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"Characterization of Red Diamondback Rattlesnake Venom Proteins on Cell Death and Function"

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Characterization of Red Diamondback Rattlesnake Venom Proteins on Cell Death and Function

By:

Venus Ebrahimian

B.S. Biological Science, Wright State University, 2010

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

<u>9 January 2014</u>

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY <u>Venus Ebrahimian</u> ENTITLED **Characterization of Red Diamondback Rattlesnake Venom Proteins on Cell Death and Function** BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF <u>Master of Science</u>.

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ABSTRACT

Ebrahimian, Venus. M.S., Department of Pharmacology/Toxicology, Wright State University, 2013. Characterization of Red Diamondback Rattlesnake protein on Cell Death and Function.

Snake venom is composed of many different toxins and protein components such as metalloproteases and phospholipase A2. Together, these proteins cause an up regulation in cell death pathways and disrupt the overall homeostasis of a living cell. The Red Diamondback Rattlesnake is abundantly available and well-known specie and has been used as a source for development of new drugs. It has been mainly used for the purposes of making anti-venom but it has also been therapeutically used for surgical purposes to act as an anti-aggregate. Integrins derived from RDB venom has been used for cancer cell treatment as well. Specifically a hemotoxic venom, RDB venom contains high concentrations of the toxin Ruberlysin; also known as Hemorrhagic Toxin II. An animal or human bitten by a RDB snake will experience localized tissue swelling, pain, bruising, as well as necrosis at the bit site. Systematically, the venom will cause excess internal bleeding, nausea, vomiting, and due to being hemotoxic venom, it will cause hemolysis. Due to its rich protein content as well as possible therapeutic purposes, RDB venom proves to hold the potential for studying cancer. The purpose of this research is to test the effects of Red Diamond Back rattlesnake venom protein components on different neuronal and lung cell lines, identify specific protein components and determine the

venom's effects on specific cellular functions, i.e., intracellular prohormone trafficking and surfactant lipid secretion. To understand how the venom components work, the venom was fractionated by Fast Protein Liquid Chromatography and desalted through membrane dialysis using ddH$_2$O. Neuro-2A and lung cells were incubated with individual snake venom fractions at different concentrations and over a 6 hour time. After analysis by specific cell death assays, i.e., MTT, LDH, propidium iodide, annexin and Hoechst, several specific fractions were observed to cause cell death, while other fractions appeared to cause an increase in proliferation. Furthermore, chromogreanin A trafficking to punctate granules characteristic of the regulated secretory pathway was disupted in the Neuro-2a cells. Likewise, secretion of surfactant lipids appeared to be enhanced for several lipids from the lung cells exposed to snake venom fractions. Specific RDB venom protein fractions were tryptically digested, spotted to a MALDI-target and sequenced using a Bruker Autoflex III MALDI TOF/TOF mass spectrometer. Ion data was collected and sent to an in-house MASCOT server database, which identified Hemorrhagic toxin (II), myotoxin, and apoxin I. The results from our study suggest that FPLC-cation exchange is a good first pass scheme for separating snake venom proteins, and that these separated proteins appear to have unique characteristics for cell death, proliferation and functionality.

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I.   STATEMENT OF THE PROBLEM

The phenotype of cells is defined by functionality conferred by the expression of specific proteins. The growth and proliferation of cells varies depending on the different types of cells and their functions. Sometimes, the regulation of growth, proliferation or functionality is lost, resulting in a tumor or cancer type of cell. The problem is that these cells have lost their regulation and form the foci of a disease called cancer. Over the past century, numerous treatments have been developed to try to bring these cells under regulation or to remove them. Some treatment therapies are more effective than others and some are more invasive and life-threatening themselves than others. The goal is to try to re-regulate the cellular function or to stop or slow the proliferation of the cells so that the tumor or cancer does not progress without creating a worse condition for the patient. The goal of our research is to identify specific proteins from snake venom that act by one of these mechanisms. Lung cells derived from biopsy samples of hospital patients afflicted with adenocarcinoma and neuroblastoma cells from mouse neural crest were used to test the snake venom proteins.

II. INTRODUCTION

THE RED DIAMONDBACK RATTLESNAKE

The red diamondback rattlesnake (RDB), also known as *crotalus ruber ruber*, is a heavy set and somewhat docile snake belonging to the Viperidae family.   The versatile nature of this particular snake is further proven by its presence in islands in the Gulf of California and along the pacific coast of Baja California [Dugan, 2012]. RDB is often misidentified as the Western Diamond-backed Rattlesnake, although the habitats of these two snakes never converge to make such confusion possible and thus is attributed to the physical characteristics of the snakes. RDB has a prominently triangular head and is a relatively large sized snake, reaching approximately between 2-4.5 feet in length with characteristic shades of red that give root to its namesake [Dugan, 2012]. Like many other snakes in the Viperidae family, RDB has a rattle, growing larger with each shedding that the snake goes through and giving the characteristic rattling sound that the snake makes when offering a warning. The snake has movable and retractable fangs, which are intricately connected to venom glands that produce venom throughout the course of the snake's lifetime. Mode of reproduction for this particular snake is through live birth and baby snakes are left to fend for themselves upon birth. Their diet consists mainly of small mammals such as ground squirrels, rats, rabbits, lizards and sometimes birds [Dugan, 2012]. There have been reports however, that adult ground squirrels are immune to rattlesnake venom and will aggressively confront the snake should they feel threatened [Biardi, 2006]. Although less venomous than its close relatives, the venom of the RDB has been identified as a hemotoxic venom. Hemotoxic venom contains

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components that mainly disrupt the homeostasis of red blood cells within the afflicted patient or prey, leading to systemic hemolysis [Panfoli, 2010].

SNAKE VENOM

Snake venom type and potency is characterized based upon many characteristics, e.g., specie, geographical location, habitat, climate and age, etc. All snake venom share many components that are found to be the same through species with some comprising a larger proportion of the mixture than others. Thus, snake venom consists of a complex and unique mixture of proteins, peptides, polypeptides, peptidases, hydrolytic enzymes, metals and etc [Fry, 2005]. Each of these components not only makes venom uniquely different but also, provides a special functionality characteristic of the particular snake. Most components found within venom are heavily involved in the incapacitation of the prey of interest for that particular snake. Once the prey has been subdued, proteins found in snake venom aid in the digestion of the prey for the snake [Fry, 2005]. Based upon the method of hunting as well as toxin and enzyme components, snake venom can be categorized as either neurotoxic or hemotoxic, although there are certain snake species that have both types of toxins present within their venom.

RDB venom contains high levels of proteolytic enzymes that are mainly active as on blood cells, thus categorizing it as a hemotoxic venom. The most common protein component observed in RDB venom has been ruberlysin, a metalloprotease commonly known as Hemorrhagic Toxin II [Mori, 1987]. Hemotoxic venoms act by destroying red blood cell membrane components as well as other surrounding vascularized tissue in the process; thus leading to severe tissue necrosis and blood coagulation. Other prominently

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found proteins in RDB venom include: apoxin, phospholipase A2, annexin as well as apoptosis inducing factors (AIF) [Joshi, 2012]. Apoxin is a recently discovered apoptosis inducing factor (AIF) that is found abundantly in snake venom [Torii, 1997]. Phospholipase A2 is an anti inflammatory protein that is found ubiquitously in all snake venom [Mackessy, 2009]. And lastly, Annexin is known as an anti coagulant inducing protein that is found broadly in all organisms including fungi [Braun, 1998].

CANCER

Cancer, by definition, refers to the unmitigated corruption of normal cellular division. Regulatory pathways that ensure normal cellular angiogenesis are interrupted by various factors and cause malignant cells to not only have compromised tumor suppressor genes but continue to divide, passing on the altered genetic material to future cells and forming what is known as tumors in the affected tissue. Causes of tumor angiogenesis can vary from environmental factors such as exposure to certain chemicals or UV light to mishaps during mitotic division [Weinberg, 2013]. The current methods used in cancer treatment are somewhat effective and mainly use chemicals that not only destroy the tumor cells but also healthy cells around the effected area as well. These treatments include the use of different forms of chemotherapy using alkylating agents, topoisomerase inhibitors, and cytotoxic antibiotics to name a few. The toxicity of chemotherapy is a factor underlying why many cancer patients do not survive this form of therapeutic treatment. Another method involves surgically removing an effected area in a given organ. This surgery is most effective if the organ is affected in a specific area that can be easily removed. The prognosis of this form of cancer treatment depends on type as well as the stage of cancer [Cancer.org, 2013]. And lastly, radiation, which

usually accompanies the other listed forms of cancer treatment. These three methods have proven to be the most effective forms of treatment of cancer despite the implications that accompany the treatment itself as well as the type of cancer.

CELL DEATH

There are two well-known cell death pathways: apoptosis and oncosis (Majno, 1995). Unlike oncosis which usually involves cellular trauma that leads to an irrecoverable injury such as nucleic changes and structural disruption causing cell death; apoptosis is programmed cell death that occurs cyclically when an error is made during the life cycle of that particular cell. Apoptosis is genetically controlled and is initiated based on the length of cell cycle under normal circumstances; other chemical and physical agents, hormones, and viruses can induce programmed suicide in the cell (Buja, 1993; Majno, 1995). Involving a signal pathway, any change during cell growth will cause the cell to go through apoptosis to prevent the passing on of any erroneous genetic material. To ensure the prevention of any such mistake, there are several proteins as well as tumor suppressor genes that check for DNA normalcy and will induce programmed cell death should there be any mismatching or other errors in cellular development. The morphology of apoptotic cells is as followed: blebbing, chromatin condensation, cell rounding and loss of processes, shrinkage of cell bodies, and cleavage of DNA into 180 base pair segments (Buja, 1993; Majno, 1995). Once the cell has died, its organelles and body are packaged and then phagocytized by macrophages present within the tissue (Buja, 1993; Majno, 1995). Oncosis morphology involves cell swelling, loss of identity, rupturing and leakage of cell content and inflammation due to the presence of cytokines into the extracellular matrix (Majno, 1995). Once the cell has died, it follows the same

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pathway as that of apoptotic cells in that their cellular content are packaged and then phagocytized by macrophages. A tissue is considered necrotic after the cells have gone through apoptosis or oncosis; a postmortem effect rather than a presently observable change (Majno, 1995). Due to the fact that both cell death methods lead to necrosis, it is best to use several different test methods to identify exact method of death amongst cells. Due to apoptosis being programmed, different markers can be used that are only characteristically present when the cell commits suicide. The P53 protein, as well as different caspases that act as "toll booths" during cell suicide, can be used as markers to identify cells going through apoptosis. In this study, LDH assay as well as MTT assay were used to detect the presence of lactate dehydrogenase which is indicative of cell damage as well as cell enzyme activity and cell proliferation to identify whether or not venom treated cells are going through apoptosis or oncosis respectfully. Alternatively, proliferation is the reverse of apoptosis and oncosis. Cells are programed to increase in number, leading to division and growth. This occurs amongst normal cells that contain genetic material that have passed through the signal pathway with flying colors. However, cellular proliferation is observed amongst mutated cells such as those found containing oncogenes. The purpose of this form of proliferation is to pass on as much of the tampered genetic material as possible. Cells are programmed to continue proliferating instead of committing suicide.

VENOM AS THERAPEUTIC AGENT

The most far-reaching objective of snake venom studies has been in their use for therapeutic purposes. The use of an isolated protein from naturally produced substance such as venom that can be readily found in nature may be an interesting and exciting

alternative to synthetic SAR types of drug production. No matter what snake, the venom they produce is complex. Some proteins are even produced by bacteria that reside in the glands. To be able to properly identify proteins from snake venom requires understanding the chemical characteristics of the venom. Venoms from a wide array of animals including but not limited to snakes have been used for therapeutic purposes in medicine and are actively used today. Cobra venom has been widely used as an anticoagulant in stroke patients as well as cardiovascular surgeries [Kini, 2006]. Biomedical engineers at Washington University have discovered that alterations in an inflammatory protein found in bee venom, i.e., mellitin, can be used as a therapeutic drug delivery system for cancer treatment (Soman, 2009).. Bee venom has also shown anti-cancer effects in prostate cancer cells through the caspase pathway activation . It does so by inducing apoptosis in cells through several pathways such as activation of caspases as well as matrix metalloproteinases (Soman, 2009). Venom derived from cone snails found in the sea has shown potential cytotoxic activity in Ehrlich's ascites carcinoma cells by inducing oxidative stress [Abdel-Rahman, 2013]. Sea anemones are also currently a new topic of interest in cancer studies and according to Ramezanpour et al, the venom of 5 types of sea anemones were tested on A549 lung cancer, T47D breast cancer and A431 skin cancer cells and were proven to be effective as anti-cancer agents [Ramezanpour, 2012]. Bufalin, a toxin extracted from the skin secretions of the Chinese toad, has shown anti-cancer activity by inducing apoptosis in various cancer cells such as colon cancer and hepatocellular cancer cells. However, snake venom appears to show the most therapeutic promise in oncological diseases. This may be due to its abundant availability as there are still various species of snake being discovered today. Venom toxin derived from Vipera

Lebetina Turanica caused ROS- and JNK-mediated upregulation of death receptors in cancer cells, thus down regulating survival proteins in these cells [Park, 2012]. Zhang et al. isolated ACTX-6 from the venom of Agkistrodon acutus snake, and found that upon exposure, it can cause apoptosis involving ROS [Vyas, 2013]. Torii et al found an apoptosis inducing factor, Apoxin I from rattlesnake venom that can be therapeutic [Torii, 1997]. Tang et al discovered that MHCC97H cells are inhibited from metastasis by cystatin; a protein found in snake venom that reduces proteinase activity and epithelial mesenchymal transition [Tang, 2011]. A disintegrin known as contortrostatin, found in the venom of the southern copperhead Agkistrodon contortrix contortrix was identified at the University of Southern California Keck School of Medicine to cause a blockage in the cellular migration and communication in human breast and ovarian cancer cells [Zhou, 1999]. When injecting tumors with the disintegrin, it was noted that the tumor angiogenesis was significantly inhibited [Zhou, 1999]. These previous venom studies represent varied and important chemical pathways that may be the future of curing specific diseases such as cancer, diabetes and Alzheimer's. However, much work needs to be done to characterize specific venom proteins and properties before this can happen.

NEUROBLASTOMA CELL LINE

Neuro-2A cells are neuronal and amoeboid stem cells derived from readily occurring neuroblastoma tumors in an albino strain A mice [ATCC]. The cells are characterized by their production of microtubule protein, numerous secretory vesicles and acetylcholinesterase which may come in use during signal transduction in neuronal cells [ATCC]. They were originally grown isolated into *in vitro* culture in 1967 [Jorgensen, 1976]. The cells grow in the form of monolayer that is adherent [ATCC]. When cultured,

they tend to grow rapidly and form cellular extensions known as neurites [Jorgensen, 1976; Cool, 1994; Cool 1995]. These cells were chosen because they have a regulated secretory pathway that is representative of neuroendocrine cells in the pituitary and they have been well-characterized previously in other studies [Jorgensen, 1976]. They endogenously produce pro-enkephalin and prohormone convertase 1/3 and prohormone convertase 2 [Bamburger, 1995]. For this reason, they have been used as a model cell line for studying the intracellular trafficking of prohormones such as pro-opiomelanocortin, pro-insulin and pro-vasopressin [Cool, 1995; Norment, 1997; Cool, 2008; Yan, 2010].

ADENOCARCINOMA CELL LINE

The lung cells (NCI-H441) are derived from a human adenocarcinoma and were obtained from ATCC. Not composed of just one specific cell line, this particular tumorigenic cell line is composed of 7 lung cancer cell lines with different degrees of genomic mutation at varying levels [ATCC]. Mainly, these cells are used for understanding genetic mutations, cancer inducing genes as well as exposure to varying substances and chemicals for treatment purposes [ATCC]. Of interest for this project was the function of the lung cells to secrete surfactant. Surfactant is critical for lung function as it provides the correct environment for the lung cells to stay 'open' without collapsing and disrupting air flow in and out. In an earlier study (Retzke & Cool, personal communication) the lung cell line was used to test differences in surfactant secretion in response to 24 and 48 hour treatment with dexamethasone, a corticosteroid that is used to mature the lungs of fetuses that may be delivered early [Platzker, 1975]. Lung surfactant is a complex mixture of proteins, e.g., surfactant protein A, B, C, or D, and lipids, e.g., lecithin and sphingomyelin (Figure 1A & B). Fetal lung maturity is determined in clinical

labs as a ratio between the lecithin and sphingomyelin (LC:SM). A ratio of 2:1 LC:SM is considered mature enough for delivery. Lecithin is usually a mixture of different phospholipids with a major component identified as phosphatidylcholine [Liu, 1998].
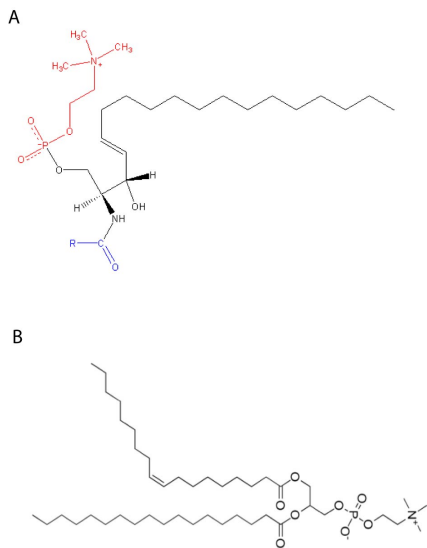
Figure 1: Structures of Lecithin (A) and Sphingomyelin (B). These are two of the main lipid components of lung surfactant.

FPLC VERSUS COLUMN CHROMATOGRAPHY

This project involved the use of a common protein separation technology that had not previously been used for separating snake venom proteins. Specifically, RDB snake venom was separated into fractions using Fast Protein Liquid Chromatography (FPLC) followed by desalting using dialysis membrane. We have used a cation exchange column on the FPLC to separate the snake venom. Cation columns are an affinity type of column that have a negative charge on the stationary phase (beads). Positively charged analytes (proteins) are bound to the column and eluted by an increasing salt gradient under a stable pH. While cation exchange FPLC separates proteins, it does not provide a high-

resolution separation whereby each protein is found in a separate fraction. This tends to be a 'first-pass' type of technology but one that yields a level of protein separation with some fractions containing multiple proteins. This can also be done in a relatively short period of time, i.e., less than 20-30 minutes compared with the older column chromatography methods that can take more than 24 hours to complete a run [Sheehan, 2004]. Column chromatography can yield higher resolution separation of individual proteins but it comes at a cost in time and starting material. Column chromatography typically uses several large columns packed with separating matrices that require a cold room or cold cabinet at 4°C and several days to run. Future FPLC work will include a sizing column which would provide a better first pass separation of protein.

HYPOTHESIS

The hypothesis is that isolated rattlesnake venom proteins can slow cell proliferation, or cause cell death in neuronal or lung cells. Furthermore, we hypothesize that some fractions can disrupt specific cell functions such as intracellular protein trafficking or release of lipids such as surfactant.

SPECIFIC AIM 1

Test the hypothesis that snake venom fractions can slow proliferation or cause cell death in lung cells or neuroblastoma cells.

SPECIFIC AIM 2

Test the hypothesis that cell functionality can be disrupted by specific snake venom proteins obtained by fractionation.

SPECIFIC AIM 3

Identify proteins in individual snake venom fractions and relate these to pathways involved in regulation of the cellular functions.

III. MATERIALS & METHODS

CELL LINE

Neuro-2A cells obtained from ATCC were originally cultured from the neuroblastoma tumors in albino strain A mice. The. lung cell line (NCI-H441) was also obtained from ATCC and was originally derived from patient biopsy samples of a human adenocarcinoma. The cells were grown and incubated following ATCC protocol. Briefly, the Neuro-2a cells were grown in DMEM media containing 10% FBS, 1X pyruvate, 1 X NaBicarbonate, and 1X PenStrep Fungizone. They were passaged when 80-90% confluent and maintained at 37°C and 5% CO_2. The CI-H441 lung cells will be grown in Waymouth's MB571/5 medium (Invitrogen) containing 2% Charcoal-stripped FBS (Hyclone) and 1.5% antibiotic/antimycotic (Cellgro; containing 10,000 unit/ml penicillin G sodium, 10,000 ug/ml streptomycin sulfate, and 25 ug/ml amphotericin B).

VENOM

Raw RDB venom was acquired from red diamondback rattlesnakes kept in the WSU Laboratory Animal Resouces facility under an approved protocol. Snakes were milked from each fang separately prior to feeding and the venom was put into test tubes indicating if venom was acquired from the left or right fang of the animal. Upon acquiring venom, it was stored at -4°C until further use.

VENOM FRACTIONS

Venom was fractionated using Fast Protein Liquid Chromatography (FPLC). Mobile phase was at 0-1 M NaCl over a 13 minute run time with a 25mM MES buffer at pH 8. The FPLC was set to run at 4 mls/min and 1ml fractions were collected. Fractions were then desalted using 1000 dalton cutoff dialysis tubing (Spectrum, Fisher Scientific). Fractions were dialyzed 4 hours changing the ddH$_2$O every hour. Upon desalting, each fraction was moved to a 1.5 ml eppendorf tube and stored at -20°C until further use.

CELL TREATMENT

Both cell types were plated in 8 and 24 well plates at a ratio of 25 ul of cells and 700 ul of complete media per well. Cells were treated upon reaching 80% confluency. The neuro-2a cells took approximately 2 days to reach that level of confluency while lung cells typically took 3-4 days. Cells were initially washed with media minus phenol red and FBS in a sterile environment before treatment. The snake venom concentration used was fixed at 100 μl of fraction combined with 700 ul of phenol red free media which was then used to treat each well with 200 μl of snake venom fraction-media mixture. A fourth well was given 200 μl of fraction-media mixture and the plates were returned to the incubator for 4 hours. Once the treatment was completed, if wells contained coverslips, they were fixed using 4% paraformaldehyde (PFA). Wells containing no coverslips were analyzed for fraction functionality using different assays based on cell type. Depending on analysis method, cell media from different treatments were collected and stored at -4°C until further use.

MTT ASSAY

Cellular proliferation post fraction treatment was detected using the TACS® MTT Cell proliferation Assay Kit (Molecular Probes Inc, Eugene, OR). The cells were seeded into 96 well flat bottomed cell culture plates at a density of 10,000 cells per well and allowed to grow overnight at 37°C and 5% CO_2. On the second day, the media was removed from the cells, the media replaced with phenol free and FBS free media containing the appropriate concentrations of snake venom fraction, and incubated for four hours at 37°C, 5% CO_2. Following incubation, 10 μl of MTT reagent was added to the media on the cells and returned to the incubator for 2-4 hours until a purple dye was visible. At that time, 100 μl of detergent reagent was added to the wells, for an overnight incubation at room temperature. The following day, the absorbance in each well was measured on a Packard Fusion Plate Reader at 570 nm. Data was averaged and the blank subtracted from the total. The results were graphed in Excel as absorbance vs time or concentration.

LDH CYTOTOXICITY ASSAY

Cellular damage from treatment was detected using the LDH Cytotoxicity Detection Kit (Clontech, Mountain View, CA). The catalyst was prepared by reconstituting the lyophilate with 1ml of distilled water. The dye solution consisted of INT solution that became readily available upon thawing. A reaction mixture was prepared based on the number of tests conducted: for 100 tests 250 μl of catalyst solution was mixed with 11.25 ml of dye solution before use. Duplicate wells were designated for controls as followed: a background control which contained 200 μl of assay medium, substance control I which contained 100 μl of test substance at the max concentration

used for experiment with 100 μl /well assay medium, low control for spontaneous LDH

release which contained 200 μl of assay medium and finally, a high control for maximum

LDH release which contained 100 ul of assay medium and 100 μl of Triton X-100

solution. The wells containing cells were pipetted repeatedly until cells detached.

Solution containing cells were transferred to ependorf tubes and were centrifuged at 250

x g for 10 min. The supernatant was carefully removed without disturbing the formed

cell pellet and transferred into labeled wells in a new 96-well-flat bottom plate. 100 ul of

reaction mixture was then added to each well and incubated for approximately 30 min in

an area void of light. The absorbance of the samples at 495 nm was done using a multi

well plate reader. Percent cytoxicity is calculated by using the following formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{Duplicate Absorbance} - \text{Low Control}}{\text{High Control} - \text{Low control}} \times 100$$

For control cells, 100 μl was added to the media in place of snake venom fractions.

PROPIDIUM IODIDE STAINING

Propidium iodide (PI) staining is typically used to distinguish between living and

non-living cells by intercalating PI into double stranded nucleic acids such as the DNA of

the dead cells and emitting a red fluorescence that is ideally detectable by a fluorescence

microscope. This stain is only permeable through the cellular membrane of dead or

dying cells, this allows for death rate analysis. The protocol was followed per the

discretion of the staining kit (Calbiochem, San Diego, CA). PI is dissolved in PBS buffer

containing PBS at a concentration of (1 μg/ml). The solution is made in the dark. with

the least presence of light. Cells are washed with PBS and treated with the stain and then

fixed on slides and kept in a dark area over night.

CHROMOGRANIN STAINING

Cells were fixed with 2% paraformaldehyde (Electron Microscopy Sciences) and 0.1% Triton X100 for 30 min and blocked with 10% goat serum in PBS for 1 hour, followed by incubation with antibodies to chromogranin A (1:250). Chromogranin A antibodies were purchased from ICN. After incubation, the cells were incubated with goat anti-rabbit IgG conjugated to biotin followed by incubation with streptavidin conjugated Texas Red (Roche, Indianapolis, IN). The coverslips were affixed to slides with GelMount mounting media (Biomeda, Foster City, CA) and images taken using a Leica digital camera system on a Leica DMR fluorescence microscope.

ANNEXIN AND HOECHST STAINING

Cells were fixed with 2% paraformaldehyde in PBS for 15 minutes. They were washed with PBS two more times before being mounted. Cells were initially washed with 1xPBS. They were then treated with Annexin diluted at 1:250 in 5 mls of 10% goat serum for 1 hour. Cells were then gently washed with PBS for give minutes, 3 times. They were then washed once with deionized water. Hoechst 33258 stain (1 μl) was combined with 10 ml of deionized water and added to the cells for 1 minute. Excess stain was then rinsed away using deionized water. mounting on slides.

THIN LAYER CHROMATOGRAPHY (TLC)

Thin layer choromatography (TLC) was used for the detection of surfactant in treated lung cells. A 9:1 ratio of chloroform to methanol was added to the wells of treated cells at equal volume. Contents of the wells were transferred to eppendorf tubes and vortexed for 30 seconds. They were then speed vacuumed for 3 minutes and the

supernatant was removed. The white lower organic phase was then dried using the speed

vacuum. The contents were resuspended in a small volume 50 ml of methanol and

HPTLC Silica Gel 60F$_{254}$ plates (EMD, Gibbstown, NJ) spotted with 5 microliters of the

extracted lipids. The HPTLC plates were resolved by running in a closed glass tank

containing a mixture of: chloroform:methanol:acetic acid:acetone:ddH$_2$O at a 51:32:9:5:3

ratio. When the solvent front reached ~0.5 cm from the top of the HPTLC plate the HP-

TLC plate was air-dried and sprayed with a mixture of primuline in water at a

concentration of 1 mg/ml and air-dried. The primuline stained plates were examined

under a DyLight 488 setting on the ChemDoc MP imaging camera (Bio-Rad, Hercules,

CA) for 1 second to 10 seconds and the best image saved for evaluation.

IMAGE CAPTURE

Stained cells were visualized on a Leica DMR epifluoresecent microscope and

images were captured using a Leica CCD digital camera system. These images were

prepared for publication using Adobe Photoshop and Adobe Illustrator CS3 software.

Non-stained cells were visualized on a light microscope.

MASS SPECTROMETRY

Snake venom fractions causing the most change in cell functionality were further

analyzed using mass spectrometry. Fractions were digested using an in-solution tryptic

digest. The material required for the preparation of the digest was: a trypsin stock, a

digestion buffer composed of ammonioum bicarbonate that is dissolved in ultra pure

water to a 50 mM concentration, a reducing buffer consisting of DDT that is dissolved in

ultra pure water to a 100 mM concentration, and an alkylation buffer prepared from

Iodoaceamide, also dissolved in ultra pure water to a 100 mM concentration. Shortly before use, dilute 2 μl trypsin working solution with 18 μl of Digestion Buffer (25 mM ammonium bicarbonate) for each sample being processed. Concentration will be ~10 ng/μl (for a total of 200 ng in 20 μl). Store activated trypsin on ice until use.

Reduction and alkylation.

Reduction solution was made by adding 15 μl of digestion buffer and 1.5 μl of reducing buffer to a 0.5 ml eppendorph tube. Add 10 μl of protein to this tube and adjust to 27 μl with ultrapure water. The tube was incubated at 95°C for 5 minutes then allowed tube to cool. After this, 3 μls of alkylation buffer was added to the sample reaction and incubated in the dark at room temperature for 20 min. For digestion, 5 μl of the trypsin stock was added to 45 μl of the digestion buffer. From this, 1 μl of the trypsin in digestion buffer was added to the reaction tube and incubated at 37°C overnight.

After digestion, cells were zip tipped to remove any remnants of salt. ZipTip- A C18 ZipTip was activated by pipetting methanol 3 times up and down, and washed by pipetting 0.1% TFA 3 times. The sample was pipetted up and down 5 times, followed by washing with 0.1% TFA, and elution with 20 μl of 95% acetonitrile containing 0.1% TFA.After digestion, cells were zip tipped to remove any remnants of salt. ZipTip- A C18 ZipTip was activated by pipetting methanol 3 times up and down, and washed by pipetting 0.1% TFA 3 times. The sample was pipetted up and down 5 times, followed by washing with 0.1% TFA, and elution with 20 μl of 95% acetonitrile containing 0.1% TFA. Purified samples were prepared for mass spec. by combining 3 μl of sample with 3

μl of alpha-Cyano-4-hydroxycinnamic acid (CHCA, Sigma) to enable the detection of protein and peptides that are around 10 kDa or less. Of this mixture, 1 ul was carefully loaded on to a well on the MS plate and dried using a vacuum. The mass spectrometer used was an Autoflex III MALDI-TOF/TOF MS (Bruker Biosciences Corporation, Billerica, MA). This is used in combination with a matrix assisted laser desorption/ionization-time of flight instrument, MALDI-TOF. Each fraction was initially analyzed by MS for peaks between 0-10,000 daltons in reflector mode and then in linear mode. The spectra data was saved to FlexAnalysis and aligned in a group. If specific peaks of high intensity were found, a LIFT was attempted to sequence the ion peaks. MS fragmentation and sequencing by MS/MS was carried out to generate a table of sequenced proteins.

III. RESULTS

FRACTIONATION OF SNAKE VENOM BY FPLC

The first objective of the project was to establish a method for fractionation of snake venom proteins. FPLC was chosen as a 'first pass' method to separate proteins found in snake venom over more traditional column chromatography methods because of their cost and time constraints. Red Diamondback rattlesnake venom was injected into the FPLC and fractionated over a 20 minute time period (Figure 2). Multiple runs of the FPLC and the same venom suggested good reproducibility of the FPLC and cation column (Figure 3 A-D). Modifying the buffers in the FPLC did change the profile (Figure 3) but it often showed less separation than the first 'simple' gradient used (Figure 3). SDS-PAGE analysis of the fractions using Coomassie staining suggested that there was some carryover on the column, potentially due to overloading with specific proteins that are in high abundance in the snake venom (Figure 4). However, there did appear to be some separation of larger and smaller proteins with the FPLC.
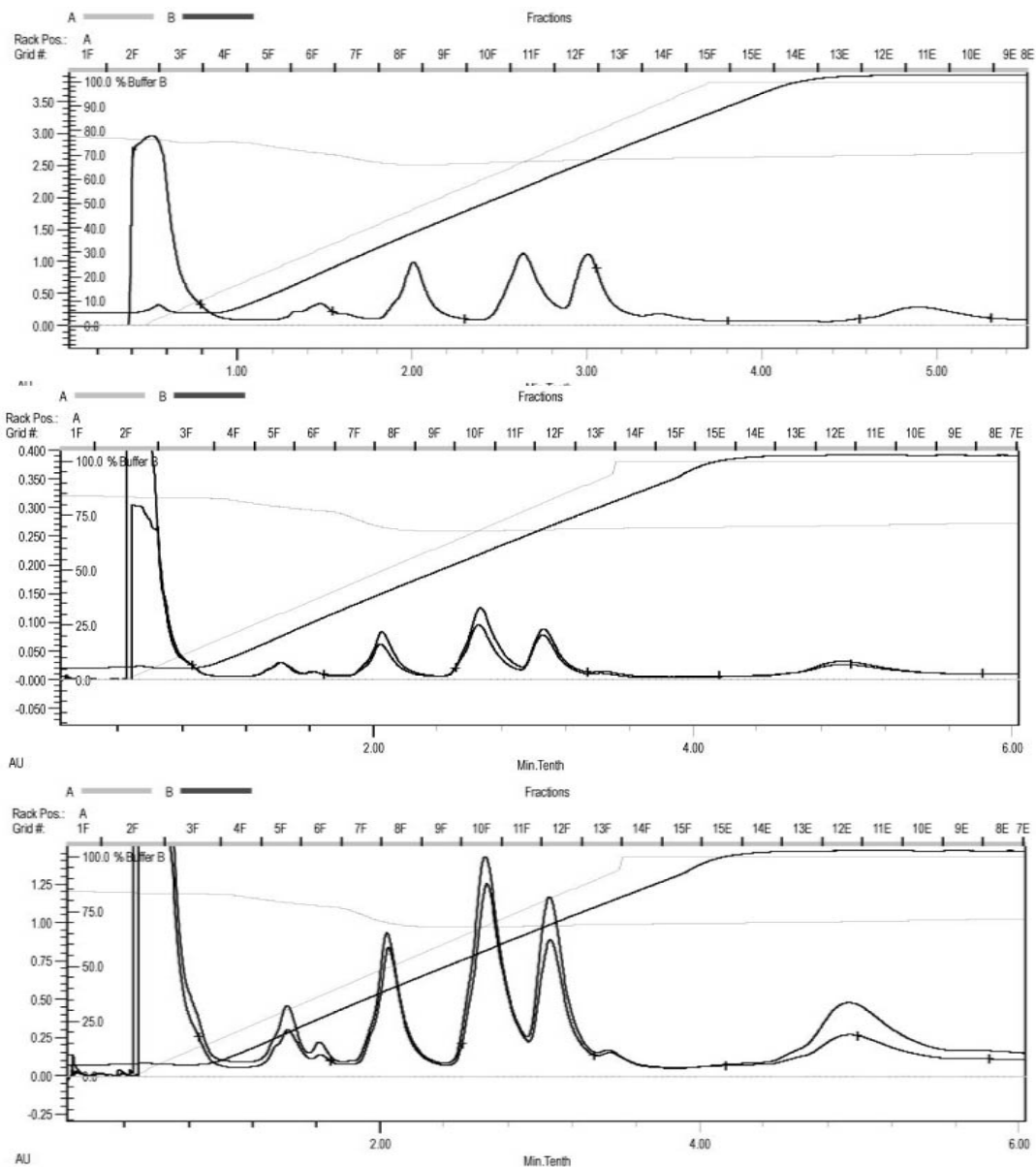
Figure 2: FPLC Fractionation of RDB rattlesnake venom. Three concentrations of RDB venom were fractionated by FPLC over the course of two days (A, B and C) with C being most concentrated. The venoms displayed a similar pattern over the run with slight shifts in retention time. The gradient from 100%A-0%B (0M NaCl, pH 8.0) to 100% B (1M NaCl, pH 8.0) and was the gradient used for the fractionation experiments in this project.
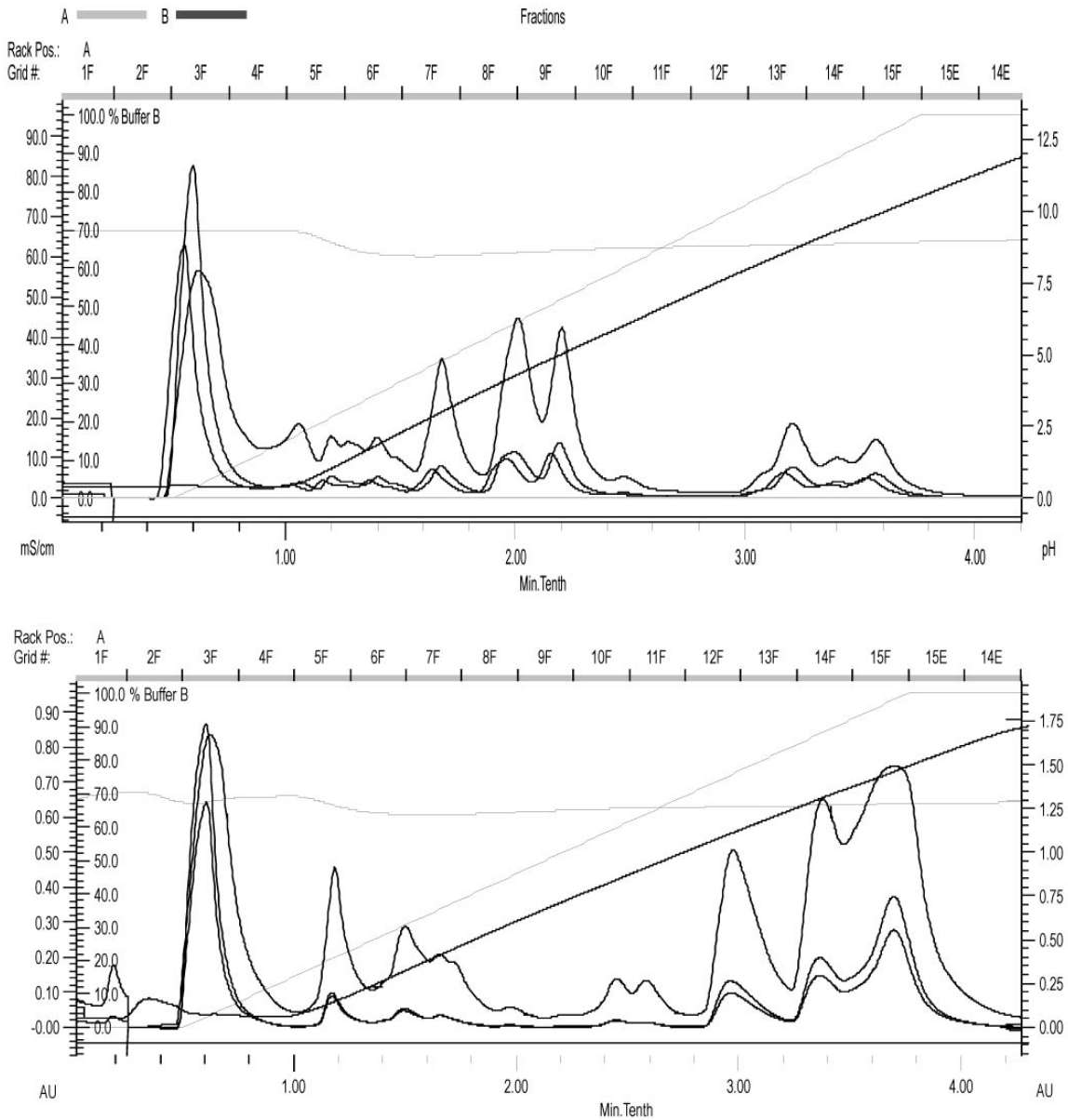
Figure 3: Observed fraction difference between venom A and B from 2011 vs. 2012

Figure 3 shows differences in snake venom from one year to the next though from the

same snake. Venom in (A) was obtained in 2010 while venom in B was obtained in 2012.

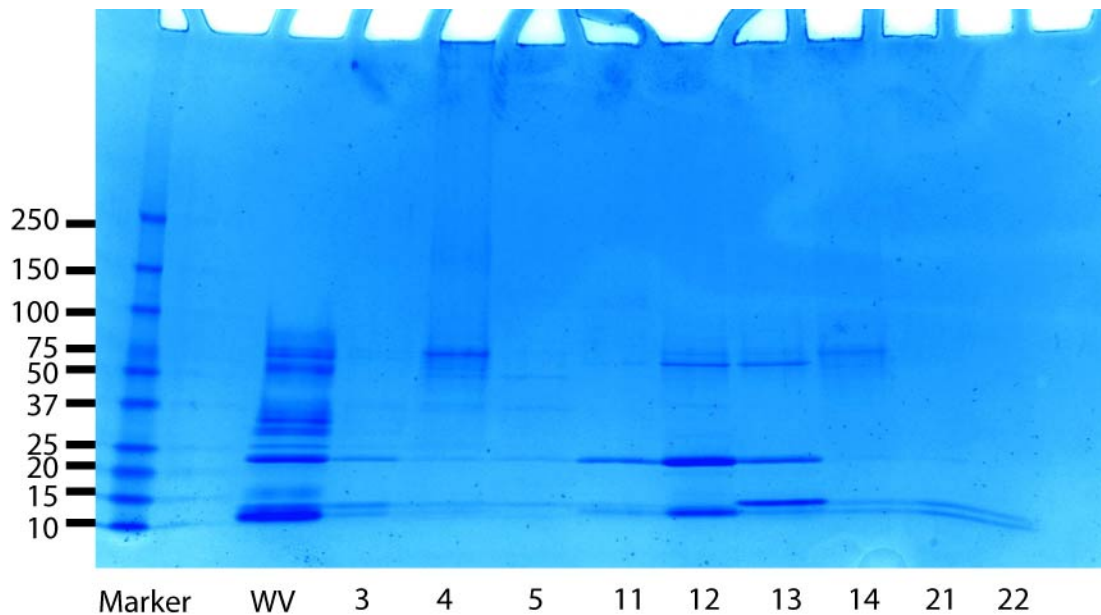Gradients were similar to those used in Figure 2.

Figure 4: SDS-PAGE of FPLC purified snake venom. This gel was stained with Coomassie stain after running. The Marker lane is indicated with MW for each marker shown on the left. WV indicates whole venom and each fraction is numbered. Only fractions showing a positive trace on the FPLC were separated.

Following the FPLC, snake venom fractions needed to be 'desalted' prior to adding them to cell culture media. If not, the media could become hyperosmotic and the changes in salt concentration could have a deleterious effect on the cells, regardless of the venom itself. The desalting was conducted using a 1000 dalton cutoff dialysis membrane stretched over the open end of an eppendorph tube. It was secured with a 'dental' type of rubber band (figure 5).
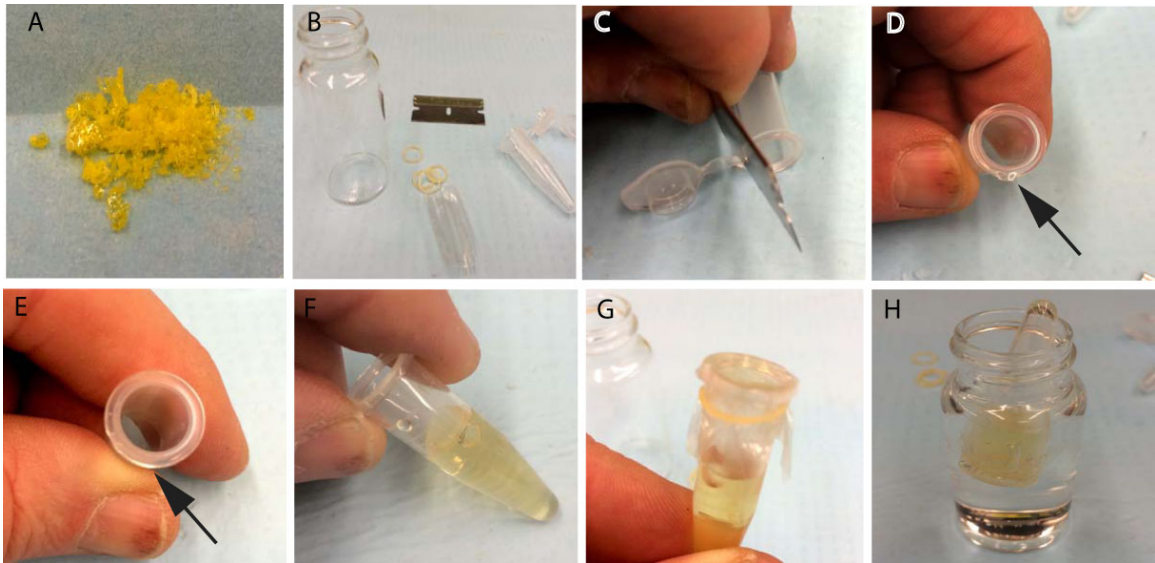
Figure 5: Steps for desalting FPLC fractions containing snake venom proteins.
(A) Shows 29 mgs of Red Diamondback snake venom after dehydration on the speed vac.
(B) shows all the parts necessary for desalting the snake venom fractions. (C) Trimming
the eppendorph lid away (arrow). (D) Removing the edge where the lid was attached so
that it does not puncture the membrane (Arrow) (E). Snake venom was pipetted into the
eppendorph (F). (G) The membrane was stretched over the eppendorph and secured with
a rubber band. (H) The eppendorph with venom and membrane was turned upside down
in a 10 ml vial containing ddH$_2$O.

SPECIFIC AIM 1

Test the hypothesis that snake venom fractions can slow proliferation or cause cell death in lung or neuroblastoma cell lines.

VENOM CYTOTOXICITY

The ability of snake venom fractions to cause cell death was analyzed by using MTT assay, LDH assay, propidium iodide assay, Hoechst and annexin staining.

<u>Neuro-2a Cell Death Assays</u>

The first analysis for cell death was a simple visual examination of the cells on the phase contrast inverted microscope (Figure 6). The neuro-2a cells were treated with one concentration of RDB fractions for 4 hours followed by observation with the inverted phase microscope. Fractions 3, 4, 5, 8, 9 and 10 displayed characteristics similar to cell death, suggesting that the venom did work. Other fractions appeared to not have an impact on cell proliferation or death.

The Neuro-2A cells were treated with one concentration of RDB fractions from 0-6 hours followed by the MTT assay. The MTT assay indicated some activity in wells containing fractions (Figure 4). Fractions 5-10 either showed small activity or did not show activity. Two wells 4 and 12 showed a reverse trend, suggesting that the SV proteins had proliferative capabilities.
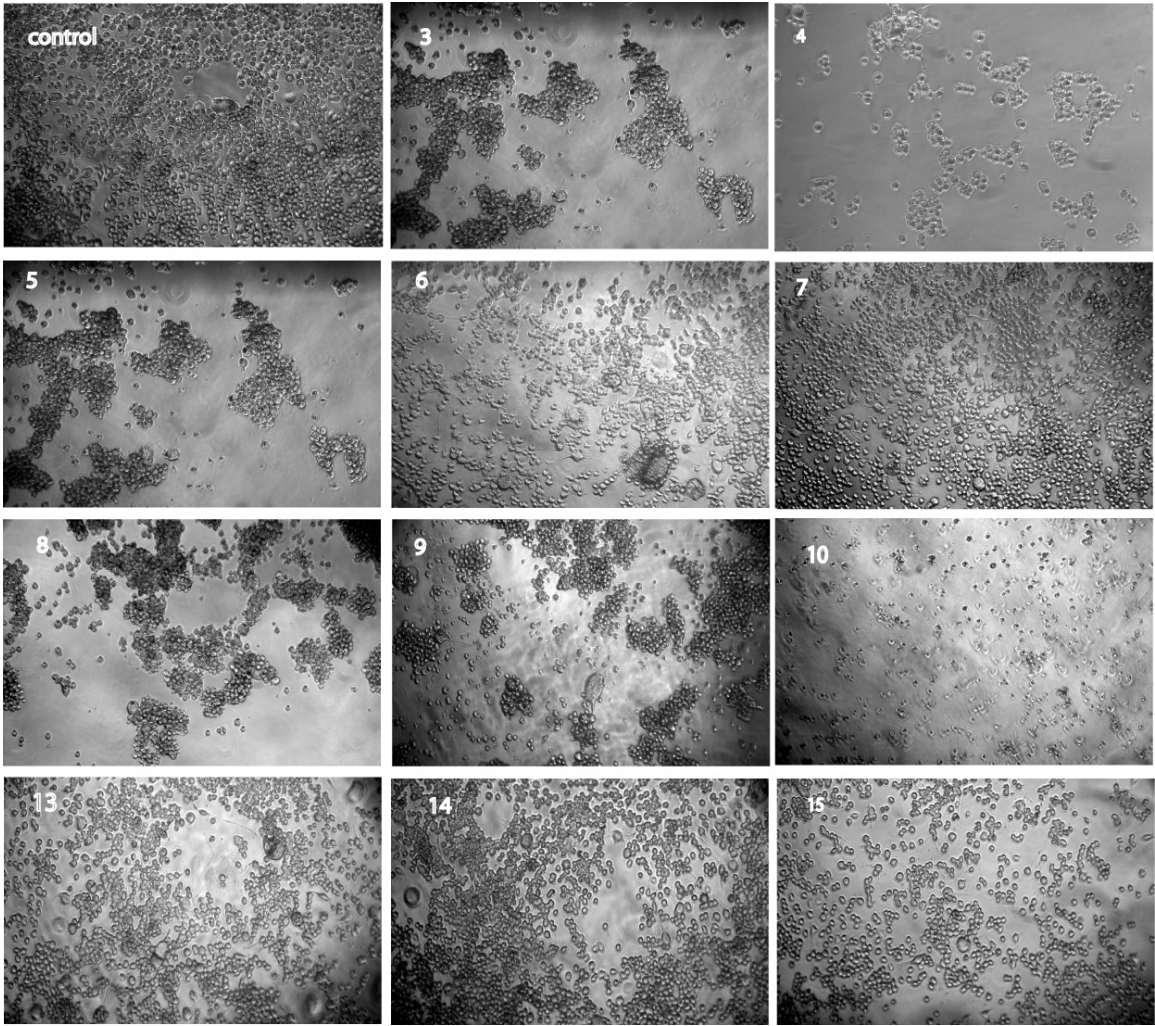
Figure 6: Neuro-2a cells post 4 hour fraction treatment. Cells were treated with a 10% solution of snake venom or water (control) in media. Cells were observed on 20X and pictures taken on an Optronics Magnafire CCD camera.

The Neuro-2A cells were treated with increasing concentrations of RDB fractions for two hours followed by the MTT assay. The MTT assay indicated some activity in wells containing fractions (Figure 7). Fractions 4, 5, 9, 10 11 and 14 showed no effect over time with the single dose of SV. However, there was an inhibitory trend in cell proliferation at several time periods in fraction 3. Likewise, there was a strong inhibition of proliferation over time in fractions 19 and 20. One fraction, 12 showed a reverse trend, suggesting that the SV proteins had proliferative capabilities. From this data, we chose to treat the cells for 2 hours with SV fractions in the rest of the experiments.

The neuro-2A and lung cells were treated with RDB fractions for four hours followed by the LDH assay. The assay indicated high LDH activity in wells containing fractions 4, 5, 9, 12, 21 and 22 showed no response to the snake venom (Figure 5). On the contrary, fractions 3, 11 and 14 showed an increase in MTT suggesting proliferation over the concentration curve. In contrast, fractions 19 and 20 showed inhibition indicating a slowing of proliferation or cell death.
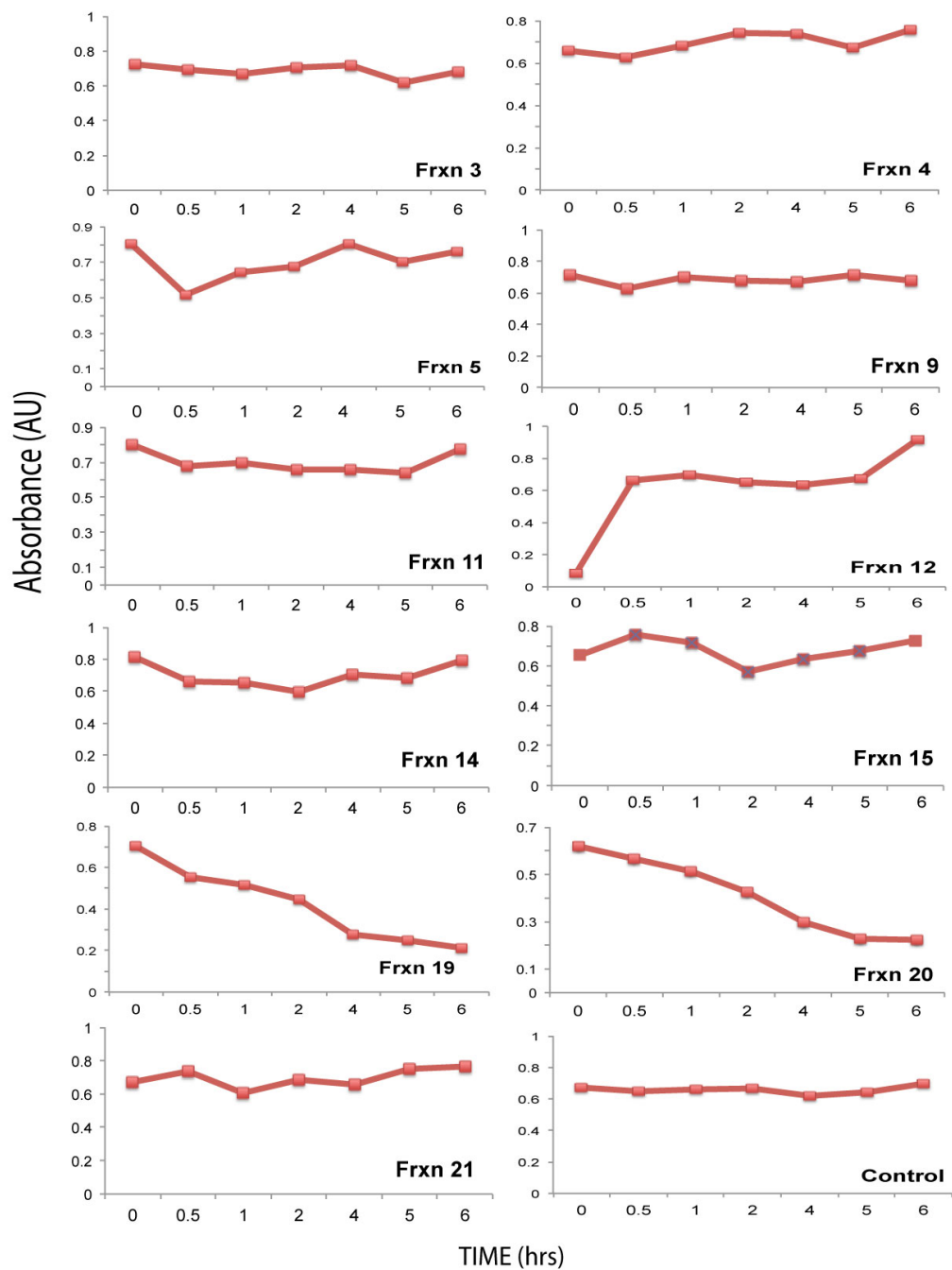
Figure 7: Effect of snake venom fractions on neuro-2a cells over time. Following the appropriate treatment time with snake venom, the cells were assayed by an MTT assay. The Control cells are shown in the lower right corner. Absorbance was determined at 595 nm on a Packard Fusion Plate reader.
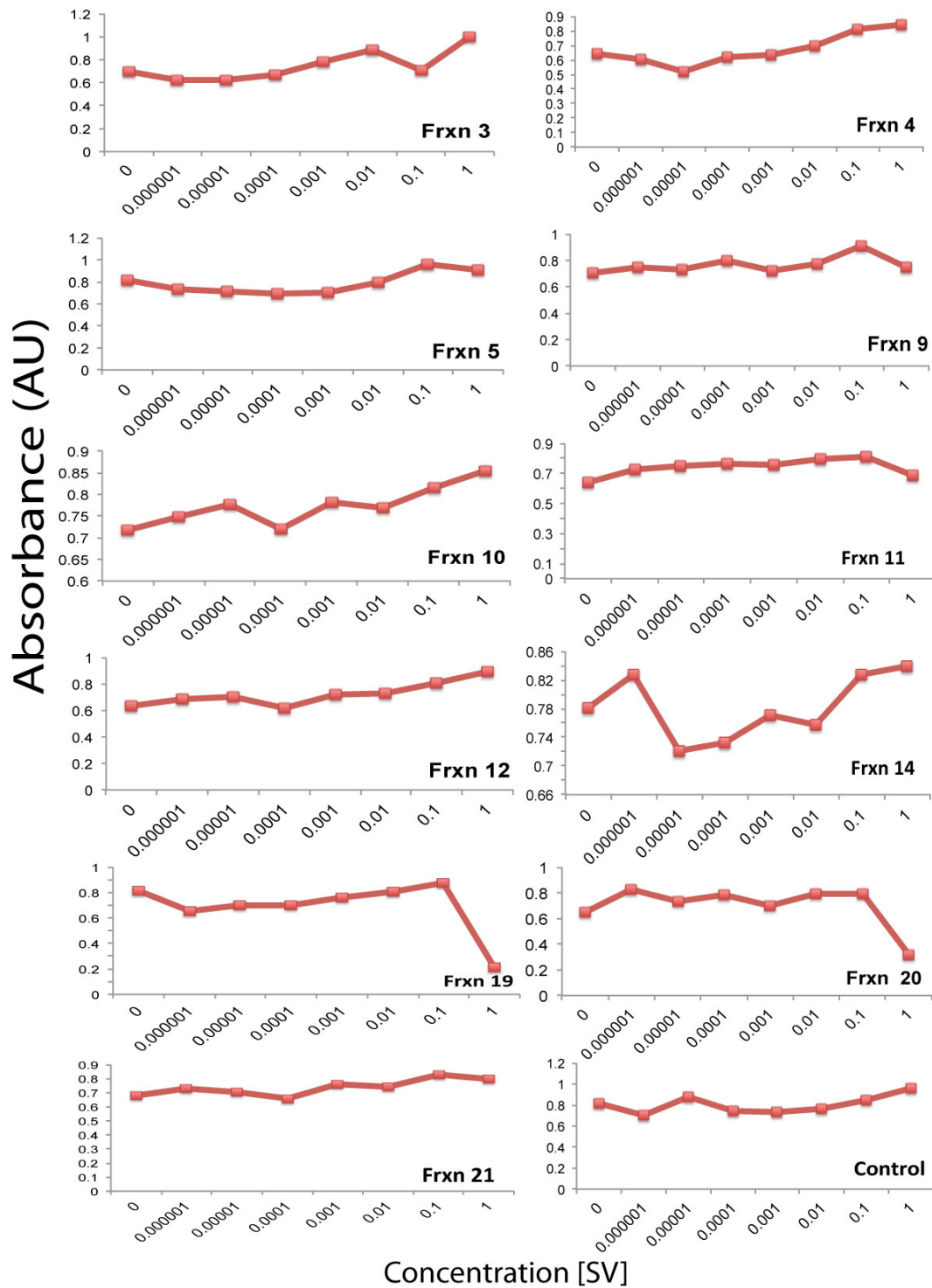
Figure 8: Effect of varying doses of snake venom fractions on neuro-2a cells.

Following 4 hour treatment with snake venom, the cells were assayed by an MTT assay.

Control cells lacking snake venom fractions are shown in the panel on the lower right.

Neuro-2a cells were tested for necrosis using the LDH assay. LDH activity was observed in specific snake venom treated cells following the 2-hour incubation (Figure 9). The figure indicates that fractions 3, 5, 9, 13, and 21-24 had varying degrees of increased LDH activity. This firmly establishes that necrosis was ongoing in these cells. Since cell death was apparent at a very early stage, we did not test for apoptosis which requires a much longer time period to work.
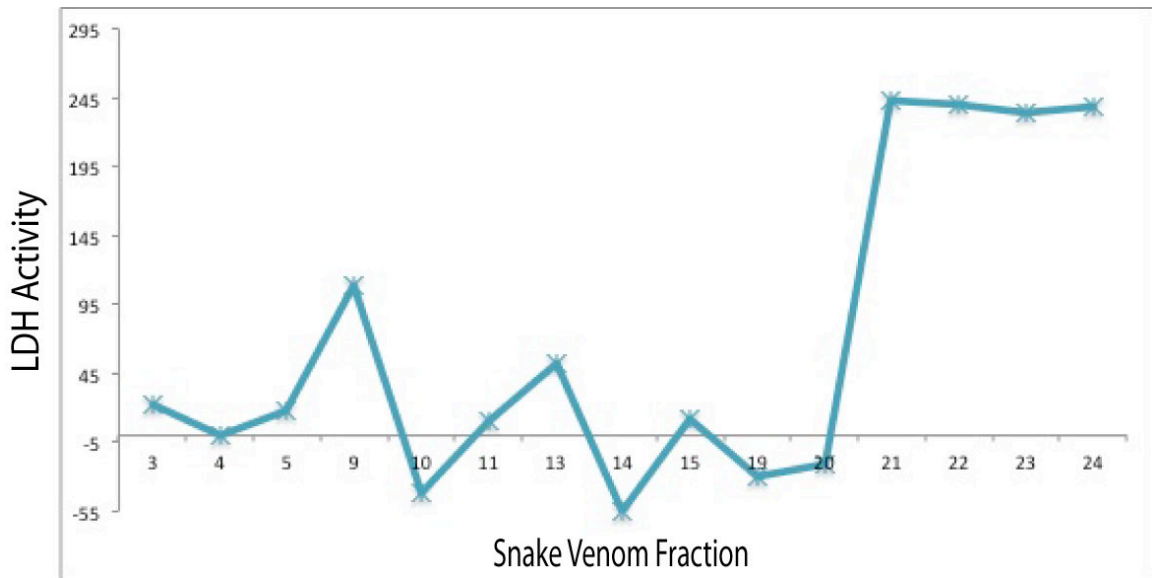
Fig. 9: LDH activity for each snake venom fraction was determined following 4 hour treatment with the fractions. LDH was assayed for each fraction. Note that the line went below zero on some fractions. This is due to the activity in the control wells being lower than the LDH in the treated cells. In these cases, subtraction of LDH gave a negative value.

Live cell membrane cannot be permeated by propidium iodide, therefore any positive fluorescence observed is indicative of cell death due to damaged cell membrane integrity. Fractions 4, 11, 12, 13, 14, and 15 showed the highest amount of fluorescence

observed. PI fluorescence was also observed at a lower amount in other fractions

suggesting that more fractions were affected than in the LDH assay.

Figure 10: Effect of snake venom fractions on cell death. Cells were treated with snake venom fractions for 2 hours at the fixed concentration of snake venom, followed by fixation and analysis with propidium iodide (Nicoletti et al., 2006). PI staining of treated Neuro-2A showed differential uptake of PI into the cytosol and nucleus of different cells.
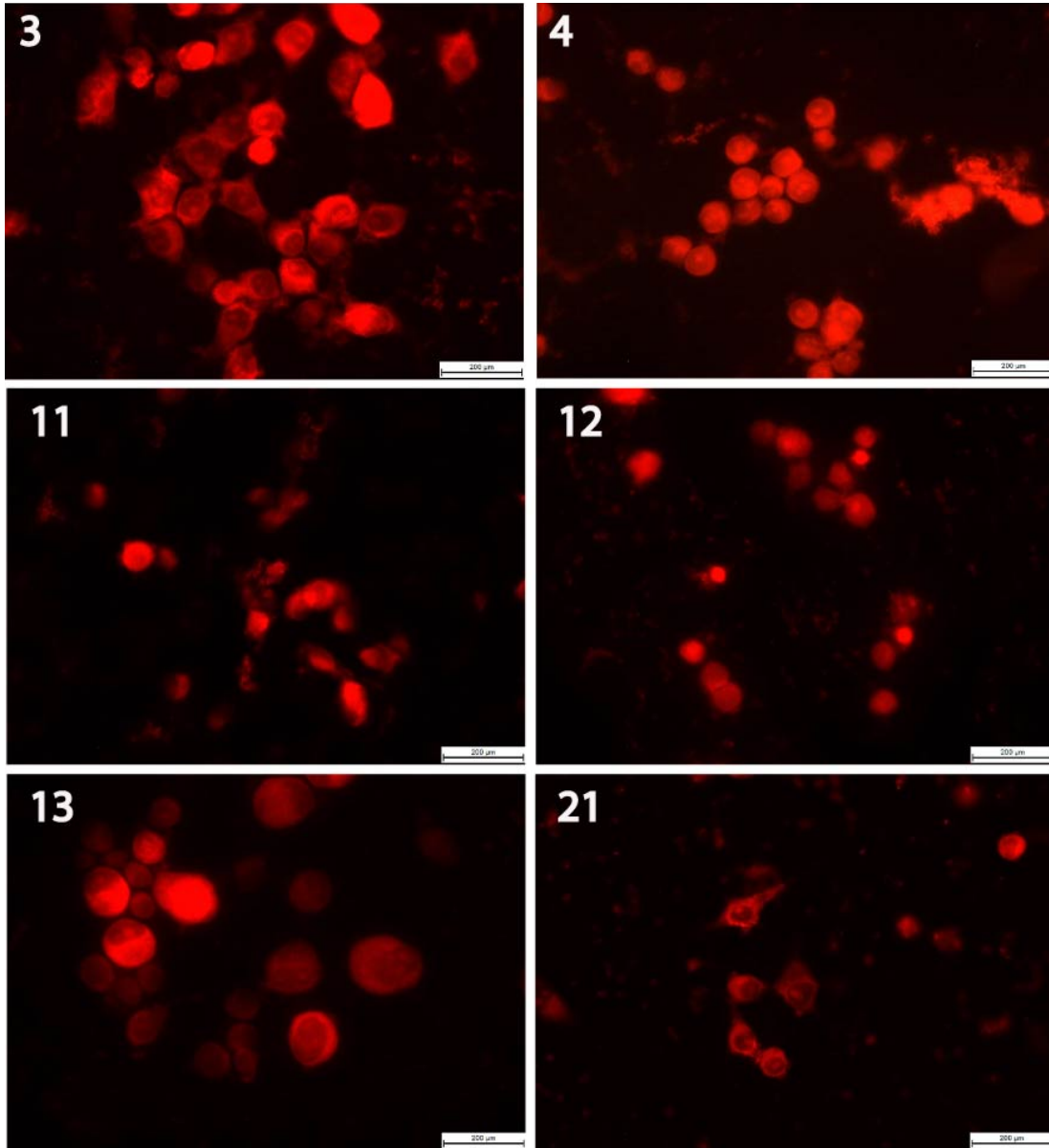
Cytotoxicity of Snake Venom on Lung Cells

Lung cells were tested by two mechanisms for cytotoxicity, annexin staining and Hoecsht staining. Annexin resides on the inner plasma membrane of the cell and is not available for staining unless the membranes have been compromised and the cell is potentially in a death state. Figure 11 shows that venom fractions 3, 9, 14, 19, 20 and 21 showed no or very low staining. Conversely, 4, 10, 11, 16 and 23 showed medium to strong staining indicating these cells were in a death state.

Hoechst is a fluorescent dye that binds to the DNA of cells and fluoresces blue under UV light. When the lung cells were stained with Hoechst dye, the nuclei were all stained (Figure 12). The results clearly show size differences in the lung cell nuclei. Particularly, fractions 3, 4, 9, 11, 13, 14, 16, 21, and 23 appeared to be larger than the nuclei from control cells. Fractions 9, 10, 11 and 14 appeared to have assymetrical or disrupted nuclei. Fraction 20 appeared to have a strong, dense staining.
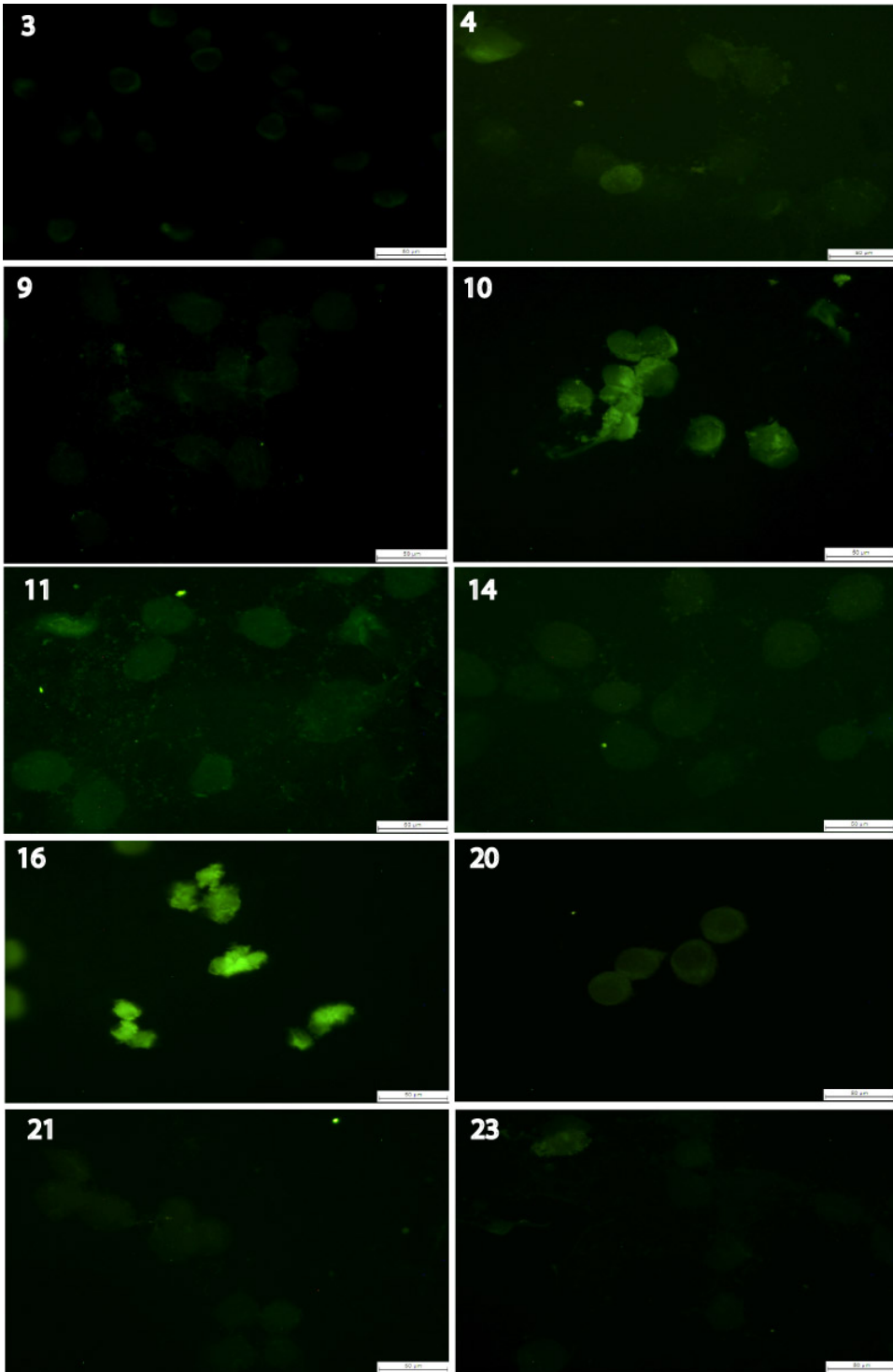
Fig. 11: Annexin staining of lung cells post treatment. Cells were treated with the SV fractions, fixed in 4% PFA and stained with annexin at 1:250 dilution. Images were taken of the cells and levels adjusted to the same threshold in Photoshop CS3. The final images were grouped in Adobe Illustrator..
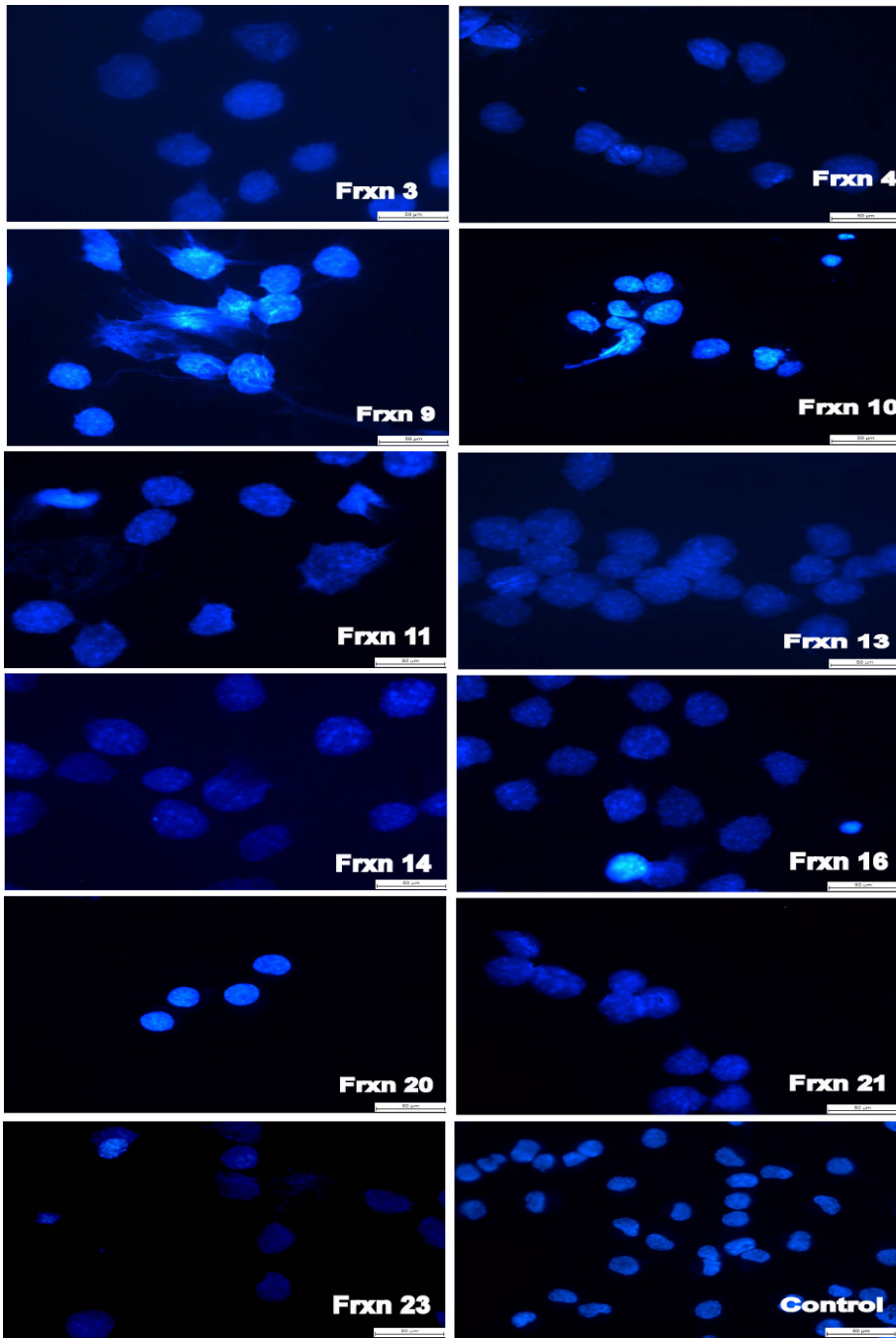
Fig. 12: Hoechst staining of lung cells post treatment. Images were captured on a Leica DMR fluorescence microscope and captured using a Leica camera. The Hoechst dye was found to stain all the nuclei, though some were brighter and some nuclei were larger than in control cells.

SPECIFIC AIM 2

Test the hypothesis that cell functionality can be disrupted by specific snake venom proteins obtained by fractionation.

FUNCTIONAL ANALYSIS OF NEURO-2A and LUNG CELLS

Cellular functionality of neuro-2A cells as well as lung cells is tested and analyzed using the TLC assay and Chromogranin staining.

CHROMOGRANIN STAINING

Chromogranin (CGA) is an endogenous protein resident in nearly every neuroendocrine cell [Lloyd, 1988]. To test the effect of snake venom on the neuro-2a cell line, which is a neuroendocrine type cell, we tested the ability of SV to perturb the intracellular targeting of chromogranin A to the regulated secretory granules. Punctate granules were observed in cells treated with fractions 3, 5, 11, 14 and 21. The other fractions displayed CGA staining, however it was not observed in punctate granules, but instead was observed in a diffuse pattern suggestive of ER staining [Cool, 2009; Yan, 2011].
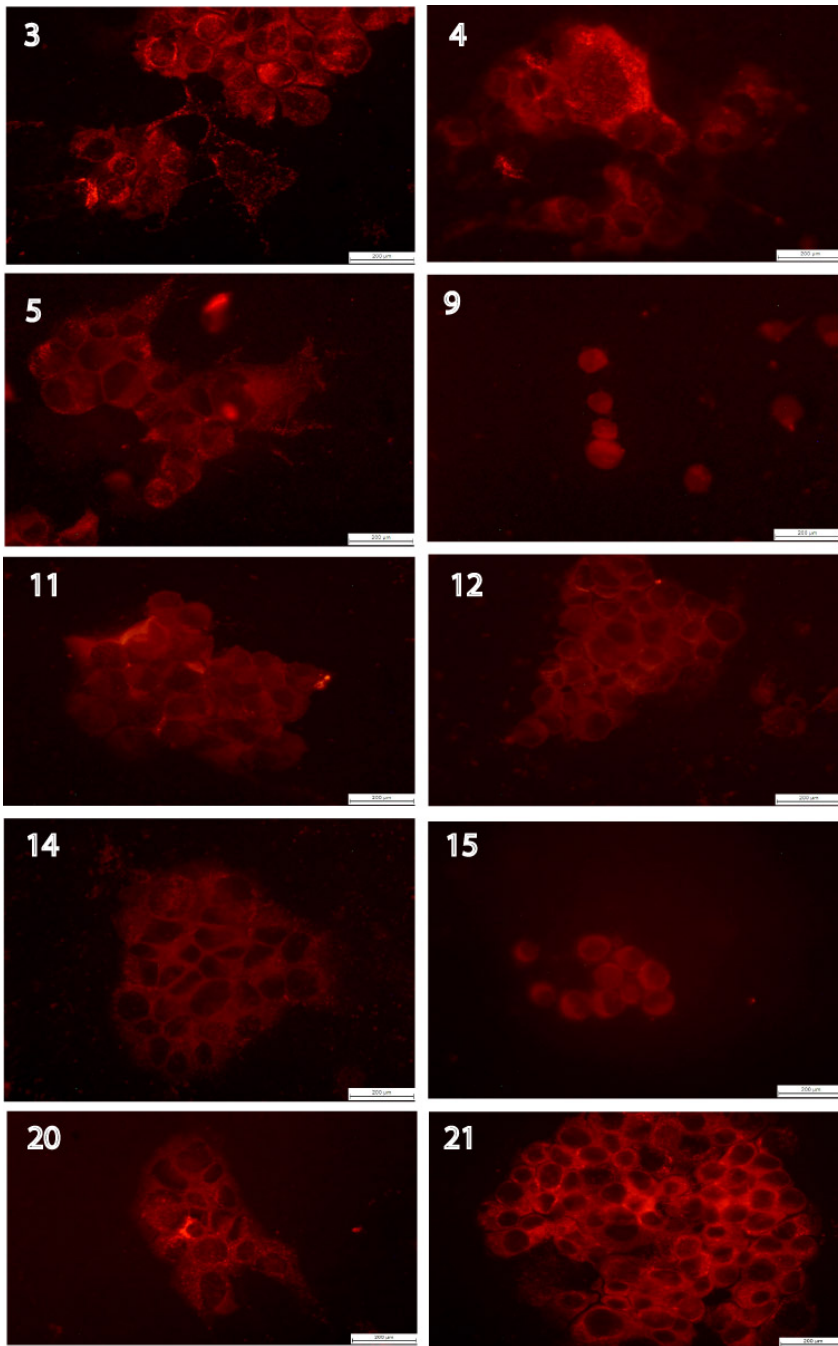
Figure 13: Chromogranin staining of Neuro-2A cells post treatment with snake venom fractions. Cells were stained using a CGA antibody at 1:250 dilution.

TLC PLATE:

A TLC plate was run with lipids extracted from the media of cells following treatment with snake venom. Following the run, the TLC plates were sprayed with a solution of primuline and viewed on the Chemdoc MP+ under a 488 nm Dylight setting. TLC plate results suggested a strong lipid release by snake venom treated cells compared with the control cells (Figure 14). In table 3, specific lipid band ratios were found to be increased in band b, as well as specific other bands. A graph (Figure 11) generated from the lipid values showed clearly in graph format the table data.

Figure 14: TLC Plate analysis of lipids secreted from lung cells. The density for each band (a-g) was determined using the Image Lab software (Bio-Rad, Hercules CA). Each lane (1-12) represents the snake venom fractions and C1, C2 and C3 indicate the control cells that were not treated with venom.

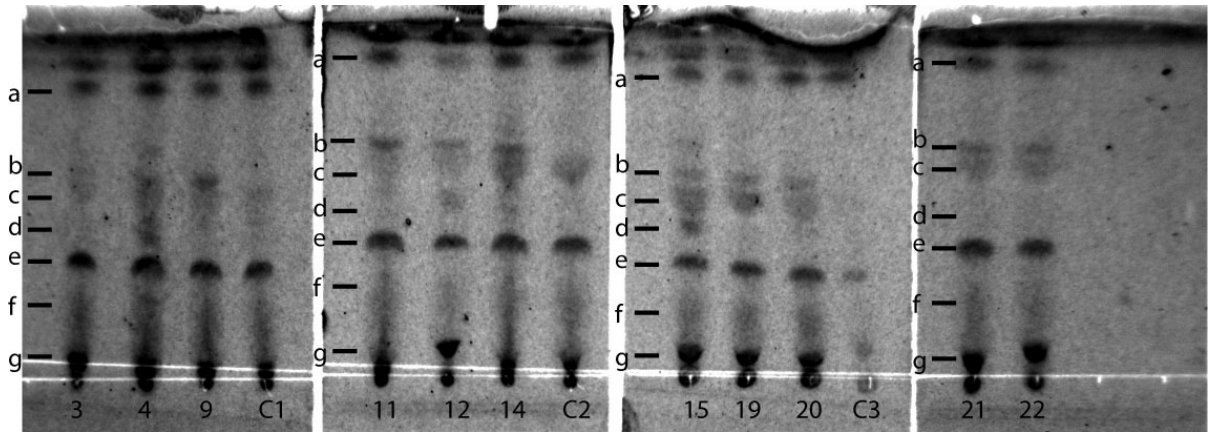| Table 1: TLC Plate Analysis of Lipids Secreted from Lung Cells in Response to Snake Venom Fractions														
Band No.	3	4	9	C1	11	12	14	C2	15	19	20	C3	21	22
a	56	70	44	57	65	32	51	132	47	42	93	111	51	42
b	236	880	1753	67	1820	1056	1337	99	498	473	506	134	632	1776
c	49	83	97	72	66	62	211	177	123	178	80	51	60	121
d	64	730	53	127	122	610	162	90	509	168	102	83	108	216
e	123	151	119	114	160	118	129	145	113	115	123	41	157	139
f	72	326	320	85	95	121	63	169	92	88	114	46	79	123
g	177	250	108	96	104	203	73	118	182	138	106	86	242	251

Grey boxes indicate control lanes which were averaged.

Percent of control = (Density/Control)X100

TLC Plates were RP-Plates run in a 51:32:9:5:3 (chloroform:methanol:acetic acid:acetone:ddH$_2$O)

TLC Plate lipids were stained with primuline.
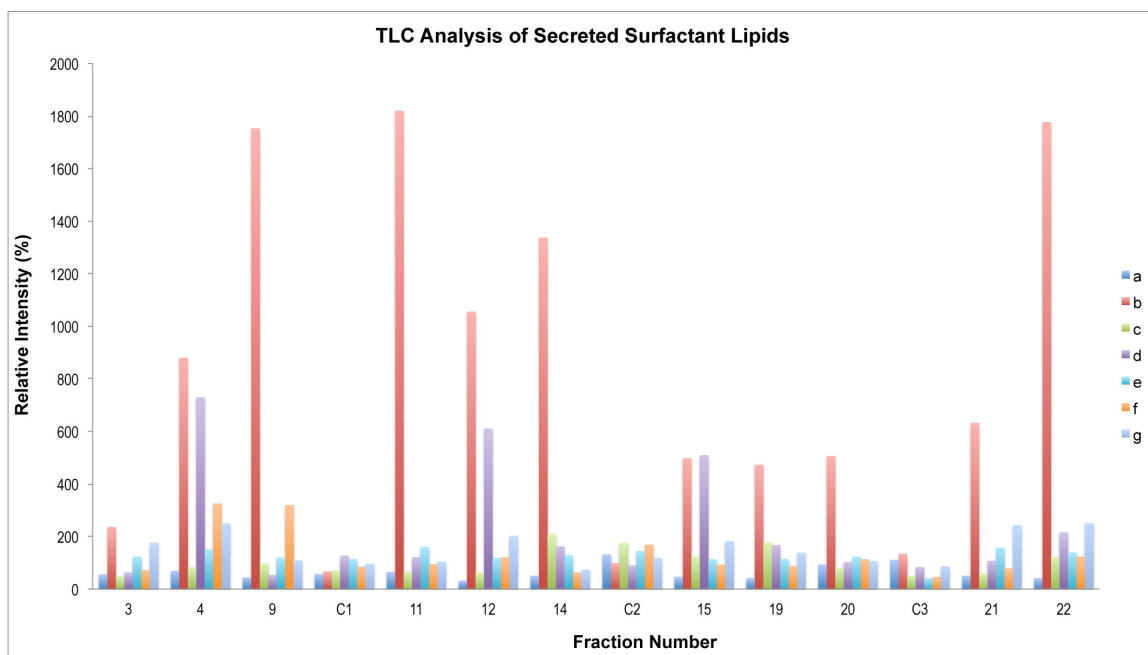
Figure 15: TLC analysis of Secreted Surfactant Lipids. Lipids bands in each lane representing each snake venom were ratioed to the average of the control cells.

SPECIFIC AIM 3

Identify proteins in individual snake venom fractions and relate these to pathways

involved in regulation of the cellular functions.

PROTEIN IDENTIFICATION:

Once specific fractions causing changes in proliferation, cell death or cellular

functionality were identified, mass spectrometry was used to specifically identify the

causative proteins. Results from the MS indicate that fractions 4, 11, 12, 13, 14, and 15

contain hemorrhagic toxin II, also known as hemorrhagic metalloproteinase HT-2. All of

the fractions causing the highest amount of cell death contained this particular protein as

indicated by MASCOT searches. Other fractions contained other proteins of interest,

such as a myotoxin, apoptosis inhibitor, and apoxin. Some of these fractions had no

apparent effects on the cell lines and thus weren't shown in every death assay or

functionality assay. However, they should be examined more closely in the future.
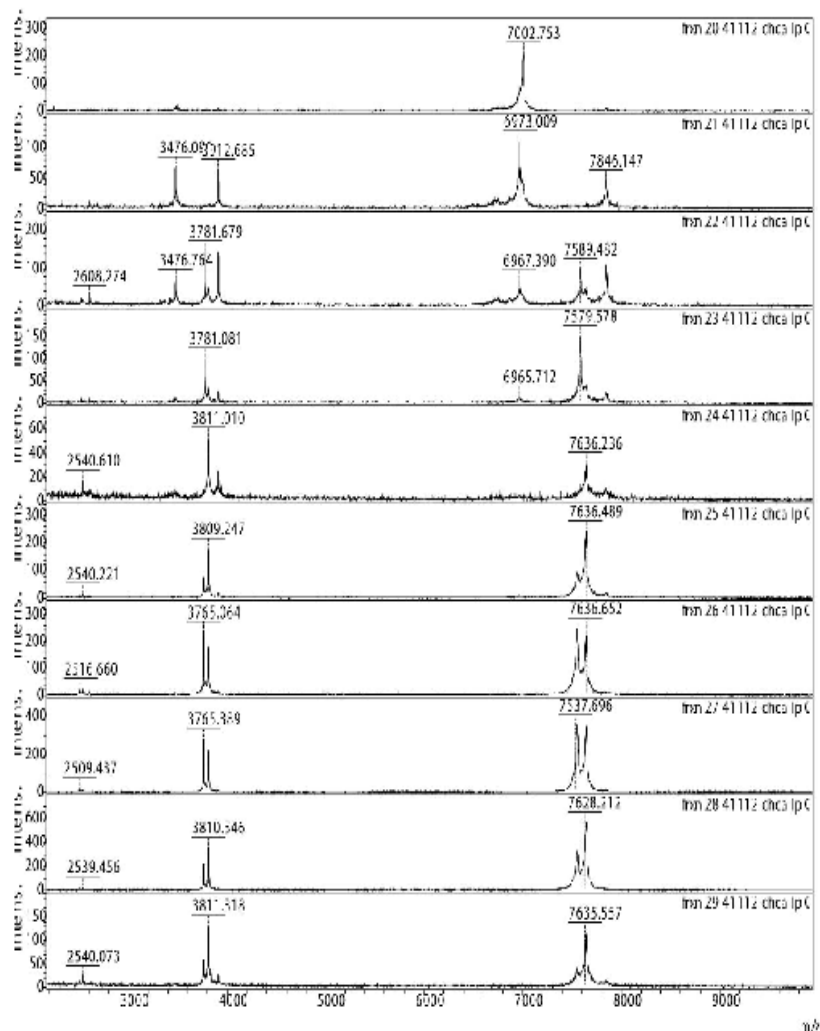
Fig 16. Peak analysis of SV fractions using MS following FPLC. Representative spectra are shown which have multiple peaks for proteins found in the snake venom fractions. Each of these spectra were digested with trypsin and subjected to LIFT MS/MS for protein sequencing.

Table 2: Snake venom sequences identified by MS sequencing.					
FPLC Frxn	Mass	Peaks m/z	MS/MS sequences	Protein/Protein Family	Score
4/5	23306	1192.4611	-SYEFSDASMR	Hemorrhagic Toxin II (Ruberlysin)	208
4/11/18	23534	1192.574	R. SYEFSDASMR. Y	Hemorrhagic metalloproteinase HT-2 (Ruberlysin)	306
9	58370	1037.455	-FWEDDGIR	Apoxin I	53
15/21	23306	1192.4611	-SYEFSDASMR	Hemorrhagic Toxin II (Ruberlysin)	104
18/19	23534	1246.6848	-K. NTLHSFGEWR. K	Hemorrhagic Toxin HT-2 (Ruberlysin)	67
18	14527	1246.6445	K. LTNCDPKKDR. Y	Phospholipase A2 (Myotoxin I)	22
20	5328	930.5320	K.KGGHCFPK. T	Myotoxin (Toxic Peptide C)	64

IV. DISCUSSION

The main purpose of this study was to determine the effects of Red Diamondback rattlesnake venom on cancerous cell lines. The top questions in consideration were: 1) Can snake venom factions cause cell death 2) what are the effects of those fractions on the cancer cells; 3) what are the causative proteins and their effect on cell functionality? To answer these questions, we examined the effects of snake venom on cell death and proliferation; cellular functionality; and identified proteins in the venom fractions.

SPECIFIC AIM I

Test the hypothesis that snake venom fractions can slow proliferation or cause cell death in lung or neuroblastoma cells.

We observed that specific fractions had an inhibitory effect on cellular proliferation while other fractions appeared to cause proliferation. When cell death was assayed similar fractions appeared to give a strong reaction that resulted in cell death. These assays were used solely as a first pass screening effort to test the snake venom fractions prior to moving on with further tests. As such, they provided some overlapping and complimentary results as well as divergent results suggesting they may have an effect on the cells, but not the same effect always. While we talk about cell death in these assays, there could be an alternative answer to whether the cells were dying as a result of the snake venom interacting with cellular death processes. We could speculate that the

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protease activity associated with the snake venom fractions was cleaving proteins expressed by the cells to specifically bind the cells to the culture dish surface. In this manner, the cells would be released, causing them to round up and die. Since they are adherent, the loss of their substrate to grow on would result in their death, and the venom would not have to enter or interact with other mechanistic functions of cell death. While it is interesting to speculate that these two cell lines might undergo cell death in the presence of specific fractions of snake venom, it is a long step towards the possibility that they could be used in a therapeutic role. Likewise, there is a potential benefit for a component in the snake venom fractions being used to help cells proliferate. This could be very important in situations like neuronal cell death or spinal cord injuries where the goal is to improve the outcome or initiate proliferation or regrowth of spinal cord cells. The venom component could be used to 'seed' the area and act as a type of chemokine to cause cells to proliferate and fill in the gap as an effective treatment for the condition.

SPECIFIC AIM II

The goal of this aim was to test the hypothesis that cell functionality could be disrupted by specific snake venom proteins obtained by fractionation.

LIPID SECRETION

Aside from their role in exchanging oxygen and CO_2, lung cells, particularly type II alveolar cells, are also responsible for secreting lipids as part of the surfactants used to help the lung function (Grgic, 2003). This is an especially critical area due to the potential for premature birth and the need to quickly increase fetal lung secretion of surfactants prior to birth. Corticosteroids have been given to pregnant women for more

than 30 years now to improve fetal lung maturity [O'Reilly, 1988; O'Reilly, 1989; O'Reilly, 1991; Vidaeff, 2004]. A standard dose of corticosteroid (dexamethasone or betamethasone) is 12 mg every 24 hours for 2 doses. However, in some circumstances, 24 hours is all the time there is before delivery, and both doses have to be given within the 24 hour period. Thus, a non-lethal chemical that could speed this process would be an advantage over the corticosteroid. Our results are the first to show stimulation of lung surfactant release due to snake venom exposure. The results suggest a fast increase in production of the lipids and subsequent secretion of lipids from the cells. On the contrary, the results could also be explained by the lipids in the lamellar bodies being released as the cell membranes are permeablized. The results suggest that snake venom components might be able to cause changes in the lungs that could cause breathing difficulty in snake bite patients, especially if more surfactant is being secreted. This is potentially an interesting finding as it could allow for an increased production of surfactants in fetal lungs. It would be interesting to speculate that a snake venom component could someday be given to pregnant women to mature the fetal lungs in the cases of probably premature delivery. However, this may be more problematic due to the fact that giving snake venom to a pregnant woman might be of great concern to safety and ethical panels for human use. Nonetheless, some of these fractions should be tested in animal models to determine if they do have a future use as therapeutics for lung types of diseases.

CHROMOGRANIN STAINING

Chromogranin A is produced in endocrine and neuroendocrine cells and is packaged in regulated secretory granules in these cells [Huttner, 198]. It is a sensitive endogenous marker for targeting proteins into the regulated secretory granules. When

there is a disruption in these cells, the regulated secretory proteins are not targeted efficiently or at all to these granules. Thus, chromogranin A staining in Neuro-2a cells is a useful marker for normal versus perturbed cell functioning. Staining treated Neuro-2a cells with chromogranin A antibodies showed that the cell function was compromised by SV fractions with six fractions showing the most change in cellular functionality. Endocrine disruption by snake venom is not normally a first concern but may be of concern later, if the patient survives the snake bite. However, if the patient lives, and depending on the location of the bite, some endocrine or neuroendocrine function may be compromised by the exposure to the venom.

SPECIFIC AIM III

Identify proteins in individual snake venom fractions and relate these to pathways involved in regulation of the cellular functions.

MASS SPECTOMETRY

Based on the results obtained from the MS, we have determined a major protein residing in the venom of RDB snakes is Ruberlysin, also known as hemorrhagic toxin II. While this metalloproteinase was present in the venom, there were other proteins that were also identified; i.e, myotoxin and apoxin. The FPLC separation was not good enough to indicate whether one or a combination of all of these were causing a disturbance in cellular integrity as well as intercellular trafficking disturbances. As speculated previously, cell death, and even lipid release may have been a result of the release of the cells from the plate or lysing of the plasma membrane. With RDB venom being mainly a hemotoxic venom, it was no surprise to find this protein to be present in

huge amounts; however, its effects on neuro-2A cells was very interesting. The presence of other components such as myotoxin and especially apoxin, as well as numerous other proteins not identified in this study, but known to exist suggest that the venom may hold some interesting keys to unlocking cellular functions, that may someday be useful for therapeutic uses.

CONCLUSION

Exposure to Red Diamondback Rattlesnake venom fractions causes cellular death in neuroblastoma and adenocarcinoma cells at a 1:8 dose ratio in 2-4 hours. The exposure to these fractions damages the cell membrane of the neuro 2A and lung cells, leading to loss of axonal communication in neuronal cells and cell death in both cell types. Apoptosis and necrosis are likely to be the form of death caused by RDB protein toxicity. Assays such as the LDH and MTT indicated the effects of the multiple proteins present within each of those fractions. Our data indicates high death rate in six of the fractions containing high amounts of the following proteins: Ruberlysin, phospholipase A2, as well as Apoxin I. These proteins have indicated that they may play an important role in the apoptotic behavior amongst treated cancer cells. Ruberlysin is mainly present in hemolytic venom and as named, is involved in the lysis and destruction of blood cells. However, in our study, ruberlysin has shown its involvement in the disruption of cellular integrity and apoptosis. Phospholipase A2 is mainly involved in inflammation and may play a key role in cellular necrosis post treatment. Apoxin I is an apoptosis inducing factor that has shown involvement in the apoptosis of fraction treated cells. Six of the twelve RDB fractions showed the highest amount of the mentioned proteins present. However, further separation of these proteins was not possible, leading to a less than

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accurate analysis of the functionality of these proteins in relation to themselves, one another and their effects on that of the treated cells.

Obtained results give reason to further research on protein specific functionality of the RDB venom on various cancer lineage cells to obtain conclusive results. Research into the therapeutic use of venoms should continued as a possible anti-cancer treatment form as well as for lung maturity and as an anti-inflammatory for surgical usage. RDB venom is not as well researched as that of other venoms; this work would provide better understanding of the protein components of this specific type of venom. It will provide a better understanding of cancer cells functionality in exposure to toxin inducing proteins as well as their possible application in cancer treatment as well.

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