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ADVANCES IN BIOTECHNOLOGY AND THE BIOSCIENCES FOR WARFIGHTER PERFORMANCE AND PROTECTION: ANTI-APTAMERS FOR ENVENOM

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AFRL-HE-BR-TR-2006-0075

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

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14. ABSTRACT This effort was focused on developing a novel, aptamer-based antivenin for treatment of envenomation by the Kurdistan Viper (*Vipera raddei kurdistanica*). The research was conducted to provide evidence to prove whether a synthetic, aptamer-based antivenin could be developed to treat snake envenomations in humans. Using PLA_2 from *Crotalus durissus terrificus* venom as a simulant of the Kurdistan viper venom, two tissue culture cell lines were examined and developed for *in vitro* cell culture models. For each cell line, an LD_{50} value was determined post PLA_2 exposure at various concentrations. Cytotoxicity activity was determined by utilizing an XTT colorimetric assay. DNA aptamers developed against the PLA2 were tested in these *in vitro* models, along with known PLA2 inhibitors. Inhibitors were tested for their effectiveness against these LD_{50} values for each cell line. However, in these assays, known LD_{50} values for PLA_2 did not prove to be toxic to the cells themselves. Higher concentrations of PLA_2, were also ineffective in killing cells the effectiveness of the inhibitor's ability to decrease PLA_2 activity, thereby preventing toxicity to cells, could not be determined. Additionally, no determination was able to be made on the efficacy of the aptamers.

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List of Acronyms

AFSOC	Air Force Special Operations Command
AMC	Air Mobility Command
ATCC	American Type Culture Collection
CMI	Conceptual MindWorks, Inc.
DESA	7,7-Dimethyl-(5Z,8Z)-eicosaienoic acid
DHS	DL-*erythro*-Dihydrosphingosine
DMEM	Dulbecco's Modified Eagle's Medium
DTPA	Diethylenetriaminepentaacetic acid
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal Bovine Serum
PLA_2	Phospholipase A_2
SOCOM	Special Operations Command

PREFACE

The scope of this effort was to address the development of a novel, aptamer-based anti-venom for treatment of envenomation by the Kurdistan Viper (Vipera raddei kurdistanica) and to provide evidence for whether or not a synthetic, aptamer-based antivenin can be developed which could be used to treat snake envenomations in humans. The development of this antivenin would be groundbreaking in the neutralization of toxins and would provide an unfilled ability to treat warfighters envenomated by this organism.

No classified information was used to generate, or is included in, this report. In accordance with AFRL program guidance, this report was submitted to the project sponsor for further disposition. Any restrictions upon the dissemination of this report, or any classification of the information contained herein, are at the discretion of the United States Air Force, Counterproliferation Branch, Brooks City-Base, Texas.

EXECUTIVE SUMMARY

This effort was focused on developing a novel, aptamer-based antivenin for treatment of envenomation by the Kurdistan Viper (*Vipera raddei kurdistanica*). The research was conducted to provide evidence to prove whether a synthetic, aptamer-based antivenin could be developed to treat snake envenomations in humans. The development of this antivenin would be groundbreaking in the neutralization of toxins and therefore provide an unfilled ability to treat warfighters envenomated by this organism.

During this 1 year effort, the Conceptual MindWorks (CMI) team was able to address two of the specific aims of the project. Using PLA_2 from *Crotalus durissus terrificus* venom as a simulant of the Kurdistan viper venom (no source available at this time), two tissue culture cell lines were examined and developed for *in vitro* cell culture models. The two cell lines used for this study were C2C12 (CRL-1772), a mouse myoblast cell line, and MDCK (CCL-34), a canine kidney cell line. For each cell line, an LD_{50} value was determined post PLA_2 exposure at various concentrations. Cytotoxicity activity was determined by utilizing an XTT colorimetric assay.

DNA aptamers developed against the PLA2 were tested in these *in vitro* models, along with known PLA2 inhibitors. Inhibitors were tested for their effectiveness against these LD_{50} values for each cell line. However, in these assays, known LD_{50} values for PLA_2 did not prove to be toxic to the cells themselves. Higher concentrations of PLA_2, 10µg/mL for C2C12 cells and 50µg/mL for MDCK cells, were also ineffective in killing cells the effectiveness of the inhibitor's ability to decrease PLA_2 activity, thereby preventing toxicity to cells, could not be determined. Additionally, no determination was able to be made on the efficacy of the aptamers.

This research was designed to extend the research, planning and integration efforts underway by the Air Force Research Laboratory (AFRL) to produce techniques for the neutralization of biowarfare agents.

Chapter 1. Program Description

1.1 Technical Approach

A major component found in the venom of the Kurdistan Viper, shown in Figure 1, known to cause altered pathophysiological effects associated with post-envenomation, such as hemorrhage and necrosis, is Phospholipase A_2 (PLA_2).[1,2]

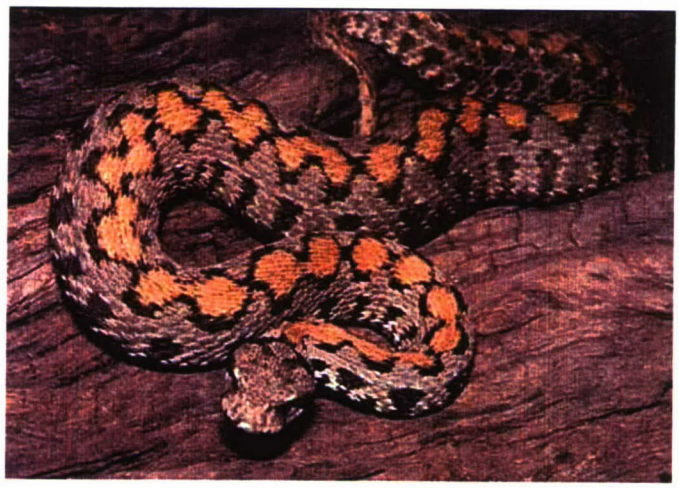


Figure 1. The Kurdistan Viper (*Vipera raddei kurdistanica*)

CMI's research team will develop highly specific and selective aptamers against PLA_2, found in the venom from the viper, using a SELEX like process. The team will determine which mouse and/or human cell lines are susceptible. Possible cell lines are from mouse muscle tissue as the final anti-PLA_2 testing will be in a mouse model. Using an *in vitro* culture model, the team will select aptamers with the greatest ability to neutralize PLA_2. Cytotoxicity will be measured using two methods: 1) enzymatic assays and 2) neutral red viability test. This will allow quantification of cell viability. Variables will be dose of PLA_2 and concentration of aptamer cocktail. During the third year of this research, the aptamer production and cell culture work will be extended to the mouse model. BALB/c mice will be used for *in vivo* assays; assuming that the *in vitro* assays demonstrate that an anti-PLA_2 toxins aptamer cocktail spares cells from the applied venom. Protection assays will involve post-treatment of mice with aptamer cocktail twice-daily by subcutaneous injections.[3]

1.2 Customer

The primary customer is the Air Force Special Operations Command (AFSOC) and US Special Operations Command (USSOCOM). In general, the Departments of Defense, Homeland Security, and Environmental Protection Agency will also benefit from this capability.

1.3 Goals and Objectives of the Research and Development

Specific aims of the project are as follows:

1. Development of DNA based aptamers against PLA_2 found in the venom from Kurdistan Viper.
2. Selection of DNA aptamers that provide the greatest protection from the pathogenic effects of PLA_2 *in vitro* cell culture model.
3. Development of a mouse model for poisonous snake envenomation.
4. Testing of aptamer cocktail in a mouse model to determine if DNA based aptamers given post exposure to PLA_2 spares the mice.

1.4 Customer Benefits/Payoffs

Testing the efficacy of aptamers selected against PLA_2 will be carried out using appropriate cell lines and a range of cytotoxicity, cell proliferation and function tests. Additional benefits of this research will be knowledge gained for similar studies on excreted toxins from bacterial select agents. A further benefit of considering the use of derivative dendrite polymers or aptamers in any studies is the possibility that these could be designed to bind to a wide range of bacteria and viruses and also block their activity.[4]

Chapter 2. Technical Discussion

2.1 Background

Venomous snakes represent a potentially serious threat to US and coalition forces deployed to Iraq. The risk of venomous snakebites will be higher for ground forces during field operations. Although total numbers of bite causalities will likely be very small, victims are at risk for sustaining serious injury, disability and possible death. Successfully treating these casualties requires knowledge of emergency first aid and management of the bite site. The most effective treatment for significant snake envenomation (the act of injecting venom by a bite) is geographic and species specific antivenin administered by medical personnel. The most important decision will likely be determining if antivenin should be administered; not all snakebites result in significant envenomation requiring antivenin. The degree of envenomation is judged according to clinical criteria such as the presence of widely distributed pain, edema progressing toward the trunk, petechiae (pinpoint reddish rash) or ecchymosis (hemorrhagic spots), and systemic symptoms including fever, nausea or vomiting. Clinicians should be aware that most antivenins are produced from horses and approximately 15

to 20 percent of patients receiving equine-based antivenins will exhibit adverse side effects[5].

Most antivenin is produced by injecting snake venom in increasingly higher doses into horses, thereby inducing the animal's immune system to produce antibodies to the venom. The horse's blood is collected and processed to manufacture antivenin, a process that can take 6 to 8 weeks. Antivenin is marketed freeze-dried in glass vials or as a liquid in ampoules. Most antivenin had a shelf-life of approximately 3 years.[6]

The quality of antivenin and efficiency of production are directly related to the age and health of both the biting snakes and horses used in manufacturing the antivenin as well as quality control practices in the facility that produces the antivenin. The World Health Organization has published guidelines for properly producing antivenin and recommends using horses between ages 5 and 10 to produce antivenin. Approximately 15 to 20 percent of recipients of horse-derived antivenin may demonstrate early adverse pyrogenic, anaphylactic or anaphylactoid reactions to current snake antivenins. Medical personnel administering antivenin should be trained and equipped to treat adverse antivenin reactions.[7]

Military personnel could sustain a wound from a snake for which no specific antivenin is available. In these instances, one option is using polyvalent antivenin that might contain genus-specific antivenin. Six of Iraq's venomous snakes are classified as true vipers. These snakes produce hemotoxic venom that causes severe damage to blood cells and tissue of bite victim,[8] as shown in Figure 2.

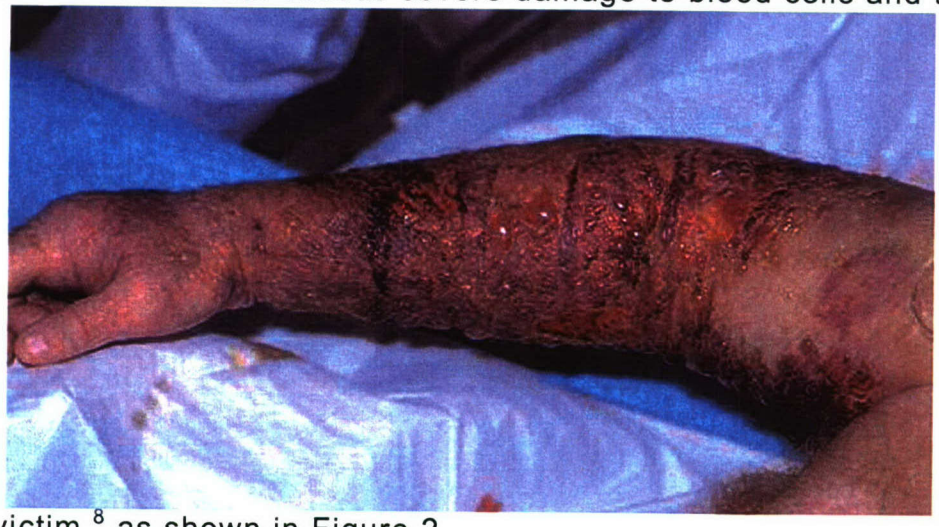


Figure 2. Extensive damage to forearm after envenomation.

In recent years several techniques have been devised for the synthesis and functional screening of large numbers of organic molecules. Libraries of peptides, antibodies or partially randomized proteins displayed on the surfaces of cells and variants with desirable binding or catalytic abilities can be iteratively selected and amplified.[9,10] Aptamers are artificial nucleic acid

ligands that can be generated against amino acids, drugs, proteins and other molecules. They are isolated from complex libraries of synthetic nucleic acids by an iterative process of adsorption, recovery and reamplification, a process know as SELEX (Systematic Evolution of Ligands by Exponential Enrichment).[11,12] Today, the SELEX process has been applied to more than a hundred different target molecules. A wide variety of molecules have been targeted by *in vitro* selection experiments that have yielded specific nucleic acid-binding sequences. Perhaps the smallest target being chelated zinc molecule (atom).[13] Literally hundreds of proteins have been targeted by SELEX selection and yielded anti-protein aptamers.[14,15] These proteins include toxins,[16,17] glycoproteins,[18] HIV 1 Rev,[19] immunoglobulins, signal molecules,[20] growth factors,[21] and toxins. Aptamers have been produced against very diverse molecules ranging from organic dyes to nucleotides, to amino acids, peptides and complex proteins.[22]

Furthermore, at Brooks City-Base, our own laboratory selected aptamers against live anthrax spores,[17,23] shiga toxin, whole virus VEE and whole bacterial cells showing that aptamers can be obtained for almost any desired target whether simple or complex.

2.2 Hypotheses

Antivenin therapy is the mainstay of medical treatment of snakebites, along with administration of plasma expanders, pain medication, diazepam, tetanus toxoid, antiseptics and antibiotics. Patients who have pain, swelling, ecchymosis, systemic symptoms or abnormal laboratory findings within 30 minutes to one hour of a bite are probable candidates to receive antivenin therapy. Before receiving antivenin therapy, the patient must be tested for hypersensitivity to the antivenin. Antivenin therapy is the most effective when given within four hours of a snakebite.[24]

Hemotoxic venom attacks the circulatory system and muscle tissue causing excessive scarring, gangrene, permanent disuse of motor skills and sometimes leads to amputation of the affected area.

An anti-PLA_2 aptamer could fulfill almost all the criteria that describe an ideal antivenin: economically affordable; possess a long shelf-life under various storage conditions; exact affinity for the specific venom selected against; zero allergenicity; synthetically manufactured.

The high affinity and specificity of nucleic acid aptamers, their lack of immunogenicity and the ability to raise aptamers against any target for which an *in vitro* selection method can be devised, makes them tempting candidates for drug discovery. In the area of potential therapeutic aptamer antagonists of the toxin ricin have been isolated with IC50 values in the nanomolar range.[16] Furthermore, a PEG-conjugated aptamer to vascular endothelial growth factor that inhibits pathogenic angiogenesis has already

reached clinical trials and is intended for the treatment of blindness induced by macular degeneration. It was also found to be safe and relatively long lasting following injection and has a half-life in plasma of little over 9 hours[25, 26]

With this background we will test the following hypotheses:

1. Anti-Kurdistan Viper PLA_2 (aKVPLA$_2$) aptamer cocktails would provide cyto-protection to Mouse C2C12 muscle cells and MDCK canine kidney cells *in vitro* against envenomation from Kurdistan viper venom toxicity.

2. An aptamer cocktail could act as a synthetic antivenin, protecting mice from snake envenomation toxicity.

Chapter 3 Materials and Methods

3.1 Materials

3.1.1 *Phospholipase A_2 and Inhibitors*

PLA_2 from *Crotalus durissus terrificus* venom (P5910) and its known inhibitors: Diethylenetriaminepentaacetic acid (DTPA D6518), DL-*erythro*-Dihydrosphingosine (DHS D6908) and 7, 7-Dimethyl-(5Z, 8Z)-eicosaienoic acid (DESA D8008) were purchased from Sigma-Aldrich. PLA_2, DHS and DESA were reconstituted in dH_2O to make a 5mg/mL, 83mM and 30mM concentration, respectively. The stock concentrations were stored in 50µL aliquots at -20°C. DTPA was stored at room temperature and reconstituted to make appropriate stock concentrations for each individual experiment.

3.1.2 *Cell Lines*

The two cell lines used for this study are shown in Figures 3 and 4. C2C12 (CRL-1772), a mouse myoblast cell line, and MDCK (CCL-34), a canine kidney cell line, were both purchased from American Type Culture Collection (ATCC). Cell lines C2C12 and MDCK, were grown in Dulbecco's Modified Eagle's Medium (DMEM, ATCC 30-2002) and Eagle's Minimum Essential Medium (EMEM, ATCC 30-2003), respectively. Both media were supplemented with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals S11150) and 5% Penicillin-Streptomycin-Glutamine 100X (Pen/Strep/Glu, Invitrogen 10378-016). In all protocols, respective media for each cell line was used and cells were incubated at 37°C with an air atmosphere of 5% CO_2. Cells were grown in 150cm^2 tissue culture flasks (Corning 430825). At near confluency, cells were either subcultured or harvested.

ATCC Number: **CRL-1772**
Designation: **C2C12**

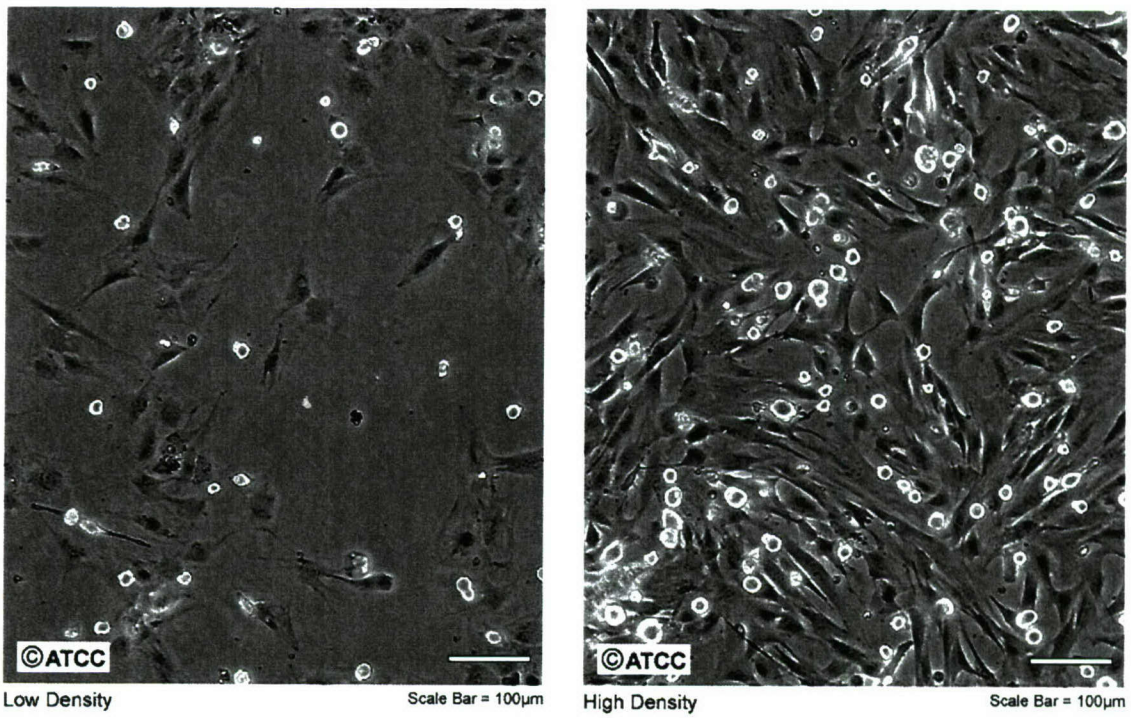
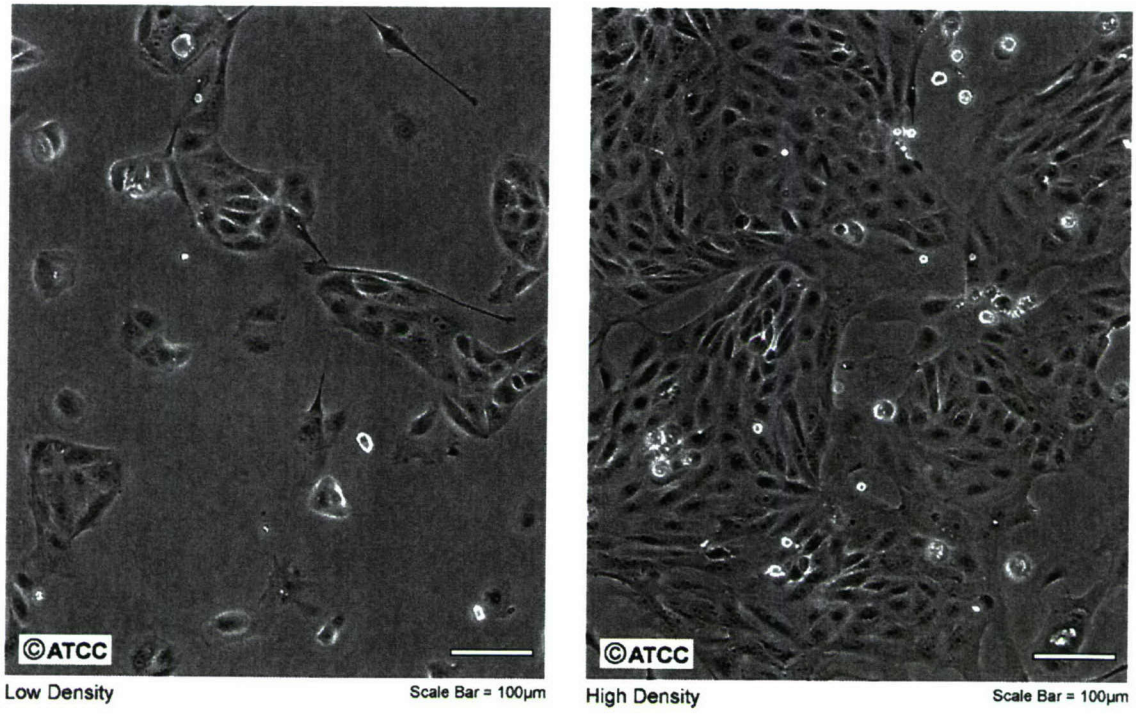


Figure 3. Phase contrast micrograph of C2C12 mouse myoblast cultured cells (ATCC: CRL-1772) at low density and high density confluency.

ATCC Number: **CCL-34**
Designation: **MDCK (NBL 2)**



Low Density Scale Bar = 100µm High Density Scale Bar = 100µm

Figure 4. Phase contrast micrograph of MDCK cultured cells (ATCC: CCL-34) at low density and high density confluency.

3.2 Methods

3.2.1 *Cell Subculturing*

To subculture C2C12 cells, media was replaced with 6mL of Trypsin-EDTA Solution 1X (ATCC 30-2101) to resuspend cells. Cells were then incubated for 5 min. Due to the tendency of multi-layer growth in MDCK cells, 10mL of Trypsin-EDTA was used to resuspend cells for 10-15 min. Once cells were observed to have been resuspended under an inverted microscope (Nikon, TMS-F), media was added to inhibit the trypsin and give a 1:4 to 1:10 subcultivation ratio for C2C12 cells and a 1:2 to 1:6 subcultivation ratio for MDCK cells.

3.2.2 *Cell Harvesting*

This protocol parallels that of *cell subculturing* with the following modifications: Once cells were observed to have been resuspended under an inverted microscope, an equivalent amount of media was added to inhibit the trypsin activity. After rinsing the cells off the flask with the added media, the mixture from all respective flasks was collected in a 50mL conical tube

(Fisher 14-959-49A). The tube was centrifuged at 15°C, 3000g for 5 min. The supernatant was properly discarded and 1mL of media was added to the cells to determine cell concentration.

3.2.3 *Determining Cell Concentration*

To determine cell concentration of a cell line, 900µL of media and 100µL of the 1mL cell/media mixture, previously mentioned, was added to a 15mL conical tube (Fisher 14-959-70C). 10µL was then pipetted into a hemocytometer (Fisher 0267110). Each grid of the hemocytometer represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to approximately 1 mL, the total number of cells per mL was determined using the following calculations: Cells/mL = average cell count per grid x dilution factor x 10^4. This value was used to determine starting concentrations for confluency testing and cytotoxicity assays.

3.2.4 *Confluency Testing*

Confluency tests were conducted for both cell lines on separate 24 well Falcon plates (Fisher 353047). Using the value of cell concentration, previously mentioned above, starting concentrations of 5.0^3, 1.0^4, 2.0^4, and 5.0^4 cells/mL were placed into six designated wells of each plate as shown in Figure 5 and incubated at 37°C with an air atmosphere of 5% CO_2.

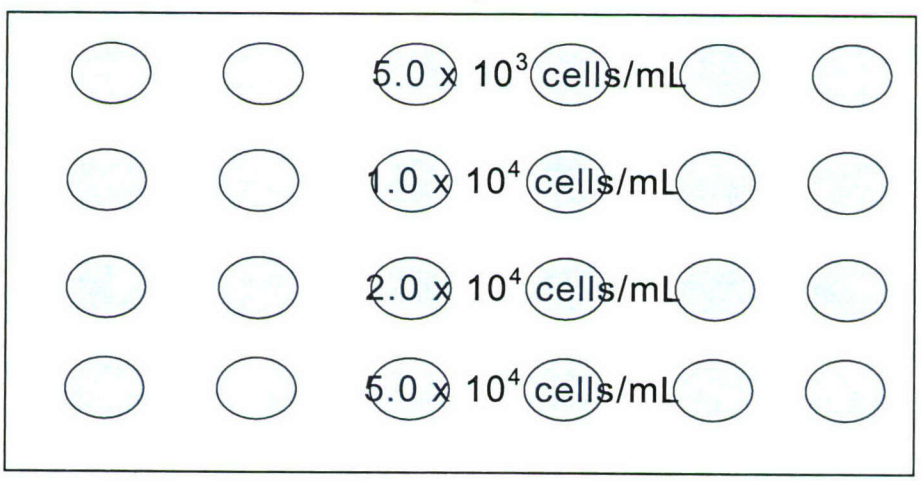


Figure 5. Experimental design for confluency tests of C2C12 and MDCK cell lines.

After incubation periods of 24- and 48-hours, each well was observed for confluency percentage on an inverted microscope. In the case of MDCK cells, an additional incubation period of 72-hours was observed. The average percentage of respective concentrations for each time point was used in determining starting concentrations for the cytotoxicity assays which followed.

3.2.5 *Cytotoxicity Assay*

Cytotoxicity activity was determined by utilizing an XTT Cell Proliferation Kit II (Roche 11 465 015 001). In brief, this assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells.[27] Therefore, this conversion only occurs in viable cells.

When cells were at near confluency, 200µL of varying PLA_2 concentrations, ranging from 3 to 60µL/mL, were pipetted into designated wells as shown in Figure 6. Each concentration was calculated from the 5mg/mL stock and diluted with media. This assay required incubation periods of 30 min, 2, 4, and 8 hours at 37°C with an air atmosphere of 5% CO_2. One 24-well plate was used for each incubation period for each cell line.

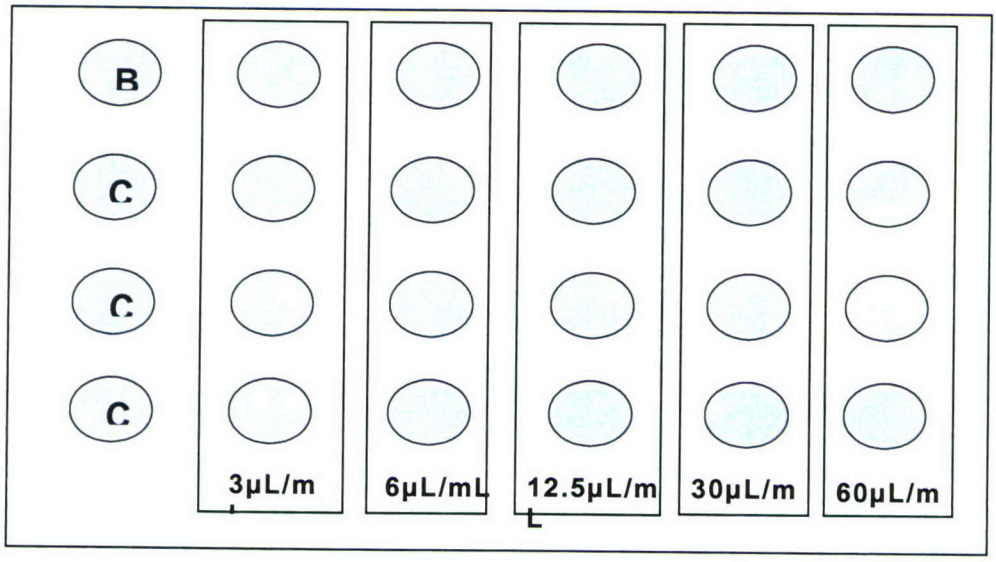


Figure 6. Experimental design of XTT assays with varying concentrations of PLA_2. (B) blank well containing only media, (C) control wells containing cells and media.

Varying concentrations of each PLA_2 inhibitor were also exposed to the cell lines. Each concentration was calculated from the 5mg/mL stock and diluted with media. Due to results demonstrating the LD_{50} for PLA_2 when exposed to cells, this assay required incubation periods of 2 and 4 hours at 37°C with an air atmosphere of 5% CO_2. One 24-well plate was used for each incubation period for each cell line. Figure 7 shows the experimental design. When combining PLA_2 and inhibitor, 100µL of each concentration for

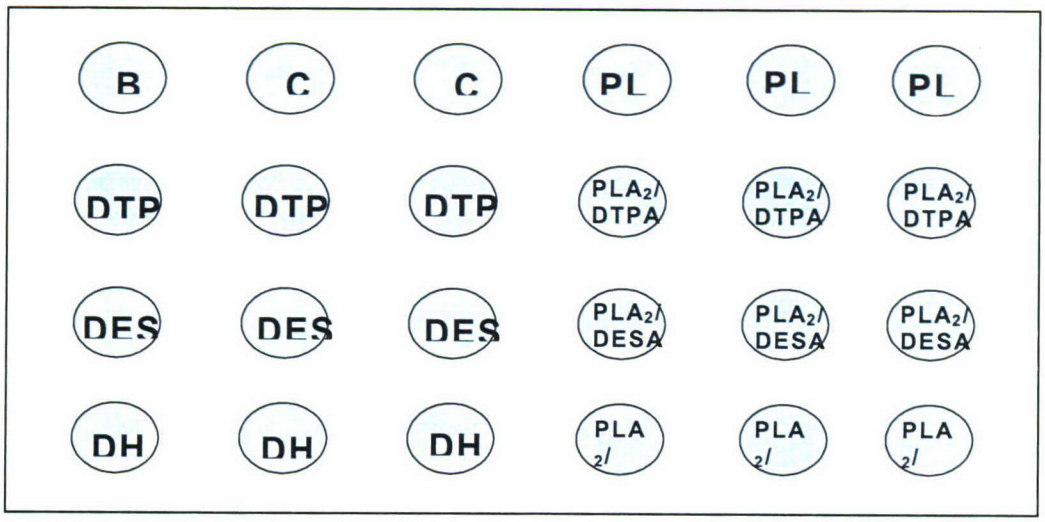


Figure 7. Experimental design of XTT assays with varying inhibitors of PLA_2. blank (B) well containing only media, control (C) wells containing cells and media, PLA_2 (PLA_2), Diethylenetriaminepentaacetic acid (DTPA), 7, 7-Dimethyl-(5Z, 8Z)-eicosaienoic acid (DESA) and DL-*erythro*-Dihydrosphingosine (DHS).

After incubation periods, 100µL cocktail mix of 2.8mL labeling reagent and 56µL electron-coupling reagent was pipetted into each well. The plate was then incubated for 30 minutes prior to being read by a microplate reader set at 490nm (BioTek, Synergy™ HT).

Chapter 4 Results

4.1 Confluency Testing

Table 1 shows the confluency rate of both C2C12 and MDCK cell lines. As shown, MDCK showed to have a lower proliferation rate than that of C2C12 cells. These results helped determine a set time frame for which the researcher could conduct XTT assays.

MDCK CONFLUENCY			Starting	C2C12 CONFLUENCY	
24 Hr	48 Hr	72 Hr	Concentration	24 Hr	48 Hr
10%	15%	20%	5.0 X 10^3	30%	45%
20%	25%	30%	1.0 X 10^4	50%	70%
40%	70%	75%	2.0 X 10^4	70%	90%
50%	80%	90%	5.0 X 10^4	90%	100%

Table 1. Results of confluency percentages from various starting concentrations after 24, 48 and 72 hour culturing periods.

4.2 LD_{50} Values

For each cell line, an LD_{50} value was determined post PLA_2 exposure at various concentrations. With regard to C2C12 cells, as seen in Figure 8, the LD_{50} value is approximately 5µg/mL for 2 hour exposure periods and approximately 4µg/mL for 4 hour exposure periods. Interestingly, an increase in cell proliferation between 30 minute and 2 hours exposure periods occurred regularly in C2C12 XTT assays.

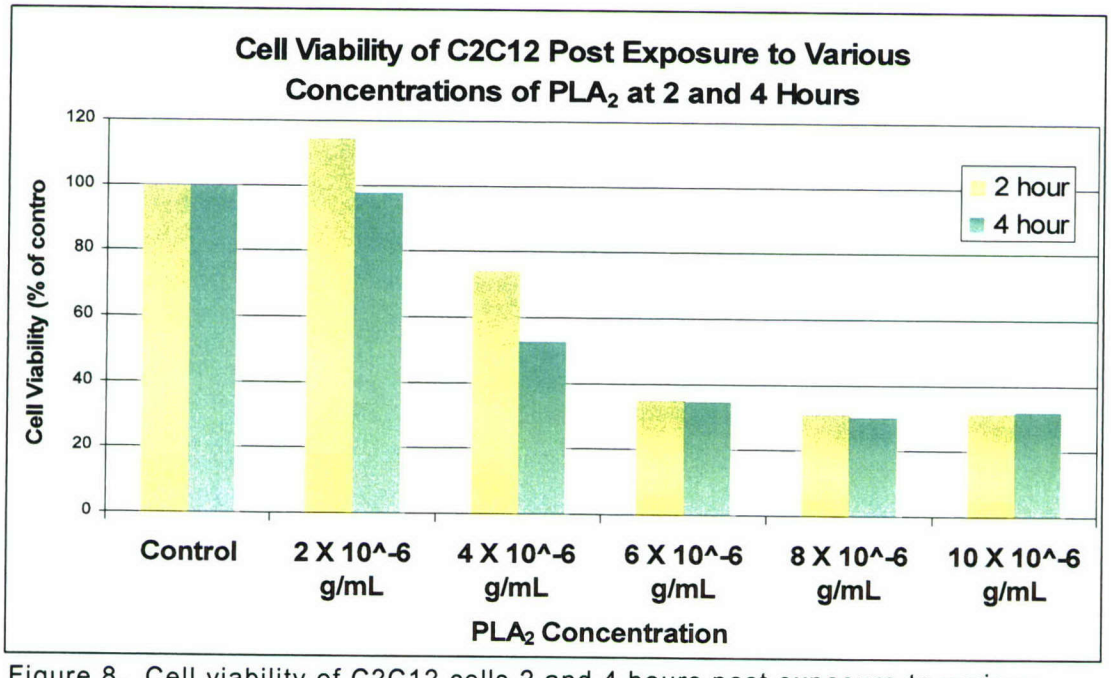


Figure 8. Cell viability of C2C12 cells 2 and 4 hours post exposure to various concentrations of PLA_2.

In the case of MDCK cells, as seen in Figure 9, the LD_{50} is approximately 30µg/mL for 2 hour exposure periods and approximately 13µg/mL for 4 hour exposure periods.

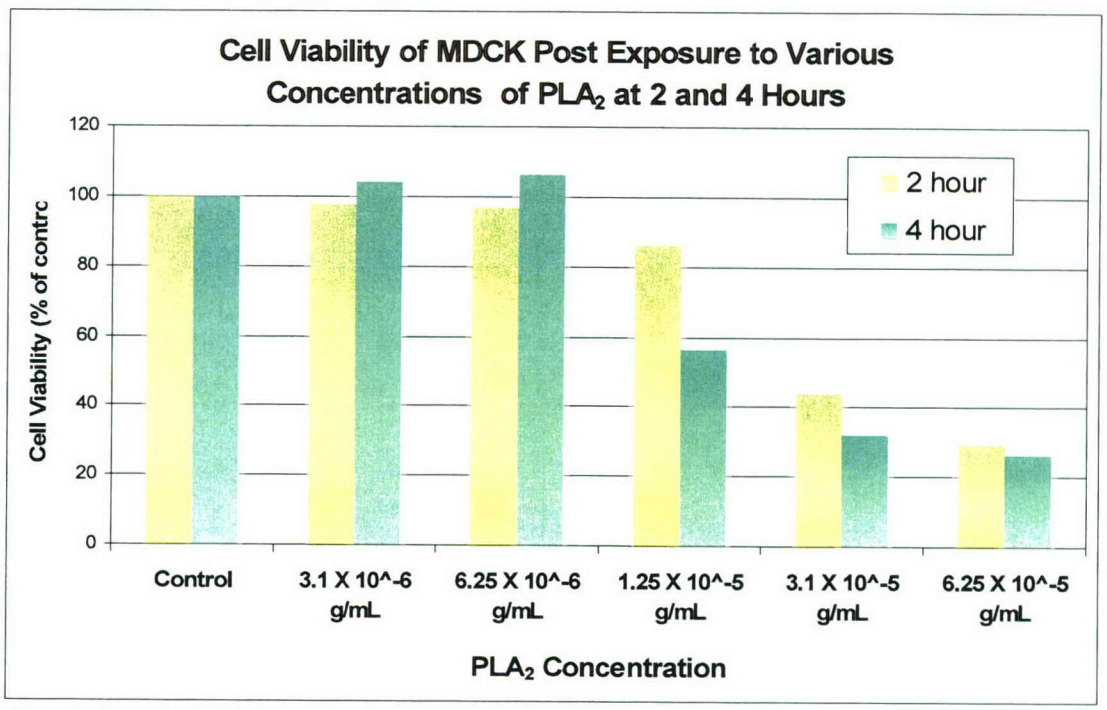


Figure 9. Cell viability of MDCK cells 2 and 4 hours post exposure to various concentrations of PLA_2.

4.3 Inhibitor Evaluation

Inhibitors were tested for their effectiveness against these LD_{50} values for each cell line. However, in these assays, known LD_{50} values for PLA_2 did not prove to be toxic to the cells themselves. Higher concentrations of PLA_2, 10µg/mL for C2C12 cells and 50µg/mL for MDCK cells, were also ineffective in killing cells as shown in Figures 10 and 11.

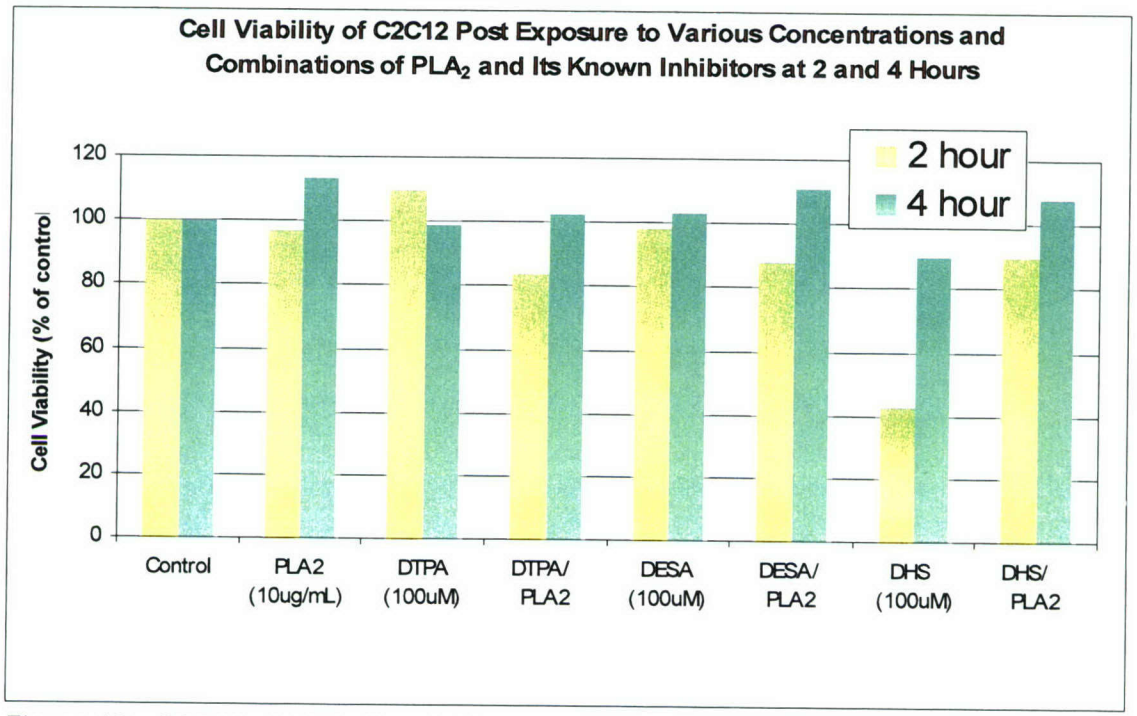


Figure 10. C2C12 viability 2 and 4 hours post exposure to PLA_2 and its known inhibitors.

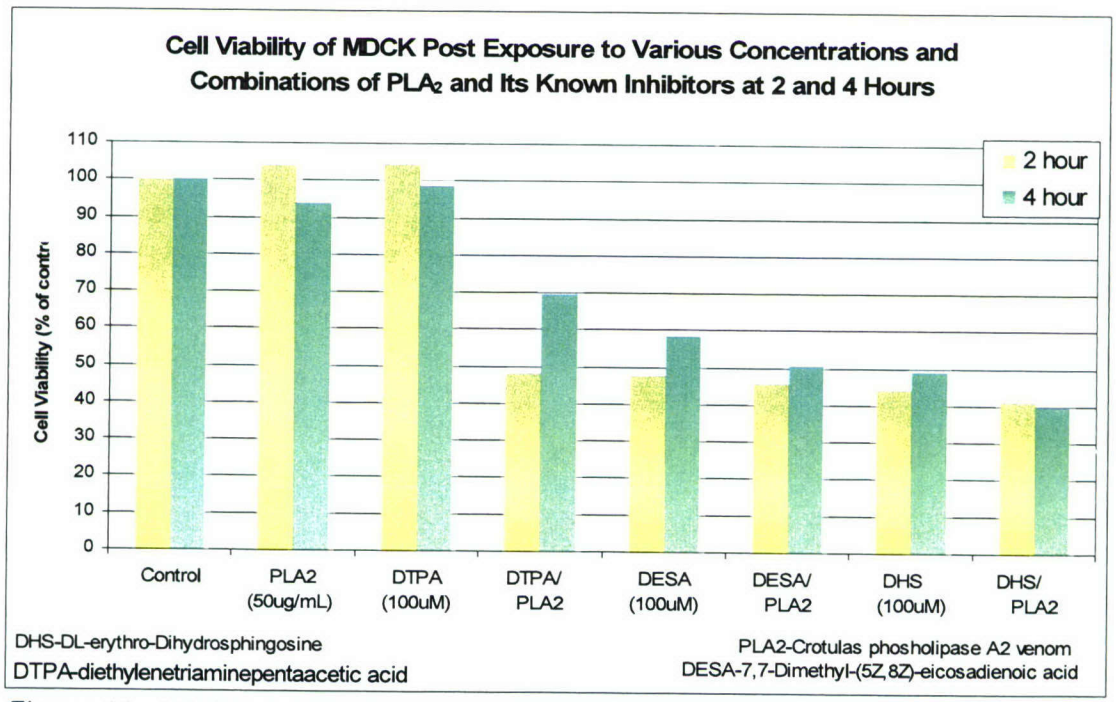


Figure 11. MDCK viability 2 and 4 hours post exposure to PLA_2 and its known inhibitors.

Based on the results presented, the effectiveness of the inhibitor's ability to decrease PLA_2 activity, thereby preventing toxicity to cells, could not be determined.

Chapter 5 MSDS

5.1 PHOSPHOLIPASE A_2 FROM CROTALUS DURISSUS TERRIFICUS

SIGMA-ALDRICH MATERIAL SAFETY DATA SHEET

Substance Name CAS # SARA 313
PHOSPHOLIPASE A(2) 9001-84-7 No
Synonyms E.C. 3.1.1.4 * Lecithinase A * Phosphatidase * Phosphatidolipase * Phospholipase A * Phospholipase A(sub 2)
RTECS Number: SZ6114900

Section 3 - Hazards Identification
EMERGENCY OVERVIEW
Poison. May be fatal if enters bloodstream. Do not breathe dust. Do not use if skin is cut or scratched. Wash thoroughly after handling.
HMIS RATING
HEALTH: 4
FLAMMABILITY: 0
REACTIVITY: 0
NFPA RATING
HEALTH: 4
FLAMMABILITY: 0
REACTIVITY: 0
For additional information on toxicity, please refer to Section 11.

Section 4 - First Aid Measures

Section 5 - Fire Fighting Measures

FLASH POINT N/A
AUTOIGNITION TEMP N/A
FLAMMABILITY N/A
EXTINGUISHING MEDIA
Suitable: Water spray. Carbon dioxide, dry chemical powder, or appropriate foam.

FIREFIGHTING
Protective Equipment: Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes. Specific Hazard(s): Emits toxic fumes under fire conditions.

Section 6 - Accidental Release Measures

PROCEDURE TO BE FOLLOWED IN CASE OF LEAK OR SPILL
Evacuate area.
PROCEDURE(S) OF PERSONAL PRECAUTION(S)
Wear self-contained breathing apparatus, rubber boots, and heavy rubber gloves.
METHODS FOR CLEANING UP
Spilled material should be carefully wiped up or moistened with water and removed. Ventilate area and wash spill site after material pickup is complete.

Section 7 - Handling and Storage

HANDLING
User Exposure: Avoid inhalation. Do not get in eyes, on skin, on clothing. Avoid prolonged or repeated exposure. Do not use if skin is cut or scratched. Wash thoroughly after handling.
STORAGE
Suitable: Keep tightly closed.
Store at -20°C

Section 8 - Exposure Controls / PPE

ENGINEERING CONTROLS
Safety shower and eye bath. Use only in a chemical fume hood.
PERSONAL PROTECTIVE EQUIPMENT
Respiratory: Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU). Where risk assessment shows air-purifying respirators are appropriate use a full-face particle respirator type N100 (US) or type P3 (EN 143) respirator cartridges as a backup to engineering controls. If the respirator is the sole means of protection, use a full-face supplied air respirator. Hand: Compatible chemical-resistant gloves. Eye: Chemical safety goggles.
GENERAL HYGIENE MEASURES
Wash contaminated clothing before reuse.

Section 9 - Physical/Chemical Properties

Appearance Physical State: Solid Property Value At Temperature or Pressure
pH N/A
BP/BP Range N/A
MP/MP Range N/A
Freezing Point N/A
Vapor Pressure N/A
Vapor Density N/A
Saturated Vapor Conc. N/A
SG/Density N/A
Bulk Density N/A

Odor Threshold N/A
Volatile% N/A
VOC Content N/A
Water Content N/A
Solvent Content N/A
Evaporation Rate N/A
Viscosity N/A
Surface Tension N/A
Partition Coefficient N/A
Decomposition Temp. N/A
Flash Point N/A
Explosion Limits N/A
Flammability N/A
Auto ignition Temp N/A
Refractive Index N/A
Optical Rotation N/A
Miscellaneous Data N/A
Solubility N/A
N/A = not available

Section 10 - Stability and Reactivity

STABILITY Stable: Stable. Materials to Avoid: Strong oxidizing agents.
HAZARDOUS DECOMPOSITION PRODUCTS
Hazardous Decomposition Products: Nature of decomposition products not known.
HAZARDOUS POLYMERIZATION
Hazardous Polymerization: Will not occur

Section 11 - Toxicological Information

ROUTE OF EXPOSURE
Skin Contact: May cause skin irritation.
Skin Absorption: May be harmful if absorbed through the skin.
Eye Contact: May cause eye irritation.
Inhalation: May be harmful if inhaled. Material may be
irritating to mucous membranes and upper respiratory tract.
Ingestion: May be harmful if swallowed.
SENSITIZATION
Sensitization: Prolonged or repeated exposure may cause allergic reactions in certain sensitive individuals.
SIGNS AND SYMPTOMS OF EXPOSURE
To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.
CONDITIONS AGGRAVATED BY EXPOSURE
Basic phospholipases in general appear to be toxic, having lethal dose of about 500 ug/kg/mouse or even considerably less as exemplified by presynaptic neurotoxins and

myonecrotoxins. Many of the acidic phospholipases A, however, are much less toxic and not lethal even at 2000 ug/kg, although there are exceptions. An acidic phospholipase a (isoelectric point 5.1) from naja nigricollis has a lethal dose of 800 ug/kg in the mouse.* The toxicological properties have not been thoroughly investigated. May be fatal if enters bloodstream.

TOXICITY DATA

Intraperitoneal Mouse 5 MG/KG LD50
Subcutaneous Mouse 1200 UG/KG LD50
Intravenous Mouse 7500 UG/KG LD50

Section 12 - Ecological Information

No data available.

Section 13 - Disposal Considerations

APPROPRIATE METHOD OF DISPOSAL OF SUBSTANCE OR PREPARATION
Contact a licensed professional waste disposal service to dispose of this material. Observe all federal, state, and local environmental regulations.

Section 14 - Transport Information DOT

Proper Shipping Name: None
Non-Hazardous for Transport: This substance is considered to be non-hazardous for transport.
IATA
Non-Hazardous for Air Transport: Non-hazardous for air transport.

Section 15 - Regulatory Information

US CLASSIFICATION AND LABEL TEXT
US Statements: Poison. May be fatal if enters bloodstream. Do not breathe dust. Do not use if skin is cut or scratched. Wash thoroughly after handling.
UNITED STATES REGULATORY INFORMATION
SARA LISTED: No
CANADA REGULATORY INFORMATION
WHMIS Classification: This product has been classified in accordance with the hazard criteria of the CPR, and the MSDS contains all the information required by the CPR.
DSL: No
NDSL: No

Section 16 - Other Information

DISCLAIMER

For R&D use only. Not for drug, household or other uses.

5.2 DIETHYLENETRIAMINEPENTAACETIC ACID

SIGMA-ALDRICH **MATERIAL SAFETY DATA SHEET**

Section 1 - Product and Company Information

Product Name DIETHYLENETRIAMINEPENTAACETIC ACID FREE&
Product Number D6518
Brand SIAL
Company Sigma-Aldrich
Address 3050 Spruce Street
SAINT LOUIS MO 63103 US
Technical Phone: 800-325-5832
Fax: 800-325-5052
Emergency Phone: 314-776-6555

Section 2 - Composition/Information on Ingredient

Substance Name CAS # SARA 313
DIETHYLENETRIAMINE PENTAACETIC ACID 67-43-6 Yes
Ingredient Name CAS # Percent SARA 313
NITRILOTRIACETIC ACID 139-13-9 0.2 Yes
Formula C14H23N3O10
Synonyms Acetic acid,
((carboxymethylimino)bis(ethylenenitrilo))tetra-*
(((Carboxymethyl)imino)bis(ethylenenitrilo))tetraa
cetic acid * CHEL 330 * CHEL 330 acid * Chel DTPA
* Dabeersen 503 * Detapac * Detarex *
Diethylenetriaminepentaacetic acid *
1,1,4,7,7-Diethylenetriaminepentaacetic acid *
(Diethylenetrinitrilo)pentaacetic acid * DTPA * Hamp-EX Acid * Monaquest CAI *
Pentetic acid * Titriplex V * 3,6,9-Triazaundecanedioic acid, 3,6,9-tris(carboxymethyl)-
RTECS Number: MB8205000

Section 3 - Hazards Identification

EMERGENCY OVERVIEW
Irritant. Dangerous for the environment. Irritating to eyes. Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
Calif. Prop. 65 carcinogen.
HMIS RATING
HEALTH: 2*
FLAMMABILITY: 0
REACTIVITY: 0

NFPA RATING
HEALTH: 2
FLAMMABILITY: 0
REACTIVITY: 0
*additional chronic hazards present.
For additional information on toxicity, please refer to Section 11.

Section 4 - First Aid Measures

ORAL EXPOSURE
If swallowed, wash out mouth with water provided person is conscious. Call a physician.
INHALATION EXPOSURE
If inhaled, remove to fresh air. If not breathing give artificial respiration. If breathing is difficult, give oxygen.
DERMAL EXPOSURE
In case of contact, immediately wash skin with soap and copious amounts of water.
EYE EXPOSURE
In case of contact, immediately flush eyes with copious amounts of water for at least 15 minutes.

Section 5 - Fire Fighting Measures

FLASH POINT
392 °F 200 °C Method: closed cup
AUTOIGNITION TEMP N/A
FLAMMABILITY N/A
EXTINGUISHING MEDIA
Suitable: Water spray. Carbon dioxide, dry chemical powder, or appropriate foam.
FIREFIGHTING
Protective Equipment: Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.
Specific Hazard(s): Emits toxic fumes under fire conditions.

Section 6 - Accidental Release Measures

PROCEDURE(S) OF PERSONAL PRECAUTION(S)
Wear respirator, chemical safety goggles, rubber boots, and heavy rubber gloves.
METHODS FOR CLEANING UP
Sweep up, place in a bag and hold for waste disposal. Avoid raising dust. Ventilate area and wash spill site after material pickup is complete.

Section 7 - Handling and Storage

HANDLING
User Exposure: Do not breathe dust. Avoid contact with eyes,
skin, and clothing. Avoid prolonged or repeated exposure.

STORAGE
Suitable: Keep tightly closed.

Section 8 - Exposure Controls / PPE

ENGINEERING CONTROLS
Safety shower and eye bath. Mechanical exhaust required.
PERSONAL PROTECTIVE EQUIPMENT
Respiratory: Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU). Where risk assessment shows air-purifying respirators are appropriate use a dust mask type N95 (US) or type P1 (EN 143) respirator.
Hand: Compatible chemical-resistant gloves.
Eye: Chemical safety goggles.
GENERAL HYGIENE MEASURES
Wash thoroughly after handling.

Section 9 - Physical/Chemical Properties

Appearance Physical State: Solid
Property Value At Temperature or Pressure
Molecular Weight 393.35 AMU
pH 2.5 23 °C Concentration: 10g/l
BP/BP Range N/A
MP/MP Range 219.0 - 220.0 °C
Freezing Point N/A
Vapor Pressure N/A
Vapor Density N/A
Saturated Vapor Conc. N/A
SG/Density N/A
Bulk Density N/A
Odor Threshold N/A
Volatile% N/A
VOC Content N/A
Water Content N/A
Solvent Content N/A
Evaporation Rate N/A
Viscosity N/A
Surface Tension N/A
Partition Coefficient N/A
Decomposition Temp. N/A
Flash Point 392 °F 200 °C Method: closed cup
Explosion Limits N/A
Flammability N/A
Auto ignition Temp N/A
Refractive Index N/A

Optical Rotation N/A
Miscellaneous Data N/A
Solubility in Water:1 g/l, 20°C
Solvent: clear, colorless 0.1M in NaOH 1M,
20°C
N/A = not available

Section 10 - Stability and Reactivity

STABILITY
Stable: Stable.
Materials to Avoid: Strong oxidizing agents.
HAZARDOUS DECOMPOSITION PRODUCTS
Hazardous Decomposition Products: Carbon monoxide, Carbon dioxide,
Nitrogen oxides.
HAZARDOUS POLYMERIZATION
Hazardous Polymerization: Will not occur

Section 11 - Toxicological Information

ROUTE OF EXPOSURE
Skin Contact: May cause skin irritation.
Skin Absorption: May be harmful if absorbed through the skin.
Eye Contact: Causes eye irritation.
Inhalation: Material may be irritating to mucous membranes and upper respiratory tract.
May be harmful if inhaled.
Ingestion: May be harmful if swallowed.
SIGNS AND SYMPTOMS OF EXPOSURE
To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.
TOXICITY DATA
Oral Rat > 2,000 mg/kg LD50
Intraperitoneal Rat 587 MG/KG LD50
Remarks: Behavioral: Convulsions or effect on seizure threshold.
Behavioral: Aggression. Lungs, Thorax, or Respiration: Chronic
pulmonary edema.
Intraperitoneal Mouse 543 MG/KG LD50
IRRITATION DATA
Skin Rabbit
Remarks: No irritation effect
Eyes Rabbit
Remarks: Moderate irritation effect

Section 12 - Ecological Information

ACCUMULATION
Bioaccumulation Potential: Indication of bioaccumulation.
ACUTE ECOTOXICITY TESTS
Test Type: flow-through bioassay
Species: Leuciscus idus
Time: 96 h
Value: > 100 mg/l
SIAL - D6518 www.sigma-aldrich.com Page 4
Test Type: Growth inhibitor on algae.
Time: 72 h
Value: 1.0 - 10.0 mg/l
Test Type: EC50 Daphnia
Species: Daphnia
Time: 48 h
Value: 245 mg/l
ELIMINATION
Elimination: 20.0 - 60.0 %

Section 13 - Disposal Considerations

APPROPRIATE METHOD OF DISPOSAL OF SUBSTANCE OR PREPARATION
Contact a licensed professional waste disposal service to dispose of this material. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber. Observe all federal, state, and local environmental regulations.

Section 14 - Transport Information

DOT
Proper Shipping Name: Environmentally hazardous substances, solid, n.o.s.
UN#: 3077
Class: 9
Packing Group: Packing Group III
Hazard Label: Class 9
PIH: Not PIH
IATA
Proper Shipping Name: Environmentally hazardous
substance, solid, n.o.s
IATA UN Number: 3077
Hazard Class: 9
Packing Group: III

Section 15 - Regulatory Information

EU ADDITIONAL CLASSIFICATION
Symbol of Danger: Xi-N
Indication of Danger: Irritant. Dangerous for the environment.
R: 36-51/53
Risk Statements: Irritating to eyes. Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
S: 26-36-61
Safety Statements: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing. Avoid release to the environment. Refer to special instructions/safety data sheets.
US CLASSIFICATION AND LABEL TEXT
Indication of Danger: Irritant. Dangerous for the environment.
Risk Statements: Irritating to eyes. Toxic to aquatic organisms,
may cause long-term adverse effects in the aquatic environment.
Safety Statements: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing. Avoid release to the environment.
Refer to special instructions/safety data sheets.
US Statements: Calif. Prop. 65 carcinogen.
UNITED STATES REGULATORY INFORMATION
SARA LISTED: Yes
NOTES: This product is or contains a component that is subject to SARA313 reporting requirements.
TSCA INVENTORY ITEM: Yes
UNITED STATES - STATE REGULATORY INFORMATION
CALIFORNIA PROP - 65
California Prop - 65: This product is or contains chemical(s) known to the state of California to cause cancer.
CANADA REGULATORY INFORMATION
WHMIS Classification: This product has been classified in accordance with the hazard criteria of the CPR, and the MSDS contains all the information required by the CPR.
DSL: Yes
NDSL: No
Section 16 - Other Information
DISCLAIMER
For R&D use only. Not for drug, household or other uses.

5.3 DL-ERYTHRO-DIHYDROSPHINGOSINE

SIGMA-ALDRICH **MATERIAL SAFETY DATA SHEET**

Section 1 - Product and Company Information

Product Name DL-ERYTHRO-DIHYDROSPHINGOSINE

Product Number D6908
Brand SIGMA
Company Sigma-Aldrich
Address 3050 Spruce Street
SAINT LOUIS MO 63103 US
Technical Phone: 800-325-5832
Fax: 800-325-5052
Emergency Phone: 314-776-6555

Section 2 - Composition/Information on Ingredient

Substance Name CAS # SARA 313
DL-ERYTHRO-DIHYDROSPHINGOSINE 3102-56-5 No
Formula $C_{18}H_{39}NO_2$

Section 3 - Hazards Identification

HMIS RATING
HEALTH: 0
FLAMMABILITY: 0
REACTIVITY: 0
NFPA RATING
HEALTH: 0
FLAMMABILITY: 0
REACTIVITY: 0
For additional information on toxicity, please refer to Section 11.

Section 4 - First Aid Measures

Section 5 - Fire Fighting Measures

FLASH POINT N/A
AUTOIGNITION TEMP N/A
FLAMMABILITY N/A

Section 6 - Accidental Release Measures

Section 7 - Handling and Storage

STORAGE Store at -20°C

Section 8 - Exposure Controls / PPE

Section 9 - Physical/Chemical Properties

Appearance Color: White

Form: Powder
Property Value At Temperature or Pressure
Molecular Weight 301.5 AMU
pH N/A
BP/BP Range N/A
MP/MP Range N/A
Freezing Point N/A
Vapor Pressure N/A
Vapor Density N/A
Saturated Vapor Conc. N/A
SG/Density N/A
Bulk Density N/A
Odor Threshold N/A
Volatile% N/A
VOC Content N/A
Water Content N/A
Solvent Content N/A
Evaporation Rate N/A
Viscosity N/A
Surface Tension N/A
Partition Coefficient N/A
Decomposition Temp. N/A
Flash Point N/A
Explosion Limits N/A
Flammability N/A
Auto ignition Temp N/A
Refractive Index N/A
Optical Rotation N/A
Miscellaneous Data N/A
Solubility Solvent: clear, colorless 20 mg/ml $CHCl_3$
N/A = not available

Section 10 - Stability and Reactivity

Section 11 - Toxicological Information

Section 12 - Ecological Information

No data available.

Section 13 - Disposal Considerations

Section 14 - Transport Information

DOT
Proper Shipping Name: None

Non-Hazardous for Transport: This substance is considered to be non-hazardous for transport.
IATA
Non-Hazardous for Air Transport: Non-hazardous for air transport.

Section 15 - Regulatory Information

UNITED STATES REGULATORY INFORMATION
SARA LISTED: No
CANADA REGULATORY INFORMATION
WHMIS Classification: This product has been classified in accordance with the hazard criteria of the CPR, and the MSDS contains all the information required by the CPR.
DSL: No
NDSL: No

Section 16 - Other Information

DISCLAIMER
For R&D use only. Not for drug, household or other uses.

5.4 7,7-DIMETHYL-(5Z,8Z)-EICOSADIENOIC ACID

SIGMA-ALDRICH **MATERIAL SAFETY DATA SHEET**

Section 1 - Product and Company Information

Product Name 7,7-DIMETHYL-(5Z,8Z)-EICOSADIENOIC ACID
Product Number D8008
Brand SIGMA
Company Sigma-Aldrich
Address 3050 Spruce Street
SAINT LOUIS MO 63103 US
Technical Phone: 800-325-5832
Fax: 800-325-5052
Emergency Phone: 314-776-6555

Section 2 - Composition/Information on Ingredient

Substance Name CAS # SARA 313
7,7-DIMETHYL-(5Z,8Z)-EICOSADIENOIC 89560-01-0 Yes
ACID
Ingredient Name CAS # Percent SARA 313
The hazards identified with this None product are those associated with the residual solvents that are used in the manufacturing process.
METHANOL 67-56-1 <= 1 Yes
Formula $C_{22}H_{40}O_2$

Section 3 - Hazards Identification

EMERGENCY OVERVIEW
Toxic. Toxic by inhalation, in contact with skin and if swallowed. Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed. Irritating to eyes and skin.
Target organ(s): Eyes. Kidneys.
HMIS RATING
HEALTH: 2*
FLAMMABILITY: 0
REACTIVITY: 1
NFPA RATING
HEALTH: 2
FLAMMABILITY: 0
REACTIVITY: 1
*additional chronic hazards present.
For additional information on toxicity, please refer to Section 11.

Section 4 - First Aid Measures

ORAL EXPOSURE
If swallowed, wash out mouth with water provided person is conscious. Call a physician immediately.
INHALATION EXPOSURE
If inhaled, remove to fresh air. If not breathing give artificial respiration. If breathing is difficult, give oxygen.
DERMAL EXPOSURE
In case of skin contact, flush with copious amounts of water for at least 15 minutes. Remove contaminated clothing and shoes. Call a physician.
EYE EXPOSURE
In case of contact with eyes, flush with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating the eyelids with fingers. Call a physician.

Section 5 - Fire Fighting Measures

FLASH POINT N/A
AUTOIGNITION TEMP N/A
FLAMMABILITY N/A
EXTINGUISHING MEDIA
Suitable: Water spray. Carbon dioxide, dry chemical powder, or appropriate foam.
FIREFIGHTING
Protective Equipment: Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.

Section 6 - Accidental Release Measures

PROCEDURE(S) OF PERSONAL PRECAUTION(S)
Wear respirator, chemical safety goggles, rubber boots, and heavy rubber gloves.
METHODS FOR CLEANING UP

Absorb on sand or vermiculite and place in closed containers for disposal. Ventilate area and wash spill site after material pickup is complete.

Section 7 - Handling and Storage

HANDLING
User Exposure: Do not breathe vapor. Avoid contact with eyes, skin, and clothing. Avoid prolonged or repeated exposure.
STORAGE
Suitable: Keep container closed.
Store at -20°C
SPECIAL REQUIREMENTS
Light sensitive.

Section 8 - Exposure Controls / PPE

ENGINEERING CONTROLS
Safety shower and eye bath. Mechanical exhaust required.
PERSONAL PROTECTIVE EQUIPMENT
Respiratory: Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU). Use supplied-air or SCBA respirators. Europe permits the use of type AXBEK full-face cartridge respirators (EN 14387).
Other: Wear appropriate government approved respirator, chemical-resistant gloves, safety goggles, other protective clothing.
GENERAL HYGIENE MEASURES
Wash thoroughly after handling.
EXPOSURE LIMITS
Country Source Type Value
Poland NDS 100 MG/M3
Poland NDSCh 300 MG/M3
Poland NDSP –

Section 9 - Physical/Chemical Properties

Appearance Physical State: Liquid
Property Value At Temperature or Pressure
Molecular Weight 336.6 AMU
pH N/A
BP/BP Range N/A
MP/MP Range N/A
Freezing Point N/A
Vapor Pressure N/A
Vapor Density N/A
Saturated Vapor Conc. N/A
SG/Density N/A
Bulk Density N/A
Odor Threshold N/A
Volatile% N/A

VOC Content N/A
Water Content N/A
Solvent Content N/A
Evaporation Rate N/A
Viscosity N/A
Surface Tension N/A
Partition Coefficient N/A
Decomposition Temp. N/A
Flash Point N/A
Explosion Limits N/A
Flammability N/A
Auto ignition Temp N/A
Refractive Index N/A
Optical Rotation N/A
Miscellaneous Data N/A
Solubility N/A
N/A = not available

Section 10 - Stability and Reactivity

STABILITY
Stable: Stable.
Conditions to Avoid: Exposure to light may affect product quality.
Materials to Avoid: Strong oxidizing agents.
HAZARDOUS DECOMPOSITION PRODUCTS
Hazardous Decomposition Products: Carbon monoxide, Carbon dioxide.
HAZARDOUS POLYMERIZATION
Hazardous Polymerization: Will not occur

Section 11 - Toxicological Information

ROUTE OF EXPOSURE
Skin Contact: May cause skin irritation.
Skin Absorption: May be harmful if absorbed through the skin.
Eye Contact: May cause eye irritation.
Inhalation: Material may be irritating to mucous membranes and upper respiratory tract. May be harmful if inhaled.
Ingestion: May be harmful if swallowed.
TARGET ORGAN(S) OR SYSTEM(S)
Eyes. Kidneys. Liver. Heart.
SIGNS AND SYMPTOMS OF EXPOSURE
To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated. May cause convulsions. Gastrointestinal disturbances.

Section 12 - Ecological Information

No data available.

Section 13 - Disposal Considerations

APPROPRIATE METHOD OF DISPOSAL OF SUBSTANCE OR PREPARATION
Contact a licensed professional waste disposal service to dispose of this material. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber. Observe all federal, state, and local environmental regulations.

Section 14 - Transport Information

DOT
Proper Shipping Name: None
Non-Hazardous for Transport: This substance is considered to be non-hazardous for transport.
IATA
Non-Hazardous for Air Transport: Non-hazardous for air transport.

Section 15 - Regulatory Information

US CLASSIFICATION AND LABEL TEXT
Indication of Danger: Toxic.
Risk Statements: Toxic by inhalation, in contact with skin and if swallowed. Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed. Irritating to eyes and skin.
Safety Statements: Keep container tightly closed. Avoid contact with skin. Wear suitable protective clothing and gloves. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).
US Statements: Target organ(s): Eyes. Kidneys.
UNITED STATES REGULATORY INFORMATION
SARA LISTED: Yes
NOTES: This product is or contains a component that is subject to SARA313 reporting requirements.
CANADA REGULATORY INFORMATION
WHMIS Classification: This product has been classified in accordance with the hazard criteria of the CPR, and the MSDS contains all the information required by the CPR.
DSL: No
NDSL: No

Section 16 - Other Information

DISCLAIMER
For R&D use only. Not for drug, household or other uses.

All information, recommendations and suggestions herein concerning this product are based upon data believed to be reliable. However it is the user's responsibility to determine the safety, toxicity and suitability for his/her own use of this product. Since the actual use of others is beyond our control, we make no guarantee expressed or implied as to the effects of such use, the results to be obtained, or the safety and toxicity of the product. This information is not to be construed as absolutely complete, since additional information may be necessary of desirable when exceptional conditions or circumstances exist or because of applicable laws or government regulations.

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