa and mu opioid receptors or after treatment with a glutamate antagonist. The most rigorous rodent studies will show reversal of the effects of chronic mild stress on the production of anhedonia and anxiety, effects similar to those shown by human depressed patients. Taken together, these experiments will form a basic foundation for proposing the development of novel KOR antagonists as rapid acting antidepressants for eventual testing in patients with treatment-resistant depression.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: Pubmed, PsychInfo, DTIC, FEDRIP or NIH RePORT

II.2.2. Date of Search: April 29, 2016

II.2.3. Period of Search: entirety of each database

II.2.4. Key Words and Search Strategy:
#1 - Kappa antagonist AND Depression AND Forced swim test AND Mice
#2 - Kappa antagonist AND Depression AND Novelty-induced hypophagia test AND Mice
#3 - Kappa antagonist AND Depression AND Chronic mild stress AND Mice

II.2.5. Results of Search:

There latest search on April 29, 2016 found 8 hits for LY2456302. Four of the studies demonstrated LY2456302 penetrated the brain and occupied kappa opioid receptors. One studied pupil diameter in rats and humans. One study involved only humans. One hit mentioned LY2456302 in a review article. The Rorick-Kehn

reference [(b)(4)] measured the effects of LY2444295 in the forced swim test of NIH Swiss mice shortly after drug administration using a different strain of mouse than used in our laboratory. The duration of effect for LY2456302 in the forced swim test or any other behavioral test, the point of these studies, was not measured. There were 3 hits for LY24444296. One of the hits involved acute effects of LY2444295 on alcohol drinking. Two hits involved the acute effects of LY2444295 in the forced swim test or the NIH test. However, there was no information at longer time intervals, as proposed to be studied in these experiments. In addition, no studies with either drug involved measuring drug effects in the UCMS procedure or with genetic mutants as proposed in these experiments.

III. OBJECTIVE\HYPOTHESIS:

Our overall goal is to evaluate the potential of two novel kappa opioid receptor antagonists to produce behavioral effects that are similar to established antidepressant drugs (fluoxetine) and to ketamine under three different rodent behavioral tests and models of antidepressant effects. The hypothesis is that the kappa opioid antagonists LY2456302 and LY2444295 will show a pattern of antidepressant-like behavioral responses.

IV. MILITARY RELEVANCE:

Major Depressive Disorder (MDD) is one of the most common psychiatric disorders, with a lifetime prevalence of 17% in the United States [(b)(4)] [(b)(4)] MDD is considered one of the most disabling medical conditions and is predicted to be a leading contributor to the worldwide burden of disease [(b)(4)] [(b)(4)] Depression and suicide are acknowledged to be important problems in military personnel, with rates of depression and suicide that are even higher in non-deployed Army soldiers than in the general population. Conventional antidepressants are only effective with 50-60% of patients. Our goal is to use data from animal behavior tests to help develop kappa opioid receptor (KOR) antagonists as a novel antidepressant medication for patients with treatment-resistant depression and suicidal ideation.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

The effects of ketamine and the novel KOR antagonists, LY2444296 and LY2456302, will be studied in models of depression and anxiety in mice. Experiment 1 will examine the effects of the drugs in the mouse forced swim test. It is also necessary to examine the effects of the drugs on locomotor activity to know whether the effects in the forced swim test are specific. Experiment 2 will evaluate the effects of the drugs on the novelty-induced hypophagia test. Experiment 3 will evaluate the effects of the KOR antagonists at blocking analgesia. Experiment 4 evaluate the effects of the drugs in mice without mu and kappa opioid receptors. Experiment 5 is the ability of AMPA antagonists to block the effects of ketamine and the KOR antagonists. Experiments 6

and 7 will evaluate the effects of the drugs in the Unpredictable Chronic unpredictable mild stress (UCMS) model.

V.1.1. Experiment 1: Forced swim test (FST) and locomotor activity.

Ketamine produces acute and protracted behavioral effects on antidepressant tests in rodents such as the FST. Acute effects occur during the first hour whereas protracted effects persist for days after a single injection. This experiment will examine whether the KOR antagonists also produce both acute and protracted behavioral effects in a rodent model of antidepressant activity. The experiment will first determine a dose-response curve (0.3, 1.0, and 3.0 mg/kg i.p.) for the acute effects of the selective KOR antagonists LY2456302, LY2444296 and ketamine at reducing immobility in the mouse FST 1 hour after injection. From these results, a dose will be selected for further testing of each drug over time. The time course of behavioral activity will be determined over days (at 24 hours, 48 hours or longer) after injection to determine whether these drugs produce protracted behavioral effects.

NUMBER OF ANIMALS REQUIRED: 360 males, 80 females; Category E

N = 10 animals/group. Different animals are required to be tested at different times because prior experience with the FST can change performance. The experiment tests how long each drug produces its effects on this behavior.

Female mice: NIH requires that female mice will be included in the research program. A group of female mice (N=80 mice) will be tested with each drug using a dose shown to be effective in male mice.

JUSTIFICATION:

Time after injection

Ketamine	1 hour	24 hours	48 hours
Saline	10	10	10
0.3	10	10	10
1	10	10	10
3	10	10	10

LY2456302	1 hour	24 hours	48 hours
Saline	10	10	10
0.3	10	10	10
1	10	10	10
3	10	10	10
Females	1 hour	24 hours	
Saline	10	10	
Ketamine	10	10	
LY2456302	10	10	
LY2444296	10	10	
LY2444296	1 hour	24 hours	48 hours

Saline	10	10	10
0.3	10	10	10
1	10	10	10
3	10	10	10

Locomotor activity

It is important for characterizing antidepressant effects in the FST that drugs do not show increases in locomotor activity. To evaluate locomotor activity for these drugs, the mice will be tested for changes in locomotor activity using the same doses and time parameters as were active in the FST. To conserve animals, the tests will be done with the same mice used in the FST one week later.

NUMBER OF ANIMALS REQUIRED: 360 males, 80 females

N = 10 animals/group

For justification, see table above.

V.1.2. **Experiment 2**: NIH test.

This experiment will determine a dose-response curve and time course for the acute effects of ketamine and selective KOR antagonists in the NIH test. Mice are first trained to eat Reese's peanut butter chips from a petri dish in daily feeding sessions lasting 15 min in individual cages. Training sessions continue until the mice approach the food in less than 30 seconds for three consecutive days.

After accomplishing the training described above, mice are presented the food in a novel cage session, with bright overhead lights and a lemon scent. Approach latency and food consumed are measured during the 30 min session. Mice will be injected with drugs 1 hour prior to testing. A complete dose-response curve for the effects of LY2456302, LY2444296 and ketamine will be obtained at: 0.3, 1.0, and 3.0 mg/kg i.p. given 1 hour prior to testing, in comparison with saline-treated control groups tested simultaneously. After selecting a dose for further testing of each drug, the time course of behavioral activity will be determined over days (at 24 hours and 48 hours) after injection to determine whether these drug treatments produce protracted behavioral effects in the NIH test.

NUMBER OF ANIMALS REQUIRED: 360 males, 80 females; Category C

N = 10 animals/group

For justification, see numbers in table above. Different animals are required to be tested at different times because the novel test cage is no longer novel after prior exposure. The experiment tests how long each drug produces its effects on this behavior.

V.1.3. **Experiment 3**: Analgesia study.

This experiment will use analgesia produced by either the MOR agonist morphine or the KOR agonist U50,488 to measure the occupation of the two KOR antagonists and ketamine at MOR and KOR during behavioral testing . Analgesia will be measured using the response of mice to lick their paw or jump off the surface of a hot plate set at 55 degrees C. If animals do not make either the licking or jumping response within 30 sec, they will be removed from the hot plate to protect them from tissue damage. The occupation of opioid receptors by the antagonists will be estimated from studies that measure the doses and time course for blockade of analgesia produced by the KOR

agonist U50,488 (10 mg/kg) or the MOR agonist morphine (20 mg/kg) at 1 hour post-injection. Sustained opioid receptor occupation over time will be determined by retesting the same animals at 24 and 48 hours post-injection. The experiments will determine: 1) whether the KOR antagonists are selective for kappa vs mu opioid receptors; and 2) whether the KOR antagonists produce acute or protracted behavioral effects using analgesia as a behavioral model that is directly related to their occupation of KORs.
NUMBER OF ANIMALS REQUIRED: 360 mice; Category D.
N = 10 animals/group

JUSTIFICATION:
LY2456302 tested at 1 hour post-injection

Dose	Saline	U50,488	Morphine
Saline	10	10	10
0.3	10	10	10
1	10	10	10
3	10	10	10

LY2444296 tested at 1 hour post-injection

Dose	Saline	U50,488	Morphine
Saline	10	10	10
0.3	10	10	10
1	10	10	10
3	10	10	10

Ketamine tested at 1 hour post-injection

Dose	Saline	U50,488	Morphine
Saline	10	10	10
0.3	10	10	10
1	10	10	10
3	10	10	10

V.1.4. Experiment 4: Studies of drug effects in opioid knockout mice.
The purpose of this experiment is to determine whether the absence of KORs in genetic mutants block the behavioral effects of the KOR antagonists and ketamine in the FST and NIH tests. Mice with genetic deletion of KORs (Oprk1$^{-/-}$ mice) will be treated with the KOR antagonists LY2456302, LY2444296 or ketamine, all at 3 mg/kg. Their effects will be evaluated at either 1 h or 48 h after injection in order to assess the role of KORs in producing the acute and protracted behavioral effects in the FST and NIH tests.
NUMBER OF ANIMALS REQUIRED: 480 mice will be used in the experiments. 240 mice will be tested in the FST; Category E. 240 mice will be tested in the NIH test; Category C.
N = 10 animals/group

JUSTIFICATION:

	WT	Oprm1$^{-/-}$	Oprk1$^{-/-}$
FST			
Saline	20	20	20
Ketamine	20	20	20
LY2456302	20	20	20
LY2444296	20	20	20
NIH test			
Saline	20	20	20
Ketamine	20	20	20
LY2456302	20	20	20
LY2444296	20	20	20

V.1.5. Experiment 5: Role of AMPA receptors in the behavioral effects of ketamine LY2456302 and LY2444296.

The protracted effects of ketamine are thought to involve the following neurochemical events: 1) activation of AMPA receptors from glutamate release, 2) activation of TrkB receptor from the release of BDNF, and the activation of mTor. The purpose of this study is to determine whether the protracted behavioral effects of the KOR antagonists involve a similar signaling cascade involving AMPA receptors.

Studies will be conducted with both the FST and the NIH tests. The AMPA receptor antagonist NBQX will be given prior to testing the effects of ketamine or the KOR antagonists LY2456302 and LY2444296 at 1 h or 48 h post-injection. The first time point evaluates the acute effects and the second time point evaluates the protracted effects of ketamine and the KOR antagonists.

NUMBER OF ANIMALS REQUIRED: 240 mice in total. 120 mice will be tested in the FST; Category E. 120 mice will be tested in the NIH test; Category C.
N = 10 animals/group

JUSTIFICATION:

	Time after injection of ketamine (3 mg/kg)	
Treatment	1 hour	48 hours
Saline	10	10
NBQX	10	10

	Time after injection of LY2456302 (3 mg/kg)	
Treatment	1 hour	48 hours
Saline	10	10
NBQX	10	10

Time after injection of

	LY2444295 (3 mg/kg)	
Treatment	1 hour	48 hours
Saline	10	10
NBQX	10	10

V.1.6. Experiment 6: Effects of acute drug treatments after exposure to UCMS.

The goal of this experiment is to determine how exposure of mice to the UCMS procedure changes expression of the protracted behavioral effects of acute single doses of ketamine (3 mg/kg) and the KOR antagonists LY2456302 (3 mg/kg) and LY2444296 (3 mg/kg). Drug effects will be measured for 3 consecutive days starting 24 hours after injection. Anhedonia will be measured using the sucrose preference test. Anxiety will be measured using the light-dark emergence test. Stress coping will be measured using the forced swim test. The behavioral effects in mice after UCMS will be compared with non-stressed controls.

NUMBER OF ANIMALS REQUIRED: 270 males, 180 females. Category E.

Note on animal numbers. In UCMS studies, mice that do not demonstrate sucrose preference greater than 65% at baseline prior to any experimental manipulation will not be entered into the study. In the past, 25% of mice fail to make this criterion. Therefore, we have estimated an additional 30 mice in the UCMS groups will be needed for this experiment. Females will only be tested with ketamine and LY2456302.

N = 20-25 animals/group

JUSTIFICATION:

UCMS		Non-stressed Controls	
Saline	25	Saline	20
Ketamine	25	Ketamine	20
UCMS		Non-stressed Controls	
Saline	25	Saline	20
LY2456302	25	LY2456302	20
UCMS		Non-stressed Controls	
Saline	25	Saline	20
LY2444296	25	LY2444296	20

V.1.7. Experiment 7: Effects of chronic drug treatments after exposure to UCMS.

Reversal of the behavioral effects of UCMS exposure usually require chronic administration of antidepressants for 4 weeks. The present experiment will determine whether the effects of ketamine and the KOR antagonists act more rapidly the SSRI

fluoxetine when given chronically. In the present study, the effects of chronic administration of the KOR antagonists LY2456302 and LY2444296 will be compared with the SSRI fluoxetine and saline controls after their chronic administration for 28 days in a UCMS paradigm. The behavioral effects in mice after UCMS on sucrose preference, the light-dark test and the FST will be compared with non-stressed controls.

NUMBER OF ANIMALS REQUIRED: 360 males, 180 females. Category E.

Note on animal numbers. In UCMS studies, mice that do not demonstrate sucrose preference greater than 65% at baseline prior to any experimental manipulation will not be entered into the study. In the past, 25% of mice fail to make this criterion. Therefore, we have estimated an additional 40 mice in the UCMS groups will be needed for this experiment. Females will only be tested with ketamine and LY2456302.

N = 20-25 animals/group

JUSTIFICATION:

UCMS		Non-stressed Controls	
Saline	25	Saline	20
Ketamine	25	Ketamine	20
UCMS		Non-stressed Controls	
Saline	25	Saline	20
LY2456302	25	LY2456302	20
UCMS		Non-stressed Controls	
Saline	25	Saline	20
LY2444296	25	LY2444296	20
UCMS		Non-stressed Controls	
Saline	25	Saline	20
Fluoxetine	25	Fluoxetine	20

V.2. Data Analysis: Sample size estimates are guided by the results of prior published research and preliminary data generated in our laboratory using these techniques [redacted]. Estimates of the effect size expected from different experimental groups are: FST, NIH approach latency, hot plate analgesia, locomotor activity. The effect sizes from prior studies agree with the calculated expected number of animals necessary to achieve statistically significant results with a power of 0.800. For the UCMS study, data for the dependent measures (sucrose preference, preference and approach latency for the dark side in the dark/light emergence test, FST immobility) will be analyzed by a 2-way ANOVA, with stress and

drug treatment independent factors. Significant interactions will be followed by Neuman-Keuls test.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: The effects of ketamine and the kappa opioid antagonists, LY2444296 and LY2456302, will be studied in models of depression and anxiety in mice. The overall goal is to provide sufficient evidence for potential efficacy in treating depression to justify and stimulate the performance of clinical trials with patients. Behavioral studies of antidepressant drugs in mice using the validated procedures described in this protocol are believed to provide information pertinent toward understanding the effects of these drugs on depression and stress in humans and their underlying neurochemical mechanisms. These complex interactions governing mood and behavior cannot be adequately studied with in vitro systems or lower organisms such as invertebrates.

V.3.2. Animal Model and Species Justification: The studies are proposed in mice as a model organism that measures the behavioral effects of antidepressant drugs and environmental stress in a manner similar to those shown by other mammals, including humans. There are no lower species that provide the same information. Because of the extensive literature using these rodent models, positive results with these tests will stimulate the field to plan corresponding clinical trials in humans.

V.3.3. Laboratory Animals

	Strain #1	Strain #2	Strain#3
V.3.3.1. Genus & Species:	*Mus musculus*	*Mus musculus*	*Mus musculus*
V.3.3.2. Strain/Stock:	C57BL/6	Oprm1$^{-/-}$	Oprk1$^{-/-}$
V.3.3.3. Source/Vendor:	(b)(4)	In-house	In-house
V.3.3.4. Age:	6-8 weeks	6-8 weeks	6-8 weeks
V.3.3.5. Weight:	Appropriate for age	Appropriate for age	
V.3.3.6. Sex:	Male and female	Male	Male
V.3.3.7. Special Considerations:	Pathogen free		

V.3.4. Number of Animals Required (by Species): 2,950

V.3.5. **Refinement, Reduction, Replacement (3 Rs)**:

V.3.5.1. **Refinement**: 1) Mice are handled daily and acclimatized to the testing room on the days prior to conducting a behaviorel procedure. 2) An observer watches all mice in the forced swimming test for signs of distress. They will prevent any animal from drowning by removing them from the cylinder. 3) Mice will be anesthetized prior to decapitation to immobilize the animal.

V.3.5.2. **Reduction**: The same groups of male mice used to measure the forced swim test will be used to assess the effects of drugs on locomotor activity. Power calculations have been used to estimate the minimum number of mice required to reach statistical significance.

V.3.5.3. **Replacement**: There is no alternative to live animal use to study the effects of environmental stress on behavior. If an alternative to live animal use for the study of environmental stress becomes available, this alternative will be used.

V.4. **Technical Methods**:

V.4.1. **Pain / Distress Assessment**:

Analgesia: Mice are allowed to remain on the hot plate for a maximum of 60 seconds to prevent the development of tissue damage.

Forced swim test: Mice are ordinarily good swimmers and swim through the 6-min test without difficulty. An observer watches all mice in the forced swimming test for signs of distress, such as failing to swim on the surface of the water. The observer will intervene and remove any animal from the cylinder on the rare occasion that they show signs of distress.

UCMS procedure: Mice are weighed and observed daily. Any mouse showing intractable weight loss or signs of distress will be removed from the experiment in consultation with LAM.

V.4.1.1. **APHIS Form 7023 Information**:

V.4.1.1.1. **Number of Animals**:

	Strain #1	Strain #2	Strain#3
V.4.1.1.1.1. **Column C**:	800		
V.4.1.1.1.2. **Column D**:	360		
V.4.1.1.1.3. **Column E**:	1790		

V.4.1.2. **Pain Relief / Prevention**:

V.4.1.2.1. **Anesthesia/Analgesia/Tranquilization**: N/A

V.4.1.2.2. **Pre- and Post-procedural (not surgery) Provisions**: N/A

V.4.1.2.3. **Paralytics**: None.

V.4.1.3. **Literature Search for Alternatives to Painful or Distressful Procedures**:

V.4.1.3.1. **Sources Searched**: PubMed, PsychInfo

V.4.1.3.2. **Date of Search**: February 14, 2016

V.4.1.3.3. **Period of Search**: PubMed 1995 through present; PsychInfo 1965 through present.

V.4.1.3.4. **Key Words of Search**: (alternative or refine), distress, mice and forced swim test; alternative, distress and NIH test; alternative, distress and chronic mild stress.

V.4.1.3.5. **Results of Search**: The search procedure examined alternatives to the forced swim test (by searching for (alternative or refine) and chronic mild stress procedure or the NIH test (by searching for (alternative or refine) and distress and NIH test or novelty-induced hypophagia). Two databases were searched, Pubmed and Psychinfo. There were no alternative procedures located through this search. There were no hits for the keyword combinations in either PubMed or PsychInfo.

V.4.1.4. **Unalleviated Painful or Distressful Procedure Justification**: Mice tested with the forced swim test were placed in Category E. This test measures the behavioral coping responses evoked in mice when they are placed in cylinder of water for 6 min. Mice either will react passively to the stress and develop behavioral immobility or they will react actively with increased swimming and reduced behavioral immobility. Antidepressant drugs cause mice to favor an active response to the unmitigated swim stress. Mice must be tested without alleviation of the stress in order to determine how the effects of stress or drug treatments have affected their response. The mice are tested only for a limited period of time because the test is timed at 6 min

V.4.2. **Prolonged Restraint**: N/A

V.4.3. **Surgery**: N/A

V.4.3.1. **Pre-surgical Provisions**: N/A

V.4.3.2. **Procedure**: N/A

V.4.3.3. **Post-surgical Provisions**: N/A

V.4.3.4. **Location**: N/A

V.4.3.5. **Surgeon**: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: Injections will be performed with a 1 cc syringe, 26 g, ½ inch needle, and 10 cc / kg approx. volume for i.p. injections. For the FST and NIH studies, the drugs that will be used are: ketamine (3 mg/kg, i.p.) and the KOR antagonists LY2444296, and LY2456302 at a dose range of 1-3 mg/kg, i.p. Drugs will be given either 1 hour or 24 hours prior to behavioral testing. In the analgesia studies, mice will be injected with morphine or U50,488 at 10 mg/kg i.p. 30 min prior to testing. For studies with the AMPA receptor blocker NBQX, the dose of 1 mg/kg i.p. NBQX will be given 10 min prior to ketamine or the KOR antagonists. In the UCMS procedure, mice will be injected with saline, ketamine, LY2456302, LY2444296, or fluoxetine (5 mg/kg, i.p.) once daily in the UCMS procedure.

V.4.4.2. Biosamples: Brain tissue samples (cortex, hippocampus, amygdala and striatum) will be taken from all mice participating in the UCMS studies.

V.4.4.3. Adjuvants: None.

V.4.4.4. Monoclonal Antibody (MAbs) Production: None.

V.4.4.5. Animal Identification:

Ear tag in mice.

Cage card also will be labeled with animal number

A log book containing the animal number, gender / sex; dates of behavioral testing, dates of euthanasia; dates of injections, will be maintained for each mouse.

V.4.4.6. Behavioral Studies:

Hot plate analgesia. The nociceptive threshold for analgesia will be examined with a hotplate analgesia meter (Columbus Instruments, Columbus, Ohio). The hot plate provides a constant 55 degree (C) surface, a temperature low enough to avoid harming the mice, but high enough to be uncomfortable for a wild-type saline-treated animal. A small plastic cage around the hot plate prevents the animal from leaving the plate surface. Animals will be placed on the hot plate and the latency (s) to lick their hind-paw or jump will be recorded. After displaying either of these behaviors, or upon reaching the predetermined cut-off time (60 s), the animals will be removed from the hot plate. For drug studies, mice will be injected with drug 30 min prior to behavioral testing. Behavior is expressed either as the latency to lick or jump or the percentage analgesia. Percentage analgesia is calculated by taking the mean latency for saline treated mice

(s) minus the latency of the test animal (s) divided by 60 s (maximum time animals can spend on the plate) minus the mean latency for saline-treated mice (s).

Forced swim test (FST). This test is a recognized model of depressive behavior induced by inescapable stress. Because the studies model the way that antidepressant drugs alleviate stress in clinical depression, the stress cannot be alleviated by sedatives or analgesics. All experimental sessions will be conducted between 1200 and 1800 hours. Swim test sessions are conducted by placing mice in individual glass cylinders (46 cm tall, 21 cm in diameter) filled with water (23-25°C water) to a depth of 15 cm. Only one test is given with the mouse for a standard 6-min test duration. The water in the cylinder is changed after every test. Antidepressant drugs are administered only once, either 1 hour, 24 hours or 48 hours prior to testing, and the post-injection interval will be manipulated as a variable in the present experiments. A digital recorder positioned directly above the cylinders records the swim sessions. Digital video files are analyzed for immobility by a trained observer blind to the experimental treatment condition. Immobility is defined as the absence of movement on the water surface, except that necessary to keep afloat. The swimming test will be conducted only once for each animal. Injection of mice with standard antidepressant drugs produces a significant reduction of immobility by delaying the time required for mice to adopt a passive immobility behavior during the FST (b)(4) Digital files will be stored on a secured lab server.

Locomotor activity. Locomotor activity will be measured in cages resembling the home cage environment (28.9 x 17.8 x 12 cm). The cages are placed inside an infrared-sensitive motion detection system (30 x 24 x 8 cm) with an 8-beam photocell array strip (Med Associates Inc, St. Albans, VT). Total ambulatory activity, crossing the longitudinal axis and rearing is measured by counting the number of beam breaks. The test will be performed only once for each animal.

Novelty-induced hypophagia (NIH) test. Mice are exposed to 8 training sessions in a familiar feeding cage prior to testing in a novel environment. The training consists of daily sessions (15 min) in which mice are given access to a highly palatable food (peanut butter chips; Nestle, Glendale, CA) delivered in a clear plastic petri dish in a familiar feeding cage. The latency to approach and initiate ingestion and the amount of food consumption is measured regularly and mice come to approach the food within 30 sec. The novel cage environment consists of an empty clear polycarbonate cage without bedding located under bright overhead illumination located in a room different than training. The novel cage test session duration is 15 min and is videotaped. The latency to ingestion and the amount of food consumed during the novel cage session are the endpoints measured. The test is given only once for each animal in the novel cage. Drugs will be administered either 1 hour or 24 hours prior to testing. Antidepressant treatments reduce the latency to approach food in the novel environment without effects in the home cage (b)(4)

Sucrose preference test. During an 8 h session, mice are given a free choice between two bottles, one with water and one with a 1% sucrose solution. To avoid side preference, bottles are switched halfway through the test. Bottles are weighed before

and after the test. Preference is calculated as the percentage of consumed sucrose from the total amount of liquid drunk (sucrose and water).

Dark-light emergence test. The dark/light emergence test is conducted in an apparatus consisting of a two-chambered box (17 × 20 cm for each side) made of plexiglas with an opening (5 x 5 cm) connecting both chambers. One side is colored black (room light entry is limited) and the other side is white. Room illumination is 600 lux. Mice are placed into the dark side and then allowed to freely explore either the light or dark side for 5 min. Anxiety-like behavior is measured as the time required to emerge from the dark side and the time spent on the white side during the 5 min session. The apparatus is cleaned with 70% EtOH between animals.

Unpredictable Chronic unpredictable mild stress (UCMS). Mice will be exposed to a variable sequence of mild, unpredictable stressors for a duration of 21 days called the UCMS procedure. Mice are exposed to three physical or psychosocial stressors per day for a period of one hour for a duration of up to five weeks. The stressors used are: cage rotation, lights on overnight, cage on gentle shaker for 30 min, cage tilt (45 degrees), isolation for 10 hours, exposure to cold, introduction of a new partner, restraint for 30 min, exposure to rat odor, lights off (during the day), stroboscopic lighting (in the dark) and wet bedding. The stressors will be presented in a procedure room or specialty room (walk-in refrigerator) and last up to 30 min, except for stressors presented overnight. After the stressor is presented in the procedure room, the mice are returned to the animal colony. Mice are weighed every day. Mice that lose more than 10% of their body weight will be eliminated from the study. The schedule of stressor exposure in the UCMS procedure is presented below in the Table.

After exposure to the UCMS procedure for 14 days, the effects of the experimental antidepressants will be evaluated. In Experiment 6, the effects of a single acute exposure to ketamine and the KOR antagonists will be evaluated. Experiment 7 will evaluate the effects of drugs given chronically for for 7 days prior to the start of behavioral testing (see timeline below). After 7 days of treatment, mice are given three behavioral tests: sucrose preference, dark-light emergence and the forced swim test. The UCMS procedure causes reduced sucrose preference (anhedonia), increased emergence time from the dark (anxiety) and increased immobility in the forced swim test (decreased stress resilience). Non-stressed controls are weighed every day and injected with the same drugs, but not subjected to any of the stressors. Antidepressant drug treatments reverse these effects of stress without producing any effect in non-stressed controls.

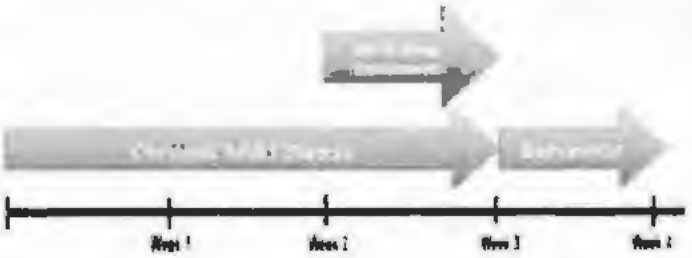
Table 1S

Schedule of Stressor Presentations in the UCMS Procedure

Day	Morning	Afternoon	Overnight
0	Rotate		Lights on
1	Cage on shaker	Rat odor	Cage tilt
2	Restraint	Cold	Stroboscopic light

3	Swim	Lights off	Isolation
4	Rat odor	Restraint + lights off	Wet bedding
5	Cage on shaker	New partner	Lights on + white noise
6	Cold	Lights off + cage tilt	Wet bedding
7	Cold swim	Restraint on shaker	Cage tilt
8	Rat odor + white noise	Cold + isolation	Lights on
9	Lights off, cage tilt, wet bedding	Home cage switch	Stroboscopic light
10	Rat odor + shaker	Restraint + lights off	Isolation
11	Swim	Cage on shaker	Wet bedding + white noise
12	Cage tilt	New partners	Lights on + white noise
13	Restraint	Lights off	Stroboscopic light
14	Home cage switch	Rat odor	No stress (Sucrose habituation)
15	Cage on shaker	Cage tilt	Wet bedding
16	Restraint	Cold	Stroboscopic light
17	Swim	Lights off	Isolation
18	Rat odor	Restraint + lights off	Wet bedding
19	Cage on shaker	New partner	Lights on + white noise
20	Cold	Lights off + cage tilt	Wet bedding
21	Cold swim	Restraint on shaker	Cage tilt

Timeline for UCMS Procedure and Drug Administration



V.4.4.7. Other Procedures: None.

V.4.4.8. Tissue Sharing: No tissue sharing is planned at this time. However, we welcome investigators to utilize tissue from these animals following completion of the study protocol. We will do whatever we can, in coordination with the IACUC, to accommodate requests for tissue.

V.4.5. Study Endpoint: Mice will be euthanized after the completion of the behavioral tests described in Experiments 1-7. PI will consult with CLAM veterinarians to evaluate animals in the UCMS procedure. Animals deemed to be in distress will be euthanized by CLAM personnel, the PI or his staff.

V.4.6. Euthanasia: Mice deemed in distress will be anesthetized with isofluorane and decapitated in Rm (b)(6) by PI or his staff or euthanized by CLAM personnel with CO_2 followed by cervical dislocation or bilateral thoracotomy.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room: Space for Dr (b)(6) laboratory has not yet been assigned.

Building(s) _____ Room Number(s) _____

V.5.1.2. Special Husbandry Provisions: None.

Food Restriction:	Yes _____	No X
Fluid Restriction:	Yes _____	No X

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: No special veterinary medical care is anticipated for these experiments. Animals deemed to be in distress will be treated or euthanized by CLAM personnel or the PI. PI will anesthetize with isoflurane followed by decapitation. If CLAM personnel observe a mouse in distress, PI should be contacted before euthanasia for possible experimental use of the mouse. PI will provide work and home contact information to CLAM.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents.

V.5.3.2. Enrichment Restrictions: Enrichment will be withheld from mice subjected to UCMS because enrichment could alter experimental outcomes. Since all mice in control and experimental groups need to be treated identically to enable a valid comparison, enrichment will be withheld from all mice in the UCMS protocol.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Behavioral testing, IP injections, Isoflurane Administration, Decapitation	(b)(6)	PI, 35 yr experience with mouse research, including behavioral testing, including the FST, light-dark box, NIH test and sucrose drinking, and decapitation.	Completed iInvestigator training at U Penn, 2000.
Behavioral testing, IP injections, Isoflurane Administration, Decapitation		Co-PI, 12 yr experience with mouse research, including behavioral testing, including the FST, light-dark box, NIH test and	Completed iInvestigator training at U Penn, 2013.

		sucrose drinking, and decapitation.	
Behavioral testing, IP injections, Isoflurane Administration, Decapitation	To be hired	Research technician. Will be trained by PI or Co-PI as needed.	

VII. **BIOHAZARDS/SAFETY**: PPE, including gowns, masks and gloves, will be worn by all personnel to reduce allergen exposure. No special precautions are necessary for working with the drugs used in these studies.

VIII. **ENCLOSURES**: none.

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

Principal Investigator Signature _____ Date _____

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

(b)(6) _____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6)

Principal Investigator Signature

4/29/16

Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress WILL or **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature

4/29/16

Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number:

B. Animal Protocol Title: Kappa Receptor Antagonists as Rapid Acting Antidepressants

C. Principal Investigator: (b)(6)

D. Performing Organization: USU

E. Funding: NIMH

F. Objective and Approach: There is an unmet medical need for new drugs that could treat comorbid depression and anxiety, especially for treatment-resistant patients. Depression is one of the most common psychiatric illnesses, especially in military personnel. An increasing number of patients (30-40%) are unresponsive to current antidepressant medications. In the past few years, low dose ketamine has been shown to produce antidepressant effects in treatment-resistant patients, showing that novel antidepressants can be developed. The effects of ketamine are a medical breakthrough because they occur immediately and last for about a week, unlike most antidepressants that require a month or longer before they even begin to work. However, the widespread use of ketamine has been limited because it is a drug of abuse and causes unwanted side effects.

The proposed studies will evaluate the effects of two novel kappa opioid receptor antagonists, LY2456302 and LY2444295, using animal behavior tests for their potential to be developed into antidepressant and anxiolytic drugs that could be used in humans. Studies will determine whether the kappa opioid receptor antagonists produce behavioral effects in mice similar to those of established antidepressants but only more rapidly and sustained over days. Their effects will be compared with low dose ketamine as a standard. The behavioral tests used will be: the forced swim test and novelty-induced hypophagia, tests that are sensitive to traditional antidepressant drugs. The experiments focus on comparing the time of onset or duration of activity for ketamine, LY2456302 and LY2444295 with the known delayed action for SSRI antidepressants. Other experiments will establish the mechanism of action of the kappa opioid receptor antagonists using mice with constitutive deletion of kappa and mu opioid receptors or after treatment with a glutamate receptor antagonist. The most rigorous evidence for antidepressant potential will come from rodent studies that will show reversal of the effects of chronic mild stress on the production of anhedonia and anxiety, symptoms similar to those shown by human depressed patients. Taken together, these experiments will form a basic foundation for evaluating the development of novel kappa opioid receptor antagonists as rapid acting antidepressants and for eventual testing in patients with treatment-resistant depression.

G. Indexing Terms (Descriptors): animals, mice, depression, anxiety, antidepressants, ketamine, kappa opioid receptors, stress,

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
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May 11, 2012

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF PSYCHIATRY

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on May 11, 2012:

Animal Protocol Title: "Stress and Drug Seeking In Rats: Role of the Endocannabinoid System and Caffeine"

USUHS Protocol Number: (b)(6)

Expiration Date: May 10, 2015

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)

(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: *Stress and drug seeking in rats: Role of the endocannabinoid system and caffeine*

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: CSTS

EARLIEST ANTICIPATED FUNDING START DATE: February, 2012

PRINCIPAL INVESTIGATOR:

(b)(6) — Principal Investigator Signature
(b)(6) PhD

PSY — Department
(b)(6) — Office/Lab Telephone
2/06/12 — Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) — Research Unit Chief / Dept. Head Signature
Typed Name (b)(6) MD

Chairman — Title
(b)(6) — Telephone
2/9/12 — Date

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) — Statistician Signature
Typed Name (b)(6) PhD

PSY — Department
(b)(6) — Telephone
2/07/2012 — Date

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) — [illegible]arian Signature
Typed Name (b)(6) DVM

LAM — Department
(b)(6) — Telephone
24 Feb 12 — Date

ANIMAL PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: (b)(6) PhD

ANIMAL PROTOCOL TITLE: *Stress and drug seeking in rats: Role of the endocannabinoid system and caffeine*

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER:

CO-INVESTIGATOR(S): Drs. (b)(6)
(b)(6)

TECHNICIANS(S): Research Assistant: (b)(6) BSc

I. NON-TECHNICAL SYNOPSIS: Posttraumatic stress disorder (PTSD) is a debilitating anxiety disorder affecting military service members and civilians in the U.S. Among the individuals with PTSD, the rate of nicotine dependence is quite high. Approximately 28.5% of the population is current smokers and 47.1% have smoked at some time in their life in the U.S. (Lasser, Boyd et al. 2000). Of those with a history of PTSD, 63.3% had smoked daily at some point in their lifetime and 45.3% were current smokers – rates considerably higher than those with no history of PTSD (47.1% lifetime and 28.5% current). Also, PTSD patients are more likely to be heavy smokers and relapse to smoking tobacco (Fu, McFall et al. 2007). These suggest that nicotine dependence is a serious problem in the individuals with PTSD. Among the potential biological mechanisms, the endocannabinoid system (ECS) has been implicated in both anxiety disorder (Picciotto and Corrigall 2002) and nicotine dependence (Cohen, Kodas et al. 2005). Thus, developing drugs targeting the ECS may have therapeutic potential on treating PTSD and nicotine dependence. Using well-established rat models of stress and nicotine self-administration, we'll investigate the effects of three stress models on anxiety and depression behavior as well as relapse to nicotine seeking. We will further investigate the effects of the cannabinoid receptor 1 agonist and caffeine during stress exposure on subsequent anxiety/depression behavior and relapse to nicotine seeking in rats. The outcome of these studies will enhance our understanding of the biological mechanisms by which the ECS regulates mood, anxiety and drug seeking, and provide a novel treatment strategy for comorbid PTSD and nicotine dependence.

II. BACKGROUND:

II.1. Background:

Post-Traumatic Stress Disorder (PTSD) and substance abuse

PTSD is a debilitating anxiety disorder that can develop in response to traumatic stress exposure such as combat or other physical and/or psychological stress. The estimated lifetime prevalence of trauma exposure is 60.7% for men and 51.2% for women with men more likely to report physical attacks, combat experience, being threatened with a weapon, while women likely to report sexual assault, childhood neglect and childhood

physical abuse (Kessler, Sonnega et al. 1995). It is well established that the exposure to traumatic stressors predisposes persons to an increased probability of use of psychoactive and addictive drugs (Brown and Wolfe 1994). The use of psychoactive drugs such as nicotine, marijuana and caffeine continues to be one of the more damaging squeal to deployment-associated PTSD in military personnel. However, biological mechanisms underlying the substance use disorder displayed in PTSD subjects are not well understood, and therapeutic interventions to reduce subsequent drug abuse in traumatized personnel are empirical and of uncertain efficacy.

PTSD and the endocannabinoid system (ECS)

The endocannabinoid system (ECS) has recently emerged as a possible target for the treatment of mood and anxiety disorders including PTSD (Chhatwal and Ressler 2007; Hill and Gorzalka 2009; Moreira and Wotjak 2010; Alger and Kim 2011). For example, a cannabinoid receptor 1 (CB1R) agonist prevented the development of behavioral and endocrine alterations in a rat model of intense stress (Ganon-Elazar and Akirav 2012). Rats were treated with the CB1R agonist WIN55,212-2 (WIN) immediately after intense stress exposure and were tested 1 week later for anxiety and hypothalamic-pituitary-adrenal (HPA) axis function. Exposure to stress enhanced anxiety behavior and HPA axis function and treatment with WIN after stress exposure prevented anxiety and HPA axis function. These effects were blocked by administration of the CB1R antagonist AM251, suggesting the involvement of the CB1R. This study suggested that the preventive effects induced by WIN are mediated by an activation of CB1 receptors in the brain and that cannabinoids could serve as a pharmacological treatment of PTSD.

PTSD and nicotine dependence

Nicotine use is associated with exposure to traumatic events and PTSD (Feldner, Babson et al. 2007). Individuals with PTSD show higher rates of smoking and worse smoking cessation outcome. For instance, almost 94% of individuals with PTSD relapsed in the first week after quitting in a community sample of daily smokers (Zvolensky, Gibson et al. 2008). Other studies have shown that smokers exposed to a traumatic event who develop PTSD tend to smoke more than those exposed to trauma that do not develop PTSD, a relationship consistent across age and trauma type (Shalev, Bleich et al. 1990; Lipschitz, Rasmusson et al. 2003; Hapke, Schumann et al. 2005). Smoking among trauma-exposed adults has been found to range between 32% (Weinbaum, Stratton et al. 2001) and 58% (Weaver and Etzel 2003). Compared to the non-smokers, smokers were more likely to develop posttraumatic stress symptoms (Ganz 2000), which is in line with the findings that traumatic event exposure facilitates smoking behavior (Feldner, Babson et al. 2007).

Growing evidence suggests a relationship between trauma exposure and subsequent smoking behavior in a military population. Beckham et al (1997) found that the number of Vietnam combat veterans who smoked was not significantly different between those with PTSD (53%) and those without PTSD (45%) (Beckham, Kirby et al. 1997). However, they found that veterans with PTSD were more likely to be heavy smokers (48% smoked $\geq$ 25 cigarettes daily vs. 28% in veterans without PTSD). Also, Vietnam veterans with PTSD who smoked reported more PTSD symptoms than non-smokers with PTSD (Beckham, Roodman et al. 1995). Among treatment-seeking veterans with PTSD (N=826) who were referred to a VA clinic for comprehensive health assessment, 73% smoked $\geq$ 20 cigarettes per day (Buckley, Mozley et al. 2004),

indicating a high prevalence of nicotine dependence in the military personnel after the trauma exposure.

The ECS and nicotine dependence

The ECS appears to play a critical role in mediating the reinforcing effects of nicotine and relapse to nicotine-seeking behavior in abstinent subjects. However, the results from the previous studies have been inconsistent. A study, using a CB1R agonist WIN 55,212-2, on nicotine self-administration with a progressive-ratio (PR) schedule of reinforcement and a reinstatement paradigm, showed that CB1R stimulation facilitates motivation for nicotine and nicotine seeking in rats (Gamaleddin, Wertheim et al. 2012). These effects were reversed by the CB1R antagonist rimonabant, but not by the CB2R antagonist AM630 indicating CB1R specific effects. Another study reported that the CB1R antagonist SR141716 reduced nicotine self-administration by antagonizing dopamine release in the mesolimbic system (Cohen, Perrault et al. 2002). These studies indicate that the CB1Rs in the brain play an important role in nicotine self-administration. However, studies using indirect agonists for the ECS reported conflicting results. For instance, the endocannabinoid reuptake inhibor VDM11 attenuated relapse to nicotine-seeking in rats (Gamaleddin, Guranda et al. 2011). Another means of elevating the endocannabinoid levels is by inhibiting activity of the hydrolase fatty acid amide hydrolase (FAAH), an enzyme responsible for breakdown of the endocannabinoids. It has been shown that a FAAH inhibitor URB597 attenuated nicotine withdrawal symptoms in rats (Cippitelli, Astarita et al. 2011). These results suggest that direct stimulation the CB1Rs facilitate nicotine self-administration while indirectly increasing the levels of the endocannabinoids attenuate nicotine self-administration in rats. Thus, drugs targeting the CB1Rs may have therapeutic potential for the treatment of nicotine dependence.

PTSD and caffeine

Caffeine is the most widely used licit drug with psychoactive effects, used by 80% of the world's population (James 1997). Although the effects of caffeine on learning and memory have been well understood (Abreu, Silva-Oliveira et al. 2011; Leite, Wilhelm et al. 2011; Nebes, Pollock et al. 2011; Panlilio, Ferre et al. 2011), the effects of caffeine on trauma-related memory and subsequent drug-seeking behavior remain largely unknown. There has been a recent increase in the availability and the use of "Energy Drinks" containing higher levels of caffeine (Reissig, Strain et al. 2009). For example, energy drink consumption of 1 or more times a day by the U.S, Army service members are 17.78% and energy drink consumption with alcohol of 1 or more times a week are 13.5% within a 30 day period (unpublished data). An animal study reported that caffeine exerts anxiogenic effects in rats tested on an unstable elevated exposed plus maze (Jones, Duxon et al. 2002). Interestingly, co-administration of caffeine and THC (an active ingredient of marijuana) worsened memory deficits induced by THC alone (Panlilio, Ferre et al. 2011). The authors demonstrated that this effect was due to A1 receptor, but not A2 receptor, blockade in the brain. However, little is known on the effects of caffeine during trauma exposure and subsequent anxiety/depression and drug seeking behavior. We hypothesize that caffeine use during trauma exposure may strengthen fear memory and subsequent stress responses. We will investigate the effects of caffeine on stress responses and drug seeking using rat models. Understanding the biological mechanism by which caffeine modulates fear memory and

drug seeking may contribute to the development of novel treatments for a comorbid PTSD and nicotine dependence.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: DoD BRD, NIH RePorter (CRISP), PubMed, AGRICOLA, AltWeb

II.2.2. Date of Search: January 12, 2012

II.2.3. Period of Search: BRD: 1997-2011 (the latest year available); NIH RePorter: entire database; PubMed: entire database; AGRICOLA: entire database; AltWeb: entire database

II.2.4. Key Words and Search Strategy: Stress, trauma, nicotine self-administration, nicotine dependence, relapse, nicotine seeking, cannabis/cannabinoid, endocannabinoid system, caffeine, cannabinoid receptors

II.2.5. Results of Search:
Stress AND nicotine AND rat 121
Stress AND nicotine self-administration AND rat 4
Stress AND nicotine seeking AND rat 0

Stress AND cannabinoid/cannabis AND rat 78
Stress AND cannabinoid/cannabis AND rat AND nicotine self-administration 0

Stress AND caffeine AND rat 74
Stress AND caffeine AND rat AND nicotine self-administration 0

Cannabinoid AND nicotine self-administration AND rat 2
Cannabinoid AND nicotine self-administration AND rat AND stress 0
We found no previous studies reporting the experiments that we proposed in this protocol.

III. OBJECTIVE\HYPOTHESIS: The driving hypothesis for this project is that exposure to aversive stress affects the endocannabinoid system in brain regions associated with reward pathways in ways that tend to increase drug seeking and drug taking behaviors in stressed individuals. We will compare the effects of three stress models on subsequent anxiety/depression behavior and relapse to drug seeking in rats. Different stress models will be compared to study changes in genes and proteins in the brain and drug seeking that are related to stress in general, not to one specific stress paradigm. One model is the "*traumatic stress*" which includes restraint and tail shock over three days developed by Dr. (b)(6) It has been shown that this paradigm produces physiological and behavioral abnormalities, such as elevations in basal plasma corticosterone levels, exaggerated acoustic startle responses and reduced body weight (Servatius, Ottenweller et al. 1995). A second stress model is the "*warrior stress*" paradigm developed by the (b)(6) lab. This model combines predator odor stress with unpredictable environmental stimuli. Unpredictable stress has face validity in relation to human stress and has been shown to reliably produce elevations in stress hormones including CORT and CRH (Weinstock,

Matlina et al. 1992) and behavior (Gonzalez Jatuff, Berastegui et al. 1999) in rodent studies. A third model is classical fear learning. This model has been used extensively in studying consolidation and retrieval of fear memory in rats (LeDoux 2003). We will test whether retrieval of fear memory triggers relapse to nicotine seeking in rats.

Recent studies have shown that CB1R agonist WIN55,212-2 attenuates stress and anxiety symptoms (Ganon-Elazar and Akirav 2012) while facilitating relapse to nicotine seeking (Gamaleddin, Wertheim et al. 2012) in rats. However, these studies failed to investigate the effects of CB1 agonist on stress-induced nicotine seeking. Since nicotine self-administration has been shown to reduce certain anxiety and stress symptoms in humans (Ziedonis, Hitsman et al. 2008), this may explain why CB1R stimulation facilitates nicotine self-administration and reduces stress responses simultaneously. Interestingly, co-administration of caffeine and THC (an active ingredient of marijuana) worsened memory deficits induced by THC alone (Panlilio, Ferre et al. 2011). However, little is known on the effects of caffeine on trauma-related memory and subsequent drug seeking behavior. Thus, we will investigate the effects of the cannabinoids and caffeine on stress-induced anxiety and relapse to nicotine seeking in rats.

Using intravenous drug self-administration in rats, we will investigate

Specific Aim 1: the effects of three stress models (traumatic stress, warrior stress and fear learning) on depression, anxiety and relapse to nicotine seeking in rats.

Specific Aim 2: the effects of CB1R agonist during stress exposure on depression, anxiety and relapse to nicotine seeking in rats.

Specific Aim 3: the effects of caffeine during stress exposure on depression, anxiety and relapse to nicotine seeking in rats.

IV. MILITARY RELEVANCE: There is a high comorbidity of PTSD and substance use disorders in the military population (Beckham, Wiley et al. 2008; Boden, Kimerling et al. 2011; Booth, Mengeling et al. 2011). A substantial number of military veterans and active duty service members relies on licit and illicit substances such as caffeine, nicotine, alcohol and marijuana to alleviate stress and anxiety symptoms as an active coping strategy (Boden, Kimerling et al. 2011). Thus, the outcome from this study will contribute to the development of more effective therapeutic interventions aimed at preventing or treating a comorbid PTSD and substance use disorders in the military population.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures: To determine the biological mechanisms of different stressors on nicotine seeking, and the role of CB1R agonist and caffeine, it is necessary to use well-established animal models of stress and an intravenous nicotine self-administration paradigm.

Anxiety and depression measures

Open field locomotor activity will be measured using an Accuscan Electronics Digiscan infrared photocell system. Animals are placed singly in a 20 x 20 x 30 cm clear Plexiglas

arena and a Plexiglas lid with multiple ventilation holes placed on top of the arena. Data are automatically gathered and transmitted to a computer operating the Fusion 3.4 software system for data collection and analyses. The interfaced software measures two dozen activity variables, including total distance and horizontal and vertical activity. Chambers are cleaned between subjects with Clidox-S base, water, and activator in a 1:18:1 preparation. The duration of locomotor activity testing is a maximum of 60 min per session. Sixteen open field activity boxes from the Dr (b)(6) Lab will be used.

Open field center time: Center time is measured using the Accuscan open field activity system during the open field activity session. Anxiety is considered to be inversely related to amount of time spent in the center zone of the open field chamber.

Rearing: Vertical activity is measured in the Accuscan open field activity system to provide an index of depression. Rearing (or vertical activity) is considered to be inversely related to major depressive symptoms.

Intravenous nicotine self-administration

Apparatus

Eight operant chambers (Med Associates Inc., St. Albans, VT, USA) for nicotine self-administration are contextually different from the animals' home cage, and located in a different room (b)(6) Each chamber is equipped with an infusion pump assembly consisting of a Razel Model A pump (Stamford, CT, USA) and 10 ml syringe connected to a fluid swivel (Instech, Plymouth Meeting, PA, USA) by Teflon tubing. Tygon® tubing enclosed by a metal spring connected the swivel to the animal's catheter exit port and was secured to Teflon threads on the catheter assembly. Each operant chamber contains two levers, and either one (Fixed Ratio 1) or five (Fixed Ratio 5) 20 g lever press responses on the active lever delivered an i.v. injection of sterile nicotine solution (Sigma/Aldrich, MO, USA) dissolved in 0.9% saline. During each injection, a cue light above the active lever is illuminated, and the house light is extinguished. Each injection is followed by an additional "time-out" (TO) period in which the cue light is extinguished; lever press responses during the entire injection-TO period are recorded but had no programmed consequences. Responses on the inactive lever are recorded but had no scheduled consequence. A detailed procedure on intravenous drug self-administration in rats has been described previously (b)(6),(b)(4) (b)(6),(b)

Lever press training

Animals are initially trained to press a lever for sucrose pellets in ventilated operant chambers under restricted diet conditions for three days. This procedure will facilitate initial nicotine self-administration training. This procedure requires an initial 16–20 hrs of food restriction followed by ad lib feeding for a few hrs after each test session. This procedure allows transient weight gain overnight, but animals return to 90% initial body weight prior to the next test session on the following day.

Intravenous (IV) catheter surgery

Please see section V.4.3 below (page 18).

Intravenous nicotine self-administration and reinstatement

After a minimum 6 day recovery from IV catheter surgery, animals are placed in operant test chambers and allowed to acquire nicotine self-administration (0.03 mg/kg/0.1 ml in a 1 s injection) on one lever press/injection (Fixed Ratio 1: Time Out 20 sec) reinforcement schedule in a daily 2-h self-administration test sessions for 5 days per week. Animals are weighed before each nicotine self-administration session to adjust nicotine dose (0.03 mg/kg). After 2-3 weeks of nicotine self-administration, animals will be tested on extinction and reinstatement paradigm. Reinstatement test will measure the ability of specific experimenter delivered stimuli to induce relapse to drug seeking behavior as described previously (Sutton, Schmidt et al. 2003). Prior to reinstatement testing, nicotine self-administration behavior will be extinguished through extinction training when lever press responses are no longer reinforced by drug injections. When lever press responding is extinguished, reinstatement testing commences with experimenter delivered injections of nicotine, drug associated stimuli (lights associated with drug injections during self-administration), and a brief period of intermittent mild footshock stress. These stimuli induce relapse as indicated by an increase in non-reinforced responding at the lever that previously delivered the drug injections during self-administration training. Reinstatement procedures in rats are conducted in a between session paradigm. In this paradigm, self-administration, extinction, and reinstatement occur over consecutive daily test sessions. The between session paradigm is used for drug pre-treatments that are given on one or each occasion, and reinstatement is tested over a 5 day period following the pretreatment. Some animals in reinstatement studies will be exposed once to mild footshock stress in the operant test chambers at commonly used parameters (0.5 mA/0.5 sec every 30 seconds for 15-30 minutes). Footshock stress is used to reinstate drug seeking behavior, and is thought to represent an animal model of stress induced drug craving and relapse (Self and Choi 2004).

Three stress models in rats

1. Traumatic stress

Animal model has been developed in which exposure to stress provoke a wide range of physiological responses. Intense stress, aversive challenges, and situational reminders of traumatic stress in animal models have resulted in long-term effects on behavioral, autonomic, and hormonal responses that mimic many of the clinical symptoms observed in those with PTSD. We will use an intense stress paradigm developed by Dr. (b)(6) in Psychiatry department. Stress exposure consists of a two-hour per day session of immobilization and tail-shocks. The rat is placed in a clear Plexiglas tube which enables gentle, but secure form of restraint. The size of the tube restricts the animal turning or escaping, but natural posture can be maintained. Animals can breathe naturally through the holes in the tube. Forty electric shocks to the tail (2 mA, 3 s duration) are applied at varying intervals (140 to 180 s) over the course of two hours on three consecutive days. Animals will be weighed between each stress exposure. This stress protocol was adapted from the "learned helplessness" paradigm in which animals undergo an aversive experience under conditions in which they cannot perform any adaptive responses (Seligman and Maier 1967; Seligman 1968). Considering the life span difference between humans and rats, three days of intense stress exposure in rats

are equivalent to three months of stress in humans. This protocol has been approved for Dr. (b)(6) by the IACUC (b)(6)

2. Warrior stress

The Warrior Stress Paradigm combines predator stress with unpredictable environmental stimuli. Predator stress is a non-painful but effective stressor used in rodent models investigating the effects of stress. Predator stress can be manipulated by presenting the actual predator or the odors of the predator. Exposure to predator stress produces increases in stress hormones (Hayley, Borowski et al. 2001). Exposure to predator stress also produces behavioral changes in rodents including differences in food consumption, elevated plus maze, startle response, freezing behavior, withdrawal behavior, and exploratory behavior (Belzung, El Hage et al. 2001; Endres, Apfelbach et al. 2005; Masini, Sauer et al. 2005; Takahashi, Nakashima et al. 2005; Adamec, Head et al. 2006). Predator stress is presented by introducing a cotton ball with commercially available fox urine into a test cage with the rat subject. Unpredictable, non-painful stressors include noise, flashing light, and cage shaking. Unpredictable stress are included because it is a face-valid model of human stress and also has been shown to reliably produced alterations in stress hormones (Fride, Soreq et al. 1986; Weinstock, Matlina et al. 1992), and behavior (Gonzalez Jatuff, Berastegui et al. 1999) in rodent studies. The animals are transferred from their home cage and housing room to the "stress cage" (covered with a Plexiglas lid) to be exposed to the stressors. The stress procedure lasts 20 minutes for 5 days. Fox urine (15mL) is placed on a large cotton ball and placed in varying spots in the stress cage. A bright florescent overhead light remains on during the procedure. Animals will be weighed between each stress exposure. This protocol has been approved for Dr. (b)(6) by the IACUC.

3. Pavlovian fear learning

Sprague-Dawley rats will be placed in a Plexiglas rodent conditioning chamber with a metal grid floor (model E10-10; Coulbourn Instruments, Lehigh Valley, PA, USA), dimly illuminated by a single house light, and enclosed within a sound-attenuating chamber (model E10-20). Rats will be divided into two groups. The paired group will be presented with five pairings of a tone for 20 s (CS: conditioned stimulus; 5 kHz, 75 dB) that co-terminates with a foot shock (US: unconditioned stimulus; 0.5 s, 1.0 mA). The unpaired group (control) will receive five non-overlapping presentations of the CS and US. The unpaired group will be included into the design in order to assess how contextual stimuli not associated with the phasic tone in the paired group differ in freezing response. Animals will be weighed between each stress exposure. This protocol has been approved for Dr. (b)(6) by the IACUC (b)(6)

Flow chart and time line of the study

Animals arrive at the USUHS Animal Facility (CLAM)

↓

Animals acclimate to USUHS Animal Facility — 1 week

↓

Baseline open field activity testing — 1 day

↓

Lever press training (sucrose pellets) — 3 days

↓

IV catheter surgery and recovery — 1 week

↓

Nicotine self-administration — 2-3 weeks

↓

Stress exposure ← drug/vehicle injections before stress — 3-5 days

↓

Post-stress open field activity testing — 1 day

↓

Between session extinction — 5 days

↓

Reinstatement testing — 5-6 days

##Estimated length of one iteration is approximately 7-8 weeks.

Exp. 1: Effects of traumatic stress on anxiety, depression and relapse to nicotine seeking (n=56)

Rationale: The purpose of this experiment is to determine role of traumatic stress exposure on anxiety, depression and relapse to nicotine seeking in rats.

Experimental design (see Table of timeline for details): Rats (n=14 per group) will be tested using open field activity and undergo surgery for implantation of an IV catheter. Following 1 week of recovery, animals will be trained to self-administer nicotine (0.03 mg/kg/infusion) or saline for 2 hr/day in operant chambers for 3 weeks. Animals will be tested again in an open field activity paradigm. After acquisition of nicotine self-administration, rats will be exposed to 3 days of traumatic stress and tested on an open field activity paradigm. Animals will be tested on extinction from nicotine self-administration (across 5 days) and reinstatement of drug seeking using drug associated cue light, priming injection of nicotine (0, 0.1 and 0.5 mg/kg, s.c.) and unpredictable footshock to trigger relapse to nicotine seeking in rats (across 5 days). In order to study potential long-term effects of traumatic stress on drug seeking behavior, same animals will be re-tested on the extinction and reinstatement paradigm 1 week after the initial testing.

Group	IV surgery	Mood/Anxiety behavior	Traumatic Stress	Reinstatement	Pain category	N
Control	Yes	Yes	No	No	D	14
Control	Yes	Yes	Yes	No	E	14
Nicotine	Yes	Yes	No	Drug cue Drug priming Mild footshock	E	14
Nicotine	Yes	Yes	Yes	Drug cue Drug priming Mild footshock	E	14

Exp. 2: Effects of warrior stress on anxiety, depression and relapse to nicotine seeking (n=56)

Rationale: The purpose of this experiment is to determine role of warrior stress exposure on anxiety, depression and relapse to nicotine seeking in rats.

Experimental design (see Table of timeline for details): Rats (n=14 per group) will be tested in open field activity apparatus and undergo surgery for implantation of an IV catheter. Following 1 week of recovery, animals will be trained to self-administer nicotine (0.03 mg/kg/infusion) or saline for 2 hr/day in operant chambers for 3 weeks. After acquisition and maintenance of nicotine self-administration, rats will be exposed to warrior stress over 5 days and tested in open field activity apparatus. Animals will be tested on extinction from nicotine self-administration (across 5 days) and reinstatement of drug seeking using drug associated cue light, priming injection of nicotine (0, 0.1 and 0.5 mg/kg, s.c.) and unpredictable footshock to trigger relapse to nicotine seeking in rats (across 5 days).

Group	IV surgery	Mood/Anxiety behavior	Warrior Stress	Reinstatement	Pain category	N
Control	Yes	Yes	No	No	D	14
Control	Yes	Yes	Yes	No	D	14
Nicotine	Yes	Yes	No	Drug cue Drug priming Mild footshock	E	14
Nicotine	Yes	Yes	Yes	Drug cue Drug priming Mild footshock	E	14

Exp. 3: Effects of fear memory on anxiety, depression and relapse to nicotine seeking (n=56)

Rationale: The purpose of this experiment is to determine role of fear learning on anxiety, depression and relapse to nicotine seeking in rats.

Experimental design (see Table of timeline for details): Rats (n=14 per group) will be tested in open field activity and undergo surgery for implantation of an IV catheter. Following 1 week of recovery, animals will be trained to self-administer nicotine (0.03 mg/kg/infusion) or saline for 2 hr/day in operant chambers for 3 weeks. After acquisition and maintenance of nicotine self-administration, rats will be exposed to fear learning paradigm and tested on open field activity. Animals will be tested on extinction from nicotine self-administration (across 5 days) and reinstatement of drug seeking using drug associated cue light, tone associated with fear memory, priming injection of nicotine (0, 0.1 and 0.5 mg/kg, s.c.) and unpredictable footshock to trigger relapse to nicotine seeking in rats (across 6 days).

Group	IV surgery	Mood/Anxiety behavior	Fear Learning	Reinstatement	Pain category	N
Control	Yes	Yes	No	No	D	14
Control	Yes	Yes	Yes	No	E	14
Nicotine	Yes	Yes	No	Drug cue Fear Cue Drug priming Mild footshock	E	14
Nicotine	Yes	Yes	Yes	Drug cue Fear Cue Drug priming Mild footshock	E	14

Exp. 4: Effects of CB1 agonist during stress exposure on subsequent anxiety, depression and relapse to nicotine seeking (n=112)

Rationale: The purpose of this experiment is to determine effects of CB1R agonist (WIN) during stress exposure on subsequent anxiety, depression and relapse to nicotine seeking in rats. Based on the experiments 1, 2 and 3 described above, we'll determine which stress paradigm has the most robust effects on subsequent anxiety, depression and relapse to nicotine seeking in rats. In this experiment, we'll test the effects of CB1R agonist during stress exposure on subsequent anxiety, depression and relapse nicotine seeking in rats.

Experimental design (see Table of timeline for details): Rats (n=14 per group) will be tested in open field activity paradigm and undergo surgery for implantation of an IV catheter and recover for 5-7 days after the surgery. Animals will be trained to self-administer nicotine (0.03 mg/kg/infusion) or saline for 2 hr/day in operant chambers for 3 weeks. After acquisition of nicotine self-administration, rats will be exposed to a stress paradigm with or without CB1R agonist WIN 55,212-2 (1 mg/kg, i.p.). Animals will be tested on extinction from nicotine self-administration (across 5 days) and reinstatement of drug seeking using drug associated cue light, priming injection of nicotine (0, 0.1 and 0.5 mg/kg, s.c.) and unpredictable footshock to trigger relapse to nicotine seeking in rats (across 5 days).

Group	IV surgery	Mood/Anxiety behavior	Stress	CB1R agonist	Reinstatement	pain	N
Control	Yes	Yes	No	No	No	D	14
Control	Yes	Yes	No	Yes	No	D	14
Control	Yes	Yes	Yes	No	No	E	14
Control	Yes	Yes	Yes	Yes	No	E	14
Nicotine	Yes	Yes	No	No	Drug cue Drug priming Mild footshock	E	14
Nicotine	Yes	Yes	No	Yes	Drug cue Drug priming	E	14

					Mild footshock		
Nicotine	Yes	Yes	Yes	No	Drug cue Drug priming Mild footshock	E	14
Nicotine	Yes	Yes	Yes	Yes	Drug cue Drug priming Mild footshock	E	14

Exp. 5: Effects of caffeine during stress exposure on subsequent anxiety, depression and relapse to nicotine seeking (n=112)

Rationale: The purpose of this experiment is to determine effects of caffeine during stress exposure on subsequent anxiety, depression and relapse to nicotine seeking in rats. Based on the experiments 1, 2 and 3 described above, we'll determine which stress paradigm has the most robust effects on anxiety, depression and relapse to nicotine seeking in rats. In this experiment, we'll test the effects of caffeine during stress exposure on subsequent anxiety, depression and relapse to nicotine seeking in rats.

Experimental design (see Table of timeline for details): Rats (n=14 per group) will be tested in open field activity paradigm and undergo surgery for implantation of an IV catheter and recover for 5-7 days after the surgery. Animals will be trained to self-administer nicotine (0.03 mg/kg/infusion) or saline for 2 hr/day in operant chambers for 3 weeks. After acquisition of nicotine self-administration, rats will be exposed to a stress paradigm with or without caffeine (20 mg/kg, i.p.). Animals will be tested on extinction from nicotine self-administration (across 5 days) and reinstatement of drug seeking using drug associated cue light, priming injection of nicotine (0, 0.1 and 0.5 mg/kg, s.c.) and unpredictable footshock to trigger relapse to nicotine seeking in rats (across 5 days).

Group	IV surgery	Mood/Anxiety behavior	Stress	Caffeine	Reinstatement	Pain	N
Control	Yes	Yes	No	No	No	D	14
Control	Yes	Yes	No	Yes	No	D	14
Control	Yes	Yes	Yes	No	No	E	14
Control	Yes	Yes	Yes	Yes	No	E	14
Nicotine	Yes	Yes	No	No	Drug Cue Drug priming Mild footshock	E	14
Nicotine	Yes	Yes	No	Yes	Drug Cue Drug priming Mild footshock	E	14
Nicotine	Yes	Yes	Yes	No	Drug Cue Drug priming Mild footshock	E	14
Nicotine	Yes	Yes	Yes	Yes	Drug Cue Drug priming Mild footshock	E	14

Revised

paradigm has the most robust effects on anxiety, depression and relapse to drug seeking in rats. In this experiment, we'll test the effects of caffeine during stress exposure on subsequent anxiety, depression and relapse to drug seeking in rats.

Experimental design (see Table of timeline for details): Rats (n=14 per group) will be tested in open field activity paradigm and undergo surgery for implantation of an IV catheter and recover for 5-7 days after the surgery. Animals will be trained to self-administer drug (0.1 ml infusion) or saline for 2 hr/day in operant chambers for 3 weeks. After acquisition of drug self-administration, rats will be exposed to a stress paradigm with or without caffeine (20 mg/kg, i.p.). Animals will be tested on extinction from drug self-administration (across 5 days) and reinstatement of drug seeking using drug associated cue light, priming injection of drug (0, 0.1 and 0.25 mg/kg, s.c.) and unpredictable footshock to trigger relapse to drug seeking in rats (across 5 days).

Group	IV surgery	Mood/Anxiety behavior	Stress	Caffeine	Reinstatement	Pain	N
Morphine	Yes	Yes	No	No	Drug cue Drug priming Mild footshock	E	14
Morphine	Yes	Yes	No	Yes	Drug cue Drug priming Mild footshock	E	14
Morphine	Yes	Yes	Yes	No	Drug cue Drug priming Mild footshock	E	14
Morphine	Yes	Yes	Yes	Yes	Drug cue Drug priming Mild footshock	E	14
Nicotine	Yes	Yes	No	No	Drug Cue Drug priming Mild footshock	E	14
Nicotine	Yes	Yes	No	Yes	Drug Cue Drug priming Mild footshock	E	14
Nicotine	Yes	Yes	Yes	No	Drug Cue Drug priming Mild footshock	E	14
Nicotine	Yes	Yes	Yes	Yes	Drug Cue Drug priming Mild footshock	E	14

Experimental endpoints

If signs of morbidity are observed over the course of the experiments, the veterinarian is notified and, if necessary, rats are excluded from the study and euthanized. Signs for judging morbidity include rapid breathing; shallow, labored breathing; weight loss (more than 10% of body weight not regained within 48 hr, continuous body weight loss over 5 days of more than 5 g per day), ruffled fur (rough hair coat), hunched posture, hypothermia or hyperthermia, ulceration dermatitis or infected tumors, diarrhea, impaired ambulation, evidence of muscle atrophy or other signs of emaciation, bleeding, or inability to remain upright. In cases of body weight loss, supplemental soft and sweetened food is provided, and if necessary drug self-administration training is withheld until the animal recovers.

V.2. Data Analysis: The number of rats is determined by the number of experimental groups needed for a clear interpretation of the behavioral and neurobiological data.

Revised

Intravenous drug self-administration is a highly regulated and conditioned behavior in rats. Based on our experience, 14 animals per group allows for a sufficient statistical power to detect group differences in drug self-administration behavior as described previously (b)(6),(b)(4) We also performed a power calculation using R statistical language (http://www.r-project.org/). We expect a large effect size (0.5) between groups based on the previous studies (b)(6),(b)(4) (b)(6),(b)(4) Therefore, an effect size (0.5), high power (0.80), and alpha level of 0.05 require a total of 14 animals per group.

Behavioral data will be analyzed using Analyses of Variance (ANOVA). The between-subject independent variables for experiments are Drug (drug vs. saline), Stress (stress vs. no stress), and CB1 agonist (CB1 agonist vs. vehicle). The within-subject independent variable is Time (open field activity). For experiments 1, 2 and 3, data will be analyzed with ANOVA (Stress) with repeated measures on Time (open field activity). Experiments 4 and 5 will be analyzed with ANOVA (Stress x CB1 agonist) with repeated measures on Time (open field activity). Significant interactions will be followed by Newman–Keuls *post-hoc* tests. The level of significance is set at 0.05 for all comparisons. Statistical analyses will be conducted using multiple software such as the Prism (GraphPad, San Diego, CA, USA), the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) the R statistical language (http://www.r-project.org/).

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: This study will require the use of intact living animals; behavioral measurements, such as administration of the stress protocol, anxiety symptoms, relapse to drug seeking cannot be made on cells grown in culture or on a computer simulation or other in vitro systems. Thus, non-animal models were not considered for this project and it is not possible to replace or eliminate the use of animals in this study. Due to ethical considerations, the proposed studies cannot be done with human subjects.

V.3.2. Animal Model and Species Justification: Rats will be used in behavioral paradigms that model stress, anxiety and nicotine dependence in humans. Rats are amendable to pharmacological manipulations needed to study the biological mechanisms involved in traumatic stress and substance abuse. The experimental nature of the work necessitates the use of model organisms rather than human subjects. However, results from our laboratory studies complement data obtained from human clinics and experiments. We will continually evaluate our approaches and adapt experiments for *in vitro* or computer models whenever possible.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	R. Norvegicus	
V.3.3.2. Strain/Stock:	Sprague-Dawley	

Experimental endpoints

If signs of morbidity are observed over the course of the experiments, the veterinarian is notified and, if necessary, rats are excluded from the study and euthanized. Signs for judging morbidity include rapid breathing; shallow, labored breathing; weight loss (more than 10% of body weight not regained within 48 hr, continuous body weight loss over 5 days of more than 5 g per day), ruffled fur (rough hair coat), hunched posture, hypothermia or hyperthermia, ulceration dermatitis or infected tumors, diarrhea, impaired ambulation, evidence of muscle atrophy or other signs of emaciation, bleeding, or inability to remain upright. In cases of body weight loss, supplemental soft and sweetened food is provided, and if necessary drug self-administration training is withheld until the animal recovers.

V.2. Data Analysis: Behavioral data will be analyzed using Analyses of Variance (ANOVA). The between-subject independent variables for experiments are Drug (nicotine vs. saline), Stress (stress vs. no stress), and CB1 agonist (CB1 agonist vs. vehicle). The within-subject independent variable is Time (open field activity). For experiments 1, 2 and 3, data will be analyzed with ANOVA (Stress) with repeated measures on Time (open field activity). Experiments 4 and 5 will be analyzed with ANOVA (Stress x CB1 agonist) with repeated measures on Time (open field activity). Significant interactions will be followed by Newman–Keuls *post-hoc* tests. The level of significance is set at 0.05 for all comparisons. Statistical analyses will be conducted using multiple software such as the Prism (GraphPad, San Diego, CA, USA), the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) the R statistical language (http://www.r-project.org/).

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V.3.2. Animal Model and Species Justification: Rats will be used in behavioral paradigms that model stress, anxiety and nicotine dependence in humans. Rats are amendable to pharmacological manipulations needed to study the biological mechanisms involved in traumatic stress and substance abuse. The experimental nature of the work necessitates the use of model organisms rather than human subjects. However, results from our laboratory studies complement data obtained from human clinics and experiments. We will continually evaluate our approaches and adapt experiments for *in vitro* or computer models whenever possible.

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	R. Norvegicus	

V.3.3.2. **Strain/Stock**: Sprague-Dawley

V.3.3.3. **Source/Vendor**: (b)(4)

V.3.3.4. **Age**: 6-10 weeks

V.3.3.5. **Weight**: 250 - 350 gm

V.3.3.6. **Sex**: Male

V.3.3.7. **Special Considerations**: N/A

V.3.4. **Number of Animals Required (by Species)**: Rat 392

V.3.5. **Refinement, Reduction, Replacement (3 Rs)**:

V.3.5.1. **Refinement**: Animals will be monitored on a daily basis and be given pain medication and antibiotic if any sign of pain or distress occurs. In order to reduce stress and anxiety, animals will be acclimated to the facility for 1 week upon arrival.

V.3.5.2. **Reduction**: The number of rats is determined by the number of experimental groups needed for a clear interpretation of the behavioral and neurobiological data. In general, 14 rats are used per experimental manipulation. Based on our experience, we expect about 15% of rats (or 2 per experimental group) to be excluded due to catheter failure or unstable drug self-administration behavior. Therefore, it is expected that 12 rats per experimental group will successfully complete the experiment. Based on our experience, this number allows for a sufficient statistical power to detect group differences in behavior due to the experimental manipulations described above. However, whenever possible, we will utilize repeated measures designs to obtain multiple data points from the same animals.

V.3.5.3. **Replacement**: This study requires the use of intact living animals; behavioral measurement cannot be made on cells grown in culture or with computer models. Thus, it is not possible to replace or eliminate the use of animals in this study.

V.4. **Technical Methods**:

V.4.1. Pain / Distress Assessment during intense stress exposure: For traumatic stress model, electrodes will be attached to the tail of the animal by means of a spring clip in order to minimize any painful irritation that might result from repeated taping of the tail. All animals are monitored by a technician continuously during the two hours when tail shocks are being administered to assure that they do not get into positions that do not allow them to breathe properly. Animals will be monitored daily after the stress sessions

according to CLAM SOPs and continue to be monitored daily for any indication of pain. While the animals are expected to arrest weight gain during the three day stress exposure, any animal that would lose > 20% of its body weight as measured on Day -1 would be withdrawn from the study and euthanized. The stress protocol (Dr [(b)(6)] has been approved by the IACUC [(b)(6)]

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	0	
V.4.1.1.1.2. Column D:	112	
V.4.1.1.1.3. Column E:	280	

The number of animals in Column D indicates that these animals are subjected to either IV catheter surgery or warrior stress (no physical stress) as explained in page 9. The number of animals listed in Column E is based on the premise that these animals are exposed to either stress paradigm or nicotine self-administration which includes reinstatement using mild unpredictable electric footshock. The unalleviated pain and distress resulting from the electric shocks are momentary and cause no physical injury to the tissue of tail or feet.

V.4.1.2. Pain Relief / Prevention: N/A

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:
For IV catheter surgery, rats will be anesthetized with sodium pentobarbital (60 mg/kg, i.p.) based on a previous study (Choi, Edwards et al. 2011) or ketamine/xylazine (40-80 mg/kg ketamine, 5-10 mg/kg xylazine, i.p.) and penicillin G procaine (200,000 Units/kg, s.c.) will be given as a prophylactic. Rats will also receive atropine sulfate (0.04 mg/kg, s.c.) to reduce pulmonary congestion and facilitate breathing.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Animals will be acclimated for 1 week to the USUHS CLAM. During acclimation to CLAM, animals will be handled briefly each day to acclimate them to being handled; this will reduce any stress that might result from handling of the animals during the procedures described in this study. Following exposure to the stress protocol, animals will be monitored according to CLAM SOPs.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PubMed (MeSH), AltWeb, AGRICOLA

V.4.1.3.2. Date of Search: January 13, 2012

V.4.1.3.3. Period of Search: AltWeb: Entire database, AGRICOLA: Entire database PubMed: Entire database

V.4.1.3.4. Key Words of Search:

Pain and PTSD and rat
- PubMed (MeSH): 5 references, none related to present proposal
- AGRICOLA: "0 Records"
- AltWeb: Web (0)

Pain and nicotine self-administration and rat
- PubMed (MeSH): "No items found"
- AGRICOLA: "0 Records"
- AltWeb: Web (0)

Distress and nicotine self-administration
- PubMed (MeSH): 1 reference, not related to current proposal
- AGRICOLA: "0 Records"
- AltWeb: Web (0)

Foot shock and nicotine reinstatement and rat
- PubMed (MeSH): 0 reference
- AGRICOLA: "0 Records"
- AltWeb: Web (0)

Restraint stress and nicotine reinstatement and rat
- PubMed (MeSH): 0 reference
- AGRICOLA: "0 Records"
- AltWeb: Web (0)

Corticosterone and nicotine reinstatement and rat
- PubMed (MeSH): 0 reference
- AGRICOLA: "0 Records"
- AltWeb: Web (0)

V.4.1.3.5. Results of Search: No overlapping studies discovered

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification: A rat model of traumatic stress includes a young adult male rat that is restrained in a Plexiglas tube of appropriate size and receives a series of 40 tail shock (2 mA constant current, 3 sec duration) over a period of two hours daily on three consecutive days. A coiled silver electrode is attached to the tail with a spring clip so that there is no irritation of the tail either from the shocks or from adhesive materials being applied and removed each day. The number and strength of the shocks proposed has been optimized to yield a model

of chronic stress as measured by changes in behavior and by elevated plasma levels of corticosterone. This stress protocol has been approved for Dr. (b)(6) by the IACUC (b)(6) (b)(6) (b)(6)

V.4.2. Prolonged Restraint: All animals are monitored by a technician during the two hours when tail shocks are being administered to assure that they do not get into positions that do not allow them to breathe properly. Also, the electrode can become loose over the two hour period so that the animal no longer receives the shock; monitoring by a technician is required to ensure that the animals receive all of the forty tail shocks.

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: Rats will be maintained on a light-dark cycle (12 hr light/12 hr dark). The habituation period prior to surgery is 5-7 days during which the rats are group-housed.

V.4.3.2. Procedure: All surgery will be performed under aseptic conditions in a clean area. Each surgery will be done on a separate, clean and autoclaved sheet of Whitman Benchkote paper. Surgical instruments are sterilized in a glass bead sterilizer and cleaned/sterilized between successive surgeries. Sterile gloves are used for each rat's surgery and instruments are sterilized before each rat's surgery. Rats (250-350 g) are anesthetized with sodium pentobarbital (60 mg/kg, i.p.) or ketamine/xylazine (40-80 mg/kg ketamine, 5-10 mg/kg xylazine, i.p.) and atropine sulfate (0.04 mg/kg, s.c.) to reduce pulmonary congestion and facilitate breathing. Surgery starts after the rat fails to show the withdrawal reflex after pinching the rear toe (a procedure that is repeated several times during the surgery to ensure full anesthesia level). Supplemental anesthetic dose (0.2-0.4 ml) is given as needed. Intravenous silastic catheter is implanted into the right jugular vein and attached to either the head or the back of the animal.

For the head mount, the surgical areas (the top of the skull and the area above the right jugular vein) are shaved and cleaned with 70% alcohol and betadine scrub for three times, alternating 70% alcohol with betadine scrub. The jugular vein is exposed and the catheter is inserted into the vein and secured to it with a silk suture. The catheter then passes subcutaneously to the top of the skull where it exits into a connector (a modified 22-gauge cannula) mounted to the skull with 4 jeweler's screws and dental cement. The wound areas are sutured, and an antibiotic wound spray is applied to these areas. Rats are also given 2 ml of saline (injected SC) to replace lost fluid during surgery. After surgery, a plastic cap is placed over the open end of the connector. The analgesic buprenorphine (0.1 mg/kg, SC) is given approximately 30 min following the end of surgery. The sutures are removed 10-14 days after surgery.

For the back mount, two incisions will be made, one on the back (2.5 cm), and one on the neck (1 cm). The jugular vein is isolated by teasing apart fatty tissue above the vein with small scissor tips, just above where the vein passes over the clavicle. After isolation, the vein will be punctured with a needle (22 gauge) and the sterile Silastic catheter will be inserted to the level of the sinus vena cava just outside the right atrium. The entry point of the catheter will be secured in place with a 0.5 cm x 0.5 cm mersilene surgical mesh secured to the catheter. The remaining catheter will be pulled from the neck area subcutaneously to the back incision. Then the catheter will exit via premade

22 gauge stainless steel tubing cemented into place with bell shaped dental cement and the bell base (about 0.8 cm diameter) cemented to a 1.5 x 1.5 cm Marlex surgical mesh comprising the back mount. The incisions will be closed using two stainless steel wound clips and treated with topical antibiotic Neosporin ointment (Swabbed To Back Mounted Catheter Exit Area). Animals will receive antibiotic gentamycin sulfate (5-8 mg/kg, s.c.) and the analgesic buprenorphine (0.1 mg/kg s.c.) after each surgery. Animals will receive buprenorphine (0.1 mg/kg s.c.) once every 12 hrs for next 48 hrs and then as needed.

V.4.3.3. Post-surgical Provisions: During recovery (about 30-60 min), the rats are placed in a cage that is kept warm by a heating pad (located under the cage) to prevent hypothermia. The investigators are responsible for post-operative care. The post-operative care includes continuously monitoring the rats until they are fully awake and the animals' health will be monitored and recorded in the animal's medical record for at least 3 days after surgery or until the animal appears normal. The surgery log will be kept in a drawer in the surgery room. If pain or distress such as difficulty in breathing and hypothermia is observed at any time rats will be treated with buprenorphine (0.1 mg/kg s.c.). After the IV catheter surgery, rats are individually housed in the animal facility for 5-7 days. During this time period, the rats are handled and the catheters are flushed daily with 0.2 ml of heparinized (20 U/ml), bacteriostatic saline containing gentamycin sulfate (0.33 mg/ml, i.v.) as described previously (b)(6),(b)(4)

Catheter patency will be verified by infusion of a sodium methohexital (Anesthetic, 7-15 mg/kg, i.v.) solution. Patency is indicated by a brief (about 1 minute) but rapid anesthesia. Using these procedures, catheter life averages about 6–8 weeks for rats. Most catheters are lost due to the growth of the venous wall around the catheter tip. When catheters are no longer patent, animals are either sacrificed as described below, or the original catheter is removed and another one implanted in the left jugular vein. We have observed no weight loss or obvious physical impairment to the animals due to one or two consecutive catheter implantations.

V.4.3.4. Location: (b)(6)

V.4.3.5. Surgeon: Please see personnel page 22

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: The drugs will be delivered by intraperitoneal (i.p.) or subcutaneous (s.c.) injection (1ml latex free syringe w/ 27G½ needle, B-D, Franklin Lakes, NJ). Injection volume will be adjusted based on body weight and no more than 1 ml/kg for i.p. and 2 ml/kg for s.c. administration. For i.v. administration, maximum volume will be 0.3 ml.

Sodium pentobarbital (60 mg/kg, i.p.)
Atropine sulfate (0.04 mg/kg, s.c.)
Buprenorphine (0.1 mg/kg s.c.)
Nicotine (0.03 mg/kg/infusion, i.v.)
Nicotine (0, 0.1 and 0.5 mg/kg, s.c.)
Ketamine/xylazine (40-80 mg/kg ketamine, 5-10 mg/kg xylazine, i.p.)
Gentamycin sulfate (5-8 mg/kg, s.c.)
Gentamycin sulfate (0.33 mg/ml, i.v.)
Sodium methohexital (7-15 mg/kg, i.v.)
WIN 55,212-2 (0.1, 0.3, 1 mg/kg, i.p.).
Caffeine (20 mg/kg, i.p.).
Equithesin (400 mg/kg, i.p.)
A mixture of ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.)

V.4.4.2. Biosamples: Trunk blood and brain tissue will be collected when each animal is euthanized. These samples will be analyzed for further biochemical and molecular biology studies.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Rats are identified by tail markings and ear punches matched with IDs on the cage cards. Immediately after the IV catheter surgery under anesthesia, small hole on the ear will be made to identify each animal. Ear punching is commonly used for identification of rodents and involves using a special punch to produce a small (0.5 to 1 mm) notch near the edge of the ear. The cutting edge of the punch is disinfected 70% ethanol after each use.

V.4.4.6. Behavioral Studies: See section V (Materials and Methods)

V.4.4.7. Other Procedures: Stress exposure consists of a two hour per day session of immobilization (restraint) and tail-shocks, for three consecutive days. Each animal is restrained in a Plexiglas tube and an electrode is attached to the tail. Forty electric shocks (2 mA, 3 s duration) are applied at varying intervals (140 to 180 s) over a two hour period; then the rats are returned to their cages. Exposure to this three day stress regimen has been shown to be more effective than a single stress session in producing lasting physiological and behavioral abnormalities, such as elevations in basal plasma corticosterone levels, exaggerated acoustic startle responses and reduced body weight (Servatius, Ottenweller et al. 1995). This stress protocol has been approved for Dr. (b)(6) (b)(6) by the IACUC (b)(6)

V.4.4.8. Tissue Sharing: Brain tissue and trunk blood samples will be collected at the study end point and shared for nucleic acid and protein assays. All personnel engaging in this procedure will receive training by IACUC/ARC staff, or trained laboratory personnel. We will examine multiple brain areas involved in stress and substance abuse such as the prefrontal cortex, nucleus accumbens, amygdala, hypothalamus, hippocampus, substantia nigra and ventral tegmental area for nucleic

acids and protein assays. We will study expression levels of the target genes and proteins related to the endocannabinoid system, nicotine and caffeine receptors following stress exposure and nicotine seeking. These genes include *CB1R, CB2R* and *FAAH* for the endocannabinoid system, *CHRNA2, CHRNA3, CHRNA4, CHRNA5, CHRNA6, CHRNA7, CHRNA8, CHRNA9*, and *CHRNA10* for the nicotinic acetylcholine receptors localized in the brain and *ADORA1, ADORA2A, ADORA2B, and ADORA3* for the adenosine receptors.

V.4.5. Study Endpoint: The study endpoint will be the day immediately after the drug seeking testing.

V.4.6. Euthanasia: Animals will be anesthetized with either chloral hydrate such as Equithesin (Sedative, 400 mg/kg, i.p.) or a mixture of ketamine (100 mg/kg) + xylazine (10 mg/kg) and subsequently are perfused with saline and 10% formalin. Thoracotomy will only be performed after failure of the withdrawal reflex and then intracardiac perfusion will be performed. Supplemental anesthetic doses (0.4 ml of Equithesin or 50+5 mg/kg ketamine+xylazine mixture) are given as needed. For the perfusion, the heart is exposed, and a 17-gage needle that is connected to a pump via tubing is inserted to the left ventricle of the heart. Physiological saline (50 ml) is perfused over 2 min with a pump, followed by 4% paraformaldehyde (100 ml). The procedure is performed on the downdraft table in a Procedure/Necropsy Room.

For the collection of fresh brain tissue, cylinder carbon dioxide (CO2) in the LAM will be used as recommended anesthetic for rat euthanasia. Induction of anesthesia with CO2 (2-5%) is conducted with the animal placed in an appropriate-sized clear chamber. The depth of anesthesia will be determined by loss of withdrawal response to rear foot pad pinch, tail pinch and loss of movement in response to eye extra orbital touching.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) (b)(6) Room Number(s) (b)(6)

Euthanasia will be performed in (b)(6) or CLAM.
Behavior testing will be performed in (b)(6)
Rats will be housed in the Central Animal Facility (CAF)

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes X	No
Fluid Restriction:	Yes	No X

For the lever press training before the intravenous nicotine self-administration, animals

are slightly food restricted for three days. This procedure requires an initial 16–20 hrs of food restriction followed by ad lib feeding for a few hrs after each training. This procedure allows transient weight gain overnight, but animals return to 90% initial body weight prior to the next lever press training session on the following day. This procedure lasts for three days and animals are fed ad lib after the training.

V.5.1.3. Exceptions: In addition, our experiments require that animals implanted with intravenous catheters be housed individually in order to prevent them from chewing and destroying each other's catheter exit assemblies. Animals with iv catheters will be housed individually in standard plastic caging. In other cases, non-operated animals will be group housed in standard plastic caging, unless experiments require direct comparison of biochemical or behavioral data with catheterized animals, in which they will be housed similar to experimental groups.

V.5.2. Veterinary Medical Care: N/A

V.5.2.1. Routine Veterinary Medical Care: Animals will be cared for in accordance with LAM SOPs. Animals will be observed daily or more frequently by the veterinary staff. The CLAM veterinary staff will be available for consultation with the PI if any unexpected medical events arise.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays. Investigators' contact information will be available to the veterinary staff in case a decision about early euthanasia or treatments of an animal needs to be made on the weekend or holiday.

V.5.3. Environmental Enrichment: There won't be any environmental enrichment because animals are housed individually after IV catheter surgery and subsequently tested on nicotine self-administration.

V.5.3.1. Enrichment Strategy: N/A

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Anxiety, depression behavior	(b)(6)	Assistant Professor 16 years of	Conducted animal research at

Stress model IV surgery Drug self-administration		experience in animal research	(b)(6)
Anxiety, depression behavior Stress model IV surgery Drug self-administration	(b)(6) BS	Research Assistant	Attended all training classes required by USU in order to be approved to conduct animal research on Oct 19, 2010. Completed IACUC investigator training (Feb 15, 2011)
Anxiety, depression behavior Stress model IV surgery Drug self-administration	(b)(6) PhD	Post-doc (9 years experience in animal research)	Trained at Oregon Health & Science University (OHSU), VA Medical Center, Portland, OR, and George Mason University. Attended IACUC training at each of the before mentioned institutions. Completed online training on Feb 19, 2009 and completed USU Animal Investigator Training on Mar 26, 2009.
Traumatic stress	(b)(6) M.D., Ph.D.	Associate Professor 12 years of experience in animal research	Developed the protocol
Warrior stress	(b)(6) PhD	Professor	Developed the

			protocol
Fear conditioning	(b)(6) PhD	Assistant Professor 19 years of experience in animal research	Trained at Oxford, Yale and New York Universities. Held a British Home Office license for animal experimentation. Conducted animal experiments at Yale University. Attended all training classes required by USU in order to be approved to conduct animal research on May 30, 2009.
Molecular biology and biochemical Assay	(b)(6) MD	Associate Professor	Over 20 years of experiences in molecular biology
Consultation of the experiments and data analysis	(b)(6) MD	Professor, Chairman	Over 30 years of experiences in PTSD and anxiety disorders research
Anxiety, depression behavior Stress model IV surgery Drug self-administration	(b)(6) PhD	Post-doctoral Researcher	Trained at University of Cincinnati and Cincinnati Children's Hospital Medical Center IACUC mandated facility training at each institution Completed USUHS IACUC investigator training 2/16/10

VII. BIOHAZARDS/SAFETY: In order to protect personnel from chemical hazards (chloral hydrate and paraformaldehyde) all personnel will wear PPE (lab coats, mask,

gloves and safety glasses) to limit exposure to both chemical hazards and also animal allergens. Transcardiac perfusions with paraformaldehyde and glutaraldehyde will be conducted in a certified chemical fume hood with drain for disposal of waste fluids. The laboratory space has been previously evaluated by EHOS and has been in continuous use by the laboratory of Dr (b)(6) (PSY). The laboratory is now a shared space between the (b)(6) labs (PSY).

VIII. ENCLOSURES: N/A

IX. ASSURANCES: As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) 1/20/2012

Principal Investigator Signature Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) 1/20/2012

Principal Investigator Signature Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress caused by the stress paradigm **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. However, the potential pain and distress caused by the IV catheter surgery **WILL** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) 1/20/2012

Principal Investigator Signature Date

X. PROTOCOL ABSTRACT:

A. **Animal Protocol Number**:

B. **Animal Protocol Title**: *Stress and drug seeking in rats: Role of the endocannabinoid system and caffeine*

C. **Principal Investigator**: (b)(6) PhD

D. **Performing Organization**: Uniformed Services University of the Health Sciences

E. **Funding**: Center for the Study of Traumatic Stress (CSTS)

F. **Objective and Approach**:

We hypothesize that the endocannabinoid system and caffeine play a significant role in trauma-induced anxiety/depression and relapse to nicotine seeking behavior. Using well-established rat models of stress and drug self-administration, we will investigate

Specific Aim 1: the effects of three stress models (traumatic stress, warrior stress and fear learning) on depression, anxiety and relapse to nicotine seeking in rats.

Specific Aim 2: the effects of CB1R agonist during stress exposure on subsequent depression, anxiety and relapse to nicotine seeking in rats.

Specific Aim 3: the effects of caffeine during stress exposure on subsequent depression, anxiety and relapse to nicotine seeking in rats.

G. **Indexing Terms (Descriptors)**: rat, nicotine self-administration, drug seeking, traumatic stress, psychological stress, fear memory, PTSD, anxiety, depression, endocannabinoid system, caffeine, relapse, reinstatement

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Institutional Animal Care and Use Committee
Phone: (b)(6)
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October 14, 2014

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF PSYCHIATRY

SUBJECT: IACUC Approval of Protocol - Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) by Designated Member Review, on October 14, 2014:

Animal Protocol Title: "Identification of Gene Expression Patterns in Brain Tissues and Peripheral White Blood Cells of Rat Model of Post-Traumatic Stress"

USUHS Protocol Number: (b)(6)

Expiration Date: October 13, 2017

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

(b)(6) Ph.D.

(b)(6) Ph.D.
Vice-Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

PROTOCOL NUMBER: Pending (b)(6)

PROTOCOL TITLE: Identification of Gene Expression Patterns in Brain Tissues and Peripheral White Bood Cells of Rat Model of Post-traumatic Stress

GRANT TITLE (if different from above): N/A

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Dept of Defense/USUHS - O/M funds

EARLIEST ANTICIPATED FUNDING START DATE: September 2014

PRINCIPAL INVESTIGATOR: (b)(6) M.D., Ph.D.

(b)(6) PSY (b)(6) 9/1/4

Principal Investigator Signature | Department | Office/Lab Telephone | Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6) Chairman (b)(6) 9/12/2014

Research Unit Chief / Dept. Head Signature | Title | Telephone | Date

Typed Name (b)(6) M.D.

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6) PSY (b)(6) 9/1/14

Statistician Signature | Department | Telephone | Date

Typed Name (b)(6) M.D., Ph.D.

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6) LAM (b)(6) 9/26/14

Attending/Consulting Veterinarian Signature | Department | Telephone | Date

Typed Name: LTC (b)(6) DVM

ANIMAL PROTOCOL NUMBER: Pending

PRINCIPAL INVESTIGATOR: (b)(6) M.D., Ph.D.

ANIMAL PROTOCOL TITLE: Identification of Gene Expression Patterns in Brain Tissues and Peripheral White Blood Cells of Rat Model of Post-traumatic Stress

GRANT TITLE (if different from above): N/A

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6) M.D. (b)(6) Ph.D., (b)(6) M.D.

TECHNICIAN: (b)(6) Ph.D.

I. I. NON-TECHNICAL SYNOPSIS:

We have demonstrated that specific gene expression patterns are present in brain tissues when symptoms such as exaggerated startle response are observed in an animal model of post-traumatic stress disorder (PTSD). We further have shown that correlated gene expression patterns are present in peripheral white blood cells; these may result from systemic responses to exposure to traumatic stress and may be useful in the development of diagnostic biomarkers of post-traumatic stress. We now hypothesize that genes that are both stress responsive and related to mitochondria structure and function play a key role in both normal and abnormal adaptive responses of individuals to traumatic stress correlated with combat or other catastrophic events. We therefore propose to:

1. Expand and confirm our gene expression discovery using mutl-well PCR with our established rat PTSD model.
2. Determine the function of stress responsive genes in metabolic and signaling pathways using Ingenuity Pathway Analysis.

To do so rats will be subjected (at USUHS) to the stress protocol developed by Dr. (b)(6) then euthanized so that brain tissues associated with central stress responses and blood samples can be obtained for analysis in Dr. (b)(6) laboratory and pathway analysis can be done in Dr. (b)(6) laboratory.

II. BACKGROUND:

Post-Traumatic Stress Disorder (PTSD) is a complex mental disorder that can develop in response to traumatic events during combat or other catastrophic events. An estimated 70% of adults in the United States have experienced a traumatic event at least once in their lives and up to 20% of these develop PTSD. Those with PTSD display alterations in behavioral (hyperresponsiveness (14), heightened acoustic startle response), emotional (irritability, emotional numbing), biochemical (increased thyroid activity(15), increased levels of corticotrophin-releasing factor (CRF) (16), and low cortisol levels (17)), and physiological function (abnormal activity of the autonomic nervous system (3,4) and of the hypothalamic-pituitary-adrenal (HPA) axis (5,6)). Acute stress has been shown to induce down-regulation of glucocorticoid receptor (GR) RNA (18) and facilitate long-lasting changes in cholinergic gene expression (19). These most

recent findings provide initial evidence that gene expression signatures following trauma identify an evolving neuropsychiatric disorder and are informative of its key clinical features and outcomes. However, large-scale searching for molecular targets underlying PTSD has not been reported to date.

II.1. Background:

PTSD Animal Models have been developed in which different stress paradigms provoke a wide range of behavioral responses. Intensely stressful experiences, aversive challenges, and situational reminders of a traumatic stress in PTSD animal models have resulted in long-term effects on behavioral, autonomic, and hormonal responses that mimic many of the clinical symptoms seen in patients with PTSD. The methodologies used to produce PTSD animal models include electric shock (20), social confrontations, stress-restress or time-dependent sensitization (21), underwater trauma (22), exposure of a rat to a predator (23), and the inescapable tail-shock model of traumatic stress (24-26). To establish a rat model of PTSD using highly stringent criteria we have successfully developed and tested the inescapable tail-shock model of stress in rats and verified that short and long-lasting behavioral, biochemical and physiological alterations result (27). **Importantly, the stress-induced behavioral and neurobiological symptoms in the rat models are very similar to those seen in PTSD patients** (Table 1). In PTSD patients, the amygdala displays hyperexcitability, which is thought to be responsible for the exaggerated startle responses of these individuals to stressful events.

In our animal model, stress exposure consists of a two-hour per day session of immobilization and tail-shocks, for three consecutive days. The animals are restrained in a Plexiglass tube, and 40 electric shocks (2 mA, 3 s duration) are applied at varying intervals (140 to 180 s) over the course of two hours on three consecutive days. This stress protocol was adapted from the "learned helplessness" paradigm in which animals undergo an aversive experience under conditions in which they cannot perform any adaptive response (28;29). We stress the rats for three consecutive days because it has been previously demonstrated that repeated immobilization and tail-shock stress sessions for three days is more effective than a single stress session in producing physiological and behavioral abnormalities, such as elevations in basal plasma corticosterone levels, exaggerated acoustic startle responses and reduced body weight (30). Further exposure to stress does not appear to result in greater physiological and behavioral changes (31). Considering the life span difference between human and rat, three days of stress exposure in a rat may be approximately equivalent to three months of stress in humans.

Our earlier studies have shown that (1)the difference in body weight between stressed and control rats was statistically significant ($p < 0.05$) and stressed rats continued to display reduced body weight gain for as long as body weight was monitored; (2)measurement of acoustic startle response (ASR) on day 7 following completion of the stress protocol revealed that stressed rats exhibited heightened ASR; and (3)tail blood samples from stressed rats had higher plasma corticosterone levels than control animals. Our results with the inescapable tail shock paradigm as a model of PTSD are consistent with those in the literature (34), and while we acknowledge that although the animal model cannot fully represent PTSD patients, it is the best available means to determine the molecular and cellular mechanisms of PTSD pathogenesis. Multi-dimensional validations, such as a combination of both behavioral and biological

criteria, are necessary to provide a solid basis for study of the molecular mechanisms using an animal model, to confirm resultant discoveries in clinical samples by association studies, and then to develop diagnosis and treatment strategies.

Table 1. Symptomatic Similarities of PTSD between Human and Animal Models

PTSD in Humans	**Inescapable tail-shock model of stress in rats**
Weight loss (Sutker et al., 1990;Myers et al., 2005;Braga et al., 2004)	Suppressed feeding and body weight loss (Hu et al., 2000;Harris et al., 2002)
Difficulty falling or staying asleep, nightmares (Maher et al., 2006;Maher et al., 2006)	Altered sleep patterns (Adrien et al., 1991)
Psychomotor numbness (Epstein et al., 1998;Lopez-Ibor, 2002)	Persistent behavioral abnormalities i.e. suppressed open-field activity, longer hanging wire latencies (Minor et al., 1984; Pare, 1994)
Poor concentration; memory deficits (Bremner et al., 2004;Isaac et al., 2006;Green, 2003)	Deficits in escape/avoidance learning and learning of an appetitive task (Maier, 2001)
Hypervigilance and/or exaggerated startle response (Orr et al., 2002;Orr and Roth, 2000;Pitman et al., 1999)	Exaggerated startle (Garrick et al., 2001;Servatius et al., 1995;Manion et al., 2007a)
Hyperresponsiveness of the noradrenergic system (Orr and Roth, 2000;Maes et al., 1999)	Hyperresponsiveness of the noradrenergic system (Simson and Weiss, 1988)

Mitochondrial dysfunction is increasingly recognized as a key component in stress-related mental disorders (35;36). The mitochondrial response to stress may include alterations in molecular pathways and networks involving i) an increase in the number of mitochondria to enhance function; ii) adaptive changes in expression and activity of oxidative phosphorylation genes and ATP production; iii) changes in signal transduction that affect expression of both mitochondrial and nuclear DNA-encoded genes; iv) production of reactive oxygen species (ROS); and v) induction of apoptosis. Most primary stress response mediators, i.e. hormones, immune factors and heat-shock proteins, exert effects on mitochondrial biogenesis, metabolism, ROS generation and apoptosis.

Prolonged physical and psychological stress can each induce long-lasting consequences for an individual and can be the cause of affective disorders such as PTSD. In a recent study, psychological stress was associated with increased blood concentrations of oxidative stress markers and genetic modification, namely telomere shortening (37). Overexpression of two antioxidant enzymes, glyoxalase and glutathione reductase 1, in the mouse brain has also been associated with increases in anxious behavior (38). Thus, oxidative stress could contribute to the complex control of anxious behavior and related conditions, such as panic disorder, post-traumatic stress disorder and social phobias. In fact, exposure of rats to chronic stress (immobilization for six hours during 21 days) inhibits the activities of the first complexes of the mitochondrial respiratory chain (inhibition of 69% in complex I-III and of 67% in complex II-III) indicating that complex intracellular cascades altered in stress-related disorders appear to be intimately associated with the metabolic integrity and capacity of mitochondria to maintain energetic parameters and ultimately cellular stability (39). The steroidogenic acute regulatory protein (StAR), a mitochondrial protein required for stress responses, exerts its activity at the outer mitochondrial membrane (40). Whether other mitochondria function-associated molecules are also altered after exposure to intensive stress is not known. Currently, the altered cellular and molecule markers

associated with PTSD brain are not known. Thus, this proposal will attempt to determine whether traumatic stress alters the expression profiles of mitochondria susceptible genes associated with pathogenesis of PTSD.

Expression patterns of mitochondria-related genes in peripheral lymphocytes and in specific brain regions have been identified in human PTSD and control post-mortem brains. Peripheral blood mononuclear cell gene expression signatures are informative and predictive of the PTSD outcome among survivors of trauma, and correlate with the essential neuropsychiatric symptoms observed in PTSD (58;59). However, a causal relationship between gene expression profiling in peripheral lymphocytes and specific brain regions associated with neuronal circuitry responding to a stressor remains unknown. Thus, determination of the expression of mitochondrial related gene profiles in brain amygdala, hippocampus, hypothalamus, prefrontal cortex and anterior cingulate cortex and in peripheral white blood cells and establishment of a causal relationship with biological and psychiatric stressors in an animal model of PTSD will greatly enhance our capability to objectively, promptly and cost effectively evaluate the status of PTSD associated symptoms. The potential to use the peripheral cells to rapidly determine the effects of stressors on the functions of mitochondria will expedite diagnosis and treatment as well as diminish suffering resulting from exposure to trauma. There is also the possibility that the proposed experiments will reveal biomarkers of PTSD, thus allowing the development of more specific therapeutic interventions that may repair cellular damage rather than just treat symptoms. To date, using a mitochondrial focused gene chip we have discovered that during the development of the exaggerated fear associated with PTSD, 48 genes are significantly upregulated and 37 are significantly downregulated in the amygdala complex based on stringent criteria ($p<0.01$). Ingenuity pathway analysis revealed up- or downregulation in the amygdala complex of four signaling networks – one associated with inflammatory and apoptotic pathways, one with immune mediators and metabolism, one with transcriptional factors, and one with chromatin remodeling..The quantitative RT-PCR (qRT-PCR) array (Chen Y. *et al.* ,2009, *BMC Genomics* 2009, **10**:407 doi:10.1186/1471-2164-10-407) which we will now employ has become commercially available since our last studies. qRT-PCR provides higher sensitivity, allowing deeper drilling into the into the expression profile of genes in response to stress.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: PowEResearch (the USU LRC system that seaches all databases in the LRC)

II.2.2. Date of Search: September 15, 2014

II.2.3. Period of Search: From beginning of each individual database to the present.

II.2.4. Key Words and Search Strategy:

1. Mitochondria AND PTSD AND Brain AND Gene Array AND Animal
2. Mitochondria AND PTSD AND Peripheral White Blood Cells AND Gene Array AND Animal.

II.2.5. **Results of Search**: Only two (2) articles (Ursano *et al.*, 2009; Pushpa *et al.* 2012), both from our group.

III. OBJECTIVE\HYPOTHESIS:

The **hypothesis** is that (1) specific gene expression patterns are present in brain tissues that are involved in the pathogenesis and neuropsychiatric phenotypes of PTSD; (2) specific gene expression patterns are present in peripheral white blood cells that are a consequence of systemic responses to traumatic stress and can be useful in the development of clinical non-invasive diagnostic biomarkers; and (3) that the mitochondria-centered and stress-responsive genes play a key role in normal and abnormal adaptive responses of individuals to traumatic stress encountered in combat or other catastrophic events.
The **objectives** of this proposal are as follows:
Specific Aim 1: To extend and deepen our currents findings by replacing the previously employed rat cDNA microarray (rMitChip3) design with the more senstivie qRT-PCR array.
Specific Aim 2: To experimentally determine the mitochondrial gene expression patterns and differentially expressed genes and biomarkers in amygdala, hippocampus, hypothalamus, prefrontal cortex, anterior cingulate cortex and peripheral white blood cells using our established rat-PTSD model and rMitChip3.
Specific Aim 3: To bioinformatically determine biochemical and molecular pathways, networks and drug targets from data collected from brain tissues and blood samples and to correlate these changes (in mitochondria associated gene expression profiles) with neuropsychiatric and/or behavioral abnormalities observed in an animal model of PTSD.

IV. MILITARY RELEVANCE: The information obtained from this research is expected to aid in the development of more efficacious therapeutic interventions aimed at preventing or treating stress-induced affective disorders such as PTSD. Active duty military, reservists and veterans exposed to combat related traumatic stress, as well as civilians exposed to traumatic circumstances such as natural disasters, terrorist attacks and auto or plane crashes, may all benefit from the knowledge thus obtained.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1: (b)(6) Laboratory) Male Sprague-Dawley rats, weighing approximately 250 gm, will be subjected to restraint in plexiglas tubes and tailshock (40 shocks (2mA, 3 sec duration) administered over the course of two hours on three consecutive days). All animals will be weighed the day before beginning the stress protocol to obtain baseline readings and on Days 0 and 30 following exposure to the stress protocol. Control and stressed animals will be sacrificed immediately following completion of the stress protocol (Day 0 following stress) or one month after completion of the stress protocol (Day 30 following stress) and brain tissues and lymphocytes will be harvested and frozen for further analysis to determine whether expression changes

of mitochondria-related genes are present and long lasting. Animals will be anesthetized by isoflurane inhalation (using 30% v/v isoflurane in propylene glycol with a nose cone), decapitated and the brains quickly removed and placed in ice-cold artificial cerebrospinal fluid. Brain tissues will be dissected out and stored at -70°C. Trunk blood will be collected and lymphocytes will be isolated from EDTA-anticoagulant treated blood (7.5 mL) by centrifugation using a sterile lymphocyte separation medium. The lymphocyte layer will be washed with phosphate-buffered saline (BioWhittaker) and stored at -70° for further processing

V.1.2. Experiment 2: (b)(6) Laboratory) mRNA will be extracted and purified from frozen pooled brain tissues and pooled lymphocyte samples and used in qRT-PCR. Databases will be compiled for each brain tissue and for lymphocytes at each time point and data will be normalized.

V.1.3. Experiment 3: (b)(6) Laboratory) Bioinformatic tools will be used to analyze the data to determine biochemical and molecular pathways, networks and possible drug targets in brain tissues and blood samples; that may be altered by stress and to correlate these changes with neuropsychiatric and/or behavioral abnormalities.

V.2. Data Analysis:

Determination of Number of Animals Needed: Based on our recently completed work using a mitochondrial focused gene ChIP, we anticipate that thirty rats will be needed in each group to generate enough region specific brain tissues and lymphocytes for RNA extraction as well as to establish a definitive clustering correlation between brain specific regions and peripheral lymphocytes for future clinical implications. The total RNA required for one qRT-PCR array is estimated to be 2 µg and an estimated minimum of 6 qRT-PCR arrays will be needed for each qRT-PCR experiment to allow for adequate statistical analysis. For example, the rat amygdala is the smallest of the region specific brain tissues that we will examine in this project. It is approximately 1 mm in diameter and weighs approximately 1 mg; approximately 2 mg of amygdala tissue can be obtained from one animal. Approximately 10 mg of amygdala tissue (or five animals) will be needed to extract 2 µg of RNA; thus, pooled rat amygdala tissue from 5 rats will be required for one qRT-PCR array. Therefore, a group of 30 rats will be needed for each experiment (5 rats per chip x 6 chips=30 rats). We have proposed a series of four experiments, each using a group of thirty rats (control, euthanize immediately after stress, control + 30 days and euthanize 30 days after stress), therefore, a total of one hundred twenty rats (4 experiments x 30 rats per experiment = 120 rats) will be needed.

A duplicate series of experiments has been proposed after data from the initial series have been analyzed; this duplication of the experiments is essential to allow adequate statistical analysis of the microarray data. Note that there are two distinct control groups in each series of four experiments; neither can be eliminated from the series. The enhancement resulting from duplication of the experiments will increase the statistical power of the project; in fact, the editors of scientific journals and reviewers of manuscripts generally require duplication of experiments to enhance biological consistency in order to convince scientists in the field of the validity of the data and thus

to enhance the impact of the research in the literature. Thus a total of 240 rats is required.

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: This study requires the use of intact living animals; behavioral measurements, such as administration of the stress protocol, cannot be made on cells grown in culture or on computer models or other *in vitro* systems. Thus, non-animal models were not considered for this project and it is not possible to replace or eliminate the use of animals in this study.

V.3.2. Animal Model and Species Justification: The rat (in this study, *Rattus norvegicus)* is the primary model used in studying the physiology of the amygdala, the brain area primarily involved in the processing of stressful stimuli; consequently, it is helpful to use the same animal in order to make comparisons with the literature (Kohda, et al, 2007). Use of the rat also replaces other animals higher up the phylogenetic scale. (b)(6) has developed and refined this particular animal model of traumatic stress over a period of ten years. It was adapted from a "learned helplessness" paradigm in which animals undergo an aversive experience under conditions in which they cannot perform any adaptive response (Servatius et al., 1995a; Braga et al., 2004; Manion et al., 2007b).

V.3.3. Laboratory Animals

	Species #1	Species #2
V.3.3.1. Genus & Species:	R. Norvegicus	
V.3.3.2. Strain/Stock:	Sprague-Dawley	
V.3.3.3. Source/Vendor:	(b)(4)	
V.3.3.4. Age:	~ 8 weeks	
V.3.3.5. Weight:	~250 grams	
V.3.3.6. Sex:	Male	
V.3.3.7. Special Considerations:	N/A	

V.3.4. Number of Animals Required (by Species): Rats 240

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: Animals will be housed in a climate controlled environment in groups of two per cage with free access to food and water, and will be maintained on a 12 hour reverse light/dark cycle at 22 °C. The use of paired housing for male rats has

not presented problems in the past with our use of this animal model of PTSD; however, it is conceivable that male rats could become aggressive toward one another, especially in the groups that are pair housed for 30 days before euthanization. We and CLAM staff members will observe the animals on a daily basis and will change to one rat per cage if observations indicate any behavior that would have a negative effect on the proposed experiments.

V.3.5.2. Reduction: We anticipate that thirty rats will be needed per group to generate enough region specific brain tissues and lymphocytes for RNA extraction as well as to establish a definitive clustering correlation between brain specific regions and peripheral lymphocytes for future clinical implications. The amygdala is very small and it is necessary to combine amygdala tissue from 30 rats for qRT-PCR array experiments and related immunohistochemistry. It will not be possible to reduce the number of control animals when the experiments are repeated as the bioinformatics tools used for data analysis require the use of matched control/stress groups of animals.

V.3.5.3. Replacement: This study requires the use of intact living animals; behavioral measurements cannot be made on cells grown in culture or with computer models. Thus, it is not possible to replace or eliminate the use of animals in this study.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals:

	Species #1	Species #2
V.4.1.1.1.1. Column C:	120	
V.4.1.1.1.2. Column D:	0	
V.4.1.1.1.3. Column E:	120	

V.4.1.2. Pain Relief / Prevention: N/A

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization:

Isoflurane anesthesia will be performed prior to euthanization of rats by decapitation with a guillotine for tissue harvesting. For rats, a mixture of 30% v/v isoflurane in propylene glycol (1,2-Propanediol USP grade Sigma Cat no. P4347) will be prepared. Anesthesia is performed in a certified ducted hood, biosafety cabinet or portable ductless hood equipped with a charcoal filter. While wearing gloves, the operator opens the bottle containing isoflurane in the hood and wets a cotton pad wet with isoflurane.

The pad is placed in a 100 mL beaker so as to not contact the rat, the beaker and then the rat are placed in a jar with a secure lid, made of non-porous material that is

sanitizable and allows for constant visualization of the animal and is of such size as to comfortably accommodate the animal, but not so large as to require excessive anesthetic. The animal is monitored closely. It becomes anesthetized in approximately 2 minutes. When a lack of righting reflex and a 50% reduction in respiratory rate are observed for at least 10 seconds, the rat is removed from the jar, and the jar lid immediately replaced. If there is no response to a noxious stimulus (ie. toe pinch), the operator proceeds (in this protocol) to decapitate the rat with a guillotine and harvest brain tissues and trunk blood.

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Animals will be housed in a climate controlled environment in groups of two per cage with free access to food and water, and will be maintained on a 12 hour reverse light/dark cycle at 22 °C. Animals will be acclimated for three days to the USUHS CLAM. During acclimation to CLAM, animals will be handled briefly each day to acclimate them to being handled; this will reduce any stress that might result from handling of the animals during the procedures described in this study. Following exposure to the stress protocol, animals will be monitored according to CLAM SOPs.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: PowEResearch (the USU LRC system that seaches all databases in the LRC)

V.4.1.3.2. Date of Search: September 15, 2014

V.4.1.3.3. Period of Search: From the beginning of each database to the present.

V.4.1.3.4. Key Words of Search:

- Stress and pain and mitochondria and rat: 0
- Pain and PTSD and mitochondria and rat: 0
- Pain and PTSD and rat: 0

V.4.1.3.5. Results of Search: No relevance

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
We have developed a stressed rat model in which a young adult male animal is restrained in a plexiglas tube of appropriate size and receives a series of forty tailshocks (2mA constant current, 3 sec duration) over a period of two hours daily on three consecutive days. A coiled silver electrode is attached to the tail with a spring clip so that there is no irritation of the tail either from the shocks or from adhesive materials being applied and removed each day. The number and strength of the shocks proposed has been optimized to yield a model of chronic stress as measured by changes in behavior and by elevated plasma levels of corticosterone. This stress protocol was adapted from the "learned helplessness" paradigm in which animals undergo an aversive experience under conditions in which they cannot perform any

adaptive response (Seligman and Maier 1967; Seligman and Beagley 1975). We stress the rats for three consecutive days because it has been previously demonstrated that repeated stress sessions for three days are more effective than a single stress session in producing physiological and behavioral abnormalities, such as elevations in basal plasma corticosterone levels, exaggerated acoustic startle responses and reduced body weight (Servatius et al. 1995; Ottenweller et al. 1989). Additional exposures to stress do not appear to result in greater physiological and behavioral changes (Servatius et al. 1995; Ottenweller et al. 1989). Other types of stressors appropriate for an animal model (e.g. restraints only) are not easily quantifiable and have resulted in at least partial habituation rather than chronic stress. Given these considerations, we feel that the painful procedure is justified.

In an earlier study, the startle response and adrenocortical release of rats was assessed following exposure of the animals to stressor presentations similar to that proposed in this study for one, two or three days (Servatius et al., 1994). Rats that were exposed to three days of stress exhibited an exaggerated startle response to an auditory stimulus 4 days poststressor as compared to nonshocked controls (Servatius et al., 1994; Manion et al., 2007). This exaggerated startle response was not evident in rats exposed to either 1 or 2 days of stress (Servatius et al., 1994). Exposure to our 3 day stress regimen produces a chronic stress state in rats, characterized by persistent exaggerated startle response and adrenocortical sensitization, which is appropriate as a model for the study of stress-related psychophysiological illnesses such as PTSD (Servatius et al., 1994).

V.4.2. Prolonged Restraint:

Prolonged restraint will be conducted IAW IACUC Policy 004 "Prolonged Restraint". All animals are monitored by a technician during the two hours when tail shocks are being administered to assure that they do not get into positions that do not allow them to breathe properly. Also, the electrode can become loose over the two hour period so that the animal no longer receives the shock; monitoring by a technician is required to ensure that the animals receive all of the forty tail shocks.

V.4.3. Surgery: N/A

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: N/A

V.4.4.2. Biosamples:
Trunk Blood Samples: **Trunk blood will be collected when each animal is euthanized. Blood lymphocytes will be isolated from EDTA-anticoagulant treated blood (7.5 mL) by centrifugation using a sterile lymphocyte separation medium. The lymphocyte layer will be washed with phosphate-buffered saline (BioWhittaker) and frozen for further analysis..**
Brain Tissue Samples: **Brain tissues will be harvested immediately after the final stress session or 30 days after the final stress session. Animals will be anesthetized by isoflurane inhalation (using 30% v/v isoflurane in propylene glycol, see detailed procedure described in Section V.4.1.2.1), then decapitated and the brains removed as quickly as possible. Brains will be dissected and kept in ice-cold ACSF; hippocampus, hypothalamus, prefrontal cortex, anterior cingulate cortex (Bergman 5.70 mm to -0.80 mm) and the amygdala (500-µm-thick transverse slices) will be cut from tissue blocks with a Vibratome Tissue Slicer (Technical Products International, St. Louis, Missouri). All tissue samples will be stored at –70°C prior to RNA extraction and purification.**

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Cage cards

V.4.4.6. Behavioral Studies: There is one behavioral component included in this study: exposure to traumatic stress.
Stress exposure consists of a two-hour per day session of immobilization (restraint) and tail-shocks, for three consecutives days. Each animal is restrained in a plexiglas tube and an electrode is attached to the tail. Forty electric shocks (2 mA, 3 s duration) are applied at varying intervals (140 to 180 s) over a two hour period; then the rats are returned to their cages. Exposure to this three day stress regimen has been shown to be more effective than a single stress session in producing lasting physiological and behavioral abnormalities, such as elevations in basal plasma corticosterone levels, exaggerated acoustic startle responses and reduced body weight (Servatius et al., 1995).

The plexiglas restraint tubes used in administration of the stress protocol are designed to accommodate animals of a specified size range. If an animal is near the lower end of the size range, there will be slightly more room for it to move in response to the shock and possibly twist itself into a position that will not allow it to breathe properly. The technician who monitors the stress protocol can release the adjustable end of the restraint tube slightly to allow the animal to return to a position that does not impair breathing, then retighten it. Animals of the size specified in this protocol (~250 gm) do

not usually exhibit severe reactions to the stress protocol. If the animals have been handled when they first arrive in USUHS animal facility, they generally do not resist being put into the restraint tubes. If animals were to exhibit symptoms of extreme respiratory distress or damage to the tail resulting from the electric shocks during the stress procedure or if any animals would lose more than 20% of their initial body weight as a result of exposure to the stress protocol, they would be withdrawn from the study and euthanized

Electrodes will be attached to the tail of the animal by means of a spring clip in order to minimize any painful irritation that might result from repeated taping of the tail. All animals are monitored by a technician during the two hours when tail shocks are being administered to assure that they do not get into positions that do not allow them to breathe properly. Animals will be monitored daily after the first two stress sessions according to CLAM SOPs and those animals that will be euthanized thirty days after the final stress session will continue to be monitored daily for any indication of pain. While the animals are expected to lose weight during the three day stress exposure, any animal that would lose >20% of its body weight as measured on Day -1 would be withdrawn from the study and euthanized by CLAM staff as described in Section V.4.6. The shock administered to the tails of the animals is 2 ma in intensity for a duration of 3 seconds. The animals typically jump and cry out during the duration of the shock and struggle to get away from it. As soon as the shock concludes, the animals lie quietly until the next shock. Four to six animals are shocked at one time (in individual tubes with a single electrode attached to each animal's tail). The size of the animals can vary by as much as 20 gm, while the restraint tubes are rigid and fixed. An animal that is slightly smaller than the ideal weight for which the tubes are designed can sometimes move into a position where its ability to breathe is compromised; if the animal is not being monitored by a technician who can release the end of the tube and allow it to right itself, then retighten the end of the tube, the animal can expire. Rats of the size listed for this protocol usually do not exhibit this difficulty, but will be monitored during the administration of the stress protocol. The electrodes are attached to the tail with a spring clip to prevent irritation of the tail by removal of adhesive stripes each day for three days and to minimize the possibility of infection through a break in the skin on the tail. We have not observed damage to the tail (i.e. burning of the tail) resulting from contact with the electrode during the shocks. Criteria for early termination of the shock experiments would include (1)signs of infection due to a break in the tail skin; (2)severe irritation or burning of the tail resulting from contact with the electrode during administration of the shocks; (3)loss of >20% of body weight as measured on Day -1 before administration of the stress protocol (Note that rats have been consistently observed to lose weight following exposure to stress, but never >20% of their body weight prior to administration of the stress protocol); and (4)observation of pain or infection in the animals that are housed for 30 days after the final stress session. Any animals that must be removed from the study will be given to CLAM personnel for euthanization as described in Section V.4.6.

After each use, the restraint tubes are washed in Alconox detergent solution and allowed to air dry and the electrodes are wiped with 95% ethanol.

V.4.4.7. Other Procedures: Animals will be weighed on Day-1 preceding stress exposure to obtain baseline values and again on Day 0 or Day 30 following exposure to the stress protocol.

V.4.4.8. Tissue Sharing: Frozen brain tissue and frozen lymphocyte samples will be transferred to the laboratory o[(b)(6)] for qRT-PCR array analysis and pathway analysis..

V.4.5. Study Endpoint: The study endpoint will be the third day of the stress protocol (Day 0) or thirty days (Day 30) after the final stress session for all animals that complete the stress protocol. Criteria for withdrawal of animals from the study would include (1)signs of infection due to a break in the tail skin; (2)severe irritation or burning of the tail resulting from contact with the electrode during administration of the shocks; (3)loss of >20% of body weight as measured on Day -1 before administration of the stress protocol; and (4)observation of pain – such as limping, immobility, protection of a body region, biting or squeeling when attempt is made to lift the animal - or infection in the animals that are housed for 30 days after the final stress session. Animals showing any of the above signs will immediately be withdrawn from the study and given over to CLAM personnel for euthanization.

V.4.6. Euthanasia: :

Euthanasia will be performed by CLAM staff per LAM SOPs for rats that are withdrawn from the study or otherwise not used.

All rats that are part of the study will be anesthetized as described in section V.4.1.2.1. When a lack of righting reflex and a 50% reduction in respiratory rate are observed for at least 10 seconds, the rat should be removed from the jar, and the jar lid immediately replaced. If there is no response to a noxious stimulus (ie. toe pinch), the operator may proceed (in this protocol) to decapitate the rat with a guillotine and harvest brain tissues and trunk blood.

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:

Building(s) Bldg Room Number(s) [(b)(6)]

V.5.1.2. Special Husbandry Provisions:

Food Restriction:	Yes		No	X
Fluid Restriction:	Yes		No	X

V.5.1.3. Exceptions: Reverse Light Cycle So that rats, being nocturnal, will be active during the experimental periods.

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: Animals will be cared for in accordance with LAM SOPs. Animals will be observed daily or more frequently by the veterinary staff. Should any animal lose >20% of its baseline body weight during the course of an experiment, it will be withdrawn from the study and euthanized by CLAM staff (see Section V.4.6). CLAM veterinary staff will be available for consultation with the PI should any unexpected medical events arise

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: All animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. Enrichment Restrictions: N/A

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience)	Specific training in this activity or procedure (e.g., rodent handling class, 1999)
Stress Protocol	(b)(6)	PI, 10 years experience	Developer
Isoflurane anesthesia Brain dissection & harvesting, trunk blood collection		PI, 15 years exp 20 years experience	NIH, USU Pitt, NIH, USU
Decapitation		20yr experience 20yr experience	NIH, USU PITT,NIH, USU

Dr (b)(6) will not be involved in any hands-on animal manipulations. Dr. (b)(6) will not be doing any animal work here at USUHS; he will be doing genetic analysis of tissues sent to his laboratory at Georgetown University. Dr. (b)(6) has extensive experience with brain tissue harvesting and dissection and trunk blood collection and with the administration of isoflurane anesthesia, both at the NIH and at

USUHS; he is expected to serve in an advisory capacity with regard to these procedures. Any new research associates or graduate students who rotate through the laboratory will be added to the protocol by request for and approval of a minor modification to this protocol before being permitted to do any animal procedures. Dates for attendance at the most recent USUHS Investigator/Animal User Training Course are as follows:

(b)(6) M.D., Ph.D. May 17, 2007
(b)(6) Ph.D. June 2010

VII. **BIOHAZARDS/SAFETY:** Personnel will use personal protective equipment (eye protection, lab coats, gloves, masks) when handling animals to reduce exposure to allergens. All needles will be disposed of in sharps containers. Although the shock administered is 2mA, all personnel will be trained to pause the program and disconnect the electrodes before making adjustments to electrode placement or to the movable end of the restraint tube.

VIII. **ENCLOSURES:** N/A

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6) [redacted]

Principal Investigator Signature

9-17-14
Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6) [redacted]

Principal Investigator Signature

9-17-14
Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to acco[redacted] this proposed experiment.

(b)(6)

Principal Investigator Signature

9-17-14

Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number: Pending

B. Animal Protocol Title: Identification of Gene Expression Patterns in Brain Tissues and Peripheral White Bood Cells of Rat Model of Post-traumatic Stress

C. Principal Investigator: (b)(6) M.D., Ph.D..

D. Performing Organization: Uniformed Services University of the Health Sciences and GenProMarker

E. Funding: DoD Awarded to Dr. (b)(6) M.D.

F. Objective and Approach:

The objectives of this proposal are as follows:

Specific Aim 1: To extend and deepen our currents findings by replacing the previously employed rat cDNA microarray (rMitChip3) design with the more senstivie qRT-PCR array.

Specific Aim 2: To experimentally determine the mitochondrial gene expression patterns and differentially expressed genes and biomarkers in amygdala, hippocampus, hypothalamus, prefrontal cortex, anterior cingulate cortex and peripheral white blood cells using our established rat-PTSD model and rMitChip3.

Specific Aim 3: To bioinformatically determine biochemical and molecular pathways, networks and drug targets from data collected from brain tissues and blood samples and to correlate these changes (in mitochondria associated gene expression profiles) with neuropsychiatric and/or behavioral abnormalities observed in an animal model of PTSD.

G. Indexing Terms (Descriptors): Rat, PTSD, Biomarkers, Gene array, Peripheral white blood cells, Brain tissue and Animal.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4712
www.usuhs.mil

May 06, 2015

MEMORANDUM FOR DR. (b)(6) DEPARTMENT OF PSYCHIATRY

SUBJECT: IACUC Approval of Protocol – Triennial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on May 06, 2015:

Animal Protocol Title: "Role of Nicotine and Opioid on Stress and Anxiety in Rats"

USUHS Protocol Number: (b)(6)

Expiration Date: May 5, 2018

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ (b)(6) to schedule a pre-protocol planning meeting (b)(6) This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

(b)(6)
(b)(6) Ph.D.
Chair, Institutional Animal
Care and Use Committee, USUHS

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Role of nicotine and opioid on stress and anxiety in rats

GRANT TITLE (if different from above): Individual differences in morphine self-administration and fear/anxiety in rats

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: USUHS

EARLIEST ANTICIPATED FUNDING START DATE: 10 May 2015

PRINCIPAL INVESTIGATOR:

(b)(6)

Principal Investigator Signature | PSY Department | (b)(6) Office/Lab Telephone | 3-23-15 Date

(b)(6) PhD

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)

Research Unit Chief / Dept. Head Signature | Chairman Title | (b)(6) Telephone | 3-30-15 Date

Typed Name (b)(6) MD

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)

Statistician Signature | PSY Department | (b)(6) Telephone | 3-23-15 Date

Typed Name (b)(6) PhD

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)

Attending/Consulting Veterinarian Signature | LAM Department | (b)(6) Telephone | 3/23/15 Date

Typed Name (b)(6) VMD

USUHS Form 3206- Animal Study Proposal Form Instructions

USUHS / DOD – SPONSORED ANIMAL RESEARCH PROPOSALS MUST USE THIS STANDARDIZED FORMAT

Reference DOD Directive 3216.1 & USUHS Instruction 3203

****

Specific information requested in the following animal-use protocol template is a result of requirements of the Animal Welfare Act regulations (AWAR), the Guide for the Care and Use of Laboratory Animals, and other applicable Federal regulations and DOD directives.

****

This document is intended to be an aid in the preparation of a USUHS DOD – sponsored animal use proposal. The instructions and written explanations provided for individual paragraphs (ref. animal-use protocol template in AR 40-33 / USUHSINST 3203, Appendix C) are coded as hidden text. To see the instructions and examples for each section, select the "**Show/Hide ¶**" button on your tool bar. To print the hidden text, select "Print" on the drop down file menu. Under the "Options" button, select "Hidden text" under the "Include with document" section. Use of a word processor makes completion of this template a "fill-in-the-blanks" exercise. Please provide all response entries in the following font: Arial, Regular, 12, Black. Please do NOT submit this page of instructions with your animal protocol submission.

With the exception of title headings, each paragraph and subparagraph in the following template must have a response. Portions of the template that are not applicable to your particular protocol, (i.e. no surgery or no prolonged restraint) should be marked "**N/A**". There are no space limitations for the responses.

Pertinent standing operating procedures or similar documents that are readily available to your IACUC may be referenced to assist in the description of specific procedures. It is critical that only animal studies or procedures documented in an IACUC – approved protocol be performed at your facility. Additionally, Principal Investigators, or other delegated research personnel, should keep accurate experimental records and be able to provide an audit trail of animal expenditures and use that correlates to their approved protocol.

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

IACUC Date Stamp

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Role of nicotine and opioid on stress and anxiety in rats

Revised

GRANT TITLE (if different from above): Individual differences in morphine self-administration and fear/anxiety in rats

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: USUHS

EARLIEST ANTICIPATED FUNDING START DATE: 1 April 2015

PRINCIPAL INVESTIGATOR:

Principal Investigator Signature	PSY Department	(b)(6) Office/Lab Telephone	Date

(b)(6) PhD

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

Research Unit Chief / Dept. Head Signature	Chairman Title	(b)(6) Telephone	Date

Typed Name: (b)(6) MD

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

Statistician Signature	PSY Department	(b)(6) ~~Telephone~~	~~Date~~

Typed Name: (b)(6) PhD

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

Attending/Consulting Veterinarian Signature	LAM Department	(b)(6) Telephone	Date

Typed Name: (b)(6) VMD

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) PhD

ANIMAL PROTOCOL TITLE: Role of nicotine and opioid on stress and anxiety in rats

GRANT TITLE (if different from above): Individual differences in morphine self-administration and fear/anxiety in rats

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6)

TECHNICIANS(S): (b)(6)

I. NON-TECHNICAL SYNOPSIS:

Posttraumatic stress disorder (PTSD) is a debilitating stress-related disorder affecting military service members and civilians in the U.S. Among the individuals with PTSD, the rate of substance use is quite high. Approximately 29% of the population is current smokers and 47% have smoked at some time in their life (Lasser et al. 2000). Of those with a history of PTSD, 63.3% had smoked daily at some point in their lifetime and 45.3% were current smokers – rates considerably higher than those with no history of PTSD. Also, PTSD patients are more likely to be heavy smokers and relapse to smoking tobacco (Fu et al. 2007). These suggest that nicotine dependence is a serious problem in individuals with PTSD. In addition to nicotine, opioid drug abuse is a growing public health problem in the U.S., especially because of an increasing number of individuals seeking prescription opioids for pain management (Garland et al. 2013). Opioid drugs now rank second only to marijuana among all illicit drugs of abuse (Department of Defense 2008). Adverse consequences of opioid misuse can include metabolic dysfunction, physical dependence, drug overdose, and suicide. A recent report indicated that a large number of active duty service members misused opioid pain medications in the past 12 months (Department of Defense 2010). Although misuse of addictive substances can affect stress and anxiety responses (Koob and Le Moal 2001), biological mechanisms of stress and substance use are not well understood. Using clinically relevant animal models of stress and substance abuse, we will investigate the interaction between stress and substance use in rats. This topic is particularly important for military service members and their long-term behavioral health. We believe that our work will provide valuable information relevant to the health of service members, in particular, and the general population as well.

II. BACKGROUND:

II.1. Background:

Posttraumatic stress disorder (PTSD)
PTSD is a debilitating anxiety disorder that can develop in response to traumatic stress exposure such as combat or other physical/psychological stress. The estimated lifetime

prevalence of trauma exposure is 61% for men and 51% for women with men more likely to report physical attacks, combat experience, being threatened with a weapon, while women likely to report sexual assault, childhood neglect and childhood physical abuse (Kessler et al. 1995). It is well established that the exposure to traumatic stressors predisposes persons to an increased probability of use of psychoactive and addictive drugs (Brown and Wolfe 1994). The use of psychoactive drugs such as nicotine, caffeine and opioids continues to be one of the more damaging squeal to deployment-associated PTSD in military personnel. However, biological mechanisms underlying substance use disorders displayed in individuals with PTSD are not well understood, and therapeutic interventions to reduce subsequent drug abuse in traumatized personnel are empirical and of uncertain efficacy.

Stress and nicotine use

Nicotine use is associated with exposure to traumatic events and PTSD (Feldner et al. 2007a). Individuals with PTSD show higher rates of smoking and worse smoking cessation outcome. For instance, almost 94% of individuals with PTSD relapsed in the first week after quitting in a community sample of daily smokers (Zvolensky et al. 2008). Other studies have shown that smokers exposed to a traumatic event who develop PTSD tend to smoke more than those exposed to trauma that do not develop PTSD, a relationship consistent across age and trauma type (Hapke et al. 2005; Lipschitz et al. 2003; Shalev et al. 1990). Smoking among trauma-exposed adults has been found to range between 32% (Weinbaum et al. 2001) and 58% (Weaver and Etzel 2003). Compared to the non-smokers, smokers were more likely to develop posttraumatic stress symptoms (Ganz 2000), which is in line with the findings that traumatic event exposure facilitates smoking behavior (Feldner et al. 2007b). Although 20% of American civilians smoke cigarettes, over 30% of military personnel currently smoke cigarettes and even more use smokeless nicotine-containing tobacco products (Bray and Hourani 2007). Many young never-smokers and occasional smokers become regular smokers after military basic training (Haddock et al. 1998), suggesting that the military environment (e.g., high stress) may be a factor in smoking initiation. Thus, nicotine consumption may be one of the ways to help attenuate the stress of military lifestyle.

Stress and opioid use

Prescription opioid pain medication is increasingly used in the military population (Holbrook et al. 2010). Iraq and Afghanistan veterans show increasing use of prescription opioid pain medications especially those who experienced traumatic injury and stress (Barry et al. 2011; Seal et al. 2012). Using a large scale samples from the OEF/OIF veteran population (n=141,029), Seal et al (2012) reported that veterans with mental health diagnoses, especially PTSD, showed an increased risk of receiving opioids for pain, high-risk opioid use and adverse clinical outcomes. Among the opioids, morphine is a potent analgesic drug that is used to relieve severe pain. Morphine is a highly addictive substance and it can cause and physical and psychological dependence as well as tolerance, with an addiction potential identical to that of heroin. Not surprisingly, many animal studies have shown that morphine is readily self-administered in rodents including rats (Self and Stein 1993; Yoon et al. 2010) and mice (Elmer et al. 2010). Various aspects of drug seeking and taking behavior were studied using morphine self-administration paradigm (Elmer et al. 2002; Hall et al. 2011; Kuzmin et al. 1996). However, potential effects of stress on morphine seeking behavior have not

been well characterized.

Sex differences in stress and substance use
A growing body of literature described sex differences in stress and substance abuse (Bobzean et al. 2014). Females tend to progress through the phase of addiction differently than men such as faster transition from casual use to addiction, and higher rates of relapse than men. In animal studies, morphine is known to be more powerful analgesic in males than in females (Baamonde et al. 1989; Cicero et al. 1996; Craft et al. 1999). Moreover, stress responses in males have been studied in animals and humans, and can be described as the "fight-or-flight response" (Cannon 1935; Faraday et al. 2005; Mason 1975; Selye 1946; 1973). Males generally become more aggressive (fight) or escape the threatening situation (flight) in response to stress. Although stress responses in females involve similar biological systems (i.e., the sympathetic branch of the autonomic nervous system and the hypothalamic-pituitary-adrenal axis), they also exhibit different behavioral responses that have been called the "tend-and-befriend" responses (Taylor et al. 2000). When a female experiences stress, the fight-or-flight response still occurs but she also increases care for her young and/or seeks social interaction. There has been relatively little experimental examination of sex differences in stress responses and opioid use. It has been shown that female rats display more anxiety- and depression-related behavior after exposure to psychological stress than do male rats (Yarnell 2012). The proposed experiments include male and female rats as subjects to investigate sex differences in effects of stress and nicotine on opioid use.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched:
Medline (PubMed), PsycInfo, F1000Biology (and electronic journal review service), BRD, and RePORT

II.2.2. Date of Search: March, 2015

II.2.3. Period of Search:
All years available on each database (ranging from past year to past 30 years)

II.2.4. Key Words and Search Strategy:
Nicotine, chronic nicotine administration, stress, rats, sex differences, locomotor activity, neurobehavioral functioning, hot plate, nociception, acoustic startle reflex, pre-pulse inhibition, fear conditioning, memory, anxiety, traumatic stress, PTSD, morphine self-administration, morphine, cognition (words are entered separately and in combination; rat is entered with each term) have been searched in data base searches.

II.2.5. Results of Search:

Nicotine AND morphine AND rat: 242
Stress AND nicotine AND morphine AND rat: 12
Stress AND nicotine AND morphine self-administration AND rat: 3

There was no study relevant to our proposal among those 3 studies. There has been extensive work on stress, nicotine, and opioid including our own work. However, there has been little work combining these variables in the ways that we propose (e.g., effects stress and nicotine on opioid use). Overall, literature searches on the topics to be covered in our experiments indicate the value of our approaches and have not revealed existing literature or data that would replicate our experiments.

III. OBJECTIVE\HYPOTHESIS:

The current proposal focuses on behavioral and cognitive effects of opioid, nicotine and stress in male and female rats related to fear, anxiety and drug addiction. The overarching aim of this project is to enhance the health of service members under stressful conditions by investigating whether nicotine and opioid drugs alter behavioral responses to stress. We will study the effects of voluntary use of opioid on fear and anxiety behaviors as well as regional glucose utilization in the brain. We will also investigate interaction between unpredictable stress and drug exposure (nicotine and opioid) in male and female rats. We will use the warrior stress paradigm, which combines predator odor stress with unpredictable environmental stimuli. Unpredictable stress has face validity in relation to human stress and has been shown to reliably produce elevations in stress hormones including corticosterone and corticotropin-releasing hormone (Weinstock et al. 1992) and behavior (Gonzalez Jatuff et al. 1999) in rodent studies.

Specific Aim 1: To determine effects of early and late withdrawal from morphine self-administration (MSA) on anxiety and fear behavior in male and female rats. We will use elevated plus maze and acoustic startle as anxiety measures, and Pavlovian fear conditioning and extinction as fear behavior following chronic morphine self-administration. We hypothesize that early and late withdrawal from MSA may have differential effects on fear and anxiety behaviors.

Specific Aim 2: To determine effects of morphine self-administration on anti-nociception and tolerance, and regional glucose utilization in brain. We will use a hot plate test to measure antinociception and ^{18}FDG-PET for regional glucose uptake in brain. We will also investigate individual differences in antinociception, morphine self-administration and locomotor sensitization in males and females. We hypothesize that individual differences in morphine antinociception and morphine self-administration may be associated with brain glucose utilization.

Specific Aim 3: To determine effects of chronic nicotine and/or psychological stress (Warrior Stress) on morphine self-administration and behavioral sensitization. Animals will be exposed to chronic nicotine via minipump and Warrior Stress, and then allowed to acquire morphine self-administration for two weeks. Following acquisition of morphine self-administration, animals will be tested with dose response function. Locomotor sensitization during morphine withdrawal will also be measured. We hypothesize that stress exposure may facilitate opioid abuse and nicotine exposure may attenuate opioid abuse.

IV. MILITARY RELEVANCE:

There is a high comorbidity of PTSD and substance use disorders in the military population (Beckham et al. 2008; Boden et al. 2011; Booth et al. 2011). A substantial number of military veterans and active duty service members relies on licit and illicit substances such as caffeine, nicotine, alcohol, opioids and marijuana to alleviate stress and anxiety symptoms as an active coping strategy (Barry et al. 2011; Boden et al. 2011; Seal et al. 2012). Iraq and Afghanistan veterans with chronic pain and PTSD may be at particular high risk of prescription opioids misuse given the high prevalence of substance abuse and PTSD among veterans (Mills et al. 2005; Seal et al. 2011). The outcome of this study will contribute to the development of more effective therapeutic interventions aimed at preventing or treating a comorbid PTSD and substance use disorders in the military population.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures: To determine the effects of stress and nicotine on opioid use, we will use multiple behavioral paradigms as shown below.

1. Drug administration

Nicotine administration

Nicotine will be administered chronically via subcutaneous mini-pump implanted in a brief surgical procedure developed by Dr. (b)(6),(b)(4) to model exposure to nicotine comparable to levels achieved by human cigarette smoking. Osmotic minipumps are used to provide animals with continuous drug delivery to establish and maintain fairly constant concentration of drug for many days without the trauma of repeated injections. Minipumps operate because of an osmotic pressure difference between a compartment within the pump and the tissue environment in which the pump is implanted. The high osmolality of the salt sleeve causes water to flux into the pump through a semipermeable membrane, which forms the outer surface of the pump. As the water enters the salt sleeve, it compresses the flexible reservoir, displacing the drug solution from the pump at a controlled, predetermined rate. This paradigm for administering nicotine had produced animals results on body weight and eating behavior comparable to studies of human smokers (Grunberg 1982). Subjects are anesthetized by inhalation anesthetic using LAM anesthetic equipment (vaporizer) and Isoflurane and oxygen. Subjects are placed inside an induction chamber saturated with Isoflurane vapor (3-5%). Subjects are removed from the chamber when tail pinch produces no reflex movement and anesthesia is maintained using a mask/nose cone attached to the anesthetic vaporizer (1-3%). A 3 x 5 cm area between the withers is shaved and cleaned with the antiseptic Betadine. A 2 cm transverse incision within the shaved region approximately 1 cm below the scapulae is made with blunt-nosed, curved-tipped Mayo surgical scissors, a pocket is created by gently spreading the subcutaneous tissues with the scissor tips, and the mini-pump is inserted. Surgical instruments are cleaned and sterilized by the glass bead germinator, 70% ethanol and sterile saline before each procedure and in between animals. Incisions are closed with two-three 9 mm stainless steel wound clips. The wound clips are removed in 2 weeks after the surgery. This procedure takes approx. 4 min per subject. The surgical area is thoroughly cleaned before and after procedures. Investigators wear surgical masks,

gloves, and laboratory coats or surgical scrubs. The procedures are performed under aseptic conditions. Subjects will be returned to their cage and observed post-surgery until they are able to demonstrate recovery such as return of righting reflex, or coordinated voluntary movement. They also will be monitored three times a day until they have completely recovered from the minor surgical procedures. Nicotine (6 mg/kg per day) will be delivered with a rate of 0.5 µl per hour using the Alzet osmotic minipump (model 2002, Durect Corporation, Cupertino, CA).

Intravenous drug self-administration

Apparatus
Eight operant chambers (Med Associates Inc., St. Albans, VT, USA) for drug self-administration are contextually different from the animals' home cage, and located in a different room (b)(6) Each chamber is equipped with an infusion pump assembly consisting of a Razel Model A pump (Stamford, CT, USA) and 10 ml syringe connected to a fluid swivel (Instech, Plymouth Meeting, PA, USA) by Teflon tubing. Tygon® tubing enclosed by a metal spring connected the swivel to the animal's catheter exit port and was secured to Teflon threads on the catheter assembly. Each operant chamber contains two levers, and either one (Fixed Ratio 1) or five (Fixed Ratio 5) 20 g lever press responses on the active lever delivered an i.v. injection of sterile drug solution (Sigma/Aldrich, MO, USA) dissolved in 0.9% saline. During each injection, a cue light above the active lever is illuminated, and the house light is extinguished. Each injection is followed by an additional "time-out" (TO) period in which the cue light is extinguished; lever press responses during the entire injection-TO period are recorded but had no programmed consequences. Responses on the inactive lever are recorded but had no scheduled consequence. A detailed procedure on intravenous drug self-administration in rats has been described previously (b)(6),(b)(4)

Lever press training
Animals are initially trained to press a lever for sucrose pellets in ventilated operant chambers under restricted diet conditions for two to three days. This procedure will facilitate acquisition of drug self-administration training. This procedure requires an initial 16–20 hrs of slight food restriction followed by ad lib feeding for a few hrs after each test session. This procedure allows transient weight gain overnight, but animals return to 90% initial body weight prior to the next test session on the following day.

Intravenous morphine self-administration
After a minimum of 1 week recovery from catheter surgery, animals are placed in operant test chambers and allowed to acquire i.v. morphine (0.5 mg/kg/0.1 ml in a 5 s injection) self-administration on one lever press/injection (Fixed Ratio 1: Time Out 20 sec) reinforcement schedule in a daily 3 to 4 hr self-administration test sessions for 5 days per week (3 weeks for Aim 1 and 2; 4 weeks for Aim 3). Animals are weighed before each self-administration session to adjust drug dosage. We limit the maximum amount of drug intake for morphine (20 mg/kg, iv) across 4 hrs in a single session so that there is a very little chance of drug over-dose during the self-administration.

2. Stress Paradigm

Warrior stress
The Warrior Stress Paradigm combines predator stress with unpredictable environmental stimuli. Predator stress is a non-painful but effective stressor used in rodent models investigating the effects of stress. Predator stress can be manipulated by presenting the actual predator or the odors of the predator. Exposure to predator stress produces increases in stress hormones (Hayley et al. 2001). Exposure to predator stress also produces behavioral changes in rodents including differences in food consumption, elevated plus maze, startle response, freezing behavior, withdrawal behavior, and exploratory behavior (Adamec et al. 2006; Belzung et al. 2001; Endres et al. 2005; Masini et al. 2005; Takahashi et al. 2005). Predator stress is presented by introducing a cotton ball with commercially available fox urine into a test cage with the rat subject. Unpredictable, non-painful stressors include noise, flashing light, and cage shaking. Unpredictable stress are included because it is a face-valid model of human stress and also has been shown to reliably produced alterations in stress hormones (Fride et al. 1986; Weinstock et al. 1992), and behavior (Gonzalez Jatuff et al. 1999) in rodent studies. The animals are transferred from their home cage and housing room to the "stress cage" (covered with a Plexiglas lid) to be exposed to the stressors. The stress procedure lasts 20 minutes for 5 days. Fox urine (15mL) is placed on a large cotton ball and placed in varying spots in the stress cage. A bright florescent overhead light remains on during the procedure. Animals will be weighed between each stress exposure.

3. Behavioral measures

Spontaneous locomotor activity
Open field locomotor activity will be measured using an Accuscan Electronics Digiscan infrared photocell system. Animals are placed singly in a 20 x 20 x 30 cm clear Plexiglas arena and a Plexiglas lid with multiple ventilation holes placed on top of the arena. Data are automatically gathered and transmitted to a computer operating the Fusion 3.4 software system for data collection and analyses. The interfaced software measures two dozen activity variables, including total distance and horizontal and vertical activity. Chambers are cleaned between subjects with Clidox-S base, water, and activator in a 1:18:1 preparation. The duration of locomotor activity testing is a maximum of 60 min per session. Sixteen open field activity boxes from the Dr. (b)(6) Lab will be used.

Elevated plus maze (EPM)
The EPM is a widely used a measure of anxiety (Hogg 1996; Lister 1987). Rats will be placed individually on the center platform facing a closed arm and allowed to explore the maze for 5 min. Behavior will be videotaped via closed circuit TV camera for scoring as described previously (Elliott et al. 2004). Behaviors scored include: percent time spent in the open arms [(time spent in open arms/total time) X100], percentage of open arms entries [(open arm entries/total arm entries) X100], and percentage of closed arm entries [(closed arm entries/total arm entries) X 100]. Percent time spent in the open arms and percentage of open arm entries are chosen to index anxiety because these behaviors repeatedly correlate with anxiety (Hogg 1996; Rodgers and Dalvi 1997). Video recording and use of data will be in compliance with IACUC Policy #25.

Pavlovian fear conditioning
Sprague-Dawley rats will be placed in a Plexiglas rodent conditioning chamber with a metal grid floor (model E10-10; Coulbourn Instruments, Lehigh Valley, PA, USA), dimly illuminated by a single house light, and enclosed within a sound-attenuating chamber (model E10-20). Rats will be divided into two groups. The paired group will be presented with three to five pairings of a tone for 20 sec (CS: conditioned stimulus; 5 kHz, 75 dB) that co-terminates with a foot shock (US: unconditioned stimulus; 0.5 sec, 0.8 mA). Animals will be weighed between each stress exposure. We will measure contextual fear and cued fear extinction in these animals. For contextual fear, animals will be reintroduced to the fear conditioning chamber and freezing to the context will be measured for 10 minutes. In another session, animals will be placed in a novel chamber which is different from the conditioning chamber and the tone (20 sec) will be played 10 times with a random interval (average interval 2 min). The whole session lasts less than 30 minutes and freezing behavior to the tone will be measured. These additional measures do not cause any pain in the animals because no footshock is involved and the animals are tested for previously formed fear memory.

Acoustic startle reflex (ASR) and pre-pulse inhibition (PPI)
The acoustic startle reflex test will be conducted with a Startle Response Acoustic Test System (Coulbourn Instruments, Columbus, Ohio, USA). The system consisted of 8 weight sensitive platforms in 4 sound-attenuated chambers. All acoustic stimuli will be given by a speaker mounted 24 cm above the test cage. During the testing, rats will be individually placed in an animal holder (to gently restrict animal movement) which will be then placed on a weight-sensitive platform within the chamber. A ventilating fan built into the chamber provided background noise. Following a 3-min acclimation period, animals will be exposed to six types of acoustic stimulus trials: 100 dB, 110 dB, 100 dB with pre-pulse (68 dB), 110 dB with pre-pulse (68 dB), pre-pulse alone and no stimulus. Duration for pre-pulse is 100 ms and for pulse (100 and 110 dB) is 20 ms. Each stimulus has a 2 ms rise and decay time such that onset and offset are abrupt, a primary criterion for startle. A frequency for the tones is 1 kHz as shown in previous studies (Acri et al. 1995; Manion et al. 2007). The same animals were tested five times and there was no sign of hearing impairment in those studies. Each trial type will be presented eight times. Trial types will be presented in random order to avoid order effects and habituation. Inter-trial intervals range randomly from 15 to 25s and the testing lasts approximately 20 minutes as described previously (b)(6),(b)(4)

Hot plate test
Hot plate response measures supraspinal nociceptive responses (Berry et al. 2007; Ding et al. 2005). The hot plate task will be performed with an Omnitech/Accuscan Electronics Analgesia Monitor (Model HPrx). Subjects are placed on a 26 x 26 cm square hot plate platform, enclosed on all sides by Plexiglas walls and covered with a removable Plexiglas top. The metal plate heated to 51°C (a temperature that will elicit a nociceptive response without tissue damage). Hotplate latencies are measured as the time from placement on the heated surface until the animal raised and licked a rear paw. To prevent tissue damage, a maximum latency of 60 sec is allowed. Animals that do not lick their paws at all during the 60-sec period are removed from the heated surface. The platform is cleaned between subjects. The total number of tests is no more than 8 with a minimum of 2 days of break between each test.

Tail flick test
A recent study showed that the effects of opioid drugs on anti-nociception measured by the hot plate and the tail flick methods are different in rats (South et al. 2009). The tail flick method is one of the most commonly used models to measure analgesic responses in rodents (Cecchi et al. 2008; Hardy et al. 1957). Animals will be placed and gently restrained by hand on the test platform (Tail-Flick Analgesia meter, Columbus Instrument) and the lower one-third of the tail will be placed on top of the small hole which gradually increases thermal radiation. When the animal flicks its tail, both the heat source and timer are stopped automatically. The average tail withdrawal time is supposed to be between 5 and 10 seconds and the maximum exposure time will be 20 seconds to prevent potential tissue damage. The total number of tests is no more than 8 with a minimum of 2 days of break between each test. We will try to use the hot plate test as a first choice and if some animals don't respond very well, we will switch to the tail flick test as an alternative way of measuring nociception in rats.

Oral stereotypy
Immediately following MSA, animals will be observed in their home cages for 30 min. The frequency of stereotypic behaviors will be recorded. For example, stereotypic self biting (biting of tail or digits) and biting other objects (bedding, cage, cage bars) will be recorded. The other behaviors, rearing (standing on hind legs), grooming (cleaning behavior), locomotion (walking from one side of the cage to another), catalepsy (immobile with eyes open), sleep (immobile with eyes closed and lying down), inactivity (immobile but not cataleptic), will be recorded as described previously (Wennemer and Kornetsky 1999).

Locomotor activity with morphine administration
Locomotor activity will be measured using an Accuscan Electronics Digiscan infrared photocell system in the (b)(6) inside the LAM. Animals will be placed singly in a 20 x 20 x 30 cm clear Plexiglas arena and a Plexiglas lid with multiple ventilation holes placed on top of the arena. Animals will be habituated to the boxes for 60 minutes as a baseline. Following MSA, locomotor activity will be monitored for 60 minutes. During morphine withdrawal, animals will be tested with either intravenous (0, 0.5 and 2 mg/kg) or subcutaneous (0, 1, and 5 mg/kg) morphine in a single session. Animals will be habituated to the open field activity boxes (60 min), ascending doses of morphine will be injected with 60 min interval and locomotor activity will be monitored continuously.

Morphine withdrawal signs
Quantifying withdrawal signs will provide information about an organism's level of addiction and dependence to drug. Animals will be observed in either a home cage or a clean plexiglass cage for 30 min. Withdrawal signs include ptosis, piloerection, mouth and teeth chattering, abnormal grooming, jumping, chewing/biting, stereotypy, wet dog shake, and locomotor activity (horizontal and vertical) as reported previously (Guitart et al. 1992; Guitart et al. 1993; Malin et al. 1992; O'Dell et al. 2004; Pinelli and Trivulzio 1997; Rasmussen et al. 1990; Stephens and Riley 2009). Each episode will be counted as one occurrence or symptom. This procedure does not cause any stress or pain in the animals.

^{18}FDG μPET/μCT imaging

The imaging will be conducted in accordance with the approved IACUC animal imaging SOP# 3 from the Translational Imaging Facility (CNRM). The animal will be anesthetized with 3% isoflurane/oxygen mixture. FDG will be administered via an i.v. catheter or a tail vein at less than 5 ml/kg. After FDG injection, the animal will either be placed back to its cages awake or placed on a heated surface and maintained under anesthesia during 45-60 minutes of FDG uptake period. After the uptake period, the anesthetized rat will be transferred to a special rodent holder (m2m Imaging) with an integrated 'flow through' nosecone and active waste gas removal via the facility vacuum. A 45-60 minute μPET acquisition will be conducted with anesthesia maintained and animals continuously monitored for signs of movement or changes in respiration. A rectal probe will be used to monitor body temperature. A 5 min μCT scan will also be performed. The bed automatically translates the animal from the μPET to the μCT without disturbing the rodent or anesthesia. After the imaging, anesthesia will be halted, the animal placed on a heating pad, and recovery monitored. The rodent will be returned to its cage, which will be labeled with the following information: injection time, activity amount, volume injected, on-cage survey results and time of survey and free release time. *This procedure will be conducted by the trained personnel at the TIF and is considered as a pain category C.*

Specific Aim 1: To determine effects of early and late withdrawal from morphine self-administration (MSA) on anxiety and fear behavior in male and female rats. We will use elevated plus maze and acoustic startle as anxiety measures, and Pavlovian fear conditioning and extinction as fear behavior following chronic morphine self-administration. We hypothesize that early and late withdrawal from MSA may have differential effects on fear and anxiety behaviors.

Experiment 1: Effects of early withdrawal from morphine self-administration on anxiety and fear behavior.

Following one-week recovery from the catheter surgery, rats will be tested on baseline open field activity (OFA) for 60 min in the morning and tested with ASR for 20 min in the afternoon. Next day, animals will be trained to self-administer intravenous morphine (0.5 mg/kg/infusion) in the operant conditioning chambers (5 days per week) for three weeks. OFA will be measured immediately after MSA on day 5, 10 and 15 to monitor drug-induced locomotor activity. During spontaneous withdrawal from morphine, animals will be tested on the EPM for anxiety behavior (withdrawal day 2) and fear conditioning for fear memory (withdrawal day 3-5).

Timeline

Animals arrive and acclimate to USUHS Animal Facility (LAM) (1 week)

↓

Lever press training in operant conditioning chambers (2-3 days)

↓

Catheter surgery (1 day)

↓

Recovery (1 week)

Baseline OFA 60 min and ASR 20 min (1 day)

Morphine self-administration (5 days/week, 3 weeks)
OFA (day 5, 10 and 15) – 60 min each
↓
OFA 60 min and ASR 20 min (withdrawal day 1)
↓
EPM 5 min (withdrawal day 2)
↓
Fear conditioning 20 min (withdrawal day 3)
Context test 10 min (withdrawal day 4)
Cue test 25 min (withdrawal day 5)
↓
Euthanasia

Sex	Self-admin	Behavioral measures	Pain category	N
Male	Saline	OFA/ASR EPM Fear Cond	E	12
Male	Morphine	OFA/ASR EPM Fear Cond	E	12
Female	Saline	OFA/ASR EPM Fear Cond	E	12
Female	Morphine	OFA/ASR EPM Fear Cond	E	12

Experiment 2: Effects of late withdrawal from morphine self-administration on anxiety and fear behaviors.

Following one-week recovery from the catheter surgery, rats will be tested on baseline open field activity (OFA) for 60 min in the morning and tested with ASR and PPI for 20 min in the afternoon. Next day, animals will be trained to self-administer intravenous morphine (0.5 mg/kg/infusion) in the operant conditioning chambers (5 days per week) for three weeks. OFA will be measured immediately after MSA on day 5, 10 and 15 to monitor drug-induced activity. During late withdrawal from chronic MSA (between 1 and 2 weeks), animals will be tested on the EPM for anxiety behavior and fear conditioning and memory test for fear memory.

Timeline

Animals arrive and acclimate to USUHS Animal Facility (LAM) (1 week)
↓
Lever press training in operant conditioning chambers (2-3 days)
↓
Catheter surgery (1 day)

↓

Recovery (1 week)

↓

Baseline OFA 60 min and ASR 20 min (1 day)

Morphine self-administration (5 days/week, 3 weeks)
OFA (day 5, 10 and 15) – 60 min each

↓

OFA 60 min and ASR 20 min (withdrawal day 7)

↓

EPM 5 min (withdrawal day 8)

↓

Fear conditioning 20 min (withdrawal day 9)
Context test 10 min (withdrawal day 10)
Cue test 25 min (withdrawal day 11)

↓

Euthanasia

Sex	Self-admin	Behavior	Pain category	N
Male	Saline	OFA ASR EPM Fear Cond	E	12
Male	Morphine	OFA ASR EPM Fear Cond	E	12
Female	Saline	OFA ASR EPM Fear Cond	E	12
Female	Morphine	OFA ASR EPM Fear Cond	E	12

Specific Aim 2: To determine effects of morphine self-administration on anti-nociception and tolerance, and regional glucose utilization in brain. We will use a hot plate test to measure antinociception and ^{18}FDG-PET for regional glucose uptake in brain. We will also investigate individual differences in antinociception, morphine self-administration and locomotor sensitization in males and females. We hypothesize that individual differences in morphine antinociception and morphine self-administration may be associated with brain glucose utilization.

Experiment: Effects of morphine self-administration on anti-nociception and regional glucose uptake in the brain.

Following one-week recovery from catheter surgery, animals will be tested on baseline OFA (morning) and nociception (afternoon) in one day. Next day, a baseline brain glucose uptake will be measured with ^{18}FDG-PET/CT. Animals will be trained to self-administer intravenous morphine (0.5 mg/kg/infusion) in operant conditioning chambers

(5 days per week) for 3 weeks. Anti-nociception will be measured in MSA day 1, 3, 5, and withdrawal day 1. OFA will be measured in day 5, 10 and 15 of self-administration, and in withdrawal day 1. Animals will be scanned with ^{18}FDG-PET/CT (second scan) in withdrawal day 2. One week later, the animals will be tested with morphine challenge (1 mg/kg) and scanned with ^{18}FDG-PET/CT for the third time.

Timeline

Animals arrive and acclimate to USUHS Animal Facility (LAM) (1 week)
↓
Lever press training (2-3 days)
↓
Catheter surgery (1 day)
↓
Recovery (1 week)
↓
Baseline OFA 60 min and hotplate test 1 min (1 day)
↓
Baseline FDG-PET scan 1 hr (1 day)
↓
Morphine self-administration (5 days/week, 3 weeks)
OFA (day 5, 10 and 15) – 60 min each
Hotplate test (day 1, 3, 5) – 1 min each
↓
OFA 60 min and hotplate 1 min (withdrawal day 1)
↓
FDG-PET scan (withdrawal day 2)
↓
Morphine challenge (1 mg/kg) and FDG-PET scan (withdrawal day 9)
↓
Euthanasia

Sex	Self-admin	Behavioral measures	Brain measures	Pain category	N
Male	Saline	OFA Hotplate	FDG-PET	D	12
Male	Morphine	OFA Hotplate	FDG-PET	D	12
Female	Saline	OFA Hotplate	FDG-PET	D	12
Female	Morphine	OFA Hotplate	FDG-PET	D	12

Specific Aim 3: To determine effects of chronic nicotine and/or psychological stress (Warrior Stress) on morphine self-administration and behavioral sensitization. Animals will be exposed to chronic nicotine via minipump and Warrior Stress, and then allowed

to acquire morphine self-administration for two weeks. Following acquisition of morphine self-administration, animals will be tested with dose response function. Locomotor sensitization during morphine withdrawal will also be measured. We hypothesize that stress exposure may facilitate opioid abuse and nicotine exposure may attenuate opioid abuse.

Experiment: Effects of nicotine and stress on morphine self-administration and behavioral sensitization.

Following one week of acclimation in the housing room (a reversed light/dark cycle), each animal will be implanted with osmotic minipump to deliver chronic nicotine (6 mg/kg/day, s.c.). During the second-week period of nicotine administration, animals will be exposed to Warrior Stress (20 min a day) for 7 days and OFA behavior will be measured. After three weeks of nicotine exposure, minipump will be removed and catheter will be implanted in the jugular vein in a single surgery. Following 1 week of recovery from catheter surgery, animals will be allowed to self-administer intravenous morphine (0.5 mg/kg/infusion, FR1) in operant conditioning chambers (3 hr/day, 5 days per week) for 2 weeks. Upon stabilization of morphine self-administration, animals will be tested with a dose-response function (0, 0.1, 0.3, 0.5 and 1 mg/kg/infusion, i.v.) for 2 weeks. Following 1-week withdrawal from MSA, animals will be tested with morphine challenge (0, 0.5 and 2 mg/kg) to measure locomotor sensitization.

Timeline

Animals arrive and acclimate to USUHS Animal Facility (LAM) (1 week)
↓
Baseline OFA 60 min and ASR 20 min (1 day)

Osmotic nicotine mini-pump implantation for 10 min (3 weeks)
↓
Warrior stress 20 min/day (7 days)
Behavioral measures – OFA and ASR (day 7)
↓
Mini-pump removal and catheter surgery (1 day)
↓
Recovery (1 week)
↓
Baseline OFA 60 min and ASR 20 min (1 day)
↓
Acquisition of morphine self-administration (5 days/week, 2 weeks)
OFA (day 5, 10) – 60 min each
↓
Morphine dose response test (5 days/week, 2 weeks)
↓
Locomotor sensitization test with morphine (0, 0.5, 2 mg/kg) (1 day)
↓
Euthanasia

Sex	Warrior Stress	Nicotine mini-pump	Behavior	Pain category	N
Male	No	No	OFA ASR	D	12
Male	No	Yes	OFA ASR	D	12
Male	Yes	No	OFA ASR	E	12
Male	Yes	Yes	OFA ASR	E	12
Female	No	No	OFA ASR	D	12
Female	No	Yes	OFA ASR	D	12
Female	Yes	No	OFA ASR	E	12
Female	Yes	Yes	OFA ASR	E	12

Experimental endpoints

If signs of morbidity are observed over the course of the experiments, the veterinarian is notified and, if necessary, rats are excluded from the study and euthanized. Signs for judging morbidity include rapid breathing; shallow, labored breathing; weight loss (more than 10% of body weight not regained within 48 hr, continuous body weight loss over 5 days of more than 5 g per day), ruffled fur (rough hair coat), hunched posture, hypothermia or hyperthermia, ulceration dermatitis or infected tumors, diarrhea, impaired ambulation, evidence of muscle atrophy or other signs of emaciation, bleeding, or inability to remain upright. In cases of body weight loss, supplemental soft and sweetened food is provided, and if necessary drug self-administration is withheld until the animal recovers.

V.2. Data Analysis:

The number of rats is determined by the number of experimental groups needed for a clear interpretation of the behavioral and neurobiological data. Intravenous drug self-administration is a highly regulated and conditioned behavior in rats. Based on our experience, 12 animals per group allows for a sufficient statistical power to detect group differences in drug self-administration behavior as described previously (b)(6),(b)(4) (b)(6),(b)(4) We also performed a power calculation using R statistical language (http://www.r-project.org/). We expect moderate to large effect sizes (0.5) for behavioral measures to drugs based on the previous studies (Choi et al. 2006; Self et al. 1994). Therefore, an effect size (0.5), high power (0.80), and alpha level of 0.05 with a two-sided test requires a total of 12 animals per group. The data will be analyzed by parametric or non-parametric tests with repeated measures as appropriate. Tukey HSD *post hoc* tests or Dunnett's t will be used to compare differences between the groups. All analyses will be two-tailed with alpha = 0.05. Statistical analyses will be conducted

using software such as the Prism (GraphPad, San Diego, CA, USA), the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA), and the R statistical language (http://www.r-project.org/).

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered:
This study will require the use of intact living animals. Behavioral measurements, such as self-administration of drug, stress and anxiety responses, addiction behavior cannot be observed on cells grown in culture or on a computer simulation. Non-animal alternatives such as computer modeling and cell cultures were considered but are not suitable to assess behaviors in organisms because there currently are no non-animal alternatives that provide information about effects of stress, drug and behavior in living organisms. Due to ethical considerations, the proposed studies cannot be done with human subjects.

V.3.2. Animal Model and Species Justification:
Rats will be used in behavioral paradigms that model stress, anxiety and substance abuse in humans. Rats are amendable to pharmacological manipulations needed to study the biological mechanisms involved in stress and substance abuse. The experimental nature of the work necessitates the use of model organisms rather than human subjects. However, results from our laboratory studies complement data obtained from human clinics and experiments. We will continually evaluate our approaches and adapt experiments for *in vitro* or computer models whenever possible.

	Species #1	Species #2
V.3.3.1. Genus & Species:	*Rattus norvegicus*	
V.3.3.2. Strain/Stock:	Sprague-Dawley	
V.3.3.3. Source/Vendor:	(b)(4)	
V.3.3.4. Age:	Adult (about 55 days)	
V.3.3.5. Weight:	Adult males (about 250 grams); adult females (about 170 grams)	
V.3.3.6. Sex:	Male and Female	
V.3.3.7. Special Considerations:	N/A	

V.3.4. Number of Animals Required (by Species): 280

Subjects. Subjects are male and female Sprague-Dawley rats, the most commonly used laboratory rats in various studies, including investigations of stress and drug

effects. Sprague-Dawley albino rats are considered to be the standard test rat for nicotine and stress experiments. Adult animals are approximately 55 days old upon arrival and in consultation with the breeder.

V.3.3. Laboratory Animals

Please note: Each experiment will be run as a series of smaller sub-experiments for logistical reasons:
Experiment 1 = 96 rats (plus 16 additional rats) = 112 rats
Experiment 2 = 48 rats (plus 8 additional rats) = 56 rats
Experiment 3 = 96 rats (plus 16 additional rats) = 112 rats

Additional animals are necessary due to loss of catheter patency in some animals before the end of the study and training students on surgery and other procedures.

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement: Animals will be monitored on a daily basis and be given pain medication and antibiotic if any sign of pain or distress occurs. In order to reduce stress and anxiety, animals will be acclimated to the facility for 1 week upon arrival. If there are any indications of pain or distress, then the LAM staff will be consulted. With regard to logistical and technical refinements, many of the behavioral assessments use automated equipment interfaced with computers to allow for the collection of multiple parameters simultaneously. The use of sophisticated software and computerized technologies allows us to collect fine-grained and complete data sets for every animal included in the experiment, eliminating the need for additional studies that might otherwise have been necessary.

V.3.5.2. Reduction:
The sample sizes for the proposed work were derived from power analyses of pilot data and past work in our laboratory and in other laboratories. These sample sizes, therefore, constitute the minimum number of animals necessary to obtain meaningful results and represent a useful application of knowledge of statistical effect size and the concept of power. In addition, in each experiment, multiple dependent variables are measured. Measurement of many variables in the same animals markedly reduces the total number of experiments conducted.

V.3.5.3. Replacement: This study requires the use of intact living animals; behavioral measurements cannot be made on cells grown in culture or with computer models. Thus, it is not possible to replace or eliminate the use of animals in the proposed study.

V.4. Technical Methods:
V.4.1. Pain / Distress Assessment:
Animals will be anesthetized during implantation of mini-pumps or catheters. Animals will be observed daily by investigators and by LAM staff to check health and well-being and to insure that there is no undue pain or distress based on behaviors and vocalizations. Any indications of pain or distress will be dealt with by alleviating the cause of the problem and/or by contacting LAM personnel for assistance and guidance

V.4.1.1. **APHIS Form 7023 Information**:

V.4.1.1.1. **Number of Animals**:

	Species #1	Species #2
V.4.1.1.1.1. **Column C**:		
V.4.1.1.1.2. **Column D**:	112	
V.4.1.1.1.3. **Column E**:	168	

The number of animals listed in Column E is based on the premise that these animals are exposed to fear conditioning experiment which requires exposing animals to mild and brief footshock (0.8 mA in 0.5 sec) three to five times or distress from the Warrior Stress paradigm. The pain and distress resulting from footshock during the fear conditioning are momentary and do not cause any physical injury to the tissue of animals.

V.4.1.2. **Pain Relief / Prevention**:

V.4.1.2.1. **Anesthesia/Analgesia/Tranquilization**:

For nicotine minipump implantation, subjects are first anesthetized by inhalation anesthetic to prevent pain. Isoflurane is mixed with oxygen (3–5% Isoflurane; with an oxygen flow rate of 0.5-1.0 liters/minute). Subjects are placed inside an induction chamber saturated with Isoflurane vapor. Subjects are removed from the chamber when tail pinch produces no reflex movement and anesthesia is maintained using a mask/nose cone attached to the anesthetic vaporizer (with Isoflurane concentration ranging from 1–3% to maintain appropriate depth of anesthesia). Animals receive buprenorphine (0.01-0.05 mg/kg i.v.) once every 12 hrs for next 48 hrs.

For i.v. catheter surgery, subjects are briefly anesthetized by inhalation anesthetic isoflurane mixed with oxygen (i.e., 3–5% Isoflurane; with an oxygen flow rate of 0.5-1.0 liters/minute) inside an induction chamber. Subjects will receive an injection of either sodium pentobarbital (40-50 mg/kg, i.p.) or ketamine/xylazine (40-80 mg/kg ketamine and 5-10 mg/kg xylazine, i.p.). For sodium pentobarbital injection, rats will also receive atropine sulfate (0.04 mg/kg, s.c.) to reduce pulmonary congestion and facilitate breathing. Animals receive buprenorphine (0.01-0.05 mg/kg i.v.) once every 12 hrs for next 48 hrs. Catheter patency will be verified by infusion of a sodium methohexital (Anesthetic, 7-15 mg/kg, i.v.) solution. Patency is indicated by a brief (about 1 minute) but rapid anesthesia. Using these procedures, catheter life averages about 8-10 weeks for rats.

V.4.1.2.2. **Pre- and Post-procedural (not surgery) Provisions**: Animals will be acclimated for 1 week to the USUHS LAM. During acclimation to LAM, animals will be handled briefly and weighed to acclimate them to being handled. This will reduce any stress that might result from handling of the animals during the procedures described in this study. The non-surgical procedure that may induce momentary discomfort (e.g., hot

plate test and tail flick test of nociceptive responses) is included for scientific purposes and is short in duration (less than 60 sec for hot plate and less than 20 sec for tail flick). Animals are observed daily by investigators and by LAM staff to check on their health and well-being and to assure that there is no undue pain or distress. Animals also are carefully monitored during behavioral testing procedures. In addition, animals are closely monitored during all procedures that involve any pain or potential distress (e.g., during and after surgery; during and after stress exposure). Pain/distress is assessed based on behaviors and vocalizations. Any indications of pain or distress are dealt with by alleviating the cause of the problem or by contacting LAM personnel for assistance and guidance.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: Medline (PubMed), PsycInfo, F1000Biology, AGRICOLA

V.4.1.3.2. Date of Search: March, 2015

V.4.1.3.3. Period of Search: All years available on each database are searched (from recent work up to 30 years in the past).

V.4.1.3.4. Key Words of Search:
osmotic minipumps, intravenous catheters, minipump implantation, catheter surgery, chronic self-administration, foot shock, pain, alternatives, analgesia, surgery, anesthesia, acute drug administration, rat (these words are searched in various combinations)

V.4.1.3.5. Results of Search: No alternatives to the minipump and the catheter implantation procedures have been found. No alternatives to the nociception procedures have been found. No milder alternative for the stress manipulation has been found. No alternatives are available to avoid using live animals for the assessment of effects of stress, opioid, and nicotine in intact rats and the procedures that are followed.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

Animals in specific aim 1 are exposed to fear conditioning experiment to study fear behavior. This experiment requires exposing animals to mild and brief footshock (0.8 mA in 0.5 sec) three to five times. The pain and distress resulting from these shocks during the fear conditioning are momentary and do not cause any physical injury to the tissue of animals.

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions:

All surgeries will be performed in the LAM facility by the (b)(6) Lab and the (b)(6) Lab and in consultation with LAM staff to insure proper preparation and safeguards. Implantation of osmotic minipumps and catheters uses aseptic technique. For the minipump, subjects will be anesthetized by inhalation anesthetic LAM equipment (vaporizer) and Isoflurane mixed with oxygen. Subjects will be placed inside an induction chamber saturated (at 3-5%) with Isoflurane vapor. Subjects will be removed from the chamber when tail pinch produces no reflex movement and anesthesia will be maintained (at 1-3%) using a mask/nose cone attached to the anesthetic vaporizer.

V.4.3.2. Procedure:

Minipump implantation
Subjects are anesthetized by inhalation anesthetic using LAM anesthetic equipment (vaporizer) and Isoflurane and oxygen. Subjects are placed inside an induction chamber saturated with Isoflurane vapor. Subjects are removed from the chamber when tail pinch produces no reflex movement and anesthesia is maintained using a mask/nose cone attached to the anesthetic vaporizer. A 3 x 5 cm area between the withers is shaved and cleaned with the antiseptic Betadine. A 2 cm transverse incision within the shaved region approximately 1 cm below the scapulae is made with blunt-nosed, curved-tipped Mayo surgical scissors, a pocket is created by gently spreading the subcutaneous tissues with the scissor tips, and the minipump is inserted. Incisions are closed with two-three 9 mm stainless steel wound clips. This procedure takes approx. 4 min per subject. The surgical area is thoroughly cleaned before and after procedures. Investigators wear surgical masks, gloves, and laboratory coats or surgical scrubs. The procedures are performed under aseptic conditions.

Intravenous catheter implantation
All surgery will be performed under aseptic conditions in a clean area. Each surgery will be done on a separate, clean and autoclaved sheet of Whitman Benchkote paper. Surgical instruments are sterilized in a glass bead sterilizer and cleaned/sterilized between successive surgeries. Sterile gloves are used for each rat's surgery and instruments are sterilized before each rat's surgery. Rats are anesthetized with sodium pentobarbital (40-50 mg/kg, i.p.) or ketamine/xylazine (40-80 mg/kg ketamine, 5-10 mg/kg xylazine, i.p.). Injection volume is 1 ml/kg for both drugs. Surgery starts after the rat fails to show the withdrawal reflex after pinching the rear toe (a procedure that is repeated several times during the surgery to ensure full anesthesia level). Supplemental anesthetic dose (0.1-0.4 ml) may be given as needed. Intravenous silastic catheter is implanted into the right jugular vein and attached to either the head or the back of the animal. Two incisions are made, one on the back (2.5 cm), and one on the neck (1 cm). The jugular vein is isolated by teasing apart fatty tissue above the vein with small scissor tips, just above where the vein passes over the clavicle. After isolation, the vein is punctured with a needle (22 gauge) and the sterile Silastic catheter is inserted to the level of the sinus vena cava just outside the right atrium. The entry point of the catheter is secured in place with a 0.5 cm x 0.5 cm mersilene surgical mesh secured to the catheter. The remaining catheter is pulled from the neck area subcutaneously to the back incision. Then the catheter exits via premade 22-gauge stainless steel tubing cemented into place with bell shaped dental cement and the bell

base (about 0.8 cm diameter) cemented to a 1.5 x 1.5 cm Marlex surgical mesh comprising the back mount. The incision is closed using three stainless steel wound clips and treated with topical antibiotic Neosporin ointment (Swabbed To Back Mounted Catheter Exit Area). Animals receive antibiotic gentamycin sulfate (5-8 mg/kg, iv) after the surgery. Gentamycin is also included in sterile saline solution that we use to flush catheter to maintain the patency. Animals receive buprenorphine (0.01-0.05 mg/kg i.v.) once every 12 hrs for next 48 hrs.

V.4.3.3. Post-surgical Provisions:

During recovery (about 30-60 min), the rats are placed in a cage that is kept warm by a heating pad (located under the cage) or under the heating lamp to prevent hypothermia. The investigators are responsible for post-operative care. The post-operative care includes continuously monitoring the rats until they are fully awake and the animals' health will be monitored and recorded in the animal's medical record for at least 3 days after surgery or until the animal appears normal. The surgery log will be kept in a drawer in the surgery room. If pain or distress such as difficulty in breathing and hypothermia is observed at any time rats will be treated with buprenorphine (0.01-0.05 mg/kg s.c.). After the IV catheter surgery, rats are individually housed in the animal facility for 5-7 days. During this time period, the rats are handled and the catheters are flushed daily with 0.2 ml of heparinized (20 U/ml), bacteriostatic saline containing gentamycin sulfate (0.33 mg/ml, i.v.) as described previously (b)(6),(b)(4)

Catheter patency will be verified by infusion of a sodium methohexital (Anesthetic, 7-15 mg/kg, i.v.) solution. Patency is indicated by a brief (about 1 minute) but rapid anesthesia. Using these procedures, catheter life averages about 6–8 weeks for rats. Most catheters are lost due to the growth of the venous wall around the catheter tip. When catheters are no longer patent, animals are either sacrificed as described below, or the original catheter is removed and another one implanted in the left jugular vein. We have observed no significant weight loss or obvious physical impairment to the animals due to one or two consecutive catheter implantations.

V.4.3.4. Location: Surgeries to implant the minipumps will be conducted in the LAM facility. Behavioral measures will be conducted in the laboratories assigned to Drs. (b)(6) in the LAM and (b)(6) at USUHS.

V.4.3.5. Surgeon: PI and all co-investigators and technicians

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations:

V.4.4.1. Injections: The drugs will be delivered by intraperitoneal (i.p.) or subcutaneous (s.c.) injection (1ml latex free syringe w/ 27G½ needle, B-D, Franklin Lakes, NJ). Injection volume will be adjusted based on body weight and no more than 1

ml/kg for i.p. and 2 ml/kg for s.c. administration. For intravenous administration, each bolus infusion volume will be less than 0.5 ml.

Sodium pentobarbital (40 - 50 mg/kg, i.p.)
Atropine sulfate (0.04 mg/kg, s.c.)
Buprenorphine (0.01 - 0.05 mg/kg i.v.)
Nicotine (0.5 - 6 mg/kg, s.c.)
Morphine (0.5 - 2 mg/kg, i.v.) & (1 - 5 mg/kg, s.c.)
Ketamine/xylazine (40 - 80 mg/kg ketamine, 5 - 10 mg/kg xylazine, i.p.)
Gentamycin sulfate (5 - 8 mg/kg, i.v.)
Sodium methohexital (7 - 15 mg/kg, i.v.)
Chloral hydrate (400 mg/kg, i.p.)

V.4.4.2. Biosamples:

Blood sampling from indwelling i.v. catheters
Animals have indwelling catheters implanted to the jugular vein and self-administer intravenous morphine on a daily basis. We maintain the catheter patency during the entire period of the study. We will draw a small volume of blood from the catheter for biochemical assays. For the volume of blood and the frequency of blood drawing, we will follow the NIH guidelines (http://oacu.od.nih.gov/ARAC/documents/Rodent_Bleeding.pdf) and published references (Evans 1994). For a 300 gram rat, approximately 1% of circulating blood volume (0.2 ml) can be safely drawn every 24 hrs according to the guidelines. We will draw 1% of circulating blood volume from each animal with a minimum of 2-day break between drawings. Each animal is gently held and catheter is flushed with 0.3 ml of heparinized saline. This is the same procedure to maintain catheter patency in animals. One cc syringe is connected to the catheter and 1% circulating blood volume (e.g. 0.2 cc in 300 g rat) is slowly drawn. The whole procedure takes less than 5 min and animal is returned to its home cage. This procedure does not cause any pain or distress in animals and they show normal weight gain after the procedure. Previous study also reported that the same procedure does not cause any pain or stress in rats (Thrivikraman et al. 2002). There is no anesthesia required for this procedure. Blood samples will be collected at baseline, during morphine self-administration and during spontaneous withdrawal. Total number of sample collection will not exceed 10. At the end of the experiment, animals will be euthanized as described below. Blood samples and brain tissue samples will be collected and stored for later analysis.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Rats are identified by tail markings and ear punches matched with IDs on the cage cards. Immediately after the IV catheter surgery under anesthesia, small hole on the ear will be made to identify each animal. Ear punching is commonly used for identification of rodents and involves using a special punch to produce a small (0.5 to 1 mm) notch near the edge of the ear. The cutting edge of the punch is disinfected 70% ethanol after each use.

V.4.4.6. Behavioral Studies: Please see section V.1. (Materials and Methods)

V.4.4.7. Other Procedures: N/A

V.4.4.8. Tissue Sharing:
Blood samples and brain tissue will be collected and shared with other investigators for molecular and biochemical assays. All personnel engaging in this procedure will receive training by IACUC/LAM staff, or trained laboratory personnel.

V.4.5. Study Endpoint: The study endpoint is euthanasia in each experiment (9 weeks for Specific Aim 1 and 2, and 10 weeks for Specific Aim 3). Each experiment will be run in counter-balanced cohorts. Considering pilot work, time between cohorts, and time to run each cohort, each experiment will require about 6 months to collect behavioral data and blood samples. Biochemical assays and data analyses will require several additional months per experiment.

V.4.6. Euthanasia:
Rats in Aim 1 will be euthanized using carbon dioxide inhalation from a compressed gas cylinder. Euthanasia will be done in a LAM designated procedure room followed by rapid decapitation by guillotine. This euthanasia procedure is in compliance with the 2013 AVMA Guidelines on Euthanasia. For brain tissue collection in Aim 2 and 3, chloral hydrate (400 mg/kg) or ketamine/xylazine (40-80 mg/kg and 5-10 mg/kg) will be administered at a dose of 1.0 ml/kg body weight via i.p. (intraperitoneal) injection with a 26 3/8 gauge, 0.5 inch, needle and 1ml syringe. The depth of anesthesia will be determined by loss of withdrawal response to rear foot pad pinch, tail pinch and loss of movement in response to eye extra orbital touching. Thoracotomy will only be performed after failure of the withdrawal reflex and then intra-cardiac perfusion will be performed. Supplemental anesthetic doses (200 mg/kg of chloral hydrate or a mixture 20-40 ketamine and 5 mg/kg xylazine) will be given as needed. After the deep anesthesia, the heart will be exposed, and a 17-gage needle that is connected to a pump via tubing is inserted to the left ventricle of the heart. Rats will be transcardially perfused with isotonic saline (50-100 ml) followed by a 4% paraformaldehyde/1% glutaradlehyde/50% phosphate buffer fixative (200-300 ml).

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol.

V.5.1.1. Study Room:
Building(s) (b)(6) Room Number(s) (b)(6) (b)(6)

V.5.1.2. Special Husbandry Provisions: Animals will be single housed.

Food Restriction: Yes X No _____

Fluid Restriction: Yes No X

For the lever press training prior to the intravenous drug self-administration, animals are slightly food restricted for two to three days. This procedure requires an initial 16–20 hrs of food restriction followed by ad lib feeding for a few hrs after each training. This procedure allows transient weight gain overnight, but animals return to 90% initial body weight prior to the next lever press training session on the following day. After the procedure, the animals are fed *ad lib* throughout the experiment.

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care: N/A

V.5.2.1. Routine Veterinary Medical Care: Animals will be cared for in accordance with LAM SOPs. Animals will be observed daily or more frequently by the veterinary staff. The LAM veterinary staff will be available for consultation with the PI if any unexpected medical events arise.

V.5.2.2. Emergency Veterinary Medical Care: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays. Investigators' contact information will be available to the veterinary staff in case a decision about early euthanasia or treatments of an animal needs to be made on the weekend or holiday.

V.5.3. Environmental Enrichment:

V.5.3.1. Enrichment Strategy: N/A

V.5.3.2. Enrichment Restrictions: Animals will be single housed without toys because these experiments are designed to determine effects of several interventions. Animals are housed individually because of indwelling catheters after catheter surgery and tested on drug self-administration.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Personnel	Procedure	Qualifications/Training
(b)(6) Ph.D. (Principal Investigator)	Animal Handling	(b)(6) (b)(6) > 20 years experience with rats and other rodents

	Drug Administration	USUHS Investigator Training and Animal Handling courses; > 20 years experience with rats and other rodents
	Surgical Implant of mini-pumps and catheters	U of A/UTSW Med Ctr training courses; > 20 years experience with rats and other rodents
	Behavioral Measures	U of A/UTSW Med Ctr training courses; > 20 years experience with rats and other rodents
	Perfusion and tissue collection	U of A/UTSW Med Ctr training courses; > 20 years experience with rats and other rodents
	Anesthetized Decapitation	U of A/UTSW Med Ctr training courses; > 20 years experience with rats
(b)(6) **MS**	Animal Handling	USUHS Investigator Training (Fall, 2014) and Animal Handling course (Fall, 2014); 1 year experience with rats; training by Dr. (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel
	Surgical Implant of mini-pumps and catheters	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Behavioral Measures	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Perfusion and biological sample collection	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Anesthetized Decapitation	Training by Dr (b)(6) and lab personnel
(b)(6) **PhD**	Animal Handling	USUHS Investigator Training and Animal Handling courses (several sessions, including sessions in 1980 and in early 1990s); NIH/NIMH training courses (several in mid 1980s); > 30 years experience with rats and other rodents
	Drug Administration	USUHS Investigator Training and Animal Handling courses; NIH/NIMH training courses; > 30 years experience with rats and other rodents
	Surgical Implant of Osmotic Mini-pumps	NIH/NIMH training courses; > 30 years experience with rats and other rodents; 1200+ surgeries performed
	Behavioral Measures	NIH/NIMH training courses; > 30 years experience with rats and other rodents
	Biological sample collection	NIH/NIMH training courses; > 30 years experience with rats and other rodents
	Anesthetized Decapitation	NIH/NIMH training courses; 25+ years experience with rats; 1000+ decapitations performed
(b)(6)	Animal Handling	USUHS Investigator Training (March, 2015) and Animal Handling course (March, 2015); training by Dr. (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel
	Surgical Implant of mini-pumps and catheters	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Behavioral Measures	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Perfusion and biological sample collection	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Anesthetized Decapitation	Training by Dr (b)(6) and lab personnel
(b)(6)	Animal Handling	USUHS Investigator Training (Dec, 2014) and Animal Handling course (Dec, 2014); 1 year experience with

		rats; training by Dr (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel
	Surgical Implant of mini-pumps and catheters	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Behavioral Measures	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Perfusion and biological sample collection	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Anesthetized Decapitation	Training by Dr (b)(6) and lab personnel
(b)(6)	Animal Handling	USUHS Investigator Training (July, 2014) and Animal Handling course (July, 2014); 1 year experience with rats; training by Dr (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel
	Surgical Implant of mini-pumps and catheters	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Behavioral Measures	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Perfusion and biological sample collection	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Anesthetized Decapitation	Training by Dr (b)(6) and lab personnel
(b)(6)	Animal Handling	USUHS Investigator Training (Oct, 2014) and Animal Handling course (Oct, 2014); 1 year experience with rats; training by Dr (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel
	Surgical Implant of mini-pumps and catheters	Training by Dr (b)(6) and lab personnel
	Behavioral Measures	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Perfusion and biological sample collection	Training by Dr (b)(6) and lab personnel; 1 year experience with rats
	Anesthetized Decapitation	Training by Dr (b)(6) and lab personnel
(b)(6) **PhD**	Animal Handling	USUHS Investigator Training (Fall, 2013) and Animal Handling course (Fall, 2013); > 18 year experience with rats; training by Dr (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel
	Surgical Implant of mini-pumps and catheters	Training by Dr (b)(6) and lab personnel
	Behavioral Measures	Training by Dr (b)(6) and lab personnel; > 18 year experience with rats
	Biological sample collection	Training by Dr (b)(6) and lab personnel; > 18 year experience with rats
	Anesthetized Decapitation	Training by Dr (b)(6) and lab personnel
(b)(6)	Animal Handling	USUHS Investigator Training (Dec, 2014) and Animal Handling course (Dec, 2014); 1 year experience with rats; training by Dr (b)(6) and lab personnel
	Drug Administration	USUHS Investigator Training and Animal Handling courses; training by Dr (b)(6) and lab personnel

IX. **ASSURANCES:** As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6)

Principal Investigator Signature

3/26/2015
Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6)

Principal Investigator Signature

3/26/2015
Date

I. Painful Procedure(s):

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress caused by the stress paradigm **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. However, the potential pain and distress caused by the IV catheter surgery **WILL** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6)

Principal Investigator Signature

3/26/2015
Date

IX. ASSURANCES: As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Course.

(b)(6)

Principal Investigator Signature

3/26/2015
Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

(b)(6)

Principal Investigator Signature

3/26/2015
Date

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress caused by the stress paradigm **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. However, the potential pain and distress caused by the IV catheter surgery **WILL** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

Principal Investigator Signature	3/26/2015 Date

X. **PROTOCOL ABSTRACT:**

A. **Animal Protocol Number:**

B. Animal Protocol Title: Role of nicotine and opioid on stress and anxiety in rats

C. **Principal Investigator:** (b)(6) PhD

D. **Performing Organization:** Uniformed Services University of the Health Sciences

E. **Funding:** USUHS

F. **Objective and Approach:**

Posttraumatic stress disorder (PTSD) is a debilitating consequence of trauma in military and civilian settings. PTSD is often comorbid with substance use and other psychological disorders. Nicotine-containing products (including cigarettes and smokeless tobacco) are used by more than 50% of military personnel, well above the 18% of civilians who smoke. This high use of nicotine among military personnel may be partially explained by the fact that nicotine and tobacco self-administration increase under stressful situation. Moreover, opioid pain medication abuse is increasing and this is of growing concern among military personnel. We will study effects of stress and two widely used drugs (nicotine and opioid) on psychological behaviors using animal models including intravenous drug self-administration in rats. In specific aims 1 and 2, we will study effects of morphine self-administration and withdrawal on behaviors such as fear, anxiety, and antinociception as well as brain glucose uptake in male and female rats. In specific aim 3, we will study interaction between psychological stress (Warrior Stress) exposure and drugs (nicotine and opioid) in male and female rats. For behavioral outcome measures, we will utilize paradigms such as elevated plus maze, classical fear conditioning, acoustic startle reflex, antinociception and spontaneous locomotor activity. The goal of this study is to better understand the biological mechanisms contributing to stress responses and substance use as well as individual differences in vulnerability and resilience to these environmental factors.

G. **Indexing Terms (Descriptors):** Stress, nicotine, opioid, behavioral responses of rats to stress, nicotine, drug self-administration, drug seeking, psychological stress, fear memory, PTSD, anxiety, depression,

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BETHESDA, MARYLAND 20814-4799

Institutional Animal Care and Use Committee
Phone: (b)(6)
Fax (b)(6)

June 09, 2015

MEMORANDUM FOR DR (b)(6) DEPARTMENT OF PSYCHIATRY

SUBJECT: IACUC Approval of Protocol - Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) by Designated Member Review, on June 8, 2015:

Animal Protocol Title: "Neurophysiology of Escalation of Aggression in a Rat Model of PTSD"

USUHS Protocol Number: (b)(6)

Expiration Date: June 7, 2018

Supporting Grant(s) Number: (b)(6)

Name of Principal Investigator: Dr. (b)(6)

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is (b)(6) The IACUC approved the above referenced application as submitted.

for (b)(6) Ph.D.

(b)(6) Ph.D.
Vice-Chair, Institutional Animal
Care and Use Committee

cc:
Office of Research

USUHS FORM 3206
ANIMAL STUDY PROPOSAL
PROTOCOL COVER SHEET

PROTOCOL NUMBER: (b)(6)

PROTOCOL TITLE: Neurophysiology of Escalation of Aggression in a Rat Model of PTSD

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

FUNDING AGENCY: Dept of Defense/USUHS - O/M funds
EARLIEST ANTICIPATED FUNDING START DATE: January 2015

PRINCIPAL INVESTIGATOR: (b)(6) M.D., Ph.D.

(b)(6)	PSY	(b)(6)	1/8/15
Principal Investigator Signature	Department	Office/Lab Telephone	Date

SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.

(b)(6)	Chairman	(b)(6)	2-10-15
Research Unit Chief / Dept. Head Signature	Title	Telephone	Date

Typed Name: (b)(6) M.D.

STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.

(b)(6)	PSY	(b)(6)	1/8/15
Statistician Signature	Department	Telephone	Date

Typed Name: (b)(6) Ph.D.

ATTENDING VETERINARIAN: In accordance with the Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

(b)(6)	LAM	(b)(6)	2/23/15
Attending/Consulting Veterinarian Signature	Department	Telephone	Date

Typed Name: MA (b)(6) DVM, MPH

ANIMAL PROTOCOL NUMBER: (b)(6)

PRINCIPAL INVESTIGATOR: (b)(6) M.D., Ph.D.

ANIMAL PROTOCOL TITLE: Neurophysiology of Escalation of Aggression in a Rat Model of PTSD

GRANT TITLE (if different from above):

USUHS PROJECT NUMBER: (b)(6)

CO-INVESTIGATOR(S): (b)(6) Ph.D., (b)(6) Ph.D., (b)(6) M.D. (b)(6) M.D.

TECHNICIANS(S):

I. NON-TECHNICAL SYNOPSIS:

Escalated aggression is a comorbidity of post traumatic stress disorder (PTSD), appearing as angry outbursts and reckless or destructive behavior. To elucidate the underlying neurophysiology and discover pharmacotherapy, an animal model is needed for experimentation. The rat is the lowest animal on the phylogenetic scale that corresponds to humans in anatomy, physiology, and behavior sufficiently to study the neurophysiology of aggression.

Stress is produced by restraint and tail shock for two hours on each of three consecutive days. This restraint/tail shock paradigm has been running in the laboratory for fifteen years and an IACUC protocol (PSY-14-667) is currently active. Sleep deprivation is to be achieved by giving the restraint/tail shock procedure during the final hours of the light cycle, when the animal would normally be in REM sleep.

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II. BACKGROUND:

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[redacted]

PTSD Animal Models (b)(4)

(b)(4)

[redacted]

To establish a rat model of PTSD we have successfully developed and tested the inescapable tail-shock model of stress in rats and verified that short and long-lasting behavioral, biochemical and physiological alterations result. Importantly, the stress-

induced behavioral and neurobiological symptoms in the rat models are very similar to those seen in PTSD patients.

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II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: PubMed, entire database; AGRICOLA, entire database; NIH RePorter, entire database; AltWeb, entire database; DoD Biomedical Research Database (BRD) entire database.

II.2.2. Date of Search: February 10, 2015

II.2.3. Period of Search: no limit

II.2.4. Key Words and Search Strategy: Rat, Aggression, and PTSD; Rat, Anger, and PTSD

II.2.5. Results of Search:

PubMed – 2 articles, one partially overlapping, none equivalent,
AGRICOLA - 0
NIH RePorter – 1 grant, none overlapping
AltWeb – 1 article, none overlapping
BRD – 5 related grants, none overlapping

III. OBJECTIVE\HYPOTHESIS:

The overarching hypothesis under which these experiments are proposed is that the distress that leads to PTSD, and the sleep deprivation that is intrinsic to PTSD, promote the anger, hostility, aggression, and violence that are frequently comorbid with PTSD.

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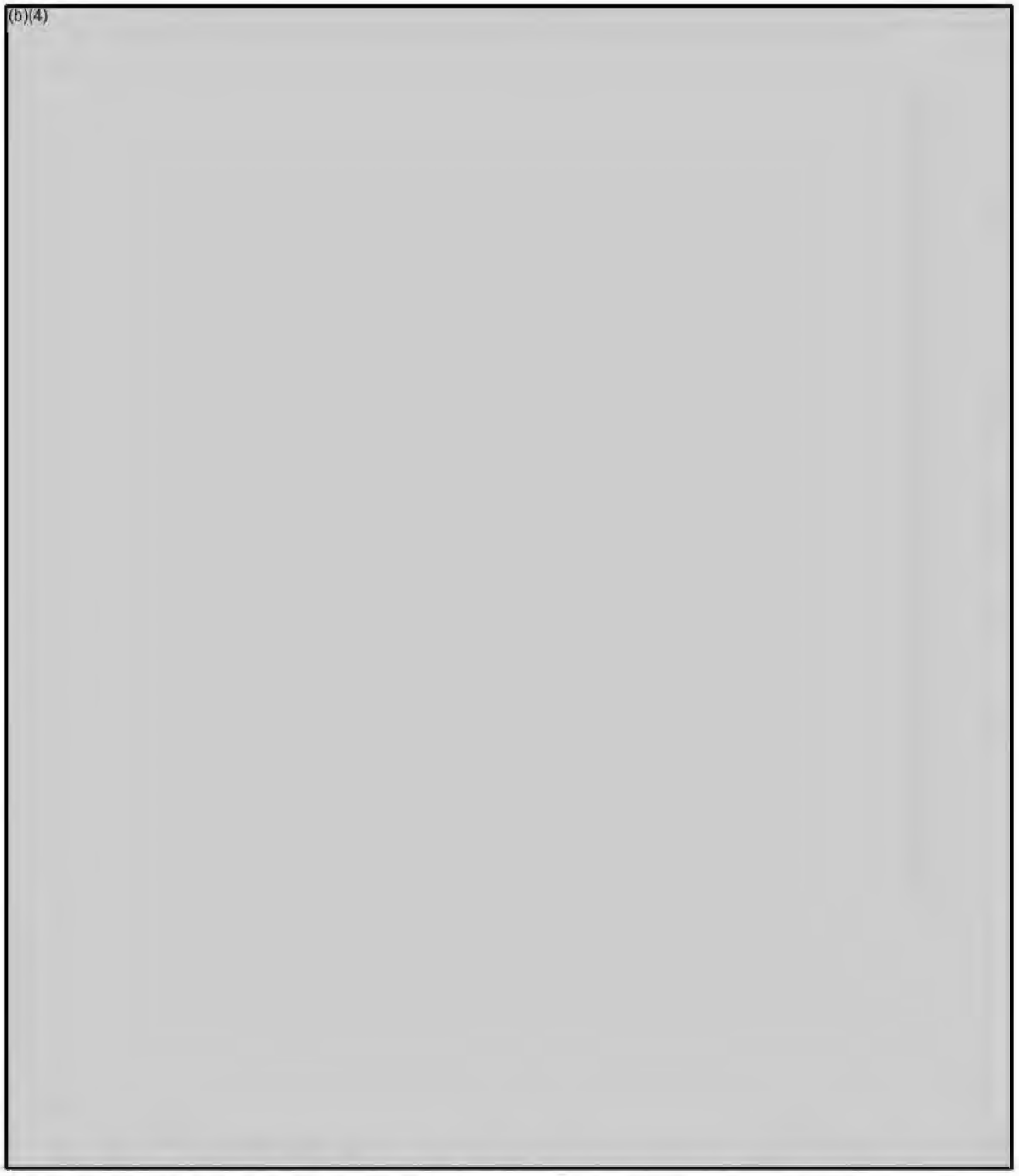

IV. **MILITARY RELEVANCE**: Irrational, angry attack stemming from PTSD presents an ever increasing danger to society and the patient as more and more troops return from Iraq and Afghanistan. The proposed experiments are intended to lay the ground work for clinical trials of pharmacotherapy for anger in PTSD.

V. MATERIALS AND METHODS:

V.1. Experimental Design and General Procedures:

V.1.1. Experiment 1: Testing Hypothesis 1 and 3.2.: (b)(4)

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Species: Rat – Fischer: 30 rats – unstressed group - Pain category C
Timeline:

- Animal arrives in LAM
- Animal remains in LAM at least one week prior to experiment
- Animal is anesthetized per V.4.1.2.1. Anesthesia/Analgesia/Tranquilization
- Animal is mounted in stereotax and electrodes lowered into place per V.4.3.2. Procedure: Experiment 1
- Electrophysiology and pharmacology are collected during a period of no more than 24 hours
- Animal is euthanized per section V.4.6. Euthanasia: and brain is saved for histology to confirm electrode positions.

Species: Rat – Fischer: 24 rats – Stressed group Pain category E – NOTE: These rats are stressed by, "social defeat" (Koolhaas *et al.*, 1997), that is, being attacked, in Experiments 2 and 3. Per V3.5 "Reduction", these rats are then to be taken after completion of Experiment 3 to complete Experiment 1.
Timeline: (Note: The following timeline may take place more than a year after the timeline just above, since Experiments 2 and 3 intervene.

- After Experiment 3 is completed, as described in section V.1.3. Experiment 3, the 24 Fischer rats are entered into Experiment 1, and electrophysiology is recorded from these rats under urethane anesthesia as follows:
- Animal is anesthetized per V.4.1.2.1. Anesthesia/Analgesia/Tranquilization
- Animal is mounted in stereotax and electrodes lowered into place per V.4.3.2. Procedure: Experiment 1

- Electrophysiology and pharmacology are collected during a period of no more than 24 hours
- Animal is euthanized per section V.4.6. Euthanasia: and brain is saved for histology to confirm electrode positions.

V.1.2. Experiment 2: Testing Hypothesis 2:

Behavior: For one (1) to two (2) months twenty four (24) male Long-Evans rats, 3 months of age or older, are to be housed four (4) to a large, 30"x24"x14" cage. These are to be the experimental animals. This group housing is meant to bring out the aggressiveness innate to male rats. During this period the experimenter will, by observation, hierarchically categorize these rats according to aggressiveness. Normal intermale aggression in rats functions to establish hierarchy and does not cause serious injury. Any rat that does not play by these rules will be removed from the experiment and either transferred to animal research protocol (b)(6) another USU rat protocol which does not entail interaction between rats, or euthanized.

Surgery: After the group housing period, Dr. (b)(6) will perform the electrode implantation in the CNRM Surgery (b)(6) as described in "V.4.3.2. Procedure: Experiment 2 and 3: Stereotaxic Surgery". Subsequent to implantation, the rat will be revived and taken care of as described in V.4.3.3. "Post-surgical Provisions. Behavior".

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Electrophysiology: As described in V.4.3.2. Procedure: "Electrophysiology"

Species: Rat:

Pain Category	Number of rats
B	288 pups (24 litters of up to 12 pups each)
C	24 females
E	24 resident males; 24 intruder males already counted for in Exp 1

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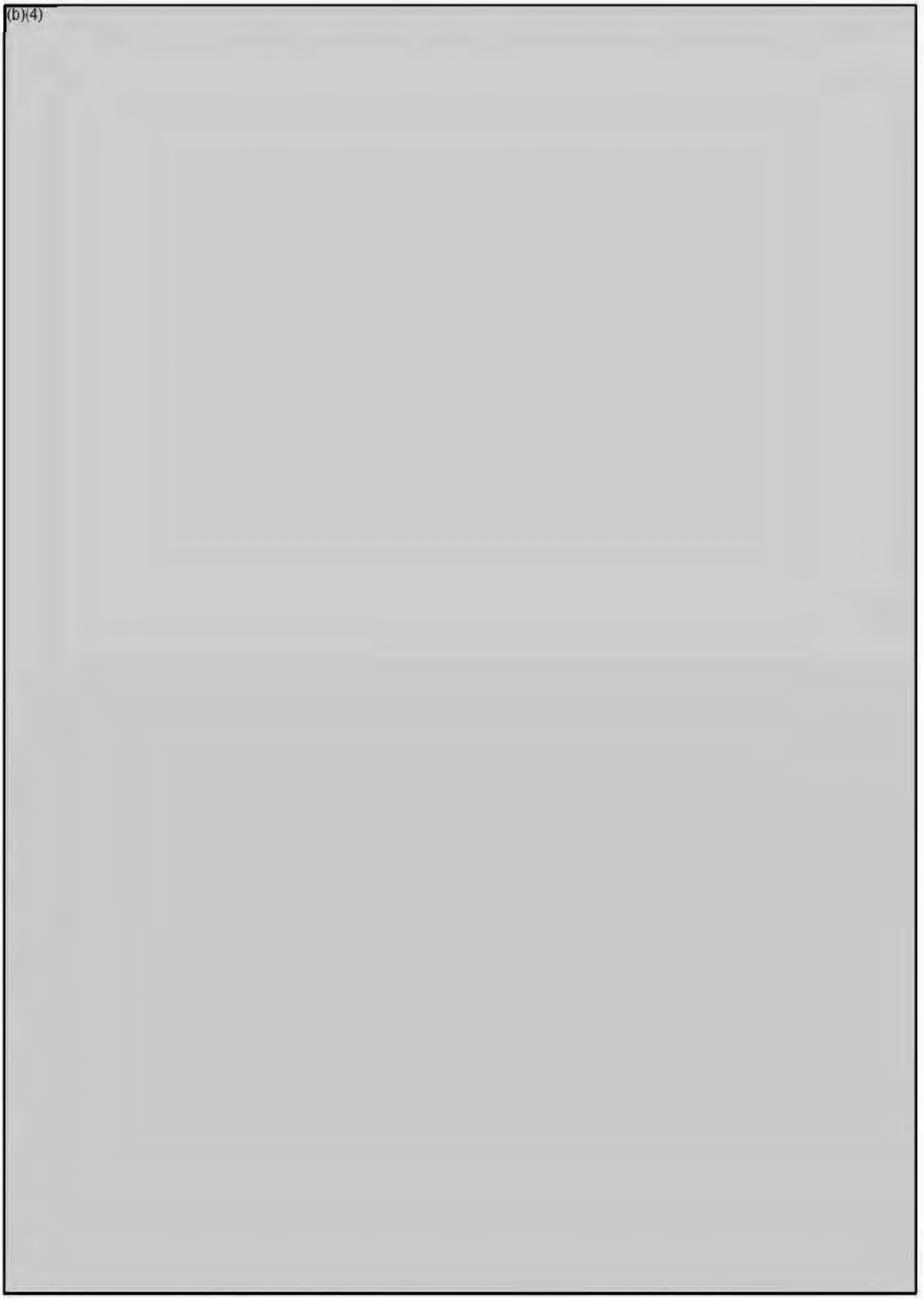

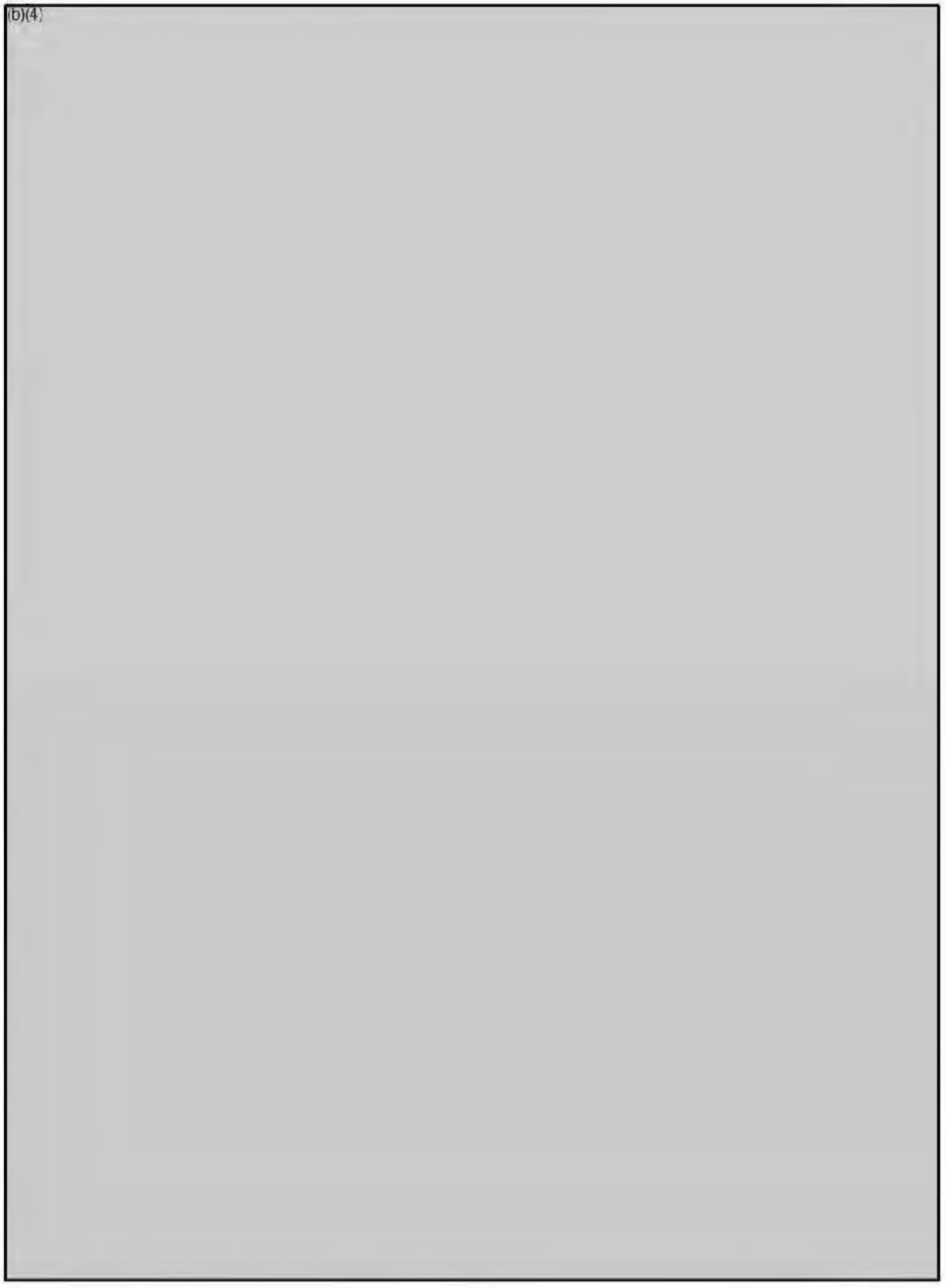
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Species: Rat:

Pain Category	Number of rats
B	288 pups (24 litters of up to 12 pups each)
C	24 females (same animals as in Experiment 2)
E	24 resident males; 24 intruder males, same animals as Experiment 2

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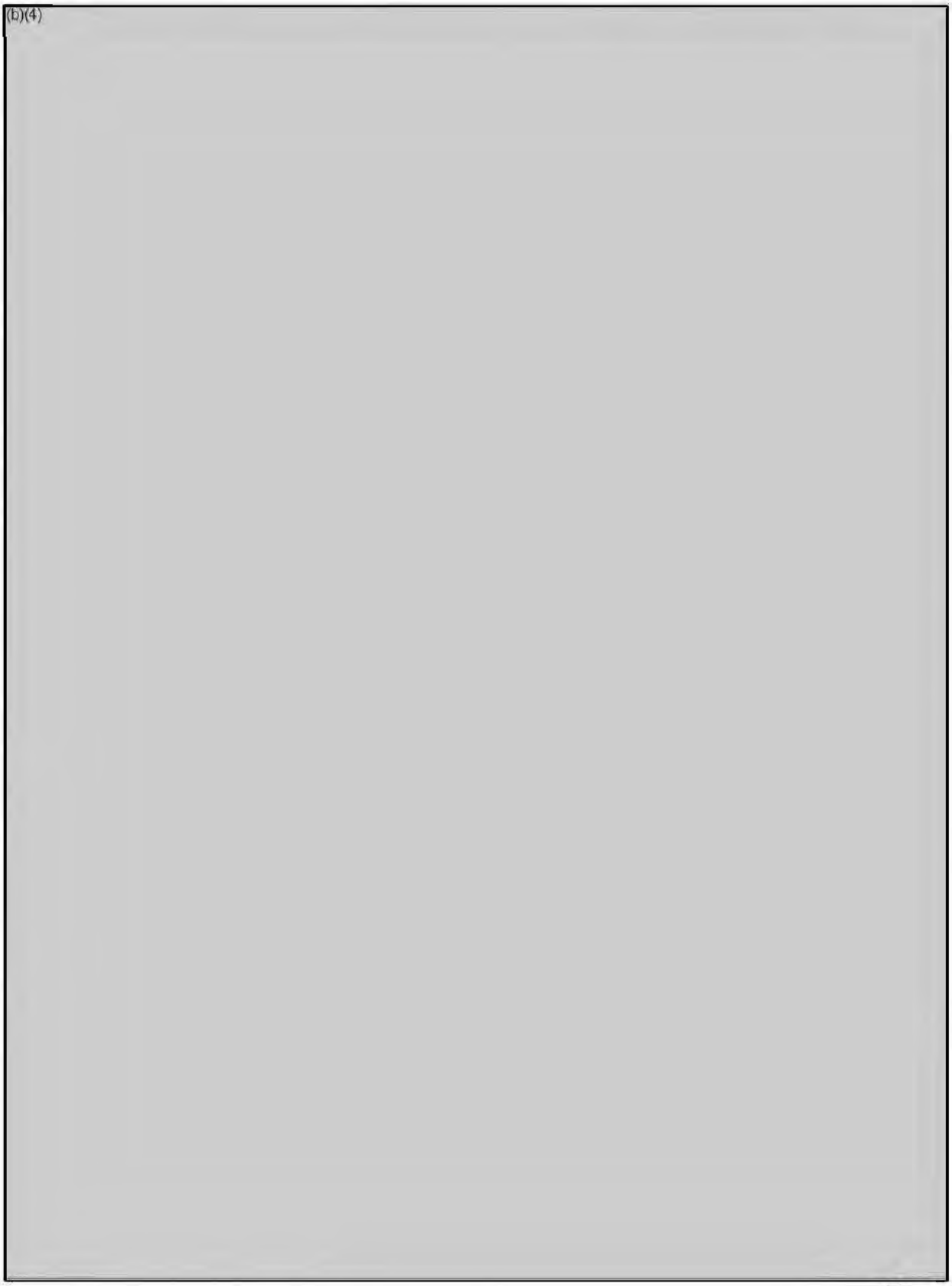

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V.2. Data Analysis: Electrophysiology is collected with the program WINwpc

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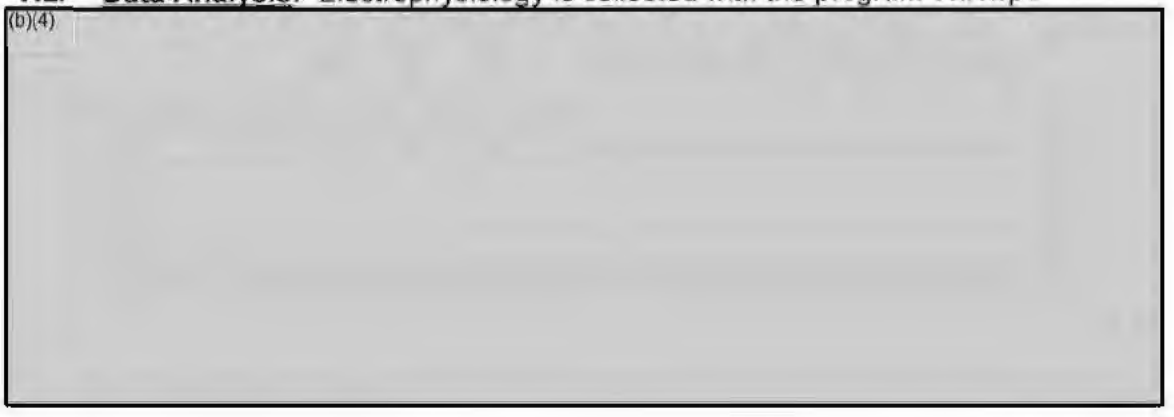

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Power: Number (n) of rats required = 16 (b)(4) experimental) rats in Experiment 2 and 16 (b)(4) (experimental) rats in Experiment 3, for power (π). Since the concern is the rejection of the null hypothesis H_0: $\mu_{experimental} = \mu_{control}$, we take $\pi = 0.8$, where $\pi = \beta/\alpha$, β = risk of false negative = 0.2, α = risk of false positive = 0.05, and a meaningful difference (Δ) = 0.25. Within group standard deviation (σ) for the different parameters is expected to be within 0.5. From experience it all comes out to about sixteen (16) rats for each experiment, Experiment 2 and Experiment 3.

Eight (8) additional experimental rats per group to fill in for any technical failures, particularly since the standard deviation for coordinates in the rat brain is 400μ (Paxinos *et al.*,1985).

V.3. Laboratory Animals Required and Justification:

V.3.1. Non-animal Alternatives Considered: Employing animals in this research is necessary to assess the effects of PTSD on brain anatomy and in vivo behavioral performance. These data will provide a means for relating behavioral responses to CNS structure and function in vivo. Non-animal alternatives do not replicate the three dimensional structure or temporal change that occur as a function of complex behavioral responses to tasks presented to a live animal. We can only do research in an animal model that displays anger and aggression.

V.3.2. Animal Model and Species Justification: The animal model is the rat (*Rattus norvegicus*), in our case the Long-Evans rat. The rat is the lowest animal in the phylogenetic scale which displays aggression relatable to human aggression and which has a basal forebrain in the pattern of the human basal forebrain. The rat, unlike the mouse, has a large enough brain in which to do the necessary multi-electrode evoked field potential electrophysiology.

V.3.3. Laboratory Animals

		Species #1	Species #2
V.3.3.1.	Genus & Species:	Rattus norvegicus	
V.3.3.2.	Strain/Stock:	Long Evans code 006 Fischer CDF code 002	
V.3.3.3.	Source/Vendor:	(b)(4)	
V.3.3.4.	Age:	Newborn - Juvenile – 3 years	
V.3.3.5.	Weight:	Newborn - 200g – 500g	
V.3.3.6.	Sex:	Male and Female	

V.3.3.7. **Special Considerations:** Colony cage < surgery.
Single cage > surgery
Male female pairing.
Wean pups

V.3.4. **Number of Animals Required (by Species):**

702 rats as follows:
Experiment 1: 54 Fischer rats. (30 of these Fischer rats are exclusively for Experiment 1. The remaining 24 Fischer rats, per V.3.5.2 Reduction, are to be used here in Experiment 1 after being used in Experiments 2 and 3.
Experiment 2: 24 Long-Evans male rats and 24 Long-Evans female rats. Plus up to 288 Long-Evans rat pups (24 liters of up to 12 pups each) to be delivered in the course of Experiments 2. (24 Fischer rats are already counted under Experiment 1.)
Experiment 3: 24 Long-Evans male rats. Plus up to 288 Long-Evans rat pups (24 liters of up to 12 pups each) to be delivered in the course of Experiments 3. (24 Fischer rats are already counted under Experiment 1. 24 Long-Evans female rats are already counted under experiment 2.)

V.3.5. **Refinement, Reduction, Replacement (3 Rs):**

V.3.5.1. **Refinement:** Animals are to be handled regularly so as to get use to the experimenter and thus minimize their stress

V.3.5.2. **Reduction:** The same female and intruder rats are used in Experiment 3 as in Experiment 2. The rat pups generated will be transferred to other protocols, when possible, rather than simply euthanized.

V.3.5.3. **Replacement:** N/A

V.4. **Technical Methods:**

V.4.1. **Pain / Distress Assessment:**

V.4.1.1. **APHIS Form 7023 Information:** N/A (APHIS Form 7023 does not list rats or mice.)

V.4.1.1.1. **Number of Animals:** Total: 702

	Species #1 Fischer	Species #2 Long-Evans
V.4.1.1.1.1. Column B:		576 (pups)
V.4.1.1.1.2 Column C:	30 (Exp.1)	24 (females in Exp 2,3)
V.4.1.1.1.3. Column D:		

V.4.1.1.1.4. Column E: 24 (Intruders) Total: 54 48 (Residents) Total: 648

V.4.1.2. Pain Relief / Prevention:

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: Urethane (general, 1.4g/Kg given IP with a 23 gauge needle) and bupivacaine (Marcaine, local given with a 25 gauge needle) for acute recording (Experiment 1). Urethane will only be used as an anesthetic for the non-recovery electrophysiologic recording to be conducted in Experiment #1. Isoflurane (general) and bupivacaine (Marcaine, local) for electrode implants preparatory to behavior (Experiments 2 and 3). The NSAID Carprofen (Zenecarp) 5 mg/Kg SC BID will be given with a 25 gauge needle during the post-operative 24 hours as a routine procedure. Deep barbiturate anesthesia (Fatal Plus preparation of pentobarbital) prior to fixation perfusion for electrode confirmation histology. Induction of all anesthesia is with isoflurane.

Induction of anesthesia with isoflurane: All anesthesias are induced with isoflurane, as follows: The rat is placed in a an induction box connected to an isoflurane vaporizer ("Matrix VIP 3000", Midmark) mounted with oxygen. Isoflurane concentration is gradually raised to 4%. When a lack of righting reflex and a 50% reduction in respiratory rate are observed for at least 10 seconds, the next stage of anesthesia commences.

Isoflurane is suitable as a general anesthetic for chronic implant based experiments (Experiments 2 and 3) because the isoflurane has departed long before the experiment, and leaves no known residual effects. Isoflurane is not suitab e for acute experiments (Experiment 1) because isoflurane drastically depresses all central brain function. Urethane is standard for acute experiments because it preserves some central brain function at the same time as producing surgical anesthesia.

Urethane as a general anesthetic for electrophysiology: As early as 1917, urethane was already "well known" to be a "very uniform anesthetic" in animal experimentation (Stewart & Rogoff, 1917). Current policy statement, from the University of Florida, for instance, describes urethane in small animal experimentation, as follows: "Urethane is a long-lasting anesthetic acceptable for use in non-recovery animal experiments only. Urethane anesthesia is recommended for neurophysiological studies that require stable autonomic physiological status, reduced depression of brainstem neural activity and in animals breathing spontaneously free of a gas anesthesia circuit. Urethane also produces long lasting anesthesia with minimal cardiovascular and respiratory system depression. Studies in rodents have shown that urethane has a rapid onset after IP administration. Advantages of urethane anesthesia are that it produces excellent muscle relaxation and analgesia sufficient for surgical manipulations. ("Urethane Anesthesia Policy", University of Florida adopted 7/24/07.)

For the complete description of anesthesia procedure see V.4.3.2. "Procedure".

V.4.1.2.2. Pre- and Post-procedural (not surgery) Provisions: Animals will be housed in a climate controlled environment with free access to food and water, and will be maintained on a 12 hour reverse light/dark cycle between 20-26°C. Animals will be

acclimated for three days to the USUHS LAM. During acclimation to LAM, animals will be handled briefly each day to acclimate them to being handled; this will reduce any stress that might result from handling of the animals during the procedures described in this study. Following protocols, animals will be monitored according to LAM SOPs.

V.4.1.2.3. Paralytics: None

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

Painful procedure: Recovery subsequent to the electrode implant is understood to expose the animal to some pain. The pain is minimized by local anesthetics given prior to the surgery and by the NSAID Carprofen (Zenecarp) given postop. There is no way to record intracerebral electric field potentials in the absence of surgical implantation of electrodes. The surgery as described meets LAM and IACUC guidelines.

Distressful procedure: The very nature of the experiment is to study stress. Restrain/tail-shock repeated on each of three days is a fundamental procedure in stress, particularly PTSD, research. Alternatives in the literature such as fox odor and one day stress do not yield a prolonged stress response indicative of PTSD.

V.4.1.3.1. Sources Searched: PubMed, entire database; AGRICOLA, entire database; NIH RePorter, entire database; AltWeb, entire database; DoD Biomedical Research Database (BRD) entire database.

PubMed – 1 journal article – not relevant
AGRICOLA - 0
NIH RePorter – 6 articles, 1 relevant, none overlapping
AltWeb – 11 articles, none relevant
BRD - 0

V.4.1.3.2. Date of Search: February 8, 2015

V.4.1.3.3. Period of Search: No limit

V.4.1.3.4. Key Words of Search: (b)(4)

V.4.1.3.5. Results of Search: Eighteen (18) journal articles, 1 relevant, none overlapping.

Literature search to check for possible new alternatives to painful and distressing procedure: The surgery as described meets LAM and IACUC guidelines. Restrain/tail-shock repeated on each of three days is a fundamental procedure in stress, particularly PTSD, research. Alternatives in the literature such as fox odor and one day stress do not yield a prolonged stress response indicative of PTSD.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:
We stress the rats for three consecutive days because it has been previously demonstrated that repeated stress sessions for three days are more effective than a single stress session in producing physiological and behavioral abnormalities, such as elevations in basal plasma corticosterone levels, exaggerated acoustic startle responses and reduced body weight (Servatius *et al.*, 1995). Additional exposures to stress do not appear to result in greater physiological and behavioral changes (Servatius *et al.*, 1995). Other types of stressors appropriate for an animal model (e.g. restraints only) are not easily quantifiable and have resulted in at least partial habituation rather than chronic stress. Given these considerations, we feel that the painful procedure is justified.

V.4.2. Prolonged Restraint: In Experiment 3 prolonged restraint will be conducted per IAW IACUC Policy 004 "Prolonged Restraint". All animals are monitored during the two hours when tail shocks are being administered to assure that they do not get into positions that do not allow them to breath properly.

V.4.3. Surgery:

V.4.3.1. Pre-surgical Provisions: None special. Rats do not vomit.

V.4.3.2. Procedure:

Experiment 1: Aseptic technique using 70% ethanol as described and justified under "Experiment 2 and 3: Stereotaxic surgery" immediately below, is to be employed. In the Center for Neuroscience and Regenerative Medicine (CNRM) Surgery (b)(6) the Fischer rat is placed in an induction box connected to an isoflurane vaporizer ("Matrix VIP 3000", Midmark) mounted with oxygen tank and isoflurane scavenger. Isoflurane concentration is gradually raised to 4%. When a lack of righting reflex and a 50% reduction in respiratory rate are observed for at least 10 seconds, the rat is removed from the box and given the IP injection urethane (1.4g/Kg in water). Urethane is an effective long duration anesthetic for experiments with rats (see Section V.4.1.2.1. "Anesthesia/Analgesia/Tranquilization") The rat is maintained on 100% oxygen for half an hour to allow time for the isoflurane to depart from the blood and fat stores since the animal may become hypoxic if left to breath plain room air. During that half hour the urethane anesthesia takes effect, which is confirmed by the absence of the pedal withdrawal reflex to pinch of the foot web. To block subsequent pain from the ear bars in the stereotax, bupivacaine (0.25%, 0.2 mL) is then injected at the anterior bulla (by following the zygomatic arch to its posterior base) and posterior bulla (palpated by the mastoid process), bilaterally. Bupivacaine (0.25%, 0.4 mL) is further injected along the scalp midline subcutaneous to the incision site prior to the incision. The rat is placed on a heating pad and temperature maintained at 37^{O}C referenced to a rectal thermometer. The rat is then mounted in the stereotax for surgery and electrode implant. Breathing rate is monitored. Increase in rate of breathing or any movement is responded to with incremental IP injections of urethane (0.1g/Kg). (b)(4)

(b)(4)

(b)(4)

Experiment 2 and 3:

Stereotaxic surgery: Stereotaxic surgery, largely following Valenstein *et al.* (1961) will be performed in the CNRM Surgery (b)(6) using aseptic technique, including surgical prep, sterile glove, sterile instruments. All surgical instruments and implantation articles - including electrodes, stainless steel screws, socket, and crimp tube - will be disinfected in 70% ethanol for 20 minutes. "Ethanol is rapidly bactericidal and fungicidal against vegetative organism. While killing spores requires a prolonged contact time, spore forming organisms only rarely cause post procedural infections, at least in humans." (Cooper *et al.* 2000). Thus, "alcohol, because of its convenience, low cost, and lack of antisepsis-associated drawbacks specifically in rats and mice, has an appropriate role in the preparation of surgical instruments for survival surgery in these species." (Huerkamp, 2002). Ethanol kills bacteria by coagulation of protein and is generally used as a 70% solution (Mitchell & Berg, page 161). The dental acrylic used for holding the implantation is sterilized by the methyl methacrylate that cures it. The stereotax will be scrubbed clean and wrapped in gauze saturated with 70% ethanol for twenty minutes prior to surgery. Cottonoid and gelfoam will be from sterile packets. Wound closure will be with wound clips, sterilized.

Long-Evans male rats (300g+) will be anesthetized with isoflurane. The rat is placed in an induction box connected to an isoflurane vaporizer ("Matrix VIP 3000", Midmark) mounted with oxygen. Isoflurane concentration is gradually raised to 4%. When a lack of righting reflex and a 50% reduction in respiratory rate are observed for at least 10 seconds the rat is removed from the isoflurane box, bupivacaine nerve blocks are applied as described in section "V.4.3.2. Procedure: Experiment 1", and the rat is replaced in the isoflurane box. Once the rat is deeply anesthetized, as judged by an absence of the pedal withdrawal reflex to a hard pinch of the foot web, the rat will be placed in a David Kopf stereotaxic apparatus, where isoflurane anesthesia is maintained at 1% through a mask that is integral to the stereotax. Any skeletomotor movement during surgery will be interpreted as response to pain; surgery will be temporarily halted, and the isoflurane concentration will be raised to 2% until response to paw web pinch ceases. A 0.5-1.0 mm hole will be drilled in the skull over each of the brain nuclei to be stimulated or recorded – five (5) holes all together. (b)(4)

(b)(4)

(b)(4) The scalp

wound will be closed around the dental acrylic using stainless steel wound clips as necessary. (b)(4)

(b)(4)

(b)(4) Finally the isoflurane anesthesia is discontinued and the rat is removed from the stereotax and returned to the anesthesia box to be maintained on 100% oxygen for half an hour. Isoflurane departing the blood into the alveoli during that half hour could render the animal hypoxic if left to breath plain room air. The rat is singly housed during recovery for one week post surgery.

(b)(4)

Recovery: See V.4.3.3. Post-surgical Provisions

Electrophysiology: Electrophysiology will be done in the (b)(6) laboratory. The rat will be

(b)(4)

(b)(4)

Histology: Following completion of Experiments 1 and 3, the rat will be anesthetized with pentobarbital (Fatal Plus preparation;100 mg/Kg, IP; Petty C., Research Techniques in the Rat, C. Thomas, Springfield IL, p. 7 provides 3 hours of anesthesia. The pentobarbital sodium will be stored in a locked cabinet bolted to the wall in (b)(6) per DEA regulation.) After deep anesthesia is verified by absence of pedal withdrawal to hard pinch of the foot web, the position of each stainless steel recording electrode is marked with an iron deposit by connecting the positive terminal of an ordinary 9V battery to the electrode and completing a circuit by connecting the negative terminal to the skull screw that serves as the reference electrode. Iron atoms (Fe^{++} and Fe^{+++}), by virtue of their charge, will move away from the electrode and into the immediate vicinity of the tissue. By Ohm's Law, V = RI, where V = voltage = 9V and R = resistance = 9 Kohm, I = current = 1mA. The sternum will then be transected sagittally, the thorax retracted, the right atrium cut to pass out fluid, and the rat will be perfused with formaldehyde containing potassium hexacyanoferrate(II) to turn the iron deposit blue via a syringe connected to an 18 gauge needle puncturing the left ventricle. Then the brain is removed and sectioned to confirm location of the blue deposit marking the position of the electrode tips. (The nichrome stimulating electrodes, which do not contain iron, are located by the impression these leave in the formaldehyde fixed tissue.)

V.4.3.3. Post-surgical Provisions:
Recovery (Experiment 2 and 3): Following surgery, animals will be kept warm and observed until conscious. Bupivacaine nerve block of the skin and fascia should last several hours. Incisions in periosteum and dura are not known to produce protracted pain. The skull and the brain do not have pain innervation. The implanted rat will be housed singly for a week of recovery. Paper chip bedding will be requested to minimize chance of wound infection. The NSAID Caprofen (Zenecarp) 5 mg/Kg SC BID will be given with a 25 gauge needle during the post-operative 24 hours as a routine procedure. There will be a follow-up evaluation of the need for analgesia again the next day, with further dosing provided if the rat does not show normal locomotor activity and appetite. This regimen is particularly effective for incisional and musculoskeletal pain (Mook D. (2005) . Thereafter the rat will be monitored at least twice daily (at least once during their dark - active - period). Infection, though unlikely, will be monitored by inspection of the implant site and be addressed by requesting appropriate antibiotics from LAM. Rats that do not move normally and eat by two days post surgery will be euthanized. Otherwise, one week will be allowed for recovery prior to proceeding with the experimental protocol.

V.4.3.4. Location: (b)(6) (CNRM Surgery)

V.4.3.5. Surgeon: Dr. (b)(6)

V.4.3.6. Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1. **Procedures**: N/A

V.4.3.6.2. **Scientific Justification**: N/A

V.4.4. **Animal Manipulations**:

V.4.4.1. **Injections**: Urethane for anesthesia (Section 4.3.2), bupivacaine for nerve block analgesia (Section 4.3.1), Caprofen post-surgical analgesia (Section V4.3.3)., pentobarbital (Fatal Plus) for histology perfusion (Section 4.3.2 – Histology), prazosin (0.1mg/Kg IP in water), paroxetine (1 mg/Kg IP in water).

V.4.4.2. **Biosamples**: Formaldehyde fixed brain slices for histologic verification of electrode position, as described in Section 4.3.2 – Histology.

V.4.4.3. **Adjuvants**: None

V.4.4.4. **Monoclonal Antibody (MAbs) Production**: N/A

V.4.4.5. **Animal Identification**: Ear punch (B.Y.T. Co, MI), "Sharpie" permanent marker on tail, and cage card.

V.4.4.6. **Behavioral Studies**:

Stress exposure consists of a two-hour per day session of immobilization (restraint) and tail-shocks, for three consecutive days. Each animal is restrained in a Plexiglas tube and an electrode is attached to the tail. Forty electric shocks (2 mA, 3 s duration) are applied at varying intervals (140 to 180 s) over a two hour period; then the rats are returned to their cages. Exposure to this three day stress regimen has been shown to be more effective than a single stress session in producing lasting physiological and behavioral abnormalities, such as elevations in basal plasma corticosterone levels, exaggerated acoustic startle responses and reduced body weight (Servatius et al., 1995).

The Plexiglas restraint tubes used in administration of the stress protocol are designed to accommodate animals of a specified size range. If an animal is near the lower end of the size range, there will be slightly more room for it to move in response to the shock and possibly twist itself into a position that will not allow it to breathe properly. The technician who monitors the stress protocol can release the adjustable end of the restraint tube slightly to allow the animal to return to a position that does not impair breathing, then retighten it. Animals of the size specified in this protocol (~250 gm) do not usually exhibit severe reactions to the stress protocol. If the animals have been handled when they first arrive in USUHS animal facility, they generally do not resist being put into the restraint tubes. If animals were to exhibit symptoms of extreme respiratory distress or damage to the tail resulting from the electric shocks during the stress procedure or if any animals would lose more than 20% of their initial body weight as a result of exposure to the stress protocol, they would be withdrawn from the study and euthanized.

Electrodes will be attached to the tail of the animal by means of a spring clip in order to minimize any painful irritation that might result from repeated taping of the tail. All

animals are monitored during the two hours when tail shocks are being administered to assure that they do not get into positions that do not allow them to breathe properly.

Animals will be monitored daily by Dr (b)(6) and LAM staff after stress sessions. The stress protocol results in a decrease of eating for a few days, during which time the rat may lose body weight, and later regain it. (Jia, Smerin,..,Li, 2014, "Corticosterone mitigates the stress response in an animal model of PTSD", *J. Psychiatric Res.* 60:29). Beyond that small loss in weight, laboratory rats normally plateau in weight at about the fourth month of age, the same time that the experiments begin, and at least maintain that weight for the next two years. Thus the loss of 20% of body weight over any period is grossly abnormal and indicative of extreme dysfunction. Whether that dysfunction is an eating disorder or other metabolic disorder is beyond the scope of this protocol or experiment to deal with. Therefore we propose euthanizing the rat in response to this criterion. Therefore, any animal that would lose >20% of its body weight as measured on Day -1 would be withdrawn from the study and euthanized per Section V.4.6. Euthanasia.

The shock administered to the tails of the animals is 2 mA in intensity for a duration of 3 seconds. The animals typically jump and cry out during the duration of the shock and struggle to get away from it. As soon as the shock concludes, the animals lie quietly until the next shock. Four to six animals are shocked at one time (in individual tubes with a single electrode attached to each animal's tail). The size of the animals can vary by as much as 20 gm, while the restraint tubes are rigid and fixed. An animal that is slightly smaller than the ideal weight for which the tubes are designed can sometimes move into a position where its ability to breathe is compromised; if the animal is not being monitored by a technician who can release the end of the tube and allow it to right itself, then retighten the end of the tube, the animal can expire. Rats of the size listed for this protocol usually do not exhibit this difficulty, but will be monitored during the administration of the stress protocol. The electrodes are attached to the tail with a spring clip to prevent irritation of the tail by removal of adhesive stripes each day for three days and to minimize the possibility of infection through a break in the skin on the tail. We have not observed damage to the tail (i.e. burning of the tail) resulting from contact with the electrode during the shocks. Criteria for early termination of the shock experiments would include (1) signs of infection due to a break in the tail skin; (2)severe irritation or burning of the tail resulting from contact with the electrode during administration of the shocks; (3) loss of >20% of body weight as measured on Day -1 before administration of the stress protocol (Note that rats have been consistently observed to lose weight following exposure to stress, but never >20% of their body weight prior to administration of the stress protocol); and (4)observation of pain or infection in the animals that are housed for 30 days after the final stress session. Any animals that must be removed from the study will be given to LAM personnel for euthanization as described in Section V.4.6.

After each use, the restraint tubes are washed in Alconox detergent solution and allowed to air dry and the electrodes are wiped with 95% ethanol.

(b)(4)

(b)(4),(b)(6)

V.4.4.7. Other Procedures:

V.4.4.8. Tissue Sharing: Once the brain of the formaldehyde perfused animal is removed for histologic verification of electrode tracks, all other tissues are available. This availability will be broadcast at USUHS by word of mouth to LAM personnel and other colleagues.

V.4.5. Study Endpoint:
Experiment 1: Recordings under anesthesia are expected to be completed within the first year. Each individual rat is ordered to arrive at about one month of age and be used during the following two months. Each recording lasts less than 24 hours, after which the rat is euthanized as described in Section V.4.3.2 – Histology.
Experiment 2 : Experiment 2 is expected to begin during the first year and conclude at the end of the second year, at which time the resident rats will be euthanized for histology. The female and intruder rats will go forward into Experiment 3.
Experiment 3: Experiment 3 is expected to begin during year 2 and conclude at the end of year 3, at which time the resident rats will be euthanized for histology of electrode positions, the intruder rats will be utilized in the Experiment 1 paradigm, and the females, now three years old, will be euthanized.
V.4.6. Euthanasia: For histology, the experiment calls for deep pentobarbital (Fatal Plus preparation) anesthesia followed by vascular perfusion (Section 4.3.2. - Histology). Rats that require euthanasia because of illness (V.4.3.3. Post-surgical Provisions; V.4.4.6.Behavioral Studies: Stress exposure) will be deeply anesthetized with isoflurane by the experimenter until the pedal withdrawal reflex to foot web pinch is absent and then decapitated with a rat guillotine. When euthanasia is requested by LAM technicians, they will follow LAM protocol which consists of carbon dioxide asphyxiation followed by a secondary method (i.e. cervical dislocation, bilateral thoracotomy).

V.5. Veterinary Care:

V.5.1. Husbandry Considerations: Except as noted below, routine animal husbandry will be provided in accordance with LAM Husbandry SOPs for each species in this protocol. Upon arrival, animals will be housed two (2) to a cage. Post surgery, rats will be housed one (1) to a cage for one week of recovery. For two (2) weeks to one (1) month, a male and a female are to cohabit in a large cage, possibly with pups, as described in Section V.1.2.Experiment 2: Testing Hypothesis 2: Behavior.

V.5.1.1. Study Room:
Building(s) USUHS (b)(6) Room Number(s) (b)(6)

V.5.1.2. **Special Husbandry Provisions**:

Food Restriction:	Yes	_____	No	X
Fluid Restriction:	Yes		No	X
V.5.1.3. **Exceptions**:		_____		X

V.5.2. **Veterinary Medical Care**:

V.5.2.1. **Routine Veterinary Medical Care**: Animals will be monitored for health, humane treatment, and husbandry considerations, twice daily by LAM personnel during routine weekday rounds and at least once daily on weekends/holidays. This is in addition to, and not in place of, the monitoring that is done by the PI and their staff. In the event of a debilitating illness or adverse reaction, the decision to treat or euthanize an animal will be made by either the veterinarian and/ or the PI.

V.5.2.2. **Emergency Veterinary Medical Care**: All emergency, weekend, and holiday care is provided by two animal husbandry technicians, one or more veterinary technicians, and an on-call veterinarian. Essential husbandry procedures and health rounds are conducted by LAM personnel once daily during weekend and holidays.

V.5.3. **Environmental Enrichment**:

V.5.3.1. **Enrichment Strategy**: Except as indicated below, all animals on this protocol will be provided with routine environmental enrichment in accordance with LAM SOPs and IACUC Policies. Examples include nestlets and tunnels for rodents; balls, toys and food enrichment treats for large animal species.

V.5.3.2. **Enrichment Restrictions**: None

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure	Name of person performing activity	Qualifications of person performing activity (e.g., research technician, 2 yrs experience	Specific training in this activity or procedure
(b)(4)	(b)(6)	Coinvestigator20yrs	In lab; USUHS PI training course, June 2010
		Coinvestigator20yrs	In lab
		Coinvestigator25yrs Coinvestigator 5yrs	Walter Reed In lab
Restraint/Tail Shock		PI – 15 years	Colburn (Manufacturer); USUHS PI training

			course, May 2007

Dr (b)(6) Dr. (b)(6) and Dr (b)(6) will not be involved in the hands-on animal manipulations. Any new research associates or graduate students who rotate through the laboratory will be added to the protocol by request for and approval of a minor modification to this protocol before being permitted to do any animal procedures.

VII. BIOHAZARDS/SAFETY: To minimize exposure to animal allergens when working with animals, lab personnel will wear lab coats and/or scrubs, gloves and masks.

VIII. ENCLOSURES: References

IX. ASSURANCES:

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Training: I verify that I have attended the USUHS Investigator/Animal User Training Cour (b)(6)

Principal Investigator Signature

2-11-15
Date

G. Training: The following personnel will attend the next USUHS Investigator/Animal User Training Course:

_____ _____

_____ _____

H. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" that the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful rese (b)(6)

Principal Investigator Signature

2-11-15
Date

I. **Painful Procedure(s):**

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL** or **WILL NOT** be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

(b)(6) [redacted] 2-11-15

Principal Investigator Signature Date

X. PROTOCOL ABSTRACT:

A. Animal Protocol Number: Pending

B. Animal Protocol Title: Neurophysiology of Escalation of Aggression in a Rat Model of PTSD

C. Principal Investigator: (b)(6)

D. Performing Organization: USUHS

E. Funding: Dept of Defense/USUHS - O/M funds

F. Objective and Approach: Objective is to elucidate the neurophysiology of stress promoted anger and aggression, particularly in PTSD, and lay the groundwork for future pharmacotherapy. The approach is to measure the escalation of aggression in rats subjected to restrain/tail shock and sleep deprivation, the two of which of which correspond to the stress and sleep disorder of PTSD. Potentiation of synaptic field potentials recorded from nuclei in the basal forebrain during escalation of aggression indicate which nuclei are involved. Serotonin and norepinephrine pharmica are tested for efficacy in diminishing the potentiation in the synaptic field potentials and mitigating the escalation of aggression.

G. Indexing Terms (Descriptors): PTSD, aggression, anger, social defeat, bullying, anxiety, panic, basal forebrain, amygdala, BNST, hippocampus, prefrontal cortex, serotonin norepinephrine, corticotrophin releasing factor, CRF, long-term potentiation, LTP, short term potentiation, STP

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