EFFECTS OF A NATURALLY DERIVED LIPID ON CELL VIABILITY AND INFLAMMATORY SIGNALING IN THE PRESENCE OF SNAKE VENOM

A Thesis

by

CLAY JORDAN LANGFELDT

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at Appalachian State University
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Abstract

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AND INFLAMMATORY SIGNALING IN THE PRESENCE OF SNAKE VENOM

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A lipid extract has demonstrated the ability to inhibit the activity of phospholipase A2 (PLA_2) found in the snake venom of the Viperidae family. This research focused on studying the effect this lipid would have in cellular systems through viability and inflammatory endpoints. Cellular models were first established with baselines in venom concentrations, lipid concentrations and time. Addition of the lipid extract at high levels, surprisingly reduced viability when in the presence of the venom. This effect was present in groups pre-incubated with the inhibitor as well as when venom was pre-incubated with the inhibitor. The amount of inhibitor (2.5 nmols) added was much larger than that of cellular phospholipids (0.4 nmols) in cell assays and so smaller amounts (0.5-0.05 nmols) were tested demonstrating no significant loss of viability but also no protection of cells. Inflammatory studies focused on the production of eicosanoids. Prostaglandin E2 and leukotriene B4 are two eicosanoids produced through different pathways from arachidonic acid metabolism following phospholipase A2 action. Concentration and time studies were carried out with the prostaglandin E2 but showed no inhibition by the addition of the inhibitor. Concentration and time trial studies of leukotriene B4

demonstrated the lipoxygenase pathway was stimulated and increased production of leukotriene B4. While venom did increase the concentration of leukotriene B4 there was no observed reduction with the introduction of the inhibitor.

Acknowledgments

First and foremost, I would like to thank the man who made this all possible. Throughout our many conversations through research and life in general, Dr. Mark Venable has been a great mentor and role model. He has always been extremely patient when I have one of my many questions but also has challenged me to think critically and grow in my quest through science and graduate school. I would also like to give thanks to my thesis committee, Dr. Michael Opata and Dr. Rachel Bleich. These two have been excellent resources for questions I have but also supportive advisors through my academic journey.

I would also like to thank all of my other teachers I have encountered through my time at App State with give more specifics appreciation to Dr. Seals and Dr. Ahmed and members of their labs, Dalton Sizemore, Austin Simmons, Gloria Rhoney and Eliza Watson. All have been vital to my projects by donating, teaching and generally assisting me in setting up cell culture and helping me troubleshoot any problem I encountered.

Another group I would like to give thanks to is many of the other graduate students in the Biology Department. Starting graduate school through the height of the COVID pandemic was difficult with many of us feeling isolated. Through different means many of us have come together and become a network of support for each other through many of our long running experiments. I would like to specifically give thanks to Tyler Olender, James Erny, Nicole Warnick, Madeline Wight, Emma Metcalf, Will Hardin, Ishani Chattopadhayay, Bronson Brown, Abbey Hockett, Ryan Whitley, and Andrew Grimes. All who have either assisted with my project directly or been a friend and I can lean on for support.

I would also like to show appreciation for my friends outside of graduate school. As the expression goes “It takes a village” and these people have been my village for my years leading up to Graduate school and more specifically through the process. I would like to thank Jake Siegal, Thomas Palmes, Alex Brashear, John Munday, Billy McLaughlin, and Maya Jarrell. All these people have been there when I returned from a long and sometimes disappointing experiment only to then boost my mood and support me through the process.

Finally, I would like to thank the establishment of Appalachian State University and mores specifically, the Biology Department and Office of Student Research who have given me the opportunity and funding to find and curate this passion I have grown to love over the past few years.

Dedication

I would like to dedicate this work to the three people who have been most inspirational throughout the entire process. First my parents, Cristy, and Lance. From a young age my mother has always supported my curiosity and helped me to grow into the person I am today. Everyday I strive to grow and better not only at what I do but who I am because of her. She has always put an emphasis on the people around her and impacted so many lives in a positive way. As for my father, he has been equally as supportive but has instilled in me a drive that I hope reflects in my work and the way I present myself. He has been someone I have always looked up to and strive to reflect his passion for life as well as his ability to inspire others. He has also had an amazing impact on many people throughout his life through his constant can make anything happen attitude and sheer will of force to succeed. Without these two and their constant support in all my endeavors through life I would not be who I am today and for that I would like to dedicate something I have worked so hard on to them.

There is a third person I would like to dedicate this work to and that is my partner Virginia. Through all the trying times of school and life I have had many friends who support me but no one truly understands or can raise my spirits like she can. Without her constant support and encouragement I do not believe I would have been able to complete this project or continued down this path in which I have so much love for. She is my best friend, and I would like to dedicate this project to her to show how immensely thankful I am for her.

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Foreword

This thesis was written in formatting that follows the structure of *Journal of Biological Chemistry.*

Introduction

Snake envenomation and the resulting necrosis and death are a neglected tropical disease caused by toxins in venom induced through bites or spraying in the eyes. Around 5 million people are bitten a year, with around 100,000 of those ending in fatalities (1). Those who are not killed by the venom can still suffer severe consequences such as necrotic loss of limbs and hospitalization (1). There are treatments for snake envenomation through therapies such as antivenin, but these are limited in availability as well as effectiveness.

Firstly, antivenom production requires acquisition of the snake's venom. This is a dangerous process and ethical problem as the snakes or handlers can be injured during the process (2). Venom is then administered to animals that then make antibodies which can be collected and used as antivenom. While the efficacy of antivenom is high, variability in the species of snake, misidentification of the species and health of the injected animal can alter the effectiveness of the antivenom (2). This process is costly, supply is limited, and it is usually unavailable to those most at risk (2).

Recent research has focused on small molecule inhibitors to neutralize specific enzymes in venom which when used in combination have showed promise (3). Snake venoms differ in composition between species, but most are composed of the same neurotoxins, hemotoxins and cytotoxins (3). Some common types of toxins are 3 finger toxin (3FTx), snake venom serine protease (SVSP), snake venom metalloproteinase (SVMP) and phospholipase A2 (PLA2) (4). These all help in the digestion of tissues and cells which help in killing or slowing prey or predator (4).

The genus of snakes, Crotalus are native to the Americas and their venom is largely composed of PLA_2s (4). There have been a few specific PLA_2 inhibitors developed, but none have passed clinical trials. Issues range from specificity to stability but the search continues (5).

Phospholipases

PLA_2 enzymes are a diverse family of enzymes branching from the larger superfamily of phospholipases. PLA_2s hydrolyze phospholipid bonds at the *sn-2* position resulting in the production of polyunsaturated fatty acids (PUFAs) and lysophospholipids (6). PLA_2s are characterized by 4 main groupings with 15 subgroups that differ based on their structure, size, source, sequencing, and function. The four groupings of PLA_2s are secretory, cytosolic, calcium-independent, and lipoprotein-associated/platelet-activating factors acetyl hydrolases (7).

Secretory PLA_2 ($sPLA_2$), were the first discovered. They have a relatively low molecular weight around 13-19 kDa. Structurally, sPLA2s have a histidine in the active site and require Ca^{2+} as a cofactor (7). This group has 6 conserved disulfide bonds with an additional 1 or 2 more dependent on subgroup. The most well studied of mammalian $sPLA_2$s is group IIA (GIIA) (7). GIIA has strong antibacterial activity and has been studied as a potential biomarker for infections like sepsis (8). $sPLA_2$s have been found to also serve nonenzymatically as receptor binding molecules (9). In mice, $sPLA_2$ can bind to M type receptors initiating immunological responses (9). A similar receptor interaction is yet to be discovered in humans, but studies have shown possible integrin-$sPLA_2$ interactions (8). This group also shares high structural and sequence homology to $sPLA_2$ GIIA derived from snake venom (7). Phospholipids form aggregates or membranes in aqueous environments and so distributions of phospholipid substrates must be considered when analyzing the kinetics of these enzymes (10). Surface dilution kinetics is used

to describe the nature of phospholipase action on the membrane and substrate in $sPLA_2$ studies (10). When targeting phospholipids $sPLA_2$s appears to show no preference in substrate (11).

Cytosolic PLA_2 ($cPLA_2$) is the largest of the PLA2s at around 80 kDa and is found intracellularly. (9). These enzymes also require Ca^{2+} but have a serine in the active site rather than a histidine (7). $cPLA_2$ also require phosphorylation as they must translocate from the cytosol to the membrane to begin enzymatic action (12). This enzyme group preferentially binds phospholipid species containing arachidonic acid (AA) (9). This PUFA is an important lipid metabolite that can form various signaling molecules, described below (13).

The third grouping of PLA_2 bears many similarities to the $cPLA_2$ grouping with larger molecular weights (~80-90 kDa) and having a serine residue in the active site (14). The primary difference of this group is that there is no requirement for Ca^2, hence the name, calcium independent PLA_2 ($iPLA_2$) (14). This group also frees AA but its lipid preference is unknown (14). Overexpression of this enzyme does lead to apoptotic signaling and cell death (15).

The fourth grouping of PLA_2 is the platelet-activating factor/lipoprotein (PAF/Lp) PLA_2. This group shares a blend of structural similarities to the other PLA_2s (16). Like $iPLA_2$ enzymatic activity does not require Ca^{2+} but a serine residue is present in its active site. Unlike other PLA_2s this group has an intermediate smaller molecular weight (20~40 kDa) but still larger than $sPLA_2$ (17). Substrate specificity of this group has been toward oxidized phospholipids, mostly found in serum lipoproteins (18). These enzymes are of great interest for their role in the physiology and detection of atherosclerosis and heart disease (19).

These four grouping demonstrate the great diversity contained in this enzyme family. Table 1 below shows all fifteen subgroups described by their Roman numeral, source, and molecular weight.

Table 1: Groupings, Locations and Molecular weight of various Phospholipase A2 isoforms

Group	Source	Molecular weight	Group	Source	Molecular weight
Secretory PLA_2			***Calcium Independent PLA_2***		
IA	Cobras and Kraits	13-15	VIA-1	Human/murine	84-85
IB	Human/porcine pancreas	13-15	VIA-2	Human/murine	88-90
IIA	Rattlesnakes; human synovial	13-15	VIB	Human/murine	88-91
IIB	Gaboon viper	13-15	VIC	Human/murine	146
IIC	Rat/murine testis	15	VID	Human	53
IID	Human/murine pancreas/spleen	14-15	VIE	Human	57
IIE	Human/murine brain/heart/uterus	14-15	VIF	Human	28
IIF	Human/murine testis/embryo	16-17	***Lipoprotein associated PLA_2***		
III	Human/murine/lizard/bee	15-18, 55	VIIA	Human/murine/porcine/bovine	45
V	Human/murine heart/lung/macrophage	14	VIIB	Human/bovine	40
IX	Snail venom	14	VIIIA/B	Human	26
X	Human spleen/thymus/leukocyte	14			
XIA, XIB	Green rice shoots (I and II)	12.4, 12.9			
XII	Human/murine	19			
XIII	Parvovirus	<10			
XIV	Symbiotic fungus/bacteria	13-19			
Cytosolic PLA_2					
IVA	Human/murine	85			
IVB	Human	114			
IVC	Human	61			
IVD	Human/murine	92-93			
IVE	Murine	100			
IVF	Murine	96			

Table adapted from (20)

While PLA_2s can have functions outside their enzymatic action, the production of AA helps in inflammation signaling and is associated with chronic inflammatory diseases such as arthritis (13). The high structural homology between $sPLA_2$ GIIA in mammals and snake venom means a potential inhibitor might work against both (21).

Inflammation

Inflammation is a term often used to describe symptoms of injury or disease. Historically inflammation has been recognized and described by its five cardinal signs: redness, swelling, pain, heat, and loss of function (22). More scientific approaches have been taken to understand and explain the mechanisms by which inflammation occurs and potential consequences of such a phenomenon (22). Inflammatory responses begin as acute or rapid reactions usually in the defense of the host. Immune or metabolic disfunctions can lead to long term or recurring, chronic problems (23).

PLA_2s can play a role in inflammation depending on if the cause is infection or injury as well as what signals are released (24). For an infection such as sepsis $sPLA_2$ is secreted as a defense mechanism. The enzyme can breakdown bacterial cell membranes leading to destruction of the organisms (24). Sterile inflammation results from physical trauma or metabolic dysfunction with an absence of infection (25). Most inflammation starts as acute but can develop or assist with chronic conditions such as diabetes, cancer and heart disease (26)

Phospholipase A2 Role in inflammation

The process of inflammation starts with the innate immune system and is triggered by a variety of stimulatory factors (27). When looking at a cellular response these factors can originate from either intracellular or extracellular components (27). Intracellular components can include nuclear proteins, organelles, or cellular chaperones which all can cause an inflammatory

response when leaked into the extracellular matrix (28). When leaked into the extracellular environment immune cells recognize them as foreign and can initiate an immune response (28).

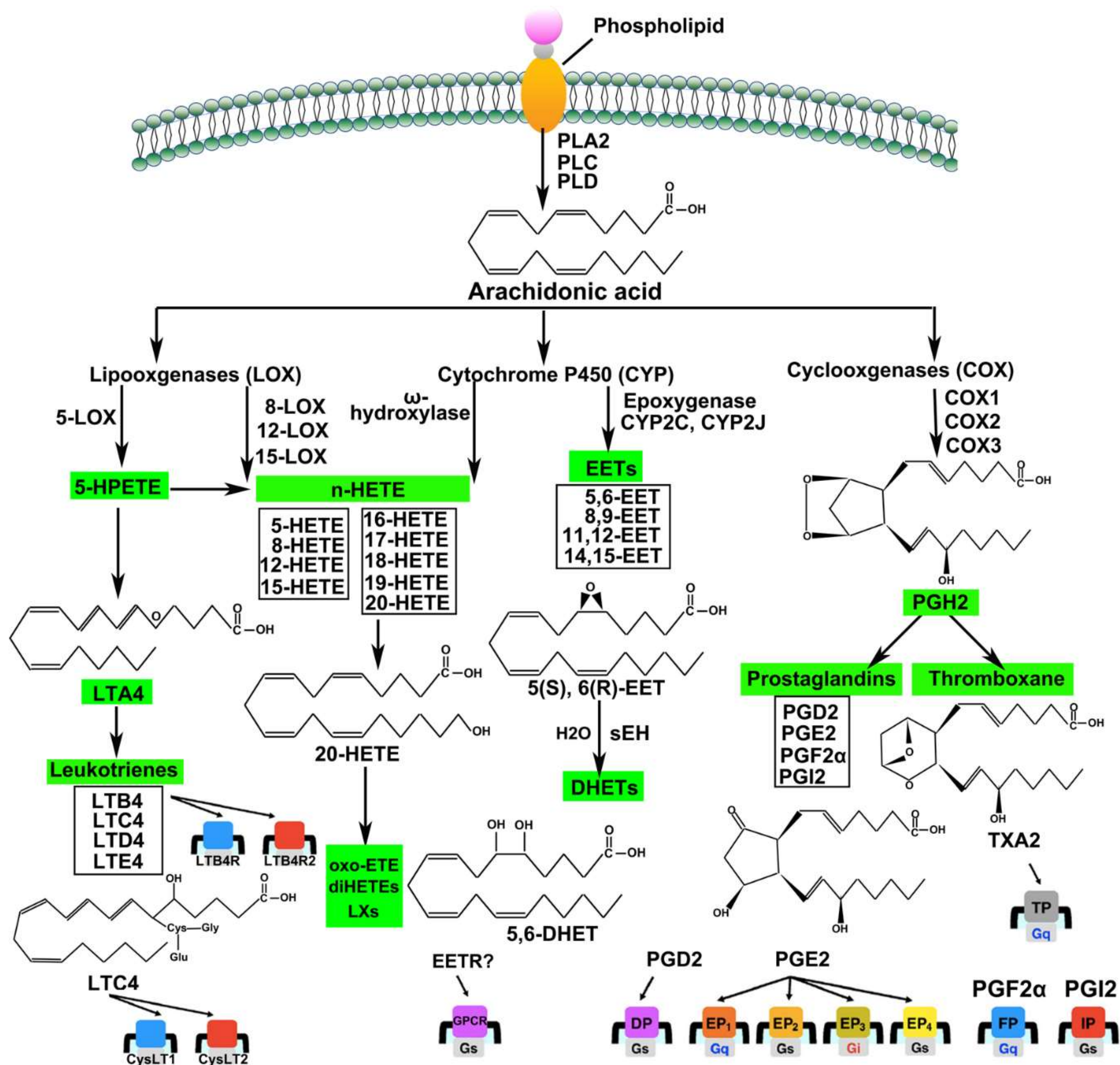
This immune response usually begins with the recruitment of other immune cells. PLA_2s can become upregulated, and AA released (13). The AA can then be metabolized by one of three different enzymatic pathways, cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP). These produce a class of lipid messaging molecules known as eicosanoids (29). These eicosanoids are rapid responders to infection not requiring transcription as do proteinaceous inflammatory molecules (30).

The COX pathway is composed of both COX 1, COX 2 and COX 3 and produces prostanoids. These can further be metabolized to produce prostaglandins (PGs) and thromboxane A2s (TXA_2) (31). COX 1 is considered a housekeeping gene and is found in almost all cell types while COX 2 is more associated with cancer and inflammatory diseases (31). Both enzymes though can contribute to autoimmune inflammatory diseases (31). A popular treatment for inflammation is non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin which target COX 2, thereby preventing the production of PGs related to pain like prostaglandin E2 (PGE_2) (32, 33).

The second pathway LOX produces leukotrienes (LTBs). This class of eicosanoids are more involved with responses to allergies (31). Successful antagonists of the enzyme, such as MK59, have been developed for treatment of seasonal allergies and asthma. Recently, inhibition of this pathway has been investigated for a potential cancer therapy (34).

The CYP pathway is the most recently discovered with its best characterized metabolite being hydroxyeicosatetraenoic acids (HETEs) (31). This class of eicosanoid also plays roles in

inflammation and vascular health (31). These three enzyme pathways shows that AA is vital to the production of various inflammatory molecules (13). Below scheme 1 shows the three pathways and the structures of some of the various eicosanoids produced.



Scheme 1: The metabolic pathways of Arachidonic Acid after release from phospholipids of the cellular membrane (31).

The inhibition of the enzymes that produce AA, PLA_2s, has been of great interest due to their central role in inflammation. Current therapies include steroids, yet those can have a range

of serious side effects (35). As described above some $sPLA_2$ inhibitors have been developed for treatment of snake venom but none have passed clinical trials (36). Although $sPLA_2$ has not been shown to have a direct link to producing high amounts of free AA it still plays a role in producing these compounds (8). Due to the nearly identical sequence and structure between the pit viper and human GIIA PLA_2s, an inhibitor of the snake venom enzyme could potentially double as an anti-inflammatory therapy (7).

Research Aims

Previous research in the Venable lab has investigated the inhibition of $sPLA_2$ in snake venom using a naturally derived lipid. This compound has demonstrated an ability to work as an inhibitor of $sPLA_2$ in an in vitro PLA_2 assay. The aim of my research was to investigate whether the inhibitor could have effects in a cellular system. My goals were two-fold, first investigating whether the inhibitor could protect and work as an anti-venom therapy by blocking the action of the $sPLA_2$. For this part of the study, I developed a model system, taking into consideration venom concentration, time, viability, and lipid concentration to effectively observe if inhibition existed. For the second part of my study, I looked at whether the lipid extract would reduce the production of inflammatory molecules like eicosanoids. If this compound inhibits the $sPLA_2$ then the amount of free AA and therefore eicosanoids would be reduced.

Results

Establishing Baseline Venom

To study the effects of venom on cell viability a baseline was to be established to determine a concentration of venom that would cause some death of cells (50% or less) but not complete loss of all cells (100%). This would allow for a measurable difference in cell survival. Initial experimentation focused on short incubations (30 min – 2 hr) using cell counts to verify cell viability. This method resulted in such high variability that it was not tenable. Figure 1 shows an example of an experiment in which cell counts were performed using a hemocytometer with trypan blue. We had indication that the stain was a primary cause of inconsistencies. The differences in the cell counts made determining accurate counts unreliable for viability measurements. Clumps of cells could be observed making counts sometimes unattainable. These clumps could be accounted to high volumes of cells (>5 x 10^5) cells.

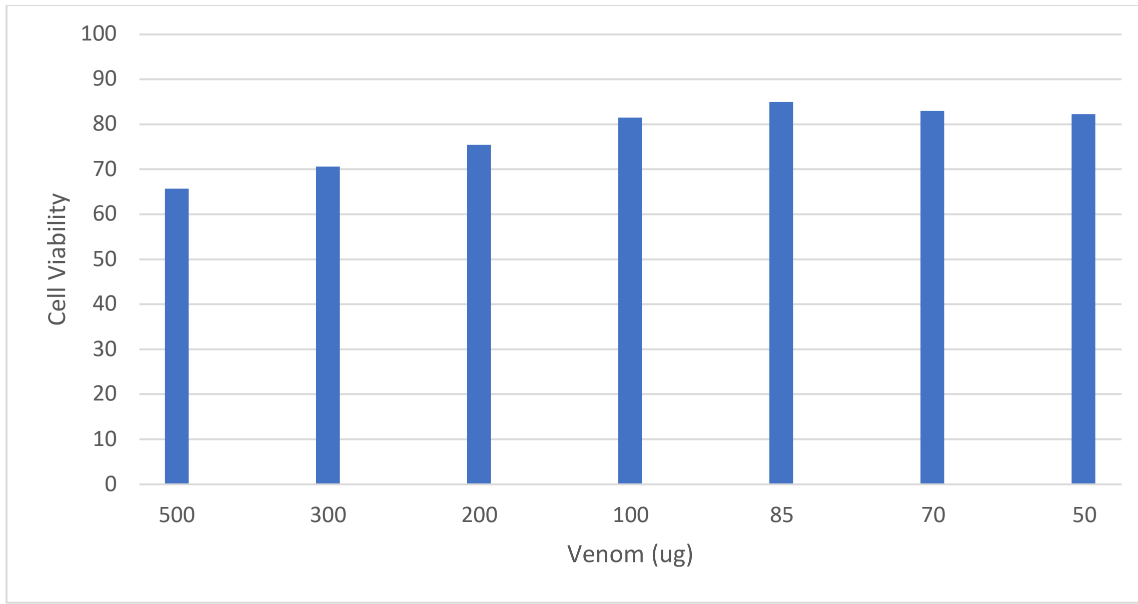


Figure 1: Cell Viability after 30-minute incubation with the addition of various amounts (50 -500 µg) of Timber Rattlesnake venom. Cells resuspended in PBS at cell density 6 x 10^5 cells/mL and cell counts by trypan blue exclusion.

To attain more reproducible results a different assay was done and so a Resazurin assay was performed to measure viability. Cells can take up resazurin salt and convert it to resorufin. This allows for a measure of metabolically active cells by absorbance and fluorescence. This also reduces discrepancies in cell counts and allows for replicate experiments to be measured at the same time. Cells were added to 96 well plates to allow for measurements of absorbance values but also for incubation with CO_2 buffer. A 2-hour incubation showed no significant difference in cell death (Figure 2).

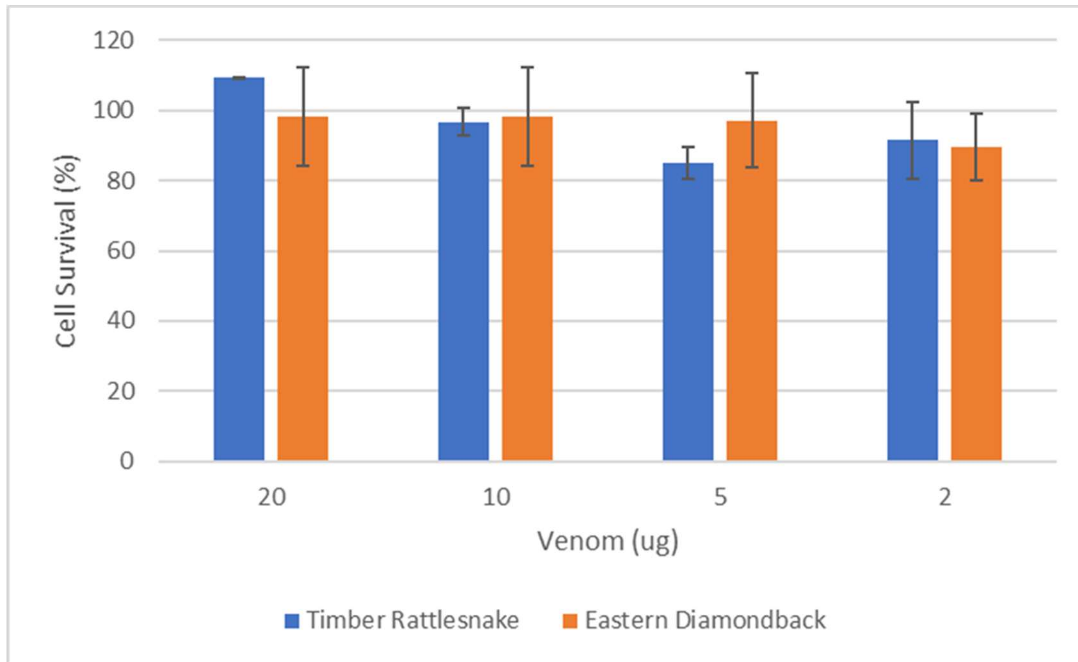


Figure 2: Cell viability after 2-hour incubations with various amounts (2-20 μg) of Timber Rattlesnake Venom and Eastern Diamondback Venom. Measurement of absorbance at 570 and 600 nm after 6-hour incubation with Resazurin solution. Cells resuspended in serum free media at 3 x 10^5 cells/mL.

To try to account for lack of cell death trials with longer incubations and lower cell concentrations were performed. Longer incubations gave more time for the enzyme to break down phospholipids and allow for cell death. Lower concentrations of cells were used since high densities showed higher amounts of cell survival. Figure 3 shows that a 24-hour incubation with a cell concentration ~1 x 10^5 demonstrated good levels of cell death that could be used as a baseline for venom treatment.

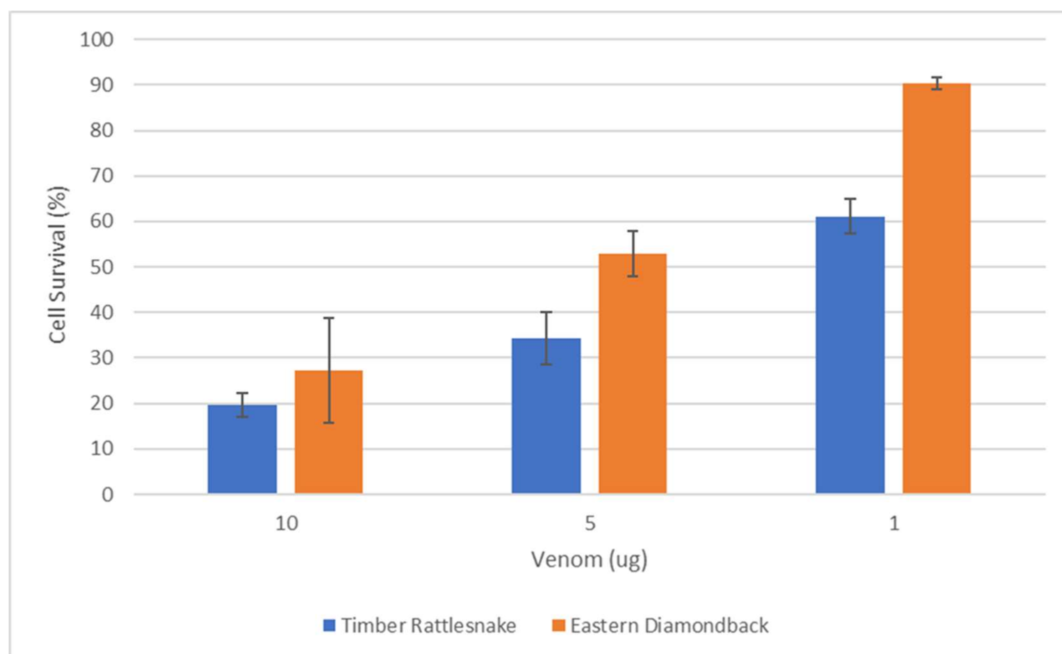


Figure 3: Cell Viability post 24-hour incubation with various amounts (1, 5 and 10 µg) of Eastern Diamondback and Timber Rattlesnake venom. Cells were in serum free media at 7 x 10^4 cells/mL. Fluorescence performed with excitation 560 nm and emission 590 nm.

Delivery of Lipids to Cells

After establishing a concentration that effectively kills cells with venom, establishing a delivery method for the lipid extract was necessary. Lipids, as mostly nonpolar compounds, do not readily dissolve into aqueous environments. To add the lipids to the cells, some vehicle would need to dissolve the lipids and deliver them into the aqueous cellular environment. Two molecules commonly used for the delivery of hydrophobic compounds to cells are Bovine Serum Albumin (BSA) and Dimethyl Sulfoxide (DMSO). BSA is the bovine homolog to human serum albumin (HSA) one of the most common protein in human blood (37). BSA acts as a natural lipid delivery system and does so through hydrophobic binding regions found in the protein (37). For this reason, BSA and HSA are both popular methods for drug delivery systems. DMSO is an

amphipathic molecule that can dissolve in both organic and aqueous environments. DMSO can readily dissolve through skin and is commonly used for transdermal delivery of medications. The first test was to see if the lipids could be delivered using either vehicle.

To see if lipids could be delivered to the cells, N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-erythro-sphingosine (NBD) labelled ceramide was suspended in either vehicle (BSA and DMSO) then incubated with cells. Fluorescent microscopy showed successful delivery of cells as the labelled ceramide glowed green with fluorescence (Figure 4).

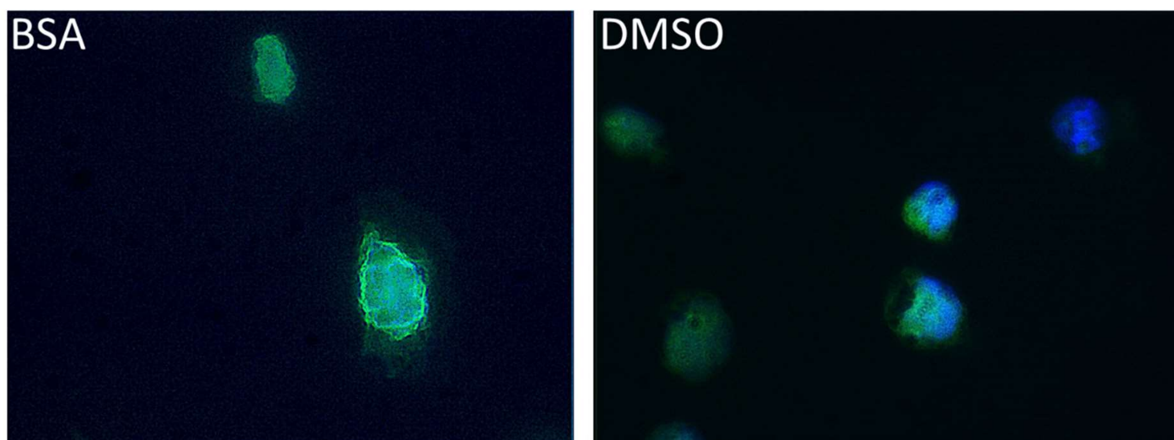


Figure 4: Fluorescent Microscopy of diphenyl(aminomethyl)phosphane labelled ceramide (NBD) in cells after delivery via DMSO and BSA. Blue represents DAPI dye and staining of nuclear proteins, Green is NBD and indicates implementation of the labelled ceramide into cell membrane.

After demonstration of both vehicles as viable delivery methods, an analysis of their effects on cell viability was done. The cell viability studies required longer incubations with the venom and so the DMSO and BSA would need to be evaluated for possible effects on cell viability. DMSO is known to decrease cell viability and so DMSO dilutions in water were made to lower the effect of the DMSO on the cell viability.

DMSO dilutions showed that an expected drop in cell viability was observed with just DMSO. Dilution of DMSO and water increased cell viability with a 1:10 dilution (10% aqueous DMSO) showing only a slight drop in viability. This method showed promise for a delivery mechanism with little loss of cell viability.

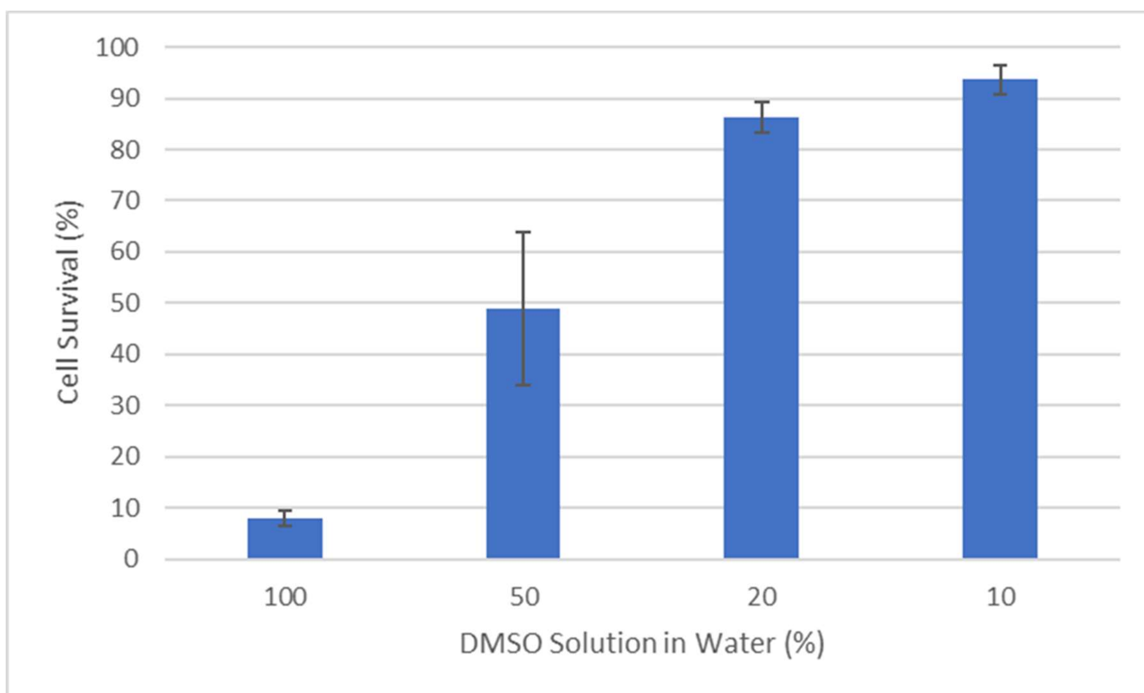


Figure 5: Effects of DMSO dilutions (1 to 1:10) on cell viability after a 24-hour incubation. Fluorescent readings were measured at 560 nm excitation and 590 emissions nm. Compared to control (100% viability). Cells at concentration 1 x 10^5 cells/mL incubated with DMSO water dilution for 24 hours.

Next, I tested a similar effect but with BSA. Figure 6 showed that without the addition of venom there was an increase in cell viability. This could be problematic with the increase in viability from the BSA potentially being mistaken as inhibition. Further research into the two different treatments revealed that BSA can affect absorbance and fluorescent reading for assays using Resazurin (38). For this reason, the DMSO dilution was selected as the preferred lipid

delivery method. The delivery method was tested again as the dilution could have affected to ability of DMSO as a lipid delivery method.

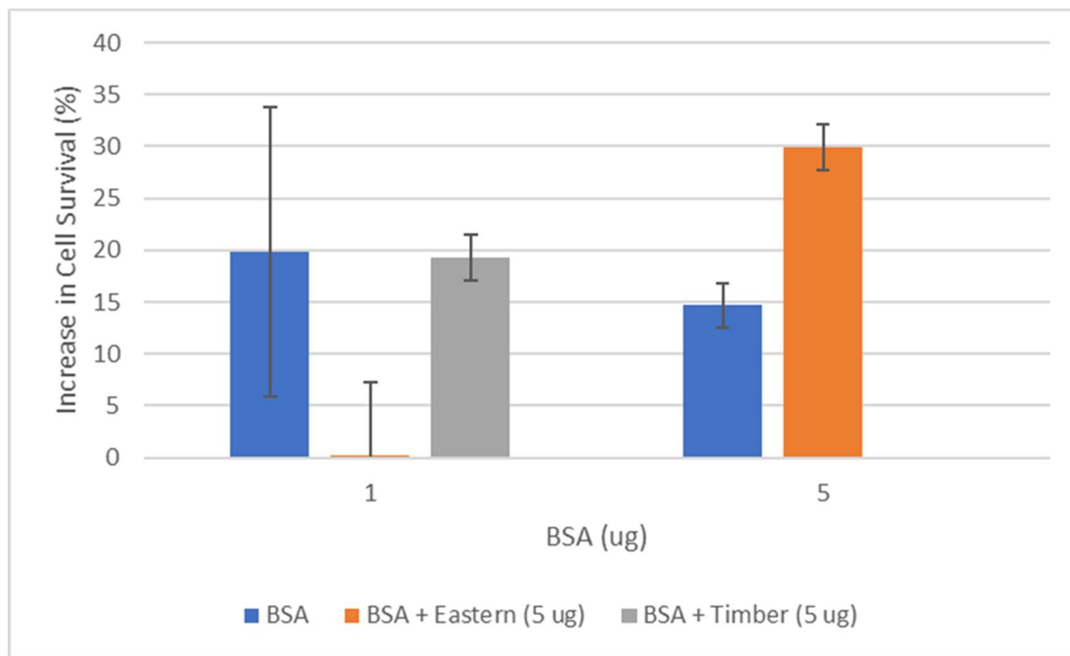


Figure 6: Effects of the addition of BSA at different concentrations on cell growth. Cells (1 x 10^5 cells/mL) were incubated with BSA (1 or 5 ug) and venom (5 µg).

Figure 4 demonstrated that the 10% aqueous DMSO worked well as a lipid delivery vehicle. To test the DMSO solution, a different lipid dye, Nile Red, was suspended in 10% aqueous DMSO and viewed under fluorescent microscopy. The red fluorescence (Figure 7) indicated the lipid dye Nile red was successfully delivered to the cells. The confirmation of the ability of lipid delivery along with the viability data of Figure 5 demonstrated the viability of the 1:10 DMSO solution. This established the lipid delivery system.

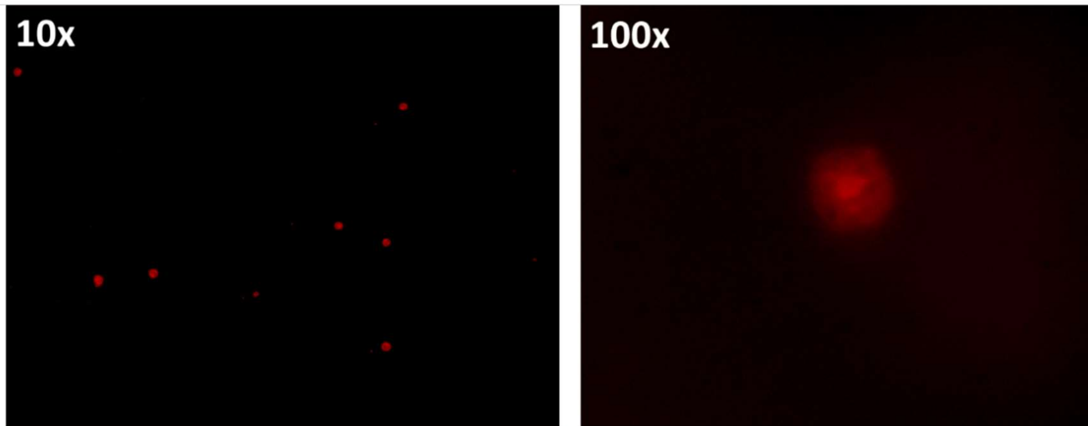


Figure 7: Fluorescent microscopy visual of Nile Red delivered to THP-1 cells via a 10% aqueous DMSO solution. Red indicates delivery of Nile red to hydrophobic regions of the cells.

Effect of Cell Differentiation

THP-1 cells are a monocytic line that can differentiate to macrophage populations in the presence of phorbol 12-myristate 13-acetate (PMA) and other stimuli. Macrophage cells produce their own PLA_2s which could lead to beneficial or detrimental effects on cell viability (39). An experiment was performed to test if there were differences in cell death of monocyte populations against macrophage populations (Figure 8). Differences in cell death from both venoms showed little difference between the monocyte and macrophage populations. This showed that if the lipid extract did induce differentiation, it should not affect the viability study. The untreated cells were chosen for the model to eliminate the extra variable of PMA.

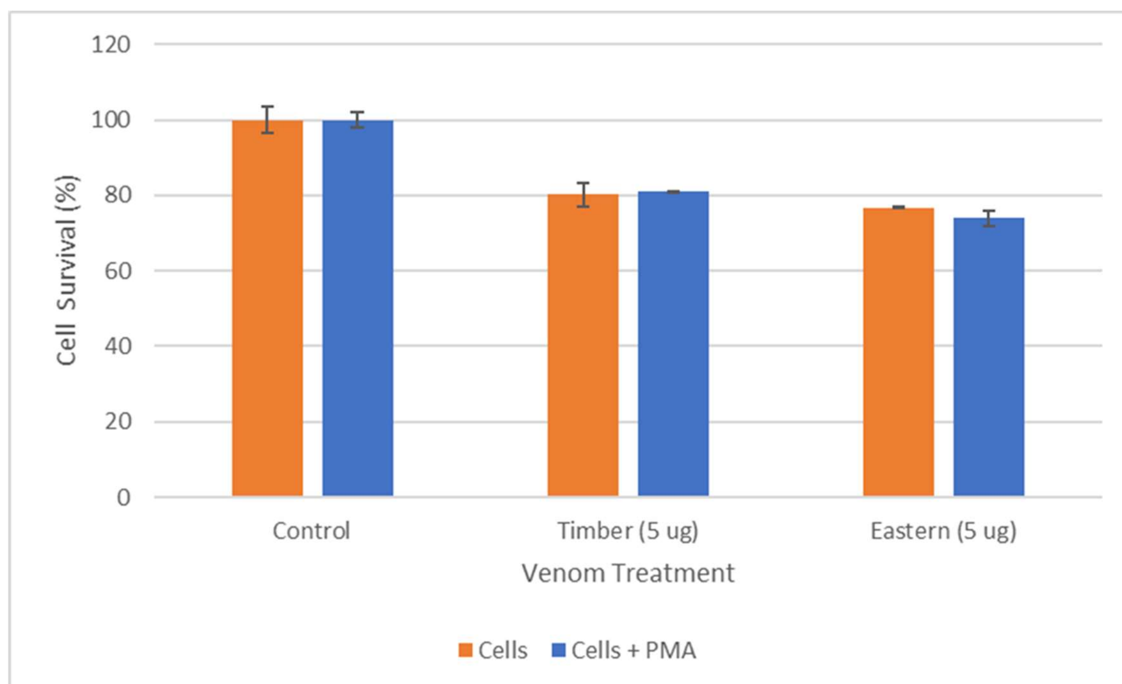
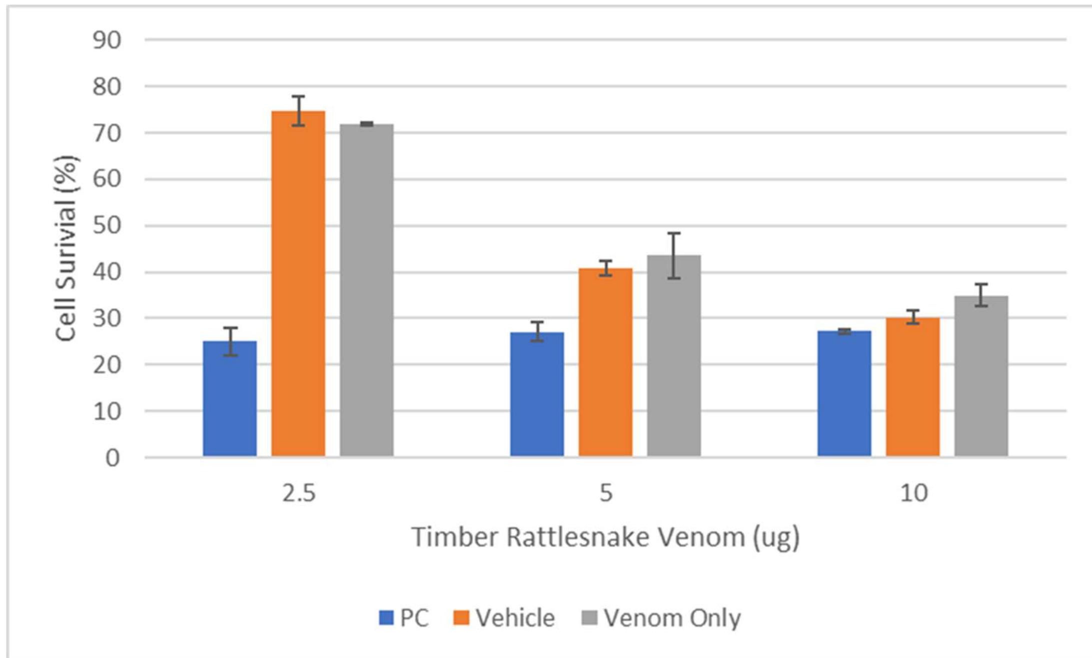


Figure 8: Macrophage and Monocyte viability differential responses to treatment with Timber rattlesnake and Eastern Diamondback venom. Cells were grown in 96 well plates at concentration 1 x 10^5 cells/mL for 24 (hrs) allowing differentiation. Cells were then treated with venom (5 µg) and allowed to incubate for an additional 24 hours. Absorbance values read 570 nm and 600 nm. Viability compared to control (100%).

Effect of the Putative Inhibitor

After establishing a system for inducing and detecting cell death by venom action we set out to study the effect of our inhibitor extract. The extract is a complex mixture of blood lipids. In vitro PLA_2 assays show inhibition of pit viper venoms using this extract. Previous studies in the Venable lab showed that the highest amount of activity resides in the phosphatidylcholine (PC) fraction from TLC. Partial purification was performed with our recent lipid extracts. The PC fraction was collected and quantitated according to lipid phosphorous content. Each phospholipid contains one atom of phosphorous, therefore the phosphorous assay reports the

concentration of phospholipid in the sample. In the whole extract there are also lipids other than phospholipids that we did not quantitate. Using the PC fraction should eliminate most of those other lipids. Addition of lipid extract PC fraction showed no protection of cells from venom but surprisingly showed an increase in cell death as contrasted with vehicle control (p values, 0.001, 0.011, 0.030) (Figure 9). We then wanted to determine if the PC being added to the cell itself influenced viability or if it was synergistic effect where the extract and venom together lowered viability. Therefore, the PC was preincubated with the venom before addition of the cells.



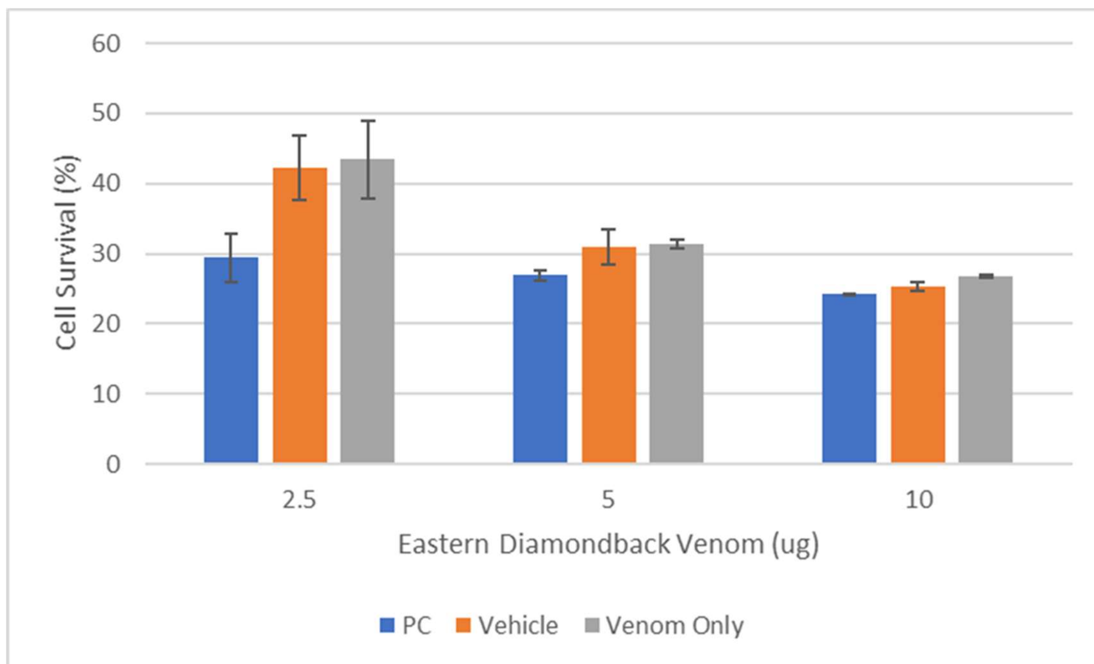


Figure 9: Cell viability following pre-incubation of inhibitor and addition of various amounts (2.5, 5 and 10 µg) of Timber and Eastern Diamondback rattlesnake venom. Cells were in serum free media and PC extract was added (2.5 nmols) and allowed to incubate

for 24 hours. Venom treatment was then added and allowed to incubate for 24 hours. Fluorescence was performed at 560 nm excitation 590 nm emission.

Addition of PC extract showed no protection of cells from venom but surprisingly showed an increase in cell death as contrasted with vehicle control. Therefore, another experiment was conducted in which the PC extract was pre incubated with the venom (1 hour) before addition to cells (Figure 10). We wanted to determine if the lipids being pre incubated affected cell viability or if it was synergistic effect where the extract and venom together lowered viability.

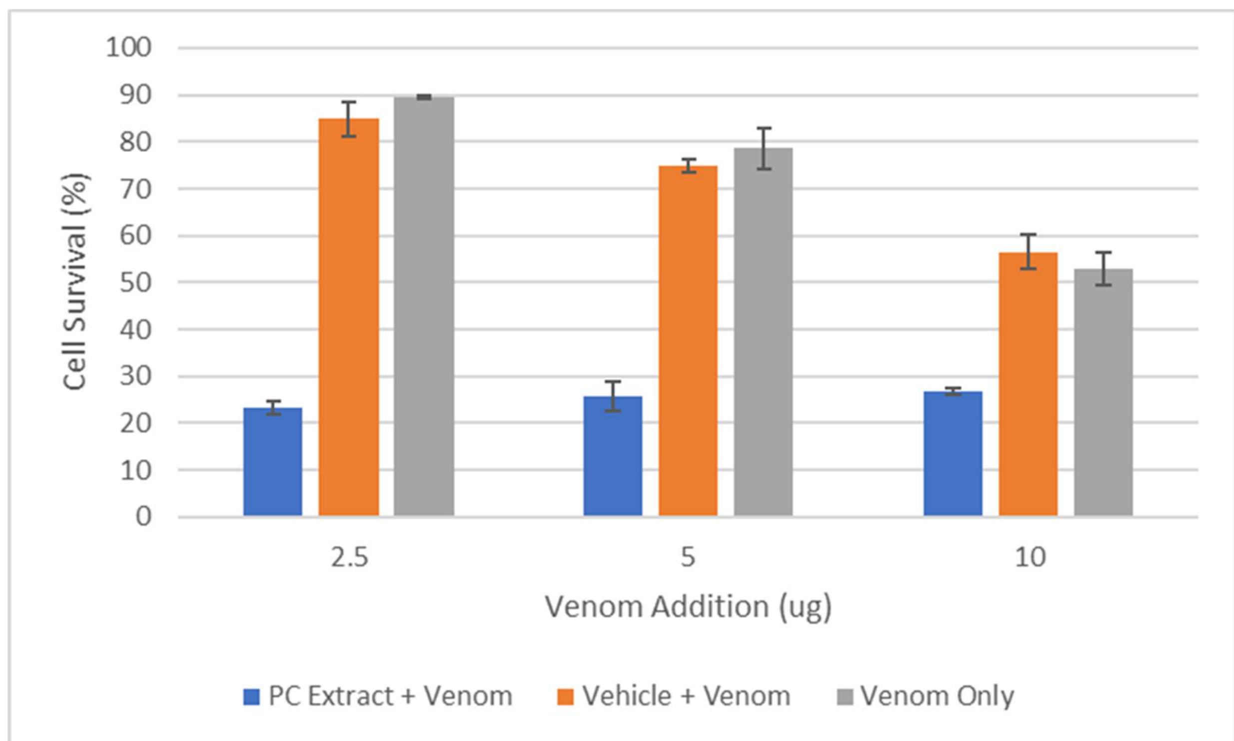


Figure 10: Preincubation of inhibitor extract with Timber Rattlesnake venom prior to introduction to cells. PC extract (2.5 nmols) was preincubated with venom (2.5, 5, and 10 µg) for 1 hour. Treatment was then added to cells, concentration 1 x 10^5 cells/mL.

Pre-incubation of the inhibitor with the venom and then addition to the cells (Figure 10) showed very similar results to those in Figure 9. The next question we asked was if the PC extract was being metabolized by the cells. The metabolism could play a role on how the cells react and therefore investigating what is happening to the PC extract and how it affects cells was the next step (Figure 11).

Testing the Lipid Extract Components

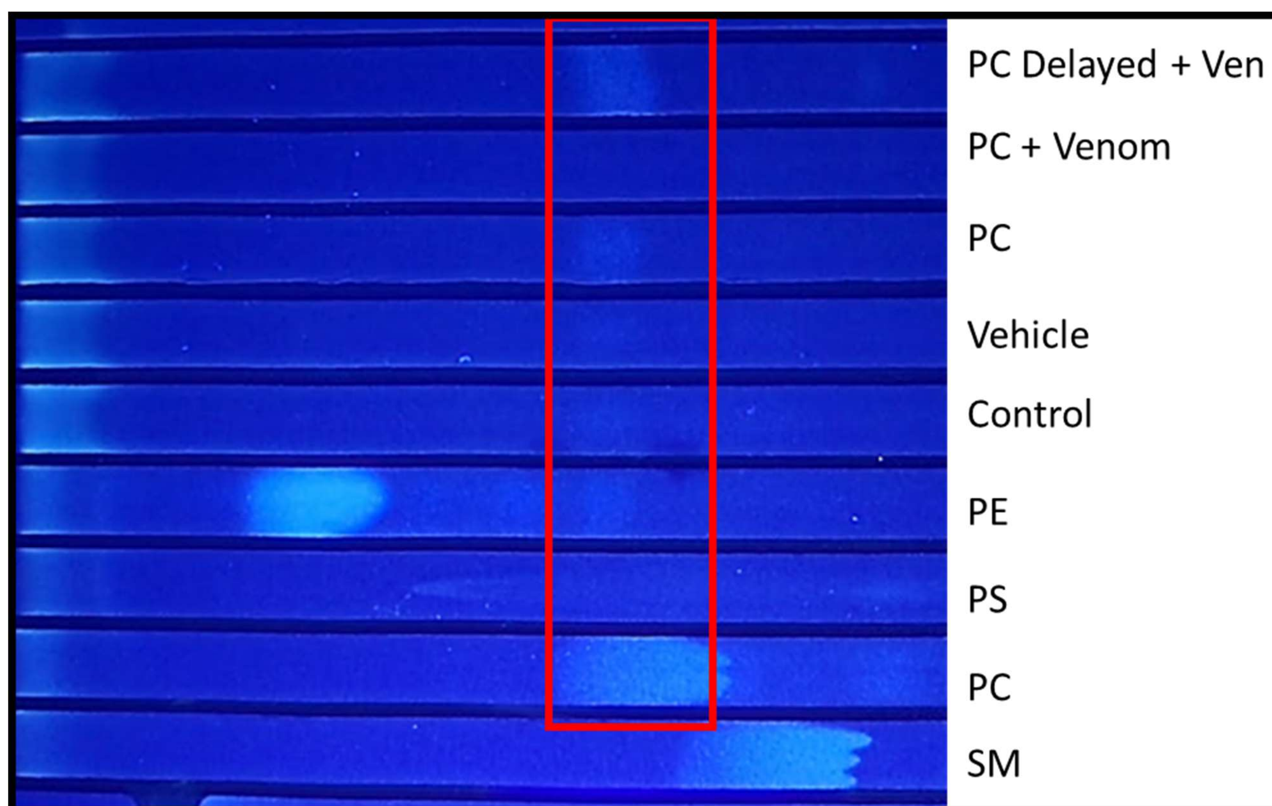


Figure 11: Thin Layer Chromatography of lipid fractions added to cells and then treated with venom. Red line shows the PC fraction in reference to the standard. There is an absence of the PC fraction in the cells treated with venom after incubation with PC.

TLC analysis of the lipids (Figure 11, red outline) showed that the PC extract was being metabolized by the venom. This means the $sPLA_2$ in the venom was metabolizing the part of the

extract added that is not the inhibitor. After showing metabolism of the lipids the next step was to determine if the addition of the PC extract itself was the cause for the decreased viability.

The PC extract lipid concentration was much higher (2.5 nmol) than the cells lipid concentration (PO_4 conc. 0.039 nmols). It was possible that the lipids themselves were leading to the cell death and so different concentrations of the lipids were incubated with cell populations and tested for viability (Figure 12). The larger amount of extract (5, 2.5 and 1.25 nmols) showed an unexpected increase in cell viability. This was most likely due to the serum starved cells using the extra phospholipids as a nutrient source. The amount of lipid was nearly 50x more than the amount of phospholipid found in the cells (0.039 nmols) and so the expected effects of cell viability would be a decrease.

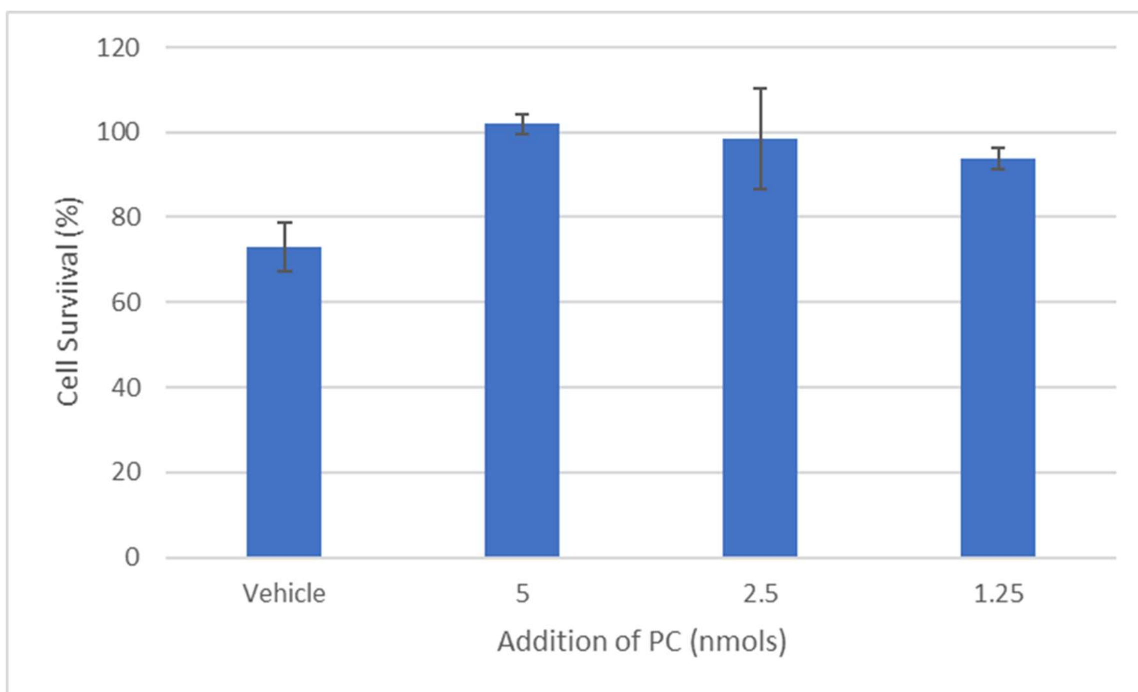


Figure 12: Effects of different concentrations of the lipid extract on viability post 48-hour incubation. Cells at concentration 1 x 10^5 in serum free media were treated with PC extract (5, 2.5, and 1.25 nmols) and allowed to incubate for 48 hours. Fluorescence read 560 nm excitation and 590 nm emission.

With the high amount of PC extract demonstrating a drop in viability but only in the presence of the venom we wanted to determine if the effect was repeated with a smaller amount of PC extract. First an experiment was done to see if the smaller amount of PC extracts influences viability (Figure 13). The data showed there was not a major difference in cell viability.

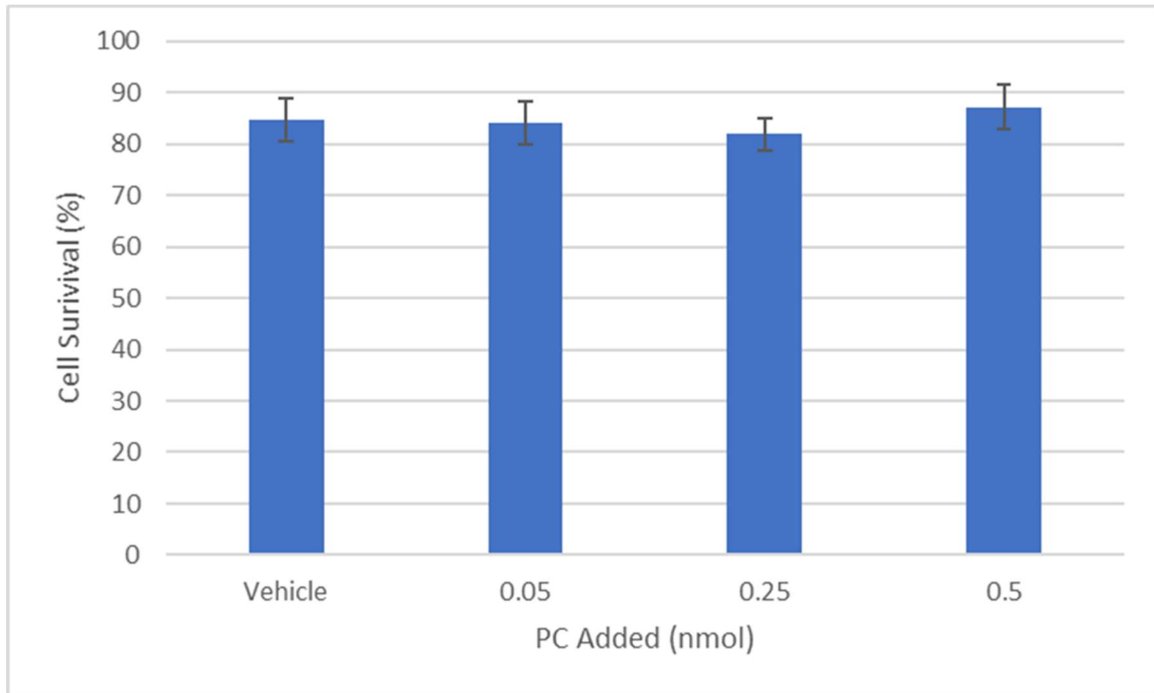


Figure 13: PC extracts effects on cell viability post 24-hour incubation. Cells at concentration 1 x 10^5 cells/mL in serum free media were treat with PC extract (0.05, 0.25, and 0.5 nmols). Cells were allowed to incubate for 24 hours then fluorescence read at 560 nm excitation and 590 nm emissions.

The smaller amount of extract added showed no decrease in cell viability. Because there was no drop in viability the small amount of additional lipid was tested with Timber Rattlesnake venom (5 µg) to see if the same drop that was observed with higher amounts reoccurred.

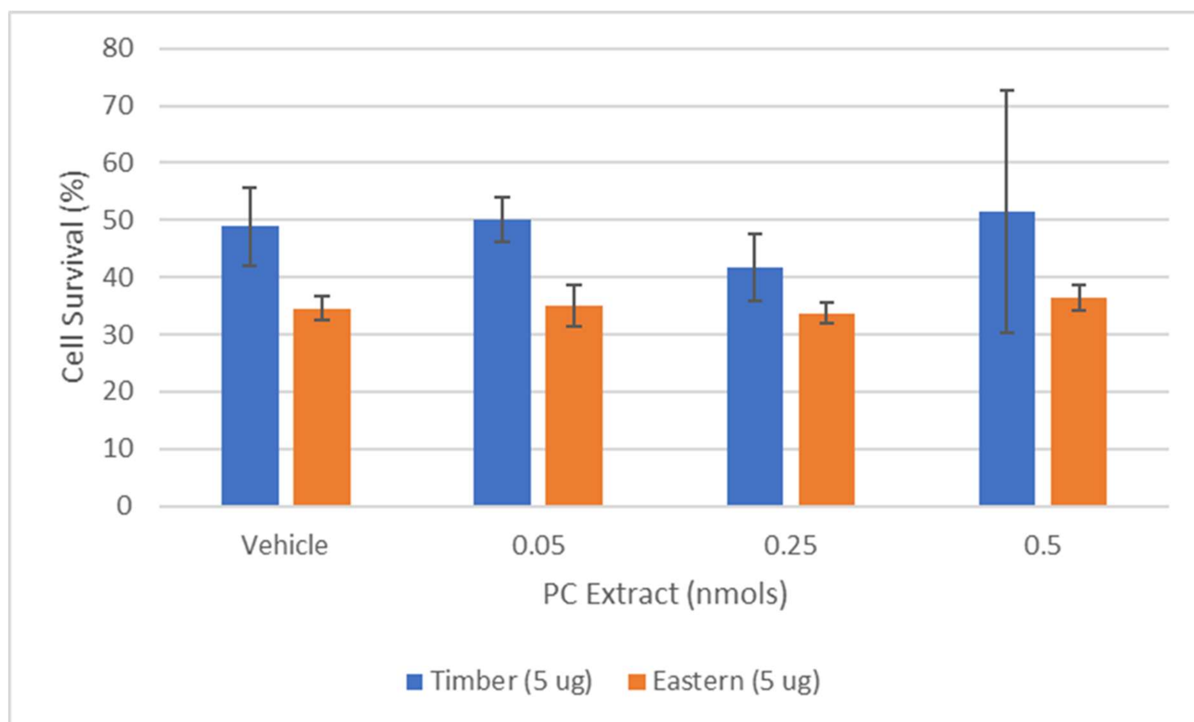


Figure 14: Lipid Effects on Cell viability after the addition of Timber Rattlesnake Venom (5 µg). Cells at concentration 1 x 10^5 cells/mL in serum free media were treated with PC extract (0.05, 0.25, and 0.5 nmols) allowed to incubate for 24 hours. Venom was then added and cells were allowed to incubated for 24 hours then fluorescence was performed at 560 nm excitation and 590 nm emission.

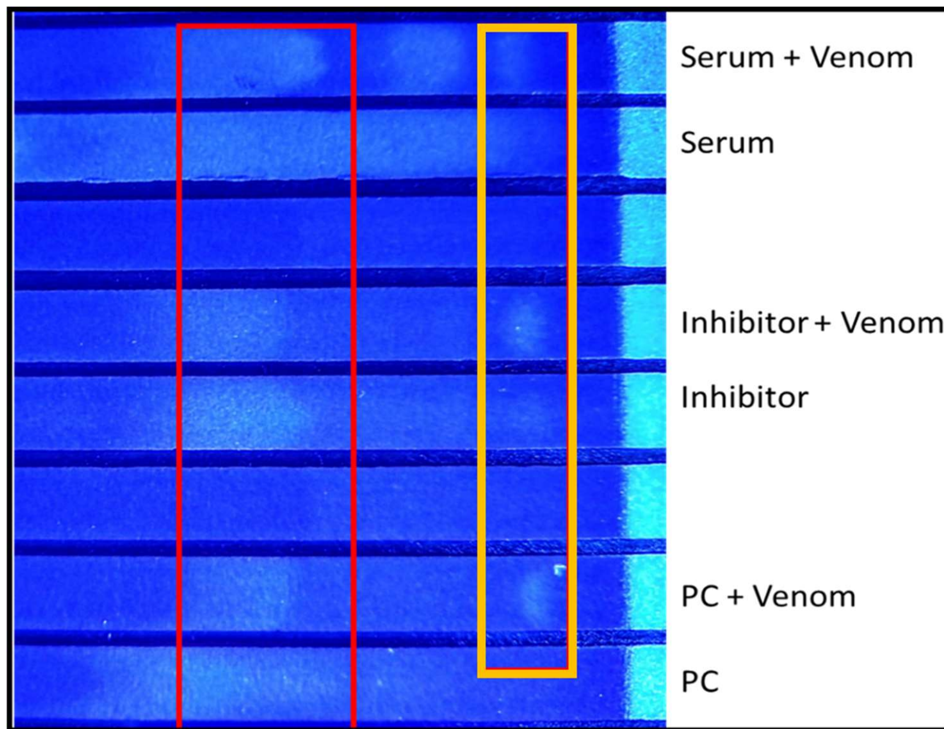


Figure 15: Testing the effects of Timber Rattlesnake venom on phosphatidylcholine standard, lipid extract and whole serum. Lipid extracts were treated with venom (1 hr) then run on TLC plates. Red outline indicates the PC chromatography. The orange outline indicates the metabolism of the PC fraction into a new band.

After showing that large amounts of the inhibitor increased the death of cells in the presence of venom the changes in lipids were observed. Figure 15 shows that there is a decrease in the phospholipid PC fraction as expected. There is also a band that forms in the venom treated categories that is more polar. This group is most likely the lyso-PC due to phospholipase action which could be responsible for the additional death of the cells.

Measurement of Inflammatory Signaling Molecules

The second objective of the project is to determine whether the PC PLA_2 inhibitor would reduce the release of the arachidonic acid and the formation of inflammatory eicosanoids in

THP-1 cells. The enzymatic action of the PLA_2 in the venom can lead to an increase in eicosanoids. We decided to focus on PGE2 and LTB4 to test both the COX and LOX pathway The inhibitory action of the PC inhibitor might then lower the production of these signaling molecules.

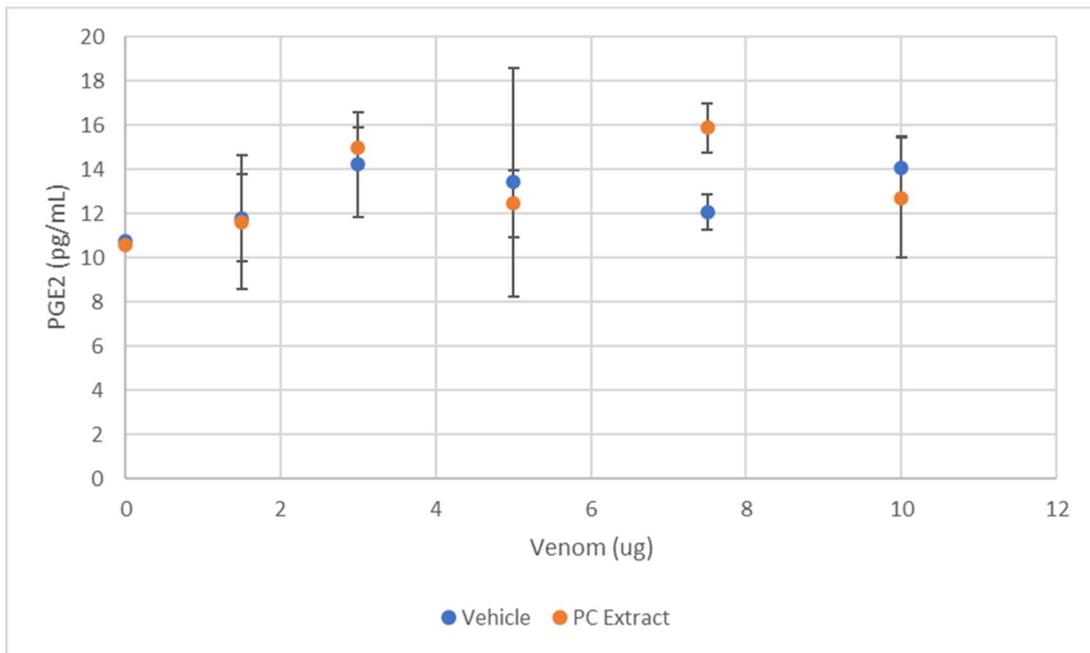


Figure 16: Differences in the Prostaglandin E2 Production of Cells with various venom concentrations with the addition of PC (2.5 nmol). Cells plated in complete media on 12 well plates at concentration 1 x 10^5 cells/mL. Cells then treated with different concentration of Timber rattlesnake venom (1.5, 3, 5, 7. 5, and 10 µg). Treatments then analyzed for PGE_2 levels.

A preliminary ELISA showed that there was a measurable signal with the addition of (5 µg) Timber rattlesnake venom on 1 mL of THP-1 cells at a concentration of 1 x 10^5 cells/mL. The first experiment was then to use different venom concentrations and measure changes in PGE_2 production with the pre incubation of PC (2.5 nmols). Figure 17 shows that there are

differences between the PGE_2 production with the pre incubation of the extract and control. The second experiment with PGE_2 was a time dependence study with fixed venom (5 µg).

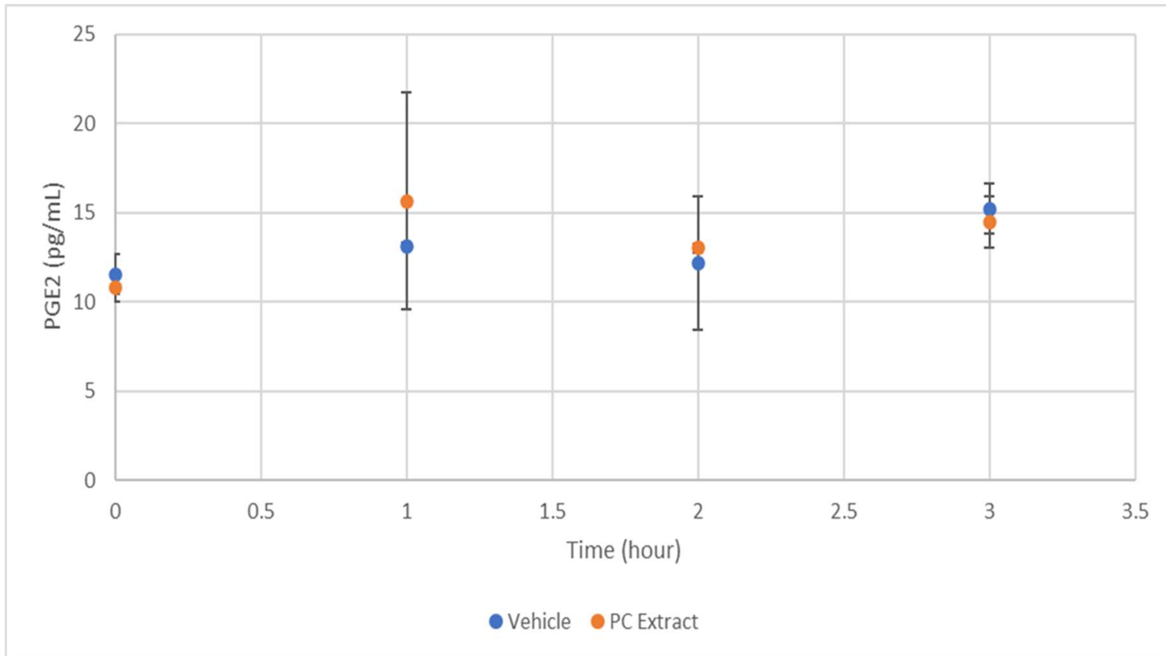


Figure 17: Differences in the Prostaglandin E2 Production of Cells with Venom (5 µg) with the addition of PC (2.5 nmol). Cells plated in complete media on 12 well plates at concentration 1 x 10^5 cell/mL. Cells were then treated at different times (1, 2, and 3 hours). Treatments then analyzed for PGE_2 levels.

The time course of PGE_2 production demonstrated very similar results to the venom concentration experiment. PGE_2 increased at 1 hr then dropped and increased somewhat. The PC did not appear to inhibit PGE_2 production.

The final two experiment were repeating a venom concentration and time study but looking at LTB_4 which is produced by a different pathway from PGE_2, lipoxygenase. The concentration study showed that the venom stimulated a response with an increase in venom

concentration correlating with an increase in LTB_4 concentration. While the venom did stimulate a response the introduction of the PC extract did not show sign of inhibitions (Figure 18).

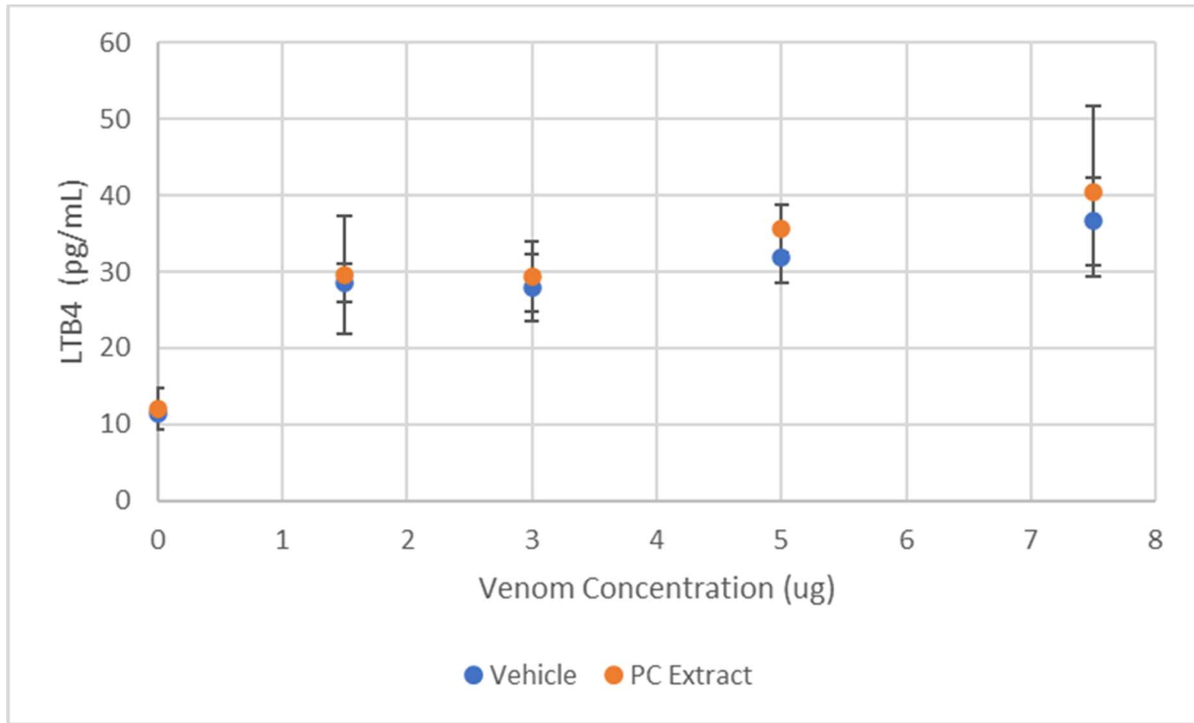


Figure 18: Differences in the Leukotriene B4 Production of Cells with Various Venom Concentrations and the addition of PC (2.5 nmol). Cells grown on 12 well plates at 1 x 10^5 cell/mL. Then pre incubated with PC extract or control before addition of venom. Samples were then analyzed for levels of LTB_4.

The final study was a time trial of LTB_4 production. Venom (3 µg) was added at different time points with PC extract pre incubated at each of the time points. The longer incubations did show increase in LTB_4 concentration but there was no decrease in production for the groups pre incubated with the PC extract (Figure 19).

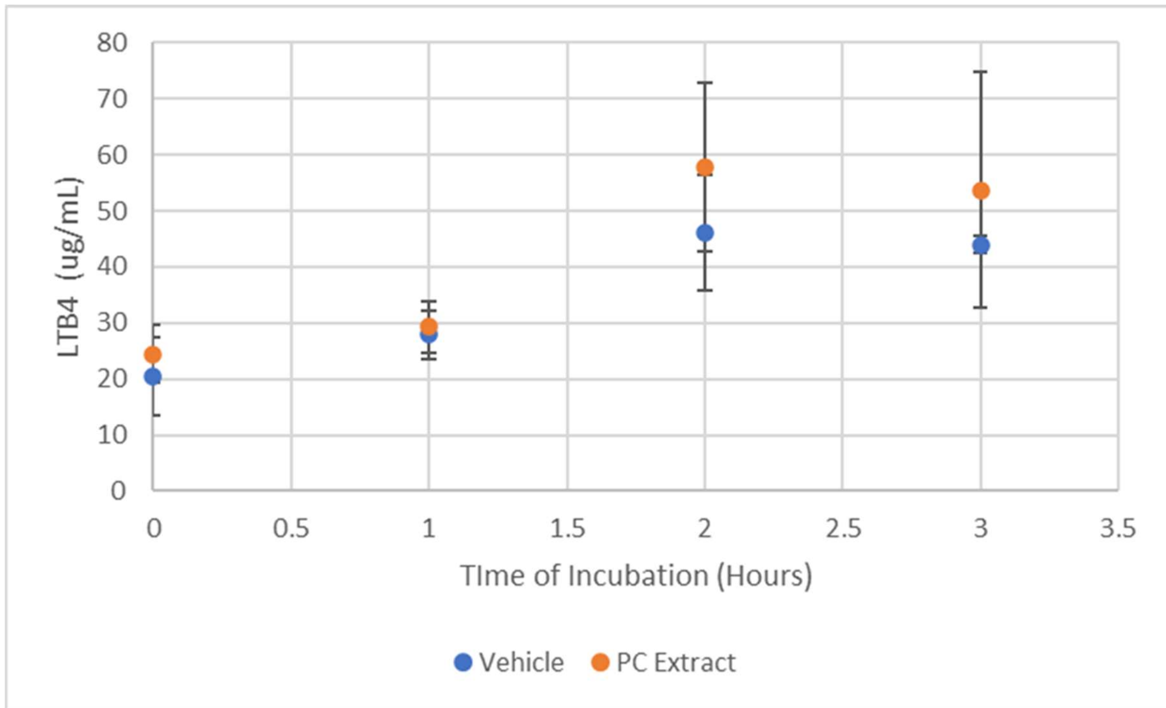


Figure 19: Differences in the Leukotriene B4 Production of Cells with Venom (3 µg) with the addition of PC (2.5 nmol). Cells plated in complete media on 12 well plates at concentration 1 x 10^5 cell/mL. Cells were then treated at different times (1, 2, and 3 hours). Treatments then analyzed for PGE_2 levels.

Discussion

We set out to begin cellular studies on a naturally derived lipid extract which has been shown to inhibit $sPLA_2$ in in vitro assays. Preliminary results from the Venable laboratory show that the extract can inhibit GIIA $sPLA_2$ from snake venom but not GIB (cobra venom) nor bee venom (GIII) or the pancreatic enzyme (GIB). There is a whole family of phospholipases and they play key roles in cells and organisms. PLA_2s play many roles in organisms including membrane maintenance and recycling, breakdown of damaged lipid, protection and repair of tissues, vesicle fusion, inflammation, infection control and more. Finding a compound or group of compounds that could specifically inhibit certain isoforms has long been a goal, and with $sPLA_2$ for its role in inflammation as discussed above. Many compounds have shown specificity in inhibition of the key GIIA PLA_2 inflammatory pathway but due to various issues have failed in cellular or preclinical trials. Finding that there is a natural extract that can inhibit snake venom GIIA PLA_2 and potentially human GIIA PLA_2 is an important discovery.

Inhibitor Effects on Cell Viability Snake Venom Treatments

The first goal of this project was to examine the effects of a naturally derived lipid inhibitor on cell viability in the presence of snake venom. To address this a cellular model was developed taking into consideration venom amount (2.5 - 10 μg), inhibitor concentration (0.05 - 5 nmols), and time (1 - 24 hours). Initial experimentation with venoms from both the Timber and Eastern Diamondback rattlesnakes demonstrated difficulty in achieving cellular death. Short incubations (10 minutes - 2 hours) were initially performed with thinking that snake venom works rapidly (within 2 hours) to incapacitate prey (40). Incapacitation of animals is due to rapid physiological changes that occur from hemolytic enzymes that cause blood clots or neurotoxins

that diminish nerve responses (40). Digestion and death of cells usually is caused by induction of apoptosis or necrosis and occurs several hours (6 - 24 hours) after initial contact with cells (40).

PLA$_2$s such as those in snake venom can help to induce the apoptosis or necrosis when in high amounts or overexpressed. Inhibiting these enzymes is potentially protective. The inhibitor that members of the Venable laboratory investigated demonstrates similar chromatographic properties to the phospholipid species phosphatidylcholine that was extracted with these lipids. After establishing baselines of venom concentration and time, an arbitrary amount of extract (2.5 nmol) was chosen to test its potential effects. Cells were observed after pre incubation with the lipid and showed no morphological signs of death. But after introduction of venom there was a drop in viability that was more than with venom alone (Figure 9). This result was unexpected, and the cause was unknown. In another experiment the PC extract was preincubated with the venom before addition to the cells (Figure 10). This again showed that there was a synergistic effect of increasing cell death requiring investigation into the cause.

First an investigation of the cellular lipids in the presence of snake venom showed that the PC fraction was being metabolized. (Figure 11). The assays were relatively small with only around 100 μL of cells at concentration 1×10^5 cells/mL. A lipid phosphorous assay showed that the amount of phospholipid presents in the cells themselves was only (0.039 nmol). Initial experiments used 2.5 nmols of PC extract, almost 100 times the amount present originally. It was expected that testing cell viability with this great increase in lipid amount would be the root cause of the increase in cell death. Instead, Figure 12 shows that longer incubations with large amounts of lipids led to an increase in cell viability. This may be due to the cells, which were in serum free media, using the extra PC for metabolism in the absence of other lipids. The question remained, what was causing the extra cell death.

Complete metabolism of the phospholipids or at least the PC fraction occurred as seen in Figure 11, but it was possible that the cells were further metabolizing these lipids. For this reason, a similar experiment was repeated but with just venom in the presence of an egg PC standard, the PC extract and then the whole serum (Figure 15). All three demonstrated some but not complete loss of the PC band showing the band was being metabolized. Furthermore, there was a smaller band closer to the origin in the lanes treated with venom. Based on the location of this banding it is most likely the lyso-PC fraction which in high concentations can induce apoptosis (41). Much of the focus of this work has been on the AA produced but the lyso-phospholipids are also important signaling molecules. Lyso-PC has specifically been found to induce apoptosis in cells when in higher concentrations (41, 42). For this reason, I believe the lyso-PC that is formed from the breakdown of the PC lipids is the cause of the extra cell death. This is possible because although the proposed inhibitor is like PC it is extracted with the PC fraction that contains other forms of PC. When added in higher amounts this allows for an increased metabolism and buildup of lyso-PC which could then induce apoptosis. This also explains why when smaller amounts of the lipid are added there is not a similar effect. This means that the inhibitor could potentially be stopping some metabolism of the lipids but with the additional PC lipids there is not a way to get a measurable difference without adding these unwanted components and potential cell death. In other research in the Venable lab there are efforts to further purify the active compounds from the bulk lipid.

Examination of inflammatory signaling molecules and the potential anti-inflammatory capabilities the lipid extract may possess

Like lyso-PC, the fatty acyl metabolites produced by PLA_2s enzymatic action are important lipid mediators that can become various signaling molecules. Eicosanoids are some

molecules directly derived from the arachidonic acid produced from the breakdown of phospholipids. Processed through different pathways the molecules produced can change depending on which enzyme pathway (COX or LOX) is upregulated. The second goal of the study was to see if the lipid extract could inhibit the production of these inflammatory molecules. The direct action of $sPLA_2$ on the production of AA is unknown but there have been studies showing that $sPLA_2$ can upregulate $cPLA_2$ (43). This upregulation does lead to an increase in AA and eicosanoid production (43).

This study looked at 2 different eicosanoids, Prostaglandin E2 (PGE_2) and Leukotriene B4 (LTB_4) as these are produced from different enzymatic pathways, COX, and LOX respectively. This way potential differences in the pathways could be measured.

Figure 14 demonstrated that the presence of a small amount of the lipid extract and venom did not synergistically affect cell viability. Additionally the cells did not need a large amount of venom to produce a signal. A preliminary study showed that a PGE_2 signal could be achieved by treating 1 mL of 1×10^5 cells with 5 μg of Timber Rattlesnake venom. This was used as a starting point for looking at a concentration and time study for PGE_2. A concentration of 2.5 nmol of PC was added 15 min prior to each venom treatment. Figure 16 shows the results of the different concentration of venom and the effects of the addition of the lipid. Treatment with venom produced a modest increase in the PGE_2 levels that varied somewhat with concentration. The inhibitor did not significantly reduce the levels of PGE_2.

Figure 17 was performed similarly but with 5 μg of venom added at different time points. This data also showed modest increase in PGE_2 that varied with time and with no significant effect from the inhibitor.

The next two experiments were for concentration and time studies and their effects on LTB_4 production. Figure 18 showed that there was an increase in LTB_4 peaking at 3 μg. Then a drop at 5 to 7.5 μg. These results showed that there was a difference in LTB_4 levels with the addition of PC with drops at all levels except the 5 μg group which instead showed a slight increase. There was not enough replication to establish a significance level for this, but it seems promising.

The final experiment demonstrated that with longer incubations there were increases in LTB_4 concentration. While there was an increase of LTB_4 production, the addition of the PC extract did not reduce this production indicating a lack of inhibitory action. This means that the use of LTB_4 as a target for inflammatory studies is viable, but the inhibitory action of the PC extract is lacking or not represented well by this model.

To conclude based on the data from Figure 16 and 17 it appears we cannot determine if the addition of the PC extract plays an inhibitory role in the production of PGE_2. The increase in the production of PGE_2 is not significant and so a decrease by inhibitory action could not be measured. On the contrary Figures 18 and 19 showed that with increase in venom concentration as well as longer incubations demonstrated an increase in LTB_4 production. While the venom did stimulate a response the introduction of the PC extract did not elicit a reduction of LTB_4 production. This suggests the PC extract does not inhibit PLA_2 activity and therefore eicosanoid production. These differences in eicosanoid production could be do to a couple of different factors that include the cell type, incubation times as well as pathway expression differences between COX and LOX enzymes. As far as the lack of inhibitory action by the PC extract the explanation could be similar to the cell viability studies. The lack of purification of the PC extract could allow for extra lipids which lack inhibitory action and therefore decrease the

inhibitory potential of the extract. Further studies should be investigated upon further purification of the extract to truly determine the inhibitory effects the PC extract may possess.

Experimental Procedures

Cell Culture and Viability Measurement

THP-1 cells, a monocytic white blood cell line, donated by Dr. Darren Seals laboratory were cultured with medium: RPMI 1640 medium (Thermo Fischer) with 4.5g/L glucose and 2mM L-glutamine and supplemented with 0.05 mM 2-mercaptoethanol; fetal bovine serum (Thermo Fischer), (10%), penicillin-streptomycin (Thermo Fischer), (1%). Cells were incubated at 37° C in a 5% carbon dioxide atmosphere. For viability studies, cells were centrifuged then resuspended in serum-free media to a concentration of ~1×10^5 cells/mL. Cells, (90 μL), were plated on 96 well plates before addition of treatment. For signaling studies, cells were centrifuged and resuspended in complete media to a concentration of ~1×10^5 cells/mL. Cells, (1 mL), were added to 12-well plates for treatment. Cell counts were confirmed using trypan blue and counting on a hemacytometer.

Lipid Extraction, Purification and Quantification

Lipids were extracted using a mixture of chloroform: methanol: water; 1:2:1 then adjusted to 2:2:2. The organic layer was evaporated under a stream of nitrogen, loaded onto silica gel G thin layer chromatography plates. Plates were eluted using chloroform: methanol: acetic acid: water, 50:25:8:3. Lipid standards of phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, and sphingomyelin were used to verify separation and lipid identity. Lipid standards were sprayed with primulin (Thermo Fischer) then viewed with UV light to verify position. Lipid extracts were visualized using water spray. Lipids were scraped and extracted from the silica using a lipid extraction method described above. Phospholipid phosphorous was quantitated according to Rouser (46)

Cell Viability Assay

For viability studies, cells were centrifuged then resuspended in serum-free media to a concentration of ~ 1 x 10^5 cell/mL. Cells (90 µL) were then plated on 96-well plates. Lipid extracts were resuspended at different concentration in 10% aqueous DMSO by sonification for 30 seconds. Lipids (5 µL) were added to each well and allowed to incubate for 24 hours. Various concentration of venom, (5 µL), were then added and allowed to incubate for 24 hours. Resazurin solution, (10 µL), was added and allowed to incubate for 6 hours then measurements were read at fluorescence, excitation 560, emission 590 and absorbances 570 and 600. Treatments were done in triplicate.

ELISA eicosanoid assay

For signaling studies, cells were centrifuged and resuspended in complete media to a concentration of ~1 x 10^5 cells/mL. Cells, (1 mL), were then added to 12 well plates. For all treatments (2.5) nmols of lipid extract were added to the cells, (50 µL), after resuspension in 10% aqueous DMSO. Cells were incubated with the lipid extract for 15 minutes prior to the addition of Timber rattlesnake venom. For concentration curve experiments 0, 1.5, 3, 5, 7.5 and 10 (µg) were added by (50 µL) solution and allowed to incubate for 1 hour prior to extractions for analysis. For time course studies, (5 µg) of venom were added at hours of 3, 2, 1 and 0 before extraction. For both studies post incubation, (500 µL) of sample were extracted into microcentrifuge tubes and centrifuged at 500 rpm for 30 secs. Samples were then plated and analyzed according to manufacturer's (Cayman) instructions.

Fluorescent microscopy

For analysis of delivery mechanisms, THP-1 cells (1 mL) were grown in 12 well plates with glass cover slips. PMA (25 mM) was added to cells and cells were allowed to incubate and adhere to plate for 2 days. The cells were then washed with phosphate buffered saline, then serum free media. NBD-ceramide was dried down and resuspended in either DMSO or BSA. NBD-ceramide (Avanti Polar Lipids), (50 µL) was added to the cells and allowed to incubate for 1 hour. The glass cover slips were then collected, and slides made and viewed using FITC on a (Olympus 1X81 microscope). Nile red was suspended in 1-% aqueous DMSO or water. Cells ,1 x 10^5 cells/mL, were resuspended in serum free media and (95 µL) added to a 96 well plate. After an incubation of 24 hours (50 µL) of cells were extracted and added to a coverslip and viewed using FITC.

Statistics

Viability differences were measured in triplicate and differences of means analyzed using students T-Test. For fluorescent/absorbance reading calculations of cytotoxicity, the protocol used is described by BioRad (47). Measurements of eicosanoid production followed manufacturers (Cayman) instructions making a logarithmic standard curve and measuring using absorbance values. Studies were done in duplicate.

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Vita

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