

ABSTRACT

Andrew N. Sayer. BREVETOXIN TOXICITY IN MAMMALIAN CELLS AS INFLUENCED BY BREVENAL. (Under the direction of Dr. James E. Gibson). Department of Biology, East Carolina University, May 2005.

Brevetoxins (PbTx) are highly potent polyether toxins produced during blooms of several species of marine dinoflagellates, most notably *Karenia brevis*. Brevenal is a nontoxic short-chain polyether that competes with PbTx for the active site on voltage-sensitive sodium channels. Human consumption of shellfish contaminated with PbTxs results in neurotoxic shellfish poisoning (NSP). Additionally, blooms of *K. brevis* are potentially responsible for adverse human health effects such as respiratory irritation and airway constriction in coastal residents. The purpose of this study was to determine if PbTx could induce DNA damage in mammalian cells, and if so, if the damage could be antagonized by brevenal. Results from the single-cell gel electrophoresis assay, inhibition of cellular proliferation assay, chromosomal aberration assay, and disruption of signaling pathways via a Western Blot analysis demonstrate that PbTx is a potent inducer of mammalian cell damage, an effect which can be reduced or alleviated by brevenal.

BREVETOXIN TOXICITY IN MAMMALIAN CELLS
AS INFLUENCED BY BREVENAL

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Andrew N. Sayer

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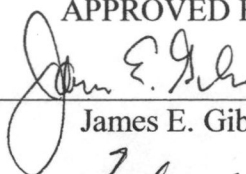
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by
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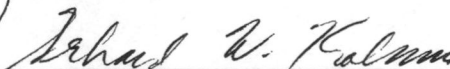
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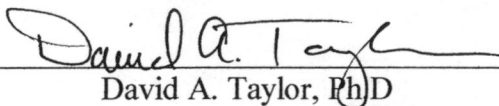
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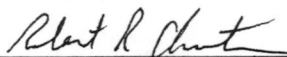
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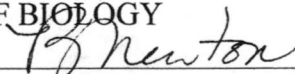
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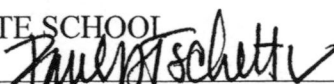
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INTRODUCTION

Harmful algal bloom (HAB) toxins are numerous, chemically complex and diverse, and produced by a variety of organisms under poorly understood environmental stimuli through even less understood molecular mechanisms. Some information is available concerning acute toxicities in humans and animals. The ingestion of tainted shellfish or finfish is responsible for human acute toxicity and the extent of the symptoms is related to the dosage of toxin ingested. It is known that the HAB toxins generally disrupt intracellular ion concentrations of sodium, calcium, or potassium with each toxin having a high affinity for specific receptor or acceptor sites. For example, the ingestion of saxitoxin or its congeners causes paralytic shellfish poisoning (PSP) (Schantz et al., 1957). Saxitoxin and all of its derivatives (at least 21 forms have been described) specifically bind to the voltage-gated sodium channel and adversely affect mainly the peripheral nervous system and to a lesser extent the central nervous system. While acute HAB toxicities often manifest as neurological effects, other effects via alternative mechanisms have been noted. Okadaic acid produced by the dinoflagellates *Dinophysis* and *Prorocentrum* causes diarrhetic shellfish poisoning (DSP). This toxin inhibits protein phosphatases, which have a wide variety of cellular functions including regulation of cell division, regulation of phosphate metabolism, regulation of many intracellular enzymes and other proteins and smooth muscle function. Okadaic acid also possesses genotoxic potential and tumor-promoting properties through the inhibition of phosphatases; but recent evidence, which shows mutations in the progeny of okadaic acid-treated cells and

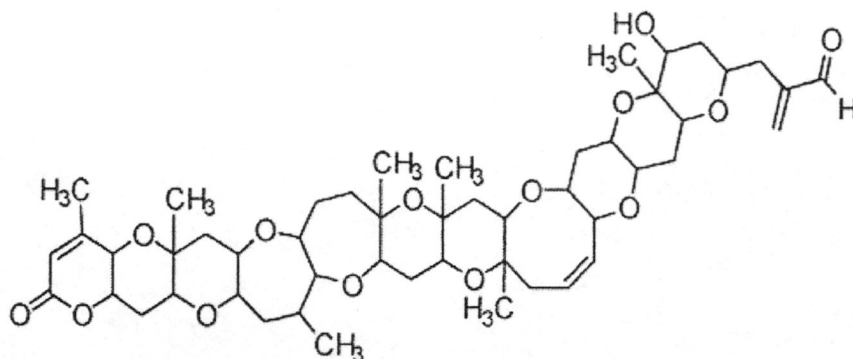
the formation of DNA-adducts, increases concerns over the hazards associated with contaminated shellfish. Okadaic acid may prove to be carcinogenic.

Brevetoxins (PbTx) are cyclic polyether compounds produced by several types of marine dinoflagellates (including *Karenia brevis* and *Chatonella antiqua*) during red tide events in the Gulf of Mexico, off the coasts of Florida and North Carolina, and other costal areas around the world resulting in millions of dollars in damage. At present, 10 PbTx isoforms (collectively PbTx 1-10) have been discovered. While considerable information is known about the neurophysiologic and biochemical properties of PbTx, there is little known about the effect on human health upon exposure. The neurotoxic compounds produced by these organisms are a serious public health concern and heavily impact the commercial fishing industry by causing massive fish kills. An estimated average of \$18 million dollars is lost each year in the U.S. fishing industry due to red tides (Anderson et al., 2000). In addition, there have been catastrophic mortalities of threatened marine species including manatees, bottlenose dolphins and sea turtles. Other animal species, such as birds, which reside in costal areas, are also negatively affected during red tides (Fairey et al., 2001). A study conducted by Kimm-Brinson and Ramsdell (2001) found PbTx-1 induced developmental abnormalities and embryo toxicity in Medaka fish embryos.

Humans are exposed to PbTx in several ways. Neurotoxic shellfish poisoning (NSP) occurs when shellfish accumulate PbTx by filter feeding (Gordon et al., 2001; LePage et

al., 2002). The shellfish are then harvested, sold at a market, and eaten. The average annual public health impact was

Figure 1. Structure of Brevetoxin



estimated at \$1 million dollars per year from shellfish poisoning due to harmful algal blooms during 1987-92 (Anderson et al., 2000). Additionally, coastal residents and visitors to coastal areas become exposed to PbTx in aerosolized form as coastal spray.

Like nearly all marine toxins, PbTx are tasteless, odorless, lipid soluble, and heat and acid stable. Upon exposure by ingestion neurological and gastrointestinal effects occur lasting several days. These include paresthesia, myalgia, vertigo, ataxia, thermal dyesthesia, bradycardia, pupil dilation, diarrhea, and mild to severe headache. In aerosolized form PbTx also cause airway irritation (Music et al., 1973), induce asthma attacks, and cause airway constriction (Abraham et al., 2002). The harmful effects of PbTx on the respiratory system trigger an immune response which may be highly sensitive in certain individuals (i.e. asthmatics, individuals with chronic obstructive pulmonary disease, and other types of pulmonary diseases). The mechanisms of brevetoxin induced immunotoxicity have yet to be elucidated.

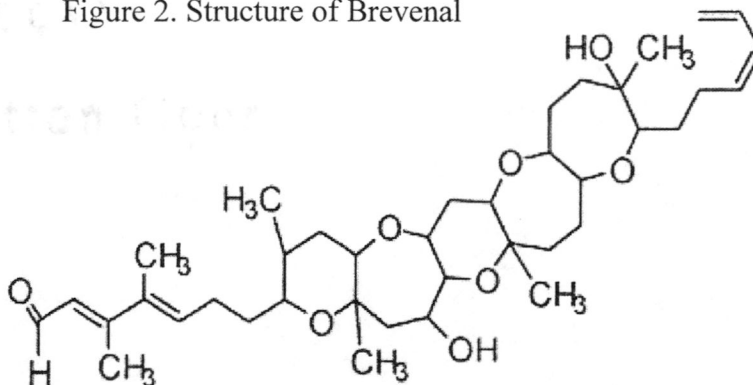
PbTx (figure 1) act on subunit 5 of the voltage-gated Na^+ channel at nanomolar concentrations

(Jeglitsch et al., 1997; Kulagina et al., 2004; Lombet et al., 1987;

Trainer et al., 1999) causing the channel to remain in the open state thus prolonging the duration of sodium transport across the membrane similar to a depolarizing neuromuscular blocking agent. Brevenal (figure 2), a naturally produced PbTx antagonist, antagonizes PbTx at the active site on the Na^+ channel (Bourdelaïs et al., 2004). It is theorized that brevenal competitively displaces PbTx at the active site, thus negating the effect of the toxin.

While PbTx elicit neurotoxic effects by prolonging the open state of voltage-gated Na^+ channels, they also cause bronchoconstriction, respiratory irritation, severe congestion, and airway constriction (Backer et al., 2003). These symptoms are an immune response likely caused by the degranulation of mast cells. While the neurotoxic effects of PbTx are well documented, there is little known about PbTx immunotoxicity. In order to study human immunotoxicity, we hypothesize PbTx may disrupt gene expression in human

Figure 2. Structure of Brevenal



lymphocytes. Since lymphocytes are crucial for immune system function, disruption of lymphocyte gene expression could harmfully affect the immune response. If PbTx does disrupt lymphocyte gene expression, could the damage be antagonized or alleviated by brevenal.

The objective of this research project was to examine brevetoxins and brevenals (toxins and antagonists, respectively) present in *Karenia* and *Chattonella*. HAB toxins were tested for their ability to induce toxicity in human lymphocytes and other mammalian cells. The following *in vitro* toxicity tests were used: (a) DNA damage (single-cell gel electrophoresis assay) in human lymphocytes, (b) cytotoxicity assay in Chinese hamster ovarian cells (CHO-K1-BH4 cells) to evaluate cell proliferation, (c) chromosomal aberration assay in CHO-K1-BH4 cells, and (d) mechanisms of brevetoxin toxicity assay involving the mitogen-activated protein kinase (MAPK) signaling pathway in CHO cells. DNA damage occurs through both endogenous and exogenous physiological and pathophysiological processes. Unrepaired or erroneously repaired DNA damage may result in gene mutation, chromosome aberration, and modulation of gene regulation, which have been associated with carcinogenesis (Clayson et al., 1994, Perera et al., 1991). These tests assessed exposure outcomes, including genotoxic and non-genotoxic events that lead to cancer or organ toxicity in mammalian cells and tissues. The results from these studies will help define the types of morphologic changes associated with HAB toxin exposure.

MATERIALS AND METHODS

The following *in vitro* assays were conducted in mammalian cells (including human) and used in accordance with the methods described by Charles et al., 2002 and other citations specifically mentioned: DNA damage assay (Single-Cell Gel Electrophoresis assay) (Clayson et al., 1994; Perera et al., 1991; Rojas et al., 1999; Tice et al., 1995; Collins et al., 1997); Inhibition of cellular proliferation assay: (Morita et al., 1992); Chromosomal aberrations assay; Mechanism of MAPK signaling pathway disruption via a Western Blot analysis (Bonifacino et al., 2004). CHO-K1-BH4 cells (CHO cells) were used in the cytotoxicity, chromosomal aberration, and mechanism of brevetoxin induced toxicity assays. Human lymphocytes were used for the DNA damage assay.

Single-Cell Gel Electrophoresis Assay

DNA damage was assessed using the alkaline single-cell gel electrophoresis assay (Comet Assay). The alkaline comet assay has been shown to be a sensitive and simple assay in monitoring DNA strand breaks in human leukocytes (Rojas et al., 1999; Tice et al., 1995) and was performed according to the procedure of Collin et al., (1997).

PbTx-2 (FW 895.1 lot # 082K1237) and PbTx-9 (FW 899.1 lot # 102K1347) both from the marine dinoflagellate *Karenia brevis* were purchased from Sigma. The Center for Marine Science at The University of North Carolina at Wilmington (UNCW) provided PbTx-2, PbTx-3, and brevenal. Heparinized blood samples were obtained by venopuncture from a healthy young female volunteer (ECU/IRB # 03-0290).

Lymphocytes were extracted using the hypaque-ficoll method of lymphocyte isolation from blood (Boyum, 1968; Collins et al., 1997). Lymphocytes were collected and counted using a Coulter counter (Beckman Coulter Z2 Cell and Particle Counter, part number: 6605700). Sigma and UNCW PbTxs (500 μg) were dissolved in 556 μl 80% ethanol (EtOH) and appropriately diluted to make 10^{-8} M and 10^{-12} M concentrations using Hanks Balanced Salt Solution (HBSS) without calcium and magnesium (IGN lot # 1810486). Brevenal (0.468 mg) was dissolved in 0.468 ml 80% ethanol and diluted into 100 $\mu\text{g}/\text{ml}$ and 1.0 $\mu\text{g}/\text{ml}$ concentrations using HBSS. Frosted slides used in the assay were dipped into a mixture containing 500 mg normal melting point agarose dissolved in 50 ml 1x Dulbecco's Phosphate Buffered Saline (Gibco lot # 21600-010).

PbTx Treatment. Human lymphocytes (approximately 500 lymphocytes were used per slide per group) were exposed to either Sigma or UNCW PbTx at 10^{-8} M or 10^{-12} M, a negative control (HBSS buffer), a positive control (2.75% hydrogen peroxide), and solvent (80% EtOH) in HBSS at 4 °C for 1 hour. Lymphocytes were allowed to incubate in 1.0 $\mu\text{g}/\text{ml}$ brevenal at 4°C for 1 hour and then exposed to 10^{-8} M Sigma or UNCW PbTxs, or hydrogen peroxide at 4°C for a second hour. Likewise, human lymphocytes were allowed to incubate in 10^{-8} M Sigma or UNCW PbTxs or hydrogen peroxide at 4°C for 1 hour, and exposed to 1.0 $\mu\text{g}/\text{ml}$ brevenal at 4°C for a second hour. Lymphocytes were also treated with the DNA polymerase inhibitor aphidicolin. Lymphocytes were exposed to 10^{-9} M aphidicolin, 10^{-9} M aphidicolin and 10^{-8} M PbTx, or 10^{-9} M

aphidicolin and 1.0 µg/ml brevenal for 1 hour followed by 10^{-8} M PbTx for a second hour.

Gel Electrophoresis. Following the treatment period the lymphocytes were removed from chemical exposure by centrifugation of the sample followed by aspiration of the supernatant, mixed with low melting point agarose, and spread onto pre-frosted slides. Once the agarose/lymphocyte layer was firmly affixed to the slide, the entire slide was immersed into a lysis solution containing salts and other detergents for a minimum of 2 hours to remove the cell membrane from the lymphocytes (Tice et al., 1995). The slides were then submersed into an alkalizing solution for 1 hour to unwind the lymphocyte DNA. Next the slides were put into an alkaline electrophoresis solution (pH>14) in a gel electrophoresis box where lymphocyte DNA undergoes separation (Singh et al., 1998). If DNA was damaged during the chemical treatment period there were many sections of fragmented DNA in the lymphocyte nucleus. If there was no damage to the DNA there were no DNA fragments. In the gel box an electric current was initiated (25 V, 300 milliamps, 40 minutes). The more fragments of DNA present in the lymphocyte nucleus, the greater number of small fragments were attracted to the negative electrode of the gel box due to the inherent charge on DNA, resulting in DNA separation. Following a 40-minute period in the gel box the slides were removed and neutralized using a tris-base solution and allowed to dry overnight (Charles et al., 2002; Rojas et al., 1999).

Fluorescent Microscopy. A fluorescent stain (SYBR green, Trevigen, Lot Number: 4250-050-05) was applied to the slide where the lymphocytes were located. The slide was then viewed under a fluorescence microscope and the comet size, head length, tail length, and amount of DNA present in the head and tail (measured by the intensity of SYBR green fluorescence) were measured using imaging analysis software (Northern Eclipse Imaging Analysis). The imaging software was able to detect SYBR green bound to DNA. The intensity of the fluorescence was assigned a numerical value where the greater the fluorescent intensity (an indicator of amount of DNA present) the greater the value. 50 comets per slide were measured and the experiment was performed in triplicate or more. The tail moment, which is defined as the percentage of DNA in the tail multiplied by the length between the center of the head and center of the tail as determined from the software, (Lee et al., 2004) was calculated and reported.

Statistical Analysis. Control and treated groups were compared using a one way analysis of variance and differences among means were determined by using the least significant difference test (LSD test), $p < 0.05$. Means and standard errors were calculated (Enterprise Guide, SAS Statistical Software, Cary, NC).

Inhibition of Cellular Proliferation Assay

The inhibition of cellular proliferation assay evaluated the ability of PbTx to inhibit cellular proliferation in CHO-K1-BH4 cells. After the incubation period, cells were evaluated for their ability to proliferate by comparing the number of cells found in treated wells compared to the number of cells in untreated (control) wells.

Cell Maintenance and PbTx preparation. First passage CHO cells were generously donated by Dr. G. Charles of The Dow Chemical Company, Midland, MI. The cells were grown in HAMS F12 culture media containing HEPES buffer, 5% fetal bovine serum, 1% fungicide, and 1% penicillin. 75 cm² flasks containing 15 ml culture media were used to grow the cells (100 µl worth of cells were used per passage). Cells were allowed to grow for approximately 7 days at 37 °C in 5% CO₂. By the end of the cellular growth phase flasks were at least 80% confluent.

PbTxS were prepared as follows: Sigma PbTx-2 and UNCW PbTxS 2 and 3 (500 µg) were dissolved in 556 µl 80% ethanol (EtOH) and appropriately diluted to make 10⁻⁴, 10⁻⁶, 10⁻⁹, 10⁻¹², and 10⁻¹⁵ M concentrations using Hanks Balanced Salt Solution (HBSS) without calcium and magnesium (IGN lot # 1810486). Sigma PbTx-9 (10 µg) was dissolved in 111.22 µl 80% ethanol and diluted using HBSS to make 10⁻⁶, 10⁻⁹, 10⁻¹², and 10⁻¹⁵ M concentrations. Brevenal was also used to evaluate the potential for PbTx antagonism. Brevenal (0.468 mg) was dissolved in 0.468 ml 80% ethanol and diluted into 1.0 µg/ml and 0.1 µg/ml concentrations using HBSS.

At the end of a CHO cell growth period, the cells were harvested for PbTx treatment. 1 ml trypsin/EDTA was used to loosen the cells from adhering to the bottom of the 75 cm² growth flask. Cells were resuspended in 1 ml culture media. Cells were counted using a Coulter counter (Beckman Coulter Z2 Cell and Particle Counter, part number: 6605700). 100,000 cells were used per control and treatment group.

PbTx and Brevenal Treatment. 6-well plates were used for PbTx treatment purposes. Each 6-well plate contained one type of PbTx (Sigma PbTx-2 or 9, or UNCW PbTx-2 or 3), and each well had a different concentration of PbTx. However, one well on each plate was designated as a control well. Culture media (2 ml) and 100,000 cells were added to each well. PbTx at 10^{-4} , 10^{-6} , 10^{-9} , 10^{-12} , and 10^{-15} M concentrations were administered to five different wells (except for Sigma PbTx-9 in which case the initial concentration used was 10^{-6} M). After PbTx treatment, plates were returned into the incubators at 37 °C in 5% CO₂ for 48 hours.

Determination of Cellular Proliferation. At the end of the treatment phase, 6-well plates were removed from the incubators. The culture media was aspirated using a vacuum pump and Pasteur pipette. Cells that had not died after PbTx treatment were considered cells with the potential to proliferate (Morita et al., 1992), and cells which had died after PbTx treatment no longer had the ability to proliferate and were aspirated away. Following aspiration, 300 µl trypsin/EDTA was used to loosen cells from the bottom of

the wells, and cells were resuspended in 1 ml culture media. Each well was counted using the coulter counter. On any 6-well plate the percentage of proliferating cells in treatment wells was compared to the percentage of proliferating cells in the control well of that plate. In this experiment, 100% of the cells found in control wells are considered capable of proliferating. The experiment was conducted in triplicate.

Cell Proliferation with Brevenal. The effect of brevenal on CHO-K1-BH4 cells exposed to PbTx was also investigated. UNCW PbTx-2 at concentrations of 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} M were used for this experiment because from the previous study this type of PbTx yielded the most consistent results. Brevenal, at 1.0 $\mu\text{g/ml}$ and 0.1 $\mu\text{g/ml}$, was used. Three 6-well plates were used, each containing a different treatment group. The wells of different plates had either 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} M UNCW PbTx-2, 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} M UNCW PbTx-2 each with 1.0 $\mu\text{g/ml}$ brevenal per well, or 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} M UNCW PbTx-2 each with 0.1 $\mu\text{g/ml}$ brevenal per well. The experiment was conducted in triplicate.

Determination of the IC50. The concentration of PbTx that inhibits 50% of the cells from proliferating (IC50) was also determined. UNCW PbTx-2 was selected for this experiment. From previous studies of UNCW PbTx-2 it was determined the IC50 value ranged between 10^{-6} and 10^{-9} M, thus UNCW PbTx-2 concentrations used in this trial were $10^{-6.5}$, 10^{-7} , $10^{-7.5}$, and 10^{-8} M. One 6-well plate was used. Cells were exposed to PbTx and left for 48 hours. After the treatment period the wells were aspirated,

trypsinized, resuspended, and counted. Percentages of proliferating cells treated with PbTx were compared to a control well on the same plate. The experiment was conducted in triplicate.

Statistical Analysis. Control and treated groups were compared using a one way analysis of variance and differences among means were determined by using the least significant difference test (LSD test), $p < 0.05$. Means and standard errors were calculated (Enterprise Guide, SAS Statistical Software, Cary, NC).

Chromosomal Aberrations Assay

The chromosomal aberration assay was conducted in CHO-K1-B4 cells maintained as for the cytotoxicity assay. This experiment tested the ability of PbTx to damage the chromosomes of CHO cells. At the time of treatment, a negative control, or the positive control chemical (mitomycin C), or PbTx at a concentration very close to the IC₅₀ was added to the CHO cell wells.

Cell Maintenance and Treatment. CHO cells were grown and maintained in the parameters set in the inhibition of cellular proliferation experiment. UNCW PbTx-2 (500 µg) was dissolved in 556 µl 80% ethanol and diluted into 10^{-7} M using HBSS. The positive control, mitomycin C (MMC), was made using 500 µg MMC (Sigma # M0503) in 4 ml culture media. The solution was then diluted 1:100 using culture media.

At the end of a CHO cell growth period, the cells were harvested for treatment. Trypsin/EDTA (1 ml) was used to loosen the cells from adhering to the bottom of the 75 cm² growth flask. Cells were resuspended in 1 ml culture media. Cells were counted using a Coulter counter (Beckman Coulter Z2 Cell and Particle Counter, part number: 6605700). 100,000 cells were used per treatment group. Three wells of a 6-well plate were used for treating the CHO cells (Charles et al., 2002). One well was a negative control (untreated), one well was a positive control (30 µl MMC), and the third well contained 10⁻⁷ M UNCW PbTx-2. After treating the cells, the plates were returned to the incubator for 48 hours at 37 °C in 5% CO₂.

CHO Cell Purification. Following the 48 hour treatment period, the 6-well plate was removed from the incubator. 5 µl/ml demecolcine solution was added to each well. The plate was placed back into the incubator for an additional 3 hours.

Following the 3 hour demecolcine treatment phase, the plates were taken out of the incubator and placed under the hood. Centrifuge tubes (3 x 15ml, one centrifuge tube per well) were also placed under the hood. A Pasteur pipette was specifically assigned to one centrifuge tube and used only for the treatment group in that centrifuge tube. The culture media was removed from an individual well using the designated Pasteur pipette for that well and put into a clearly labeled 15 ml centrifuge tube (each well corresponded with a centrifuge tube). One ml of trypsin/EDTA was then added to each well. The plate was rocked to loosen cells adhering to the bottom of the well. Once the cells were loose

(approximately 2 minutes) 1 ml of culture media was added to the wells to neutralize the trypsin. The cells were suspended and the entire trypsin/culture media/cell suspension was removed from each well and placed into the designated 15 ml centrifuge tube (Charles et al., 2002).

All three 15 ml centrifuge tubes were placed into a centrifuge. A fourth (blank) centrifuge was placed into the centrifuge to equally balance the centrifuge during centrifugation. The centrifuge was set for 1200 RPMs and activated for 10 minutes. Following this period, the centrifuge tubes were removed from the centrifuge. The supernatant of each 15 ml centrifuge tube was aspirated away using a Pasteur pipette making sure not to disturb the pellet. A different Pasteur pipette was used for each 15 ml centrifuge tube.

The pellets of each treatment group were resuspended in 4.5 ml 0.075 M KCl solution. A 1.5 M KCl solution was kept as stock, and this stock solution was diluted 1:20 to make the 0.075 M KCl solution. The pellets were again resuspended in 4.5 ml 0.075 M KCl solution for 15 minutes. Following the 15 minute waiting period, 0.5 ml of a fixative solution was added and mixed within each 15 ml centrifuge tube. The fixative solution contained a mixture of 60 ml methanol and 20 ml acetic acid (in a 3:1 combination, respectively). All treated centrifuge tubes were put into the centrifuge, again at 1200 RPMs for 10 minutes. After the centrifugation period the centrifuge tubes were removed from the centrifuge, the supernatant was aspirated with Pasteur pipettes unique for each

centrifuge tube, and 5 ml of fixative solution was added (Biswas et al., 1997). The pellet was resuspended in the 5 ml of the fixative solution and the suspension was left for 20 minutes. Following the 20 minute period, the 15 ml centrifuge tubes were placed back into the centrifuge. The centrifuge was set at 1200 RPMs for 10 minutes. After the 10 minute centrifugation period the centrifuge tubes were removed from the centrifuge, the supernatant was aspirated with Pasteur pipettes, and 5 ml of a fixative solution was added. The pellet was resuspended in the 5 ml of the fixative solution and the suspension was left for 30 minutes. Following the 30 minute period, the 15 ml centrifuge tubes were placed back into the centrifuge. The centrifuge was set at 1200 RPMs for 10 minutes. After the centrifugation period the centrifuge tubes were removed from the centrifuge and all the supernatant was aspirated away (using Pasteur pipettes exclusive for only 1 sample) until approximately 0.5 ml of fixative and the pellet remained in each 15 ml centrifuge tubes. The pellet was resuspended in the remaining 0.5 ml fixative solution.

Affixing and Staining Cells on Slides. Unfrosted slides were used and chilled overnight in distilled water at 4 °C. Excess water was gently tapped off the slides and each individual slide was clearly labeled with the sample to be affixed to it. Each individual sample from the 15 ml centrifuge tubes had a unique slide. Using a Pasteur pipette, the 0.5 fixative/cell solution sample was dripped onto a chilled slide. The distance the sample was dripped was approximately 10 inches. Once a single fixative/cell solution sample was dripped onto a slide, the chromosomes were spread by gently blowing on the slide to spread the sample. After each drop and chromosome spread, the slide was

quickly wafted over a candle flame in an effort to affix the sample in place on the slide. Approximately 10 drops per sample were administered per slide. Once the samples were affixed to the slides, the slides were allowed to dry for approximately 10 minutes (Charles et al., 2002).

After the 10 minute drying period a GIEMSA stain solution was applied to the slides. The solution consisted of 9 ml GURR buffer (Gibco # 10582-013) mixed with 1 ml GIEMSA stain (Sigma # G-3032). Using a Pasteur pipette, the stain was applied to the slides in such a manner that the slides were covered with the stain but the stain did not run off the edges of the slides. The stain was left on the slides for 10 minutes. Following the staining process the stain was removed from the slides (by pouring it away) and gently rinsed with distilled water. Slides were allowed to dry overnight.

Scoring Chromosomal Aberrations. The slides were viewed on a light microscope using an oil emersion technique to enhance image detail (Carl Zeiss, Inc., # 47-30-9901, West Germany). A 64x magnification was used. Due to the close proximity of the slide to the objective, the highest light setting possible was used to view the cells. In addition, a green lens was placed over the light source which greatly enhanced the purple-stained chromosomes. Only cells undergoing metaphase were viewed, and only those metaphase cells whose chromosomes could be distinguished were scored. Scoring of chromosomes consisted of gaps, breaks, or other clearly distinguishable aberrations (Rooney and Czepulkowski, 1986; Savage, 1999). While many types of aberrations may be present,

the main objective of the experiment was to determine if a chromosome had an aberration. Fifty distinguishable chromosomes were counted per sample, and each treatment group was conducted in triplicate.

Statistical Analysis. Control and treated groups were compared using the Mann-Whitney U-test (Wilcoxon two sample test), $p < 0.05$ (Sokal and Rohlf, 2003).

Determination of MAPK Signaling Pathway Disruption via a Western Blot Analysis

The determination of mitogen-activated protein kinase (MAPK) signaling pathway disruption via a Western blot analysis evaluated the effect of PbTx on the MAPK signaling pathway. The MAPK signaling cascade is one of many pathways a cell uses to transmit extracellular signals to intracellular and nuclear targets (Reistad et al, 2004). One or more of components of the MAPK pathway phosphorylates a subsequent step in the pathway until a final component activates the target receptor (Naranatt et al, 2003). CHO-K1-BH4 cells were isolated and purified and MAPK abundance as determined by immunoprecipitating the enzyme with antibodies to extracellular signal-regulated kinases 1 and 2 (ERK1/2), protein kinase C (PKC) and tyrosine kinase. After the immunoprecipitation and phosphorylation reactions, the samples were loaded into a gel (15% SDS-polyacrylamide gel) and run for 1 hr. using bromophenol blue dye (Biser et al., 2002). The gel was viewed on a chemiluminescence imager and the amount of MAPK activity was determined after 1, 4, 8, and 24 hours.

Total Particulate and Treatment of CHO Cells. Five 150 mm dishes (one dish for 1 hr, 4 hr, 8 hr, and 24 hr PbTx treatments and one control) were seeded with 100 μ l CHO-K1-BH4 cells and allowed to grow until 80% confluent in 25 ml culture media. At 80% confluence the dishes were treated with 10^{-7} M UNCW PbTx-2 for 1, 4, 8, or 24 hours (controls were left untreated for 24 hours).

At the end of a treatment period the media was decanted from the plate. Tris/MgCl₂ (500 μ l) solution was added to the plate (50 mM Tris solution, pH 7.4 and 5 mM MgCl₂). Plates were scraped using a rubber scraper while ensuring the Tris/MgCl₂ solution continually bathed the cells. The contents of the plate were put into a 1.5 ml Eppendorf tube. Using an 18 gauge needle and syringe, the cells in the Eppendorf tube were passed through the needle 30 times in an attempt to break up cellular membranes. The sample was frozen at -20 °C until all treatment groups were ready for homogenization.

Once all the treatment groups were passed through the 18 gauge needle, all the samples were centrifuged in 4 °C at 15,000 x g for 15 minutes. Following the centrifugation period, the samples were removed and the liquid decanted (with the pellet remaining). An additional 250 μ l Tris/MgCl₂ was added to the eppendorf tubes and the pellet was resuspended. The tubes were then again centrifuged in 4 °C at 15,000 x g for 15 minutes. Following this centrifugation period the samples were again decanted. They were resuspended in 300 μ l Tris/MgCl₂ solution. The samples were again passed through an

18 gauge needle 30 times. The samples were left on ice in preparation for the Bradford assay.

Bradford Dye-Binding Assay. A Coomassie blue dye (Bradford dye) was prepared by dissolving 100mg Coomassie Brilliant Blue in 50 ml distilled water, then adding 100ml 85% phosphoric acid (the solution will turn reddish), and then filled to 1000ml with distilled water. Bovine serum albumin (BSA) solution was prepared by putting 1 mg BSA into 1 ml distilled water (dH₂O). Serial dilutions of BSA with dH₂O were conducted; 0.50, 0.25, 0.10, and 0.05 mg/ml BSA. 100 μ l of each BSA concentration was put into duplicate cuvette tubes. This was done for all BSA samples, all protein samples collected, and an additional cuvette tube containing 100 μ l dH₂O that was used as a blank. Five ml of the Bradford dye was added to each cuvette tube and mixed with the sample. After the samples were left alone for 10 minutes, they were read using a spectrophotometer at an absorbance value of 595 nm after 2 minutes and slightly before 1 hour. The absorbance values found for the BSA serial dilution samples were used to determine the amount of protein found in the treated samples (Bradford, 1976). By comparing known amounts of protein using the BSA absorbance values, the amount of protein in the samples were found by comparison.

Gel Electrophoresis. Based on the Bradford dye binding assay, 10- μ g protein was used per well per treatment group. The concentration of protein per sample was calculated to be approximately 1 μ g/ 1 μ l. The maximum volume which could be loaded into the wells

was 15 μ l, and a minimum 2 μ l bromophenol blue (BTB) stain was needed per well. Thus, approximately 10 μ l protein sample was loaded with approximately 5 μ l BTB stain per well. The BTB stain contained 295.5 mg Tris-HCl, 0.6 g SDS, 3.0 g glycerol, and 0.0003 g bromophenol blue, and then filled to a final volume of 28.5 ml. For the working solution, 50 μ l β -mercaptoethanol was added to 950 μ l of the stock.

Once the protein and dye concentrations were determined, the electrophoresis unit was assembled. Pre-cast gels were placed into the chamber and locked into place. A 10X running buffer was diluted to 1X and used to fill the electrophoresis apparatus (the running buffer contained 30.28 g Tris-base, 150.14 g glycine, 10.0 g SDS, pH 8.6, filled to a final volume of 1000 ml for 10X stock). The center of the electrophoresis unit was filled up first to ensure no leaks were present. After this evaluation, the rest of the unit was filled half way with 1X running buffer. Each pre-cast gel had 15 wells, the first two and last wells had 10 μ l Kalidescope protein standards which served as a ladder or marker. 15 μ l of the PbTx treated samples already mixed with BTB dye were loaded into the wells in the order of 1, 4, 8, 24 hour treatments, and control. This was done two times in sequence per two gels run in the electrophoresis unit. The gel was run at 120 V until the BTB dye was near the bottom of the gel.

Gel to Nitrocellulose Membrane Transfer. Once the BTB stain had reached the bottom of the gel, the electrophoresis apparatus was turned off. Two pieces of extra thick filter paper (Bio-Rad 170-3965) and nitrocellulose membranes (cut into 3.25 x 2 in rectangles)

were prepared and pre-soaked in transfer buffer. Five paper towels soaked in transfer buffer were placed on the transfer apparatus followed by one of the two pieces of extra thick filter paper, the nitrocellulose membrane, the gel which had previously undergone electrophoresis, and finally the other piece of extra thick filter paper. An additional few pieces of paper towels soaked in transfer buffer were placed on top of the top piece of filter paper. Extra care was given to ensure no pieces of filter paper nor membrane was touched with the bare hand (only with gloves if necessary). The transfer apparatus was run at 20 V for 1 hour.

Following the gel transfer onto nitrocellulose membrane, the nitrocellulose membrane paper was removed from the gel and allowed to completely dry (approximately 15 minutes). A blocking buffer consisting of 3% dehydrated bovine milk in 50 ml dH₂O was put into a container with the membrane. The membrane remained in the blocking buffer for a minimum of 2 hours (usually 5 hours) on a rocking apparatus to ensure the entire membrane was exposed to the blocking buffer (Biser et al., 2002).

Antibody Incubations. Following the blocking buffer incubations, the blocking buffer solution was removed from the membrane and discarded. Three PBST washes were done to the membrane, each wash lasting 15 minutes. After each wash the PBST was discarded.

Following the PBST washes the primary antibody was added to the membrane. All antibodies were obtained from Abcam Inc. (ERK 1 Abcam # ab9363; ERK 2 Abcam # ab7948; PKC Abcam # ab31; tyrosine kinase Abcam # ab8076). The antibody dilutions were as follows:

Antibody	Dilution
ERK 1	1:250
ERK 2	1:250
PKC	1:500
Tyrosine Kinase	1:2000

The stock antibodies were diluted in PBST to the ratio shown in the table based on manufacturer recommendations, literature values, and trial and error. Ten ml total volume of diluted antibody was used for the treatment period. The membrane incubated in the primary antibody overnight in a rocking apparatus at 4 °C to ensure the entire membrane was covered with the antibody (Thodeti et al., 2001; Sakai et al., 1997).

The following day, the primary antibody was removed, placed into a vial, and stored for future use (the primary antibody may be used for 5 or 6 replicates). Three 10-minute PBST washes were performed on the membrane. After the final PBST wash, the PBST was discarded, and a secondary antibody incubated the membrane. The secondary antibody, goat anti-mouse for ERK1/2 and goat anti-rabbit for tyrosine kinase and PKC, was also diluted in PBST in a 1:2000 ratio. The secondary antibody was kept on the membrane for 1 hour. Following the secondary antibody incubation period the membrane washed three more times, 5 minutes per wash with PBST. After each wash, the PBST used was discarded.

Western Blot Imaging. Following the last wash with PBST the membrane was placed in a chemiluminescence solution (Pierce Biotechnology # 34075) for 5 minutes. The chemiluminescence solution attached to the secondary antibody conjugated to the horseradish peroxidase to emit light. Following the chemiluminescence treatment period, the membrane was removed and placed on a piece of aluminum foil. A piece of saran was placed on top of the membrane to prevent degradation of the membrane. The membrane was then placed in the Alpha Innotech imaging system for photographic analysis (Biser et al., 2002). Once in the Alpha Innotech imaging (Alpha Innotech, San Leandro California, FluorChem 8000) system a camera was focused on the membrane and appropriate exposure limits were set. Ten pictures were taken over a 5-minute period using different exposure intervals to maximize the detail of the picture. The light emitted by the chemiluminescence reaction was captured by the imaging system. From the

pictures taken, one picture was selected for quantification analysis using protein densitometry. The imaging system allowed for the bands of the membrane to be analyzed by band intensity in order to give an estimate of the amount of protein present.

RESULTS

Single-Cell Gel Electrophoresis Assay

The effect of different PbTx (10^{-8} and 10^{-12} M) 1-hour incubations are shown in Table 1. The results are expressed as the "tail moment" which is defined as the percentage of DNA in the tail multiplied by the length between the center of the head and center of the tail, rather than comet, head or tail length. PbTx in concentrations of 10^{-8} and 10^{-12} M cause DNA damage in human lymphocytes. Human lymphocytes exposed to a 2-hour PbTx-2 at 10^{-8} M treatment period were also investigated (Table 2). DNA damage of a magnitude similar to a 1-hour exposure was found following a 2-hour treatment period. The solvent, EtOH, did not damage the lymphocyte DNA.

Experiments conducted with 1 hour brevenal pretreatment followed by PbTx treatment for a second hour resulted in undamaged lymphocytes (Table 3). Lymphocytes were also pretreated with 10^{-8} M PbTx for 1 hour and then treated with 1.0 $\mu\text{g/ml}$ brevenal for a second hour. Brevenal at 1 and 100 $\mu\text{g/ml}$ fully antagonized the DNA damage elicited by the PbTx whether given before or after the toxins in nearly all instances. Brevenal alone caused no measurable DNA damage in human lymphocytes.

Additionally, brevenal did not prevent hydrogen peroxide induced DNA damage (Table 4), and the extent of DNA damage is not significantly different than hydrogen peroxide alone. DNA damage caused by hydrogen peroxide and brevenal is not significantly different than DNA damage caused by hydrogen peroxide alone.

To test the possibility that brevenal was antagonizing PbTx effects on DNA repair processes, an inhibitor of DNA polymerase was examined for its effect on the brevenal antagonistic effect. Aphidicolin was the inhibitor of DNA polymerase used in this study. Treatments with PbTx alone induced DNA damage that was alleviated by the addition of brevenal. By inhibiting DNA repair mechanism via aphidicolin, the extent of DNA damage seen with aphidicolin, PbTx, and brevenal is similar to PbTx alone (table 5). Figure 3 (a-f) depict several images of human lymphocyte nuclei as seen under the fluorescent microscope.

Table 1

Tail moments of human lymphocytes exposed to different PbTx treatments

Treatment	N	Tail Moment
		Mean +/- S.E.
Negative Control	30	29.2 + 0.9
H ₂ O ₂ (2.75%)	30	72.1 + 1.5*
Solvent (EtOH)	3	24.2 + 2.1
PbTx-2 10 ⁻⁸ M (Sigma)	6	41.3 + 3.6*
PbTx-2 10 ⁻¹² M (Sigma)	3	46.85 + 1.5*
PbTx-2 10 ⁻⁸ M (UNCW)	3	49.4 + 9.9*
PbTx-2 10 ⁻¹² M (UNCW)	3	41.8 + 8.1*
PbTx-3 10 ⁻⁸ M (UNCW)	6	64.0 + 6.4*
PbTx-3 10 ⁻¹² M (UNCW)	3	41.4 + 5.2*
PbTx-9 10 ⁻⁸ M (Sigma)	3	57.0 + 5.3*
PbTx-9 10 ⁻¹² M (Sigma)	3	41.0 + 2.9*

*Significantly different from the negative control, $p < 0.05$

Table 2

Tail moments of human lymphocytes treated with different sources of brevetoxins for 2 hours

Treatment	N	Tail Moment
		Mean +/- S.E.
Negative Control	30	29.2 + 0.9
H ₂ O ₂ (2.75%)	30	72.1 + 1.5*
2 Hr. PbTx-2 10 ⁻⁸ M (Sigma)	3	50.1 + 2.0*
2 Hr. PbTx-2 10 ⁻⁸ M (UNCW)	3	51.0 + 2.4*
2 Hr. PbTx-3 10 ⁻⁸ M (UNCW)	3	49.1 + 0.8*
2 Hr. PbTx-9 10 ⁻⁸ M (Sigma)	3	50.1 + 1.8*

*Significantly different from the negative control, $p < 0.05$

Table 3

The effect of brevenal on human lymphocytes exposed to brevetoxins

Treatment	N	Tail Moment
		Mean +/- S.E.
Negative Control	30	29.2 + 0.9
H2O2 (2.75%)	30	72.1 + 1.5*
Brevenal 1.0 µg/ml	9	30.8 + 2.6
Brevenal 100 µg/ml	3	36.3 + 1.7
Brevenal 1.0 µg/ml 1 hour before PbTx-2 10 ⁻⁸ M (Sigma)	3	31.3 + 2.1
PbTx-2 10 ⁻⁸ M (Sigma) 1 hour before Brevenal 1.0 µg/ml	3	33.5 + 0.3
Brevenal 1.0 µg/ml 1 hour before PbTx-2 10 ⁻⁸ M (UNCW)	3	33.9 + 1.4
PbTx-2 10 ⁻⁸ M (UNCW) 1 hour before Brevenal 1.0 µg/ml	3	40.3 + 1.9
Brevenal 1.0 µg/ml 1 hour before PbTx-9 10 ⁻⁸ M (Sigma)	3	35.5 + 2.9
PbTx-9 10 ⁻⁸ M Sigma 1 hour before Brevenal 1.0 µg/ml	3	40.1 + 2.4
Brevenal 1.0 µg/ml 1 hour before PbTx-3 10 ⁻⁸ M (UNCW)	6	34.9 + 1.25
PbTx-3 10 ⁻⁸ M (UNCW) 1 hour before Brevenal 1.0 µg/ml	3	42.4 + 0.75**
Brevenal 1.0 µg/ml simultaneous with PbTx-2 10 ⁻⁸ M (Sigma)	3	27.4 + 1.2
Brevenal 1.0 µg/ml simultaneous with PbTx-2 10 ⁻⁸ M (UNCW)	3	49.2 + 7.1

*Significantly different from the negative control, p< 0.05

** Significantly different from brevenal 1.0 µg/ml, p< 0.05

Table 4

The effect of brevenal on hydrogen peroxide

Treatment	N	Tail Moment
		Mean +/- S.E.
Negative Control	30	29.2 + 0.9
H2O2 (2.75%)	30	72.1 + 1.5*
Brevenal 1.0 µg/ml 1 hour before H2O2	3	72.1 + 3.7***
H2O2 1 hour before Brevenal 1.0 µg/ml	3	74.5 + 4.3***

*Significantly different from the negative control, $p < 0.05$ ***Significantly different from H2O2 alone, $p < 0.05$

Table 5

The effect of aphidicolin on lymphocytes exposed to brevetoxin or brevetoxin and brevenal

Treatment	N	Tail Moment
		Mean +/- S.E.
Negative Control	30	29.2 + 0.9
H ₂ O ₂ (2.75%)	30	72.1 + 1.5*
Brevenal 1.0 µg/ml	9	30.8 + 2.6
PbTx-3 10 ⁻⁸ M (UNCW)	6	64.0 + 6.4*
Brevenal 1.0 µg/ml 1 hour before PbTx-3 10 ⁻⁸ M (UNCW)	6	34.9 + 1.25
Aphidicolin 10 ⁻⁹ M	3	46.2 + 2.8**
Aphidicolin 10 ⁻⁹ M and PbTx-3 10 ⁻⁸ M (UNCW)	3	63.6 + 4.1**
Aphidicolin 10 ⁻⁹ M and Brevenal 1.0 µg/ml 1 hour before PbTx-3 10 ⁻⁸ M (UNCW)	3	64.5 + 1.3**

*Significantly different from the negative control, $p < 0.05$

** Significantly different from brevenal 1.0 µg/ml, $p < 0.05$

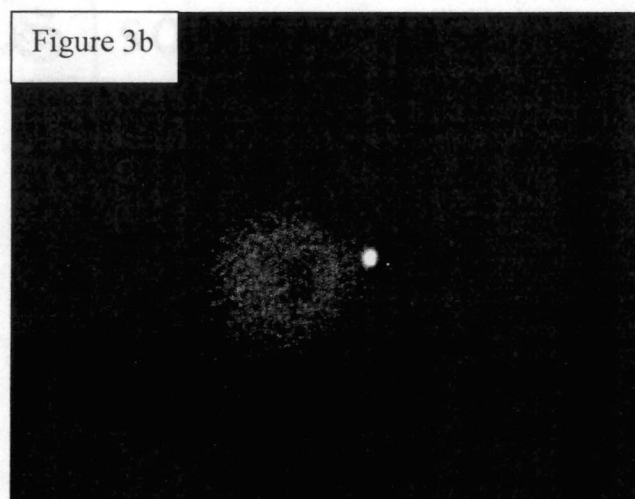
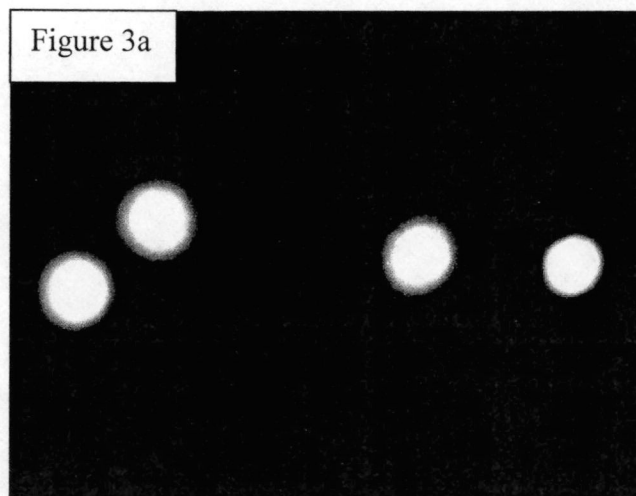


Figure 3. Human lymphocytes were left untreated or exposed to hydrogen peroxide, brevetoxin, or brevenal for 1 hour. a) Human lymphocytes not exposed to any genotoxic damaging chemical. There is no DNA damage. b) A human lymphocyte treated with hydrogen peroxide (positive control). A large amount of DNA damage is present.

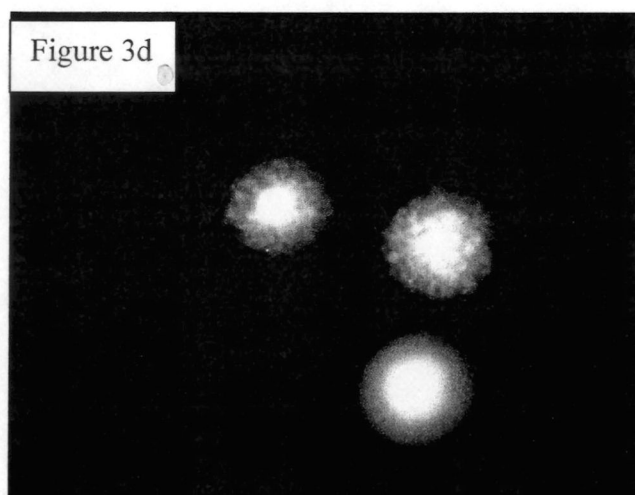
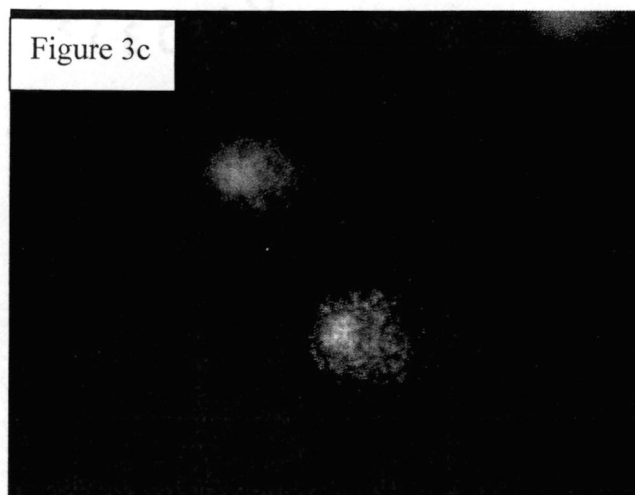


Figure 3c). Human lymphocytes treated with 10^{-8} M brevetoxin. Both sources of brevetoxin used in this experiment at a concentration of 10^{-8} M resulted in a similar degree of DNA damage. d). Human lymphocytes first treated with brevenal for 1 hour prior to treatment with 10^{-8} M brevetoxin for a second hour.

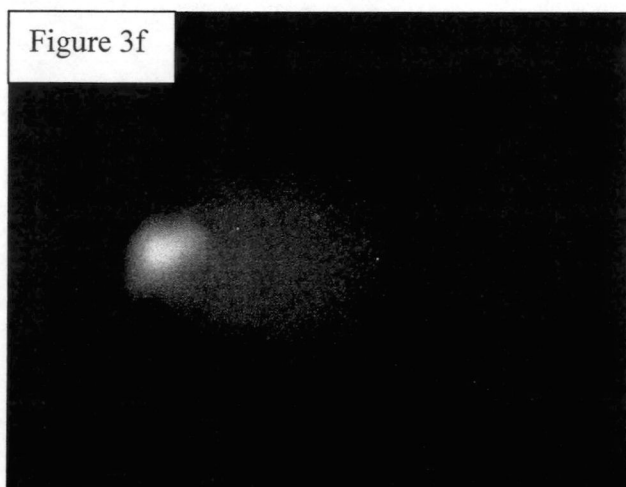
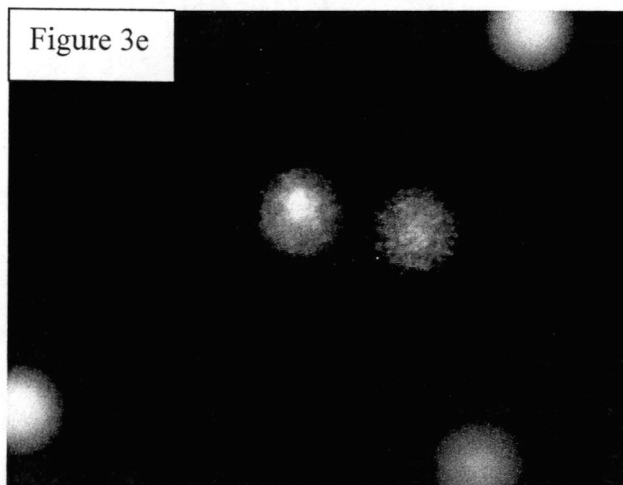


Figure 3e). Human lymphocytes first treated with 10^{-8} M brevetoxin for 1 hour prior to treatment with brevenal for a second hour. f). Human lymphocytes after treatment with the DNA polymerase inhibitor aphidicolin. Lymphocytes were treated first with 10^{-9} M aphidicolin and 1.0 $\mu\text{g/ml}$ brevenal for 1 hour, followed by 10^{-8} M PbTx for a second hour.

Inhibition of Cellular Proliferation Assay

PbTx was allowed to incubate with the CHO cells for 48 hours prior to the determination of cellular proliferation. Following the treatment phase, cells which had died during PbTx treatment were considered unable to proliferate and aspirated away with the media prior to the trypsinization process. Those cells which had survived the PbTx treatment were considered able to proliferate and were trypsinized, resuspended, and counted. The number of cells counted in each treatment well was compared to a control well (an untreated well found on the same plate). 100% of the cells in the control well were considered able to proliferate. Thus, the data is presented in terms of percentage of cells in treated wells compared to the control well.

All types of PbTx were able to inhibit cellular proliferation of CHO cells by more than 50% at high molar concentrations (10^{-4} M). At concentrations higher than 10^{-9} M the effect of the toxin varied among the PbTx types. The dose/response curve of UNCW PbTx-2 (figure 4a) and UNCW PbTx-3 (figure 4b) leveled off after 10^{-9} M and were not significantly different than controls. At 10^{-12} M, the Sigma PbTx-2 (figure 4c) made a sudden decline to approximately 55% cells capable of proliferation. This decrease at 10^{-12} M also occurs with Sigma PbTx-9 (figure 4d), although not as dramatic a decline. Still, in all instances, at concentrations of 10^{-6} M and higher, PbTx was able to inhibit the ability of CHO cells to proliferate by 50% or more.

The concentration at which 50% of CHO cells are unable to proliferate (IC50) was also evaluated. UNCW PbTx-2 was used at concentrations which were thought to be near the IC50 (figure 5) from the previous proliferation experiment. From the previous UNCW PbTx-2 dose/response curve it appeared as though the IC50 was between 10^{-6} and 10^{-9} M. From figure 5 it appears the IC50 was found to be approximately $10^{-6.75}$ M.

The effect of brevenal on the ability of CHO cells to proliferate in the presence of PbTx was also investigated. Results from the single-cell gel electrophoresis assay demonstrated that brevenal was able to reduce the amount of DNA damage generated by PbTx. One type of PbTx was used (UNCW PbTx-2) and brevenal at two different concentrations was used (1.0 $\mu\text{g/ml}$ and 0.1 $\mu\text{g/ml}$). From the data (figure 6), evidence suggests brevenal may play a role in increasing the number of CHO cells able to proliferate. Especially at the most potent concentration, 10^{-4} M, it appears as though brevenal increases the number of proliferating cells by over 20% in both brevenal treated groups. At each of the PbTx concentrations throughout the graph there is at least one or both brevenal treated groups demonstrating a significantly greater number of proliferating cells compared to the PbTx alone treated cells.

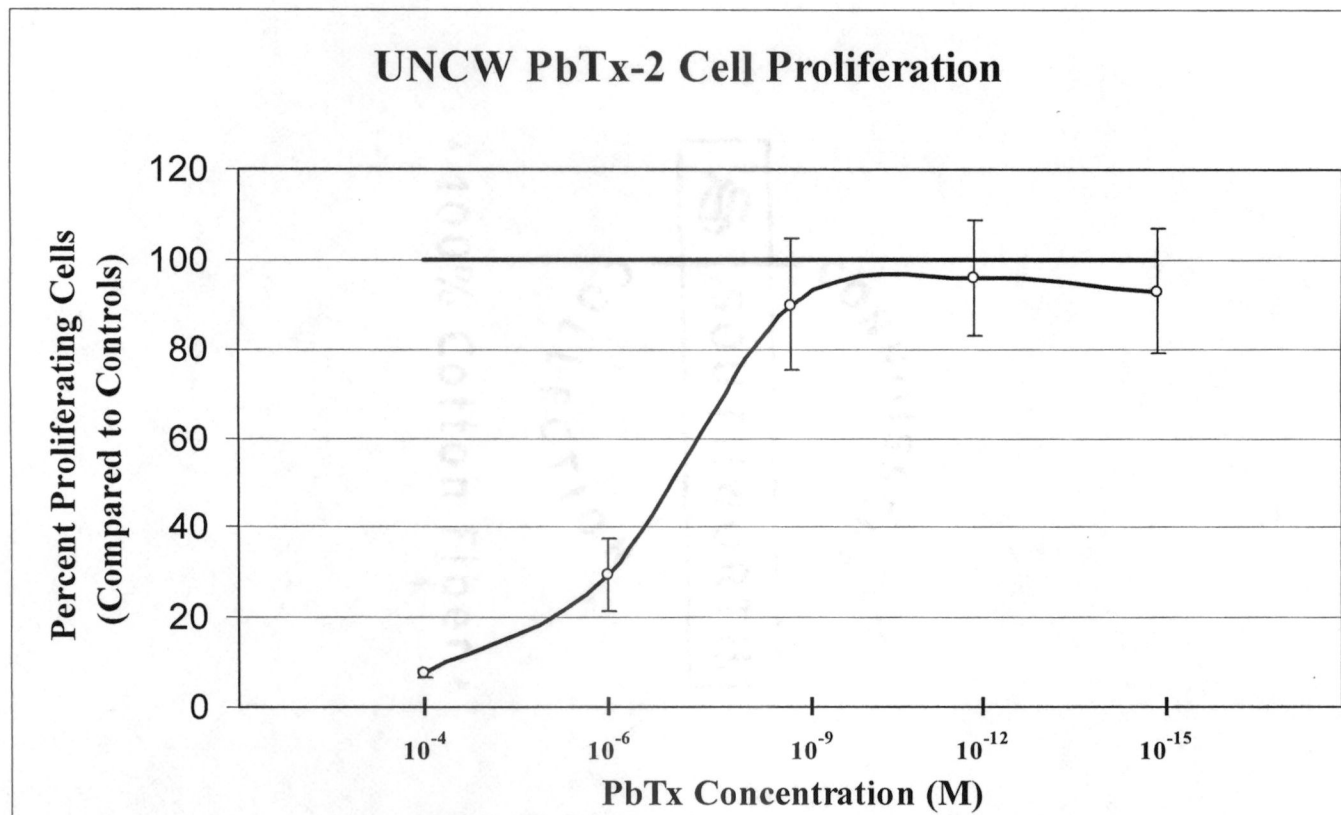


Figure 4a. The effect of UNCW PbTx-2 on CHO-K1-BH4 cells at different concentrations. CHO cells were incubated with different concentrations of PbTx for 48 hours. At the end of the treatment period, the cells were harvested and counted using a coulter counter. Dead cells were no longer able to proliferate and aspirated away when the media was removed prior to trypsinization

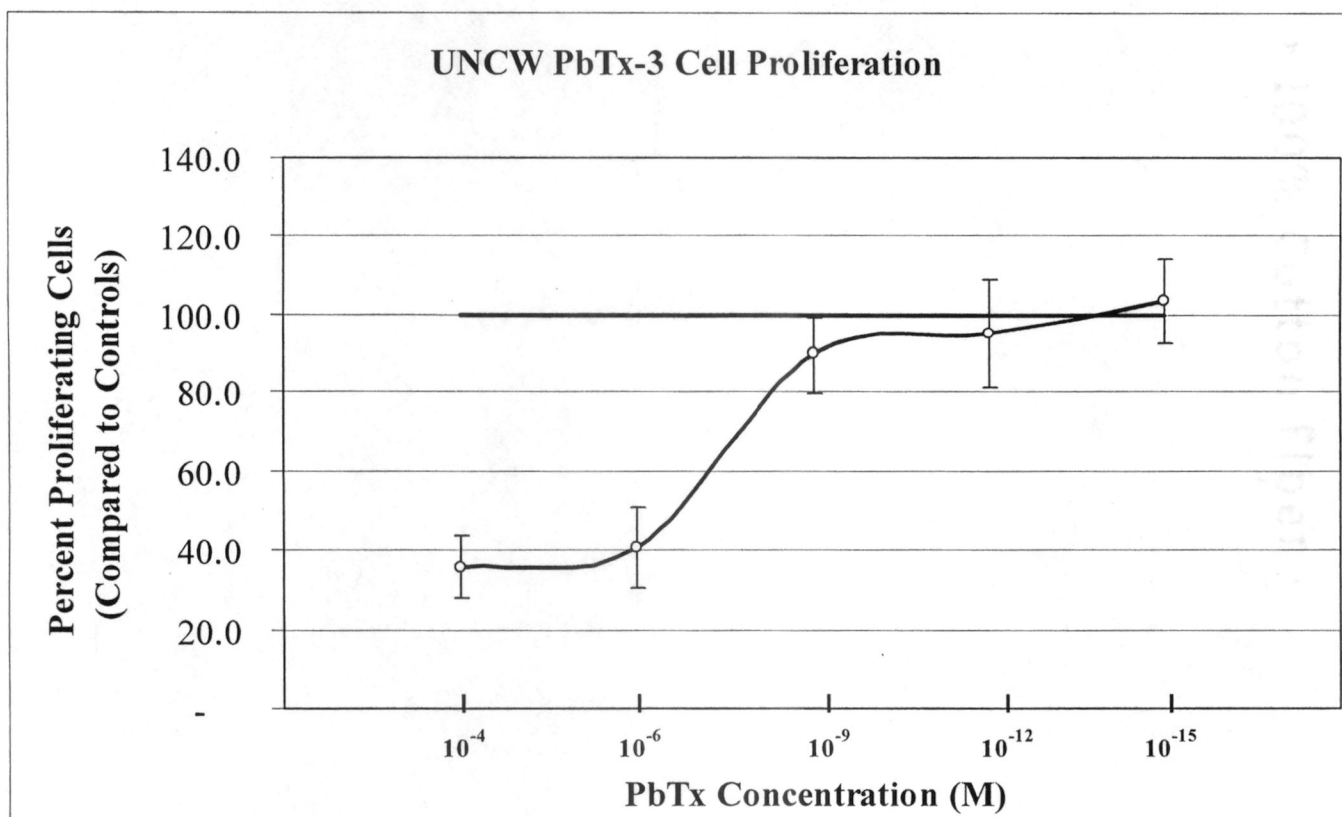


Figure 4b. The effect of UNCW PbTx-3 on CHO-K1-BH4 cells at different concentrations

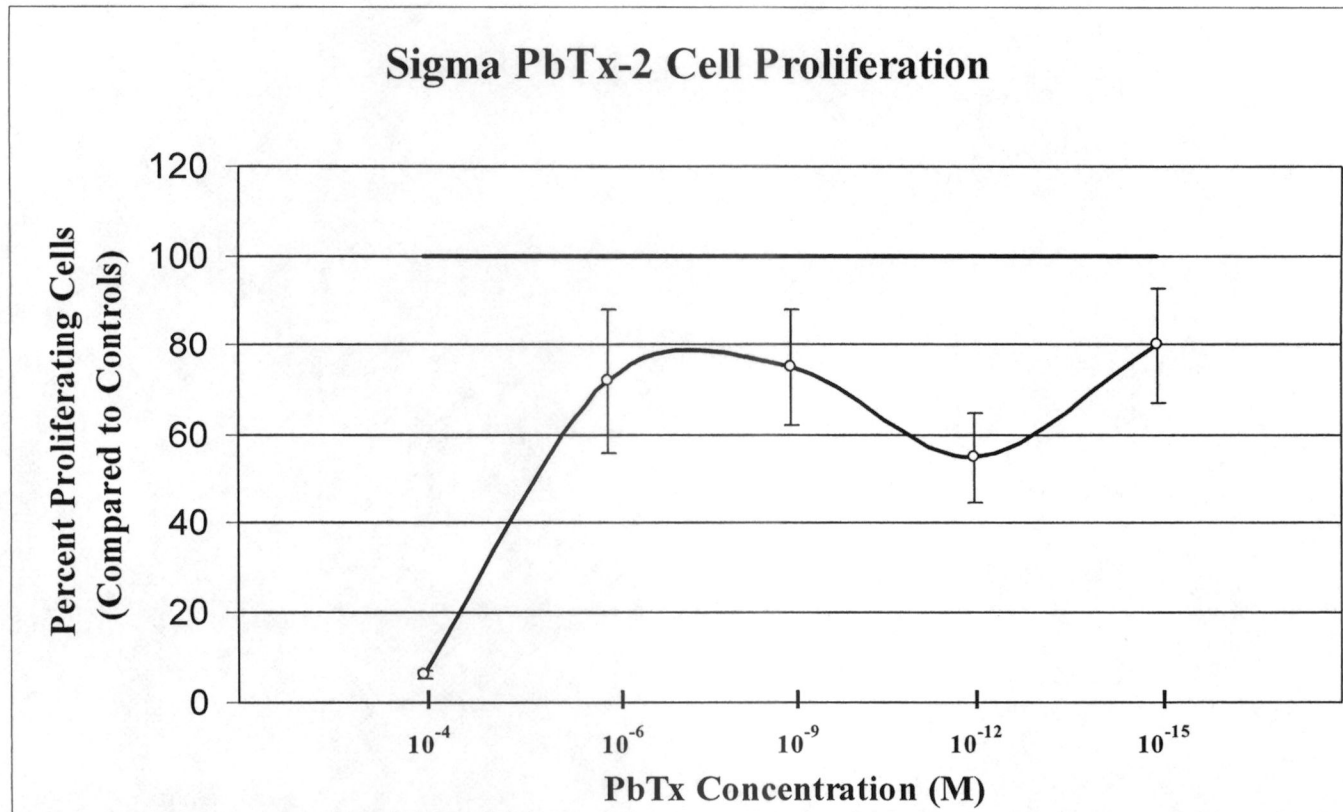


Figure 4c. The effect of Sigma PbTx-2 on CHO-K1-BH4 cells at different concentrations

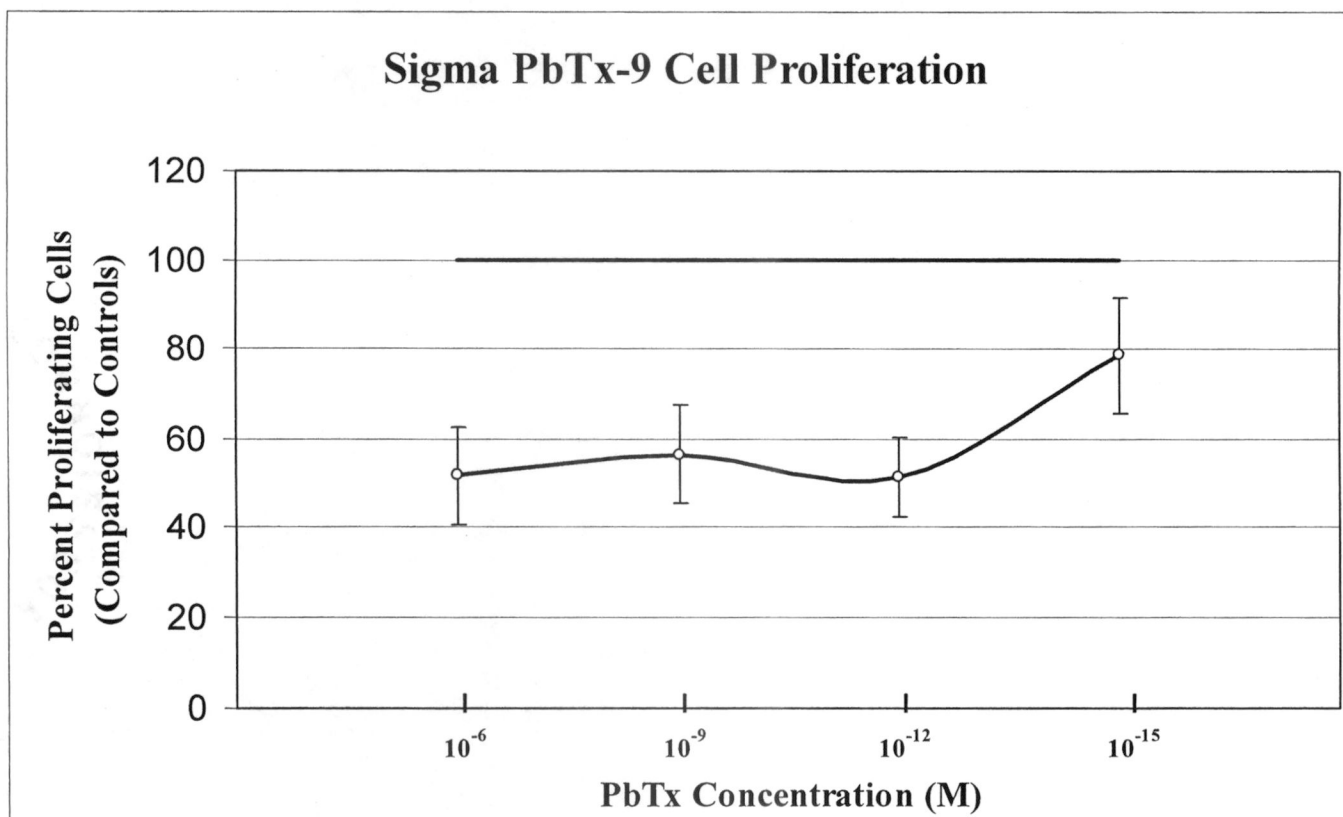


Figure 4d. The effect of Sigma PbTx-9 on CHO-K1-BH4 cells at different concentrations

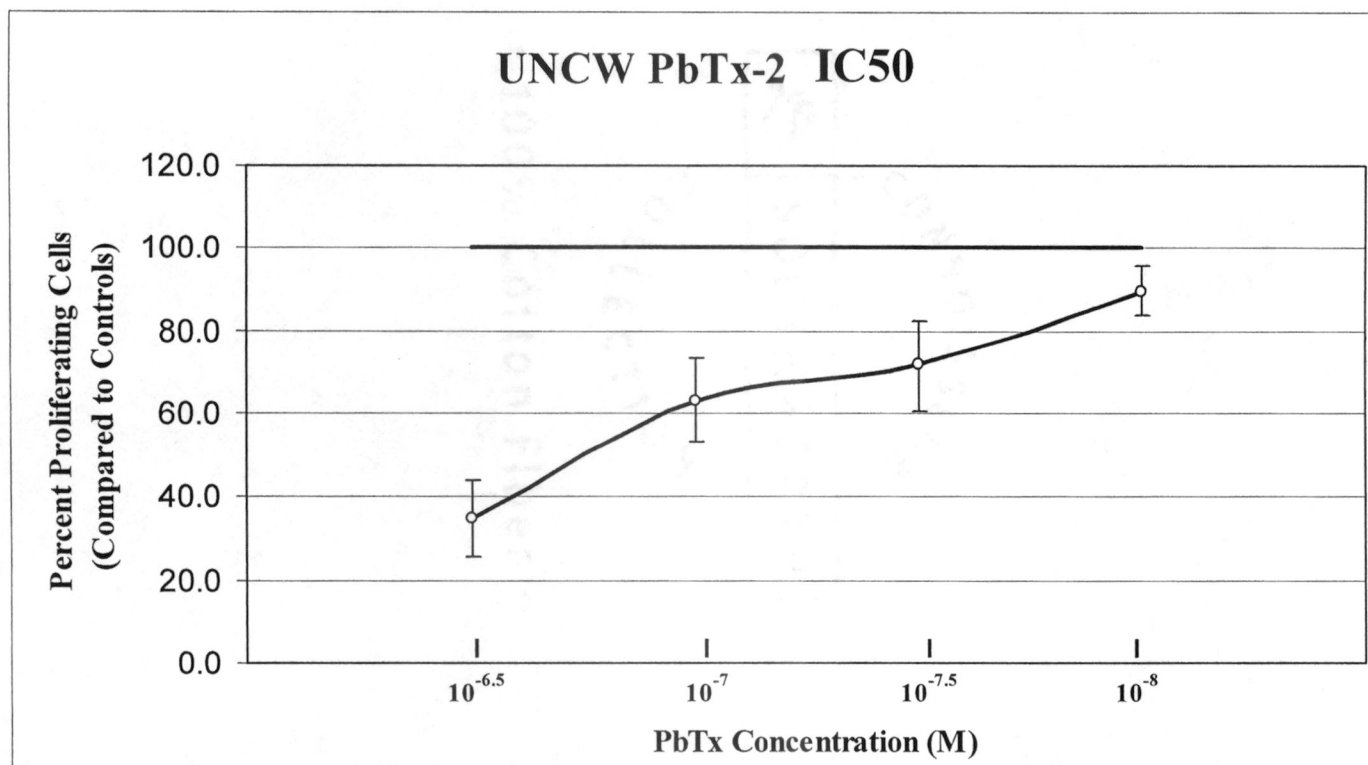


Figure 5. The lethal concentration at which 50% of CHO cells were unable to proliferate using UNCW PbTx-2 was evaluated. The ability of CHO cells to proliferate was compared to controls

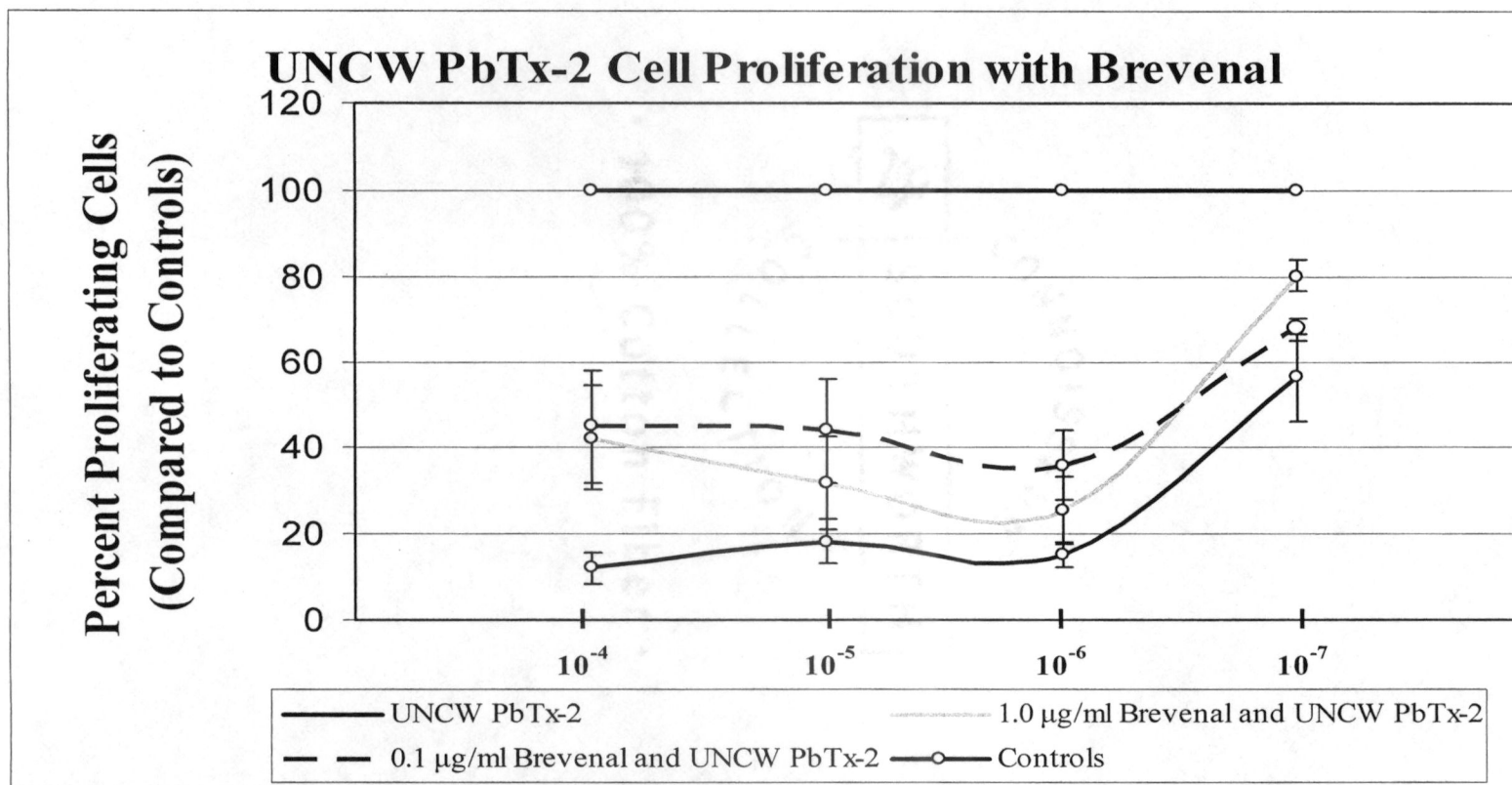


Figure 6. The effect of brevenal on the ability of CHO-K1-BH4 cells to proliferate. Two concentrations of brevenal were used, 1.0 µg/ml and 0.1 µg/ml, and were compared with UNCW PbTx at equal molar treatment concentrations

Chromosomal Aberrations Assay

The chromosomal aberrations assay was conducted to determine if PbTx had any effect on CHO cell chromosomes. Data from the single-cell gel electrophoresis assay suggested PbTx may have the potential for genotoxicity. Therefore it was hypothesized that PbTx may cause other types of gene-related damage. A negative control (untreated), positive control (Mitomycin C, MMC), and treatment group (10^{-7} M UNCW PbTx-2) were used for this assay.

While there are many types of chromosomal aberrations, the only two types of abnormalities officially categorized in this experiment were gaps and breaks. A third category, classified as 'other' contained all other types of chromosomal anomalies. These other anomalies included: aligned and displaced terminal fragments; dicentric and centric interchanges; proximal, distal, and sister non-unions; symmetrical and asymmetrical interchanges; symmetrical and asymmetrical intrachanges; monocentric and dicentric triradial breaks. Due to the complexity of the types of potential aberrations that could be found, and due to the expertise required to identify the exact type of chromosomal aberration, the three categories of gaps, breaks, and 'other' was sufficient for determining total chromosomal aberrations.

The negative control group of CHO cells (table 6a) did not contain many chromosomal aberrations. While inherent chromosome breakages do occur, a frequency of more than 1 aberration in 20 metaphase cells would be considered a high aberration occurrence for

non-treated cells (Wiley, 2005). In contrast, the positive control group (table 6b) MMC contained a very high occurrence of chromosomal defects. There were nearly two (and sometimes more) chromosomal defects per CHO cell metaphase in the group. CHO cells incubated with MMC clearly showed a very high rate of aberrations per cell. While the PbTx treated group (table 6c) did not display the amount of aberrations as the MMC treated group, there were still numerous amounts of abnormalities and irregularities found among those chromosomes. Compared to controls, the PbTx treated group contained significant damage that could not be considered inherent chromosomal defects. Examples of the chromosomal aberrations from each group are in figures 7, 8, and 9.

PbTx at a concentration of 10^{-7} M (virtually the IC₅₀ value of PbTx in CHO cell proliferation experiments) negatively affects chromosomes and induces chromosomal aberrations. While only 0.09% of negative control CHO cells exhibited chromosomal defects, 1.7% of PbTx treated groups contained aberrations, approximately an 18 fold increase in frequency. Based on the Mann-Whitney U-test ($p < 0.05$), the difference in the frequency of aberrations found between the control and PbTx treated CHO cells was significant.

Table 6a. Chromosomal aberrations scored when CHO cells untreated

	Controls			
Treatment Day	Number of Chromosome Pairs Counted (in 50 Cells)	Gaps	Breaks	Other
1	1054		1	
2	1039			1
3	1090	1		
Totals:	3183	1	1	1
Total Number of Damaged Chromosome Pairs: 3			Percentage of Chromosomes Damaged: 0.09%	
Average Number of Undamaged Chromosomes Per Break:				
1 Chromosome Break in 1061 Chromosome Pairs				
1 Chromosome Break Per 50 Metaphase Cells				

Table 6b. Chromosomal aberrations scored when CHO cells were treated with mitomycin C

	MMC Treatment				
Treatment Day	Number of Chromosome Pairs Counted (in 50 Cells)	Gaps	Breaks	Other	
1	1038	19	10	33	
2	1049	25	12	33	
3	1065	25	14	48	
Totals:	3152	69	36	114	
Total Number of Damaged Chromosome Pairs: 219*			Percentage of Chromosomes Damaged: 6.9%		
Average Number of Undamaged Chromosome Pairs Per Break:					
1 Chromosome Break in 14.39 Chromosome Pairs					
1 Chromosome Break Per 0.685 Metaphase Cells					

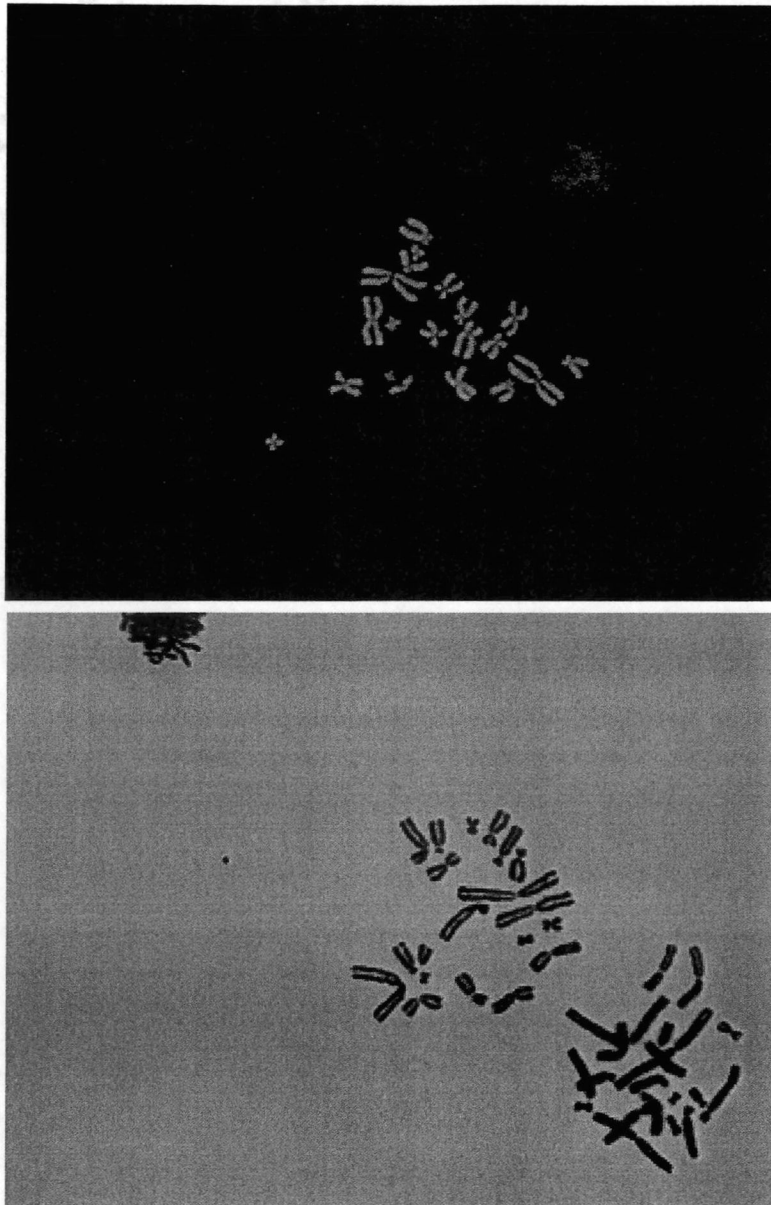
* Indicates significance compared to controls

Table 6c. Chromosomal aberrations scored when CHO cells were treated with 10^{-7} M UNCW PbTx-2

	PbTx Treatment				
Treatment Day	Number of Chromosome Pairs Counted (in 50 Cells)	Gaps	Breaks	Other	
1	1031	5	4	12	
2	1050	5	2	12	
3	1050	5	1	8	
Totals:	3131	15	7	32	
Total Number of Damaged Chromosome Pairs: 54**			Percentage of Chromosomes Damaged: 1.7%		
Average Number of Undamaged Chromosomes Per Break:					
1 Chromosome Break per 57.9 Chromosome Pairs					
1 Chromosome Break Per 2.77 Metaphase Cells					

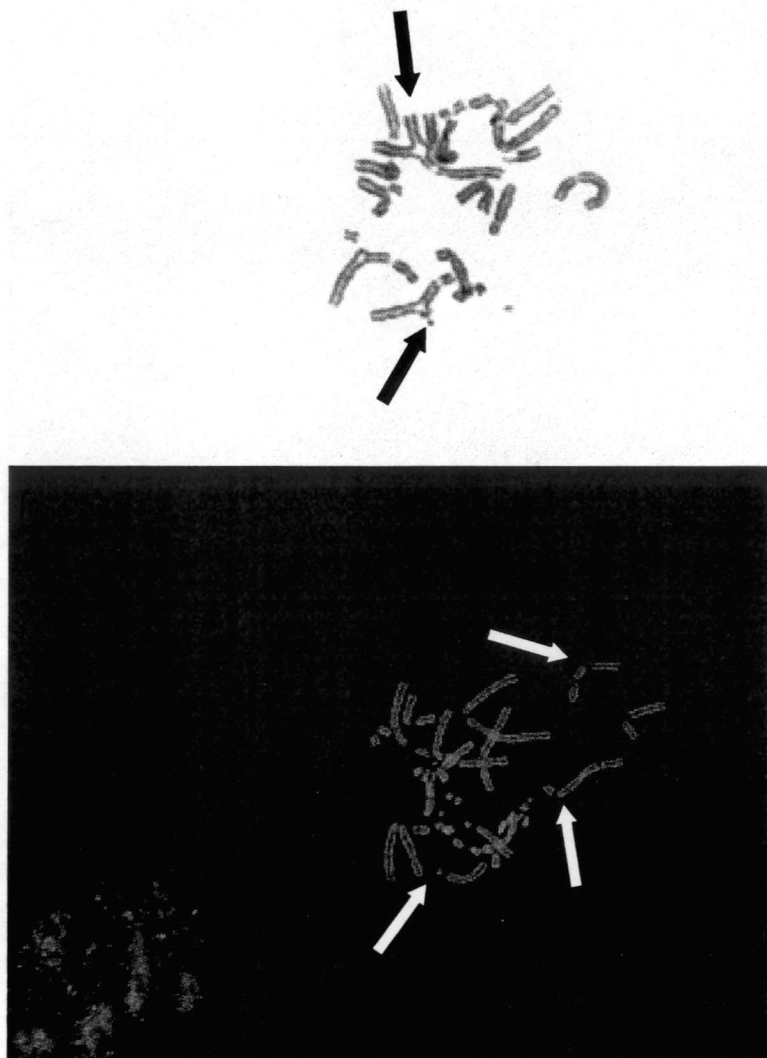
** Indicates significance compared to control and MMC treated groups

Figures 7a and 7b. Undamaged CHO Cell Chromosomes



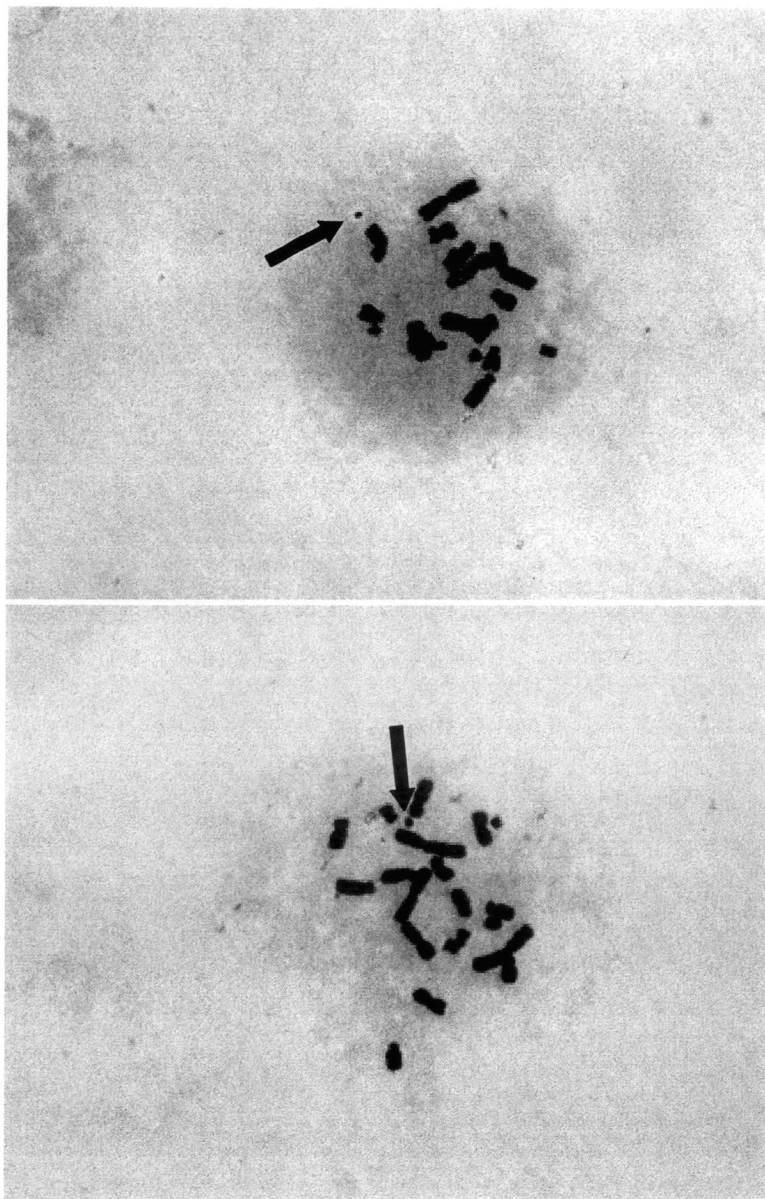
Negative control CHO cell chromosomes were undamaged and had a low incidence of inherent chromosomal damage. 0.09% of negative control CHO cell chromosomes had aberrations

Figures 8a and 8b. Mitomycin C treated CHO cell Chromosomes



Mitomycin C treated CHO cells exhibited a large number of chromosomal abnormalities. 6.9% of all MMC treated chromosomes contained abnormalities. Arrows indicate aberrations

Figures 9a and 9b. 10^{-7} M UNCW PbTx-2 Treated CHO Cell Chromosomes



CHO cell chromosomes treated with 10^{-7} M UNCW PbTx-2 contained a significant number of aberrations compared to control (untreated) CHO cell chromosomes. While the number of aberrations was not as frequent as MMC treated chromosomes, 1.7% of PbTx treated CHO cells contained chromosomal aberrations. Arrows indicate aberrations

Determination of MAPK Signaling Pathway Disruption Via a Western Blot Analysis

The determination of mitogen-activated protein kinase (MAPK) signaling pathway disruption assay was conducted to ascertain if PbTx had any effect on the following cell signaling proteins: tyrosine kinase, protein kinase C (PKC), and extracellular signal-regulated kinases 1 and 2 (ERK1/2). CHO cells were incubated with 10^{-7} M UNCW PbTx for 1, 4, 8, and 24 hour durations. After the treatment period the cells were collected, protein was isolated, and the signaling pathway analysis was initiated.

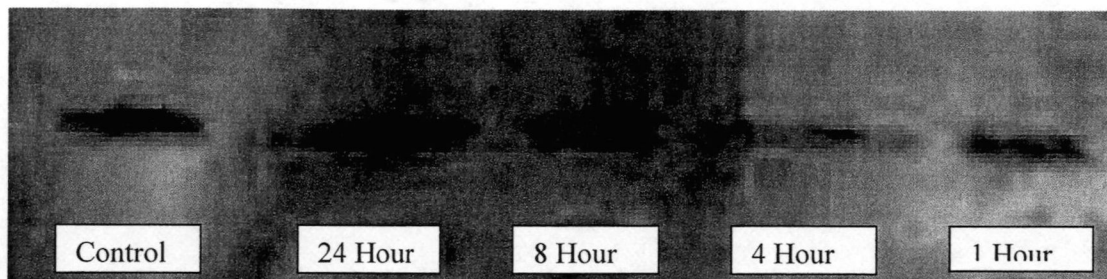
The results of the signaling pathway disruption are found in figure 10 (a-d). For each different protein selected for in this experiment, there appeared to be no difference between treated groups and controls. While certain bands appear to fade or become more intense after a certain time interval, the only information that was ascertained from this experiment was the proteins are all present after different time intervals of PbTx treatment.

While the proteins are all present at different time intervals, the amount of protein present was not certain. A protein quantification using densitometry analysis was conducted to liberally estimate how much protein was present within each band for the given time interval. The results of the quantification were inconclusive. It appeared as though band intensities at different time periods were more and less intense than controls. This would suggest that the amount of protein varied from time interval to time interval. Further investigation would be needed in order to determine the amount of protein present

throughout the duration of the experiment. Additionally, the results obtained from this experiment indicate that PbTx does not alter the MAPK signaling pathway in a way to completely eliminate the protein from the signaling pathway. While this particular pathway may be unaffected by PbTx, it would be very likely that another pathway not yet tested for would be affected by PbTx. Further investigation would be needed to isolate the exact pathway and mechanism of action for PbTx induced signaling disruption.

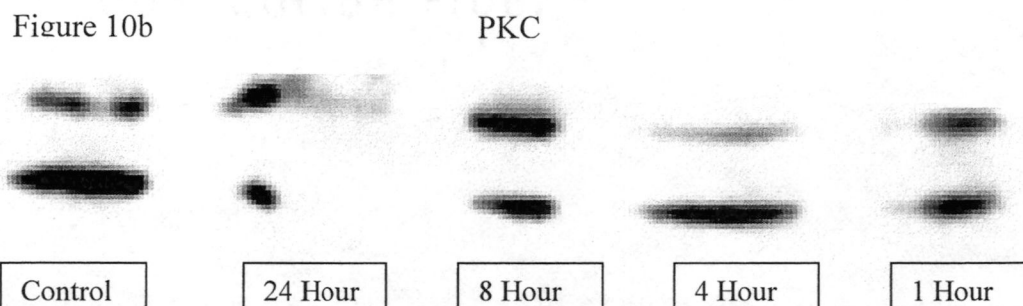
Figure 10a

Tyrosine Kinase



Western blot using the Tyrosine Kinase antibody after exposure to 10^{-7} M UNCW PbTx

Tyrosine Kinase	
Lane	Densitometry Reading
Control	2153.81
24 Hour PbTx Treatment	3454.06
8 Hour PbTx Treatment	1282.63
4 Hour PbTx Treatment	3361.13
1 Hour PbTx Treatment	2066.94

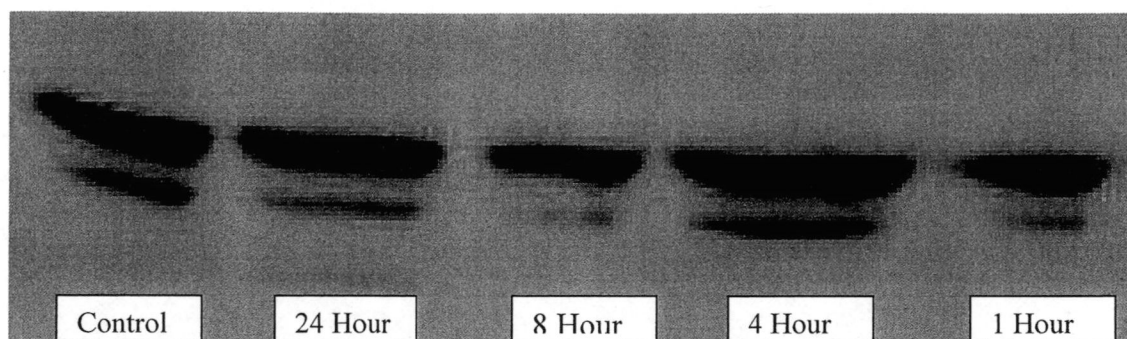


Western blot using the PKC antibody after exposure to 10^{-7} M UNCW PbTx

PKC	
Lane	Densitometry Reading
Control Band 1	162.69
Control Band 2	529.44
24 Hour PbTx Treatment Band 1	538.188
24 Hour PbTx Treatment Band 2	352.19
8 Hour PbTx Treatment Band 1	503.25
8 Hour PbTx Treatment Band 2	260.81
4 Hour PbTx Treatment Band 1	129.38
4 Hour PbTx Treatment Band 2	517.25
1 Hour PbTx Treatment Band 1	225.75
1 Hour PbTx Treatment Band 2	352.44

Figure 10c.

ERK1

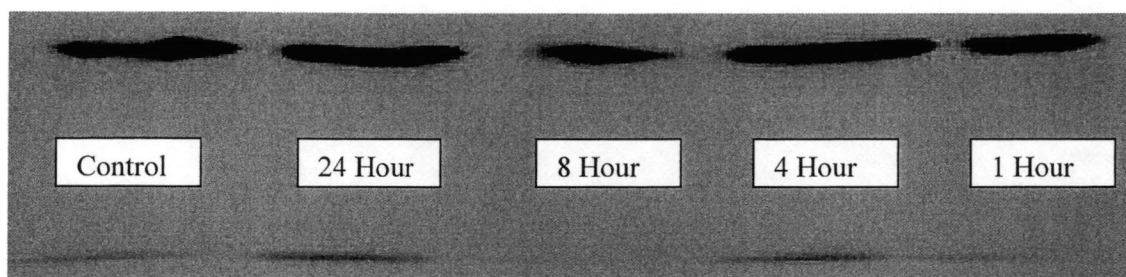


Western blot using the ERK1 antibody after exposure to 10^{-7} M UNCW PbTx

ERK1	
Lane	Densitometry Reading
Control	4095.00
24 Hour PbTx Treatment	4095.00
8 Hour PbTx Treatment	4095.00
4 Hour PbTx Treatment	4095.00
1 Hour PbTx Treatment	4095.00

Figure 10d.

ERK2



Western blot using the ERK2 antibody after exposure to 10^{-7} M UNCW PbTx

ERK2	
Lane	Densitometry Reading
Control	4095.00
24 Hour PbTx Treatment	4095.00
8 Hour PbTx Treatment	4095.00
4 Hour PbTx Treatment	4095.00
1 Hour PbTx Treatment	4095.00

DISCUSSION

Organisms involved in harmful algal blooms (HABs) produce a variety of toxins including: ciguatoxins, maitotoxins, saxitoxin, scaritoxins, okadaic acid, and palytoxins (in addition to other toxins) often in high amounts. Many of these toxins bind to ion channels in eukaryotic cells located on either cellular or nuclear membranes causing ion transport disruptions (Ito et al., 2003; Rein et al., 1999). PbTxs, at nanomolar concentrations, are known to bind to voltage-gated sodium channels on voltage gated cells (neurons) but their activity on non-voltage gated cells and nuclear membranes is unknown. PbTx has also been shown to induce a concentration dependent increase in cytosolic Ca^{2+} in cerebellar neurons (LePage et al., 2003). It is possible that brevetoxin and brevenal may bind to nuclear receptors. Binding of PbTxs to the nuclear membrane receptors in lymphocytes and CHO cells could lead to ion concentration disturbances, especially Ca^{2+} , in the nucleus resulting in damage to the nuclear DNA through Ca^{2+} activation of endonucleases or alterations in the tertiary structure of the DNA. Brevenal may work to inhibit brevetoxin-induced genotoxic damage by blocking brevetoxin's effect on nuclear receptors and therefore nuclear ion concentrations.

It has also been demonstrated that PbTxs trigger airway constriction and may induce asthma attacks (Abraham et al., 2004; Asai et al., 1982). Asthma is a hypersensitivity to an allergen or foreign substance that can trigger the immune response (Schuster et al., 2000). In addition to being toxic to neurons and the GI tract, brevetoxins cause airway irritation, airway constriction, and bronchoconstriction (Backer et al., 2003). The

harmful effects of PbTx on the respiratory system trigger an immune response that may be highly sensitive in certain individuals (i.e. asthmatics, individuals with chronic obstructive pulmonary disease, and other types of pulmonary diseases).

Human T and B-lymphocytes play an important role in immune function. These special types of white blood cells have unique cell surface receptors that are highly selective for foreign antigens capable of triggering the immune response. Less selective response elements, such as granulocytes (neutrophils and eosinophils) are also involved in the inflammatory process. These cells recognize foreign material in the body but do not recognize specific antigens. Granulocytes destroy the invading entity via phagocytosis or by other cytotoxic responses. When a foreign antigen binds to a T lymphocyte, cytokines are produced. Cytokines, such as interleukins 2, 4, 5, 10, and 13, directly stimulate B-lymphocytes, monocytes, and neutrophils. Upon activation, B cells produce antibodies to attack and destroy the foreign antigen. During a secondary exposure to a previously encountered antigen, memory T lymphocytes are able to detect the previously encountered antigen and raise the immune response more rapidly compared to the first exposure (Akdis et al., 2004). Thus, disruption of lymphocyte function could severely impair the ability of an organism to eliminate foreign antigens. If the immune system is impaired, the organism will not likely survive.

In the single-cell gel electrophoresis assay, the DNA damage measured in human lymphocytes determined the extent of single and double DNA strand breaks upon

exposure to PbTx. DNA damage was apparent upon PbTx exposure and each PbTx subtype tested caused human lymphocyte DNA damage of similar magnitude. As expected, the 10^{-8} M PbTx caused greater damage than the smaller concentration of PbTx 10^{-12} M. Still, the 10^{-12} M PbTx, a 10,000 fold more dilute concentration than 10^{-8} M, caused significant DNA damage compared to controls.

PbTx_s at 10^{-8} M and 10^{-12} M concentrations were potent inducers of DNA damage in normal human lymphocytes and the damage induced was fully antagonized by brevenal. Different sources of PbTx cause DNA damage greater than that of the controls, but not greater than that of hydrogen peroxide exposed lymphocytes. Lymphocytes not treated with any genotoxic chemicals depicted a typical undamaged circular shaped lymphocyte nucleus. Hydrogen peroxide treatment caused the greatest extent of DNA damage of all the treated groups in this experiment. Lymphocytes exposed to PbTx_s for an additional second hour induced significant damage compared to control lymphocytes.

Cell proliferation experiments revealed that PbTx inhibited the ability of cells to proliferate by 50% at concentrations greater than 10^{-6} M. While there was considerable variability among the different types of PbTx, the general trend of the dose/response curve was high cell death at high PbTx concentrations and low cell death at low PbTx concentrations. At lower concentrations, there was considerable variability between types of PbTx. UNCW PbTx-2 and 3 displayed a typical movement towards 100% cell proliferation. Sigma PbTx-2 had a sudden drop in cell proliferation at 10^{-12} M. Sigma

PbTx-9 data also demonstrated a drop in proliferating cells at 10^{-12} M and indicated a much more graduate trend towards 100% cell proliferation. The cell proliferation results of Sigma PbTx at concentrations lower than 10^{-9} M were unexpected. It was also an interesting finding that PbTx at 10^{-8} M, the concentration that caused considerable damage in human lymphocytes in the single-cell gel electrophoresis assay, did not greatly inhibit cellular proliferation. A majority of CHO cells at 10^{-8} M were able to proliferate.

The chromosomal aberrations experiment evaluated the ability of PbTx to damage chromosomes. Data from the single-cell gel electrophoresis assay suggested that PbTx have the potential to be genotoxic. UNCW PbTx-2 (10^{-7} M) was selected as the concentration used for the experiment because it was very near the UNCW PbTx-2 IC50 value ($10^{-6.75}$ M). From the results, spontaneous chromosomal damage did occur but only very rarely (0.09% of approximately 3000 chromosomes analyzed displayed aberrations). The positive control, mitomycin C, was used at a higher concentration than previous studies and caused a high amount of chromosomal aberrations. Mitomycin C is an alkylating agent that cross-links with DNA, causes strand breaks, and inhibits repair. Mitomycin C treated chromosomes displayed several types of aberrations such as interchanges and terminal fragments (although data other than gaps and breaks were recorded, they were not categorized as to the specific aberration). PbTx caused significantly greater numbers of chromosomal aberrations compared to controls. An 18 fold increase in the frequency of aberrations was found between the PbTx and control groups. While gaps and breaks occurred within the PbTx treated groups, there were

frequently terminal fragments. In several instances in PbTx and MMC groups it appeared as though a terminal fragment from one chromosome had paired up with an adjacent chromosome. The information provided by the chromosomal aberrations experiment suggests PbTx was capable of inducing significant chromosomal abnormalities at 10^{-7} M.

Damaging the genetic material of a lymphocyte or CHO cell will likely impair the function of the cell. Among the consequences of damaging genetic material are the potential for mutagenic response and/or disruption of cell signaling processes such as those signaling pathways involved with cell cycling, proliferation, migration, differentiation, plasticity, inflammation, immunomodulation, apoptosis, cell survival, and developmental processes (Clayson et al., 1994).

The PbTx antagonist, brevenal, both prevented and reversed the effect of PbTx in both the single-cell gel electrophoresis and cellular proliferation assays. Brevenal is a naturally occurring short-chain polyether that competes with PbTx at the active site of the Na^+ channel, thus negating this effect of the toxin. As a result, PbTx is competitively displaced at the active site of the Na^+ channel. In our studies with brevenal, the treatment of lymphocytes and CHO cells with brevenal prior to or along with the addition of PbTx completely reduced or alleviated damage caused by PbTx alone. More importantly, the addition of brevenal after PbTx incubations reversed PbTx induced DNA damage (single-cell gel electrophoresis assay) and increased the number of cells capable of cell proliferation (inhibition of cellular proliferation assay). In the single-cell gel

electrophoresis assay brevenal treatment reduced the amount of DNA damage to levels similar to controls in all but one instance. The cell proliferation study showed an increased number of proliferating cells at each PbTx treatment concentration in at least one of the two brevenal concentrations used in the assay. Thus, in human lymphocytes and CHO cells, brevenal either reduced or prevented the negative effects of PbTx.

It has been previously shown that brevenal competitively displaces tritiated PbTx in a synaptosome receptor-binding assay, one that considers specific sodium channel receptor site binding for natural PbTx. In that assay, however, it was clear that the concentration of brevenal needed to compete for PbTx occupied sites were over two orders of magnitude greater than for PbTx. Similarly, PbTx-induced DNA damage at 10^{-8} M in human lymphocytes was antagonized by $1.0 \mu\text{g/ml}$ (1.5×10^{-6} M) of brevenal, a 2 orders of magnitude difference (Bourdelaïs et al., 2004). However, in that study, lower concentrations of brevenal were not tested and it is possible that brevenal antagonism would be measurable at lesser amounts. In the cellular proliferation experiments, one of the two brevenal treatment groups increased the number at proliferating cells at each concentration. Therefore, the effectiveness of brevenal in the single-cell gel electrophoresis assay was limited by the concentration of PbTx used, while in the cellular proliferation assay it appeared as though brevenal was an effective agent to lessen the toxic effect of PbTx at every concentration tested. The differences between the two cell types suggest lymphocytes are a more sensitive mammalian cell type than CHO cells.

To further investigate the role of brevenal in DNA repair, aphidicolin was used in the single-cell gel electrophoresis assay to ascertain the role of DNA repair in the mechanism of brevenal antagonism (Speit et al., 2004). By inhibiting a main mechanism of DNA repair via aphidicolin, it was possible to demonstrate that brevenal allows DNA repair processes to restore PbTx induced DNA damage. The present results indicate that by inhibiting DNA polymerase there is a statistically significant amount of DNA damage even in the presence of brevenal.

A theory as to the induction of PbTx induced DNA and chromosomal damage is the alteration of the intracellular Na^+ ion concentration. Most cells have some sort of excitable voltage-gated Na^+ channel present in the cell membrane. Typically, the concentration of Na^+ ions is greater extracellularly than intracellularly due in much part to the Na^+/K^+ ATPase (Wang et al., 2003). The cell expends a vast majority of its ATP (approximately 60%) produced by the mitochondria to transfer 3 ions of Na^+ out of the cell to bring 2 K^+ ions into the cell. The Na^+ ion concentration gradient is extremely important and coupled with many different transport proteins in order to drive energetically favorable reactions. Without the established Na^+ ion gradient, these coupled transport processes would cease to exist.

PbTx, which act upon voltage-gated Na^+ channels consequently prolonging the active state, would likely disrupt the Na^+ ion concentration gradient. As a result, more Na^+ would be found intracellularly than under normal homeostatic conditions. The alteration

of the major ion responsible for driving other transport processes could be catastrophic for a cell. There would be no Na^+ ion concentration gradient established to couple with other transport proteins. For example, the Na^+/Ca^+ exchanger, which exchanges 3 ions of Na^+ into the cell to extrude 1 Ca^{2+} ion out of the cell, could no longer function. As a result, more Ca^{2+} would be found intracellularly.

While Ca^{2+} is a highly utilized ion that activates (and sequesters) a number of cellular and nuclear effectors, too much is highly toxic to the cell. Should excess Ca^{2+} enter the nucleus there would be detrimental effects. In the nucleus, Ca^{2+} activates endonucleases, proteases, alters the tertiary structure of DNA, forms insoluble complexes with phosphates (the DNA backbone for example), and causes fatty acids to aggregate into soapy globules (Petersen, 2002). Additionally, Ca^{2+} activates and suppresses gene expression in a highly regulated manner. Only 25% of all genes are not in some way regulated by Ca^{2+} (Carafoli, 2005). The unregulated activation or suppression of gene expression would cause chaos within the cell. Should an increase in Ca^{2+} be the cause for DNA and chromosomal damage, apoptosis would be a likely consequence. The cell would rather initiate regulated cell death than continue to have a catastrophic and fatal flaw.

Other organelles, such as the mitochondria, golgi, and endoplasmic reticulum (also called the sarcoplasmic reticulum in some cell types) depend on Ca^{2+} to regulate metabolic and cellular processes. While the cell can tolerate slight increases in Ca^{2+} , too much would

be very harmful. Under normal circumstances, Ca^{2+} signaling is conducted through pulses or rapid influxes of Ca^{2+} followed by its immediate removal. If the ultimate toxic effect of PbTx is a high and sustained increase in intracellular Ca^{2+} , it would be unlikely the cell would survive.

Yessotoxin, a polyether marine toxin classified among diarrhetic shellfish poisoning (DSP), is very structurally similar to PbTx. Yessotoxin is classified as DSP because it is frequently found with okadaic acid (the main toxin responsible for DSP). However, unlike okadaic acid, yessotoxin would more appropriately be classified under the same category as ciguatoxin (and maitotoxin) because they both share a more similar mechanism of action. Yessotoxin produces an intracellular Ca^{2+} increase through nifedipine-sensitive Ca^{2+} channels (de la Rosa et al., 2001). Due to the structural similarity between yessotoxin and PbTx, and because the ultimate toxic effect of PbTx may be due to an increase in intracellular and intranuclear Ca^{2+} , it may be that PbTxs also act on voltage-sensitive Ca^{2+} channels in addition to voltage-sensitive Na^+ channels.

In a multitude of cell types, activation of a variety of receptors (e.g., G-protein coupled receptors, ion channels, receptor tyrosine kinases and cytokine receptors) initiate the MAP-Kinase cascades which serve as central downstream integrators to modulate the cellular response to external stimuli. It is possible that the effects of PbTx may be measured on diverse upstream targets that can lead to changes in a common downstream effector, MAP-Kinase, and provide a basis for evaluating MAP-Kinase signaling as a

common mode of action. It is clear that MAP-Kinase signaling is an important mediator of critical cellular functions including cell proliferation, cell differentiation, and cell death. Thus, alterations in MAP-Kinase signaling might be expected to be part of the “mode of action” that leads to changes in critical cellular functions which are ultimately expressed as the adverse effect in the different tissues affected by PbTx. In particular, human inflammatory cytokines and receptor genes may be disrupted. Cytokines play an important role in mediating immune cascade reactions during the inflammatory response.

Results from the analysis of signaling pathway disruption via a Western blot analysis indicate that PbTx did not affect any of the proteins measured in the MAPK signaling cascade. The proteins selected for by the selected antibodies (tyrosine kinase, PKC, ERK1/2) were all present. Based on densitometry data there appeared to be no difference between protein concentrations throughout any of the time intervals for p42/44 (ERK1/2). Densitometry data from tyrosine kinase and PKC was variable suggesting that protein levels fluctuated throughout the course of 24 hours. Although there was no information which suggested the MAPK pathway was affected by PbTx, there may be another pathway responsible for the toxic effect of PbTx. It is also possible that alterations in the Na^+ ion concentration induced by PbTx lead to an increase in intracellular Ca^{2+} . Should this be the case, the results from the MAPK signaling pathway would be expected. This is because Ca^{2+} regulates (activates) PKC expression, and PKC activates ERK1/2 (Dravid et al., 2004). Thus the appearance of bands for these selected proteins would be present as confirmed by the results.

It has also recently been discovered that brevenal may have a therapeutic use for the treatment of pulmonary diseases such as cystic fibrosis. PbTx_s thicken mucus secretions produced by the lungs and inhibit ciliary movement. As a result, it is very difficult to clear the lungs of mucus buildup. The stagnant mucus is an ideal area for bacteria and other microscopic organisms to thrive, and individuals with cystic fibrosis often have frequent pulmonary infections. Brevenal appears to increase ciliary movement and thin out the mucus. Thus the lungs are able to clear more easily and infections are not as common. These are characteristics needed of therapeutic agents used to treat cystic fibrosis. More research will need to be conducted before the full therapeutic benefit of brevenal can be ascertained.

The information obtained in this thesis indicates PbTx_s damage human lymphocyte DNA, inhibit the ability of CHO-K1-BH4 cells to proliferate, cause chromosomal aberrations, but do not alter the MAPK signaling pathway. The genotoxic effects of mammalian cells found in the single-cell gel electrophoresis and chromosomal aberration assays suggest that exposure to PbTx_s may cause genotoxic effects at concentrations between 10^{-7} – 10^{-12} M. Furthermore, data from the cellular proliferation assay indicates PbTx_s inhibit CHO cell proliferation at concentrations as low as 10^{-12} M. Brevenal, the PbTx antagonist, appears to alleviate DNA damage and increase the number of mammalian cells able to proliferate. The results appear to indicate there may be beneficial therapeutic potential for brevenal that has yet to be discovered. The results

also indicate PbTxs may harmfully damage DNA, increase the rate of cell death, and genetically alter chromosomes. Thus, exposure to PbTxs may cause detrimental genetic abnormalities, such as uncontrolled cell proliferation and differentiation, and increase the rate of apoptosis. This indicates PbTxs as genotoxic materials which may be associated with somatic and germ cell mutations. These data taken together suggests that PbTxs are potential carcinogens.

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