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The Effects of Triptolide on Cancer Cells

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MONTCLAIR STATE UNIVERSITY

The Effects of Triptolide on Cancer Cells

by

Michael E Messina, Jr.

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

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College of Science and Mathematics

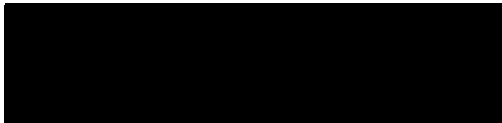
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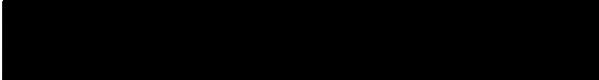


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Abstract:

Cancer is the second leading cause of death in the United States, and with the rates of breast cancer increasing steadily over time there is a need to develop new anti-cancer treatments. This research seeks to investigate how triptolide, an extract from the Chinese herb *Tripterygium wilfordii* Hook F, induces apoptosis in human breast cancer cells. Studies have shown that triptolide can act as an anti-proliferative, anti-inflammatory, and anti-cancer agent in many different clinical applications. It has been used for over two centuries in Chinese medicine and is now used to treat certain autoimmune diseases, such as rheumatoid arthritis. With breast cancer plaguing the United States as the second leading cause of cancer related deaths amongst women, as well as the adverse effects of current treatment methods, there is a need for the development of safer and alternative therapies. Triptolide is known to induce apoptosis in many cancer cells lines, but the exact mechanisms that regulate this are largely unknown. It has been suggested that triptolide activates the p53 pathway to trigger apoptosis in these cells. However, our results demonstrate that other cellular mechanics are at work in conjunction with p53, such as the differential regulation of pro- and anti-apoptotic proteins; lysosomal degradation; and a change in mitochondrial membrane permeability suggesting the release of cytochrome *c*.

Our studies examined the ability of triptolide to regulate the protein expression levels of caspase-3, caspase-7, and caspase-9, poly (ADP-ribose) polymerase (PARP), and Bcl-2 via colorimetric Western blot analysis. Apoptosis was monitored using the MTT cell viability assay and morphological observations. LysoTracker™ and MitoTracker™ were used to stain lysosomes and mitochondria, respectively. An acid

phosphatase, a marker enzyme for lysosomes, assay was conducted in order to measure the activity of lysosomal enzymes. Our results demonstrated that the amount of apoptosis, which was significantly increased by triptolide, was both time- and dose-dependent, suggesting that there may be an optimal triptolide concentration for maximal anticancer efficacy. Triptolide also differentially regulated the expression of pro- and anti-apoptotic proteins. Furthermore, the apoptotic death of cells was shown to be a lysosomal-mediated cell death. Future investigations will try to decipher the molecular mechanisms by which triptolide regulates apoptosis *in vivo* using an MCF-7 xenograft model in nude mice.

THE EFFECTS OF TRIPTOLIDE ON CANCER CELLS

A THESIS

Submitted in partial fulfillment of the requirements

For the degree of Master of Science

By

MICHAEL E MESSINA, JR.

Montclair State University

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Introduction:

Breast cancer is the second most common cancer among women in the United States and the National Cancer Institute now estimates that one in seven individuals living in the United States will develop breast cancer in their lifetime. With rates of breast cancer increasing, it is estimated there will be 1.5 million new cases worldwide in 2010 [1]. Current treatments such as chemotherapy and radiation are known to have harmful side effects on normal somatic cells therefore; it is imperative to develop safer and non-invasive treatments. The use of natural products in the fight against cancer is one possible way of slowing tumor growth and may allow us to design more effective therapies.

Cancer is a genetic disease, in part, where there is a loss of control over the cell cycle resulting in uncontrolled and unchecked cell proliferation. When the checkpoints of the cell cycle become mutated, cells are capable of reproducing at alarming rates, the danger lies when a cell that has accrued mutations is allowed to proliferate unchecked. Now these cells can continue to divide and also pass on these mutations creating clones that have the same mutations [2]. These mutations allow the cancer cells to evolve and can be lethal to the patient. Cysts, or benign tumors are non-invasive and non-cancerous, they are simply a collection of rapidly dividing cells [2]. On the other hand, malignant tumors are cancerous and can spread throughout the circulatory or lymphatic, colonize at distant sites, and eventually kill the patient [3].

Cancer is the result of an accumulation of mutations over time that leads to a cell becoming cancerous. While there is no definitive cause of cancer, several factors play key roles in triggering mutations. On a molecular level, there are several factors that can contribute to mutating the cell cycle. Proto-oncogenes, which function normally to regulate the cell cycle, when mutated, give rise to oncogenes, which are responsible for promoting excessive cell division [4]. The other category of genes that play a role are tumor-suppressor genes, such as p53, which normally block excessive cell growth and induces apoptosis. When mutated tumor suppressor genes function as tumor promoters and allow for the cells to divide unchecked [5].

Environmental factors such as smoking, exposure to UV light, and diets high in fat can also increase the risk of cancer [6, 7] as well as many genetic factors. The passing on of genetic mutations in genes BRCA1 and BRCA2 account for as much as 10% of all reported cases of human breast cancer [6, 8, 9]. It appears that women who come from families with a history of breast cancer seem to have the highest incidence of cancer caused by these genetic mutations [9], suggesting there is a genetic link between the incidence of breast cancer and familial history. While individuals who test positive for the BRCA1/2 gene are more likely to develop breast cancer it is not a guarantee [9].

Tumors are in an ever-changing environment [10, 11, 12] thus the tumor itself is constantly adapting to survive. Since the cells are always changing, treatment options are also limited to the types of cancer they can fight. When tumors change, they can adapt by becoming chemoresistant, developing resistance to radiation, or

mutating tumor-suppressor genes (i.e. p53) to give themselves a survival advantage over normal cells. Because of cancers cells resistance to many treatments and the harmful side effects of current treatments, alternative methods need to be explored.

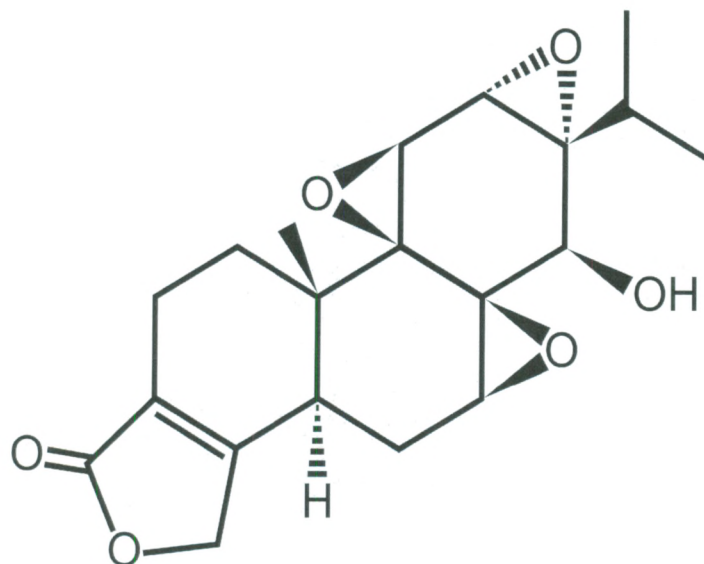


Figure 1: Structure of Triptolide

Triptolide (Figure 1), a biologically active extract from the Chinese herb *Tripterygium wilfordii* Hook F is a diterpenoid triepoxide. For centuries, the herb has been used in Chinese medicine to treat chronic pain due to inflammation [13, 14]. The properties of triptolide's unique structure are what allow it to act as an anti-inflammatory, anti-proliferative, and anti-cancer agent. Looking at the chemical composition of triptolide, it is a derivative of isoprene, which is part of the terpene family. The structure of triptolide, being a diterpenoid means its base is made up of four isoprene molecules [15] as well as other constituents. What is most interesting about the isoprene units is that through biosynthesis in some plant leaves, certain precursors to vitamins A (retinol) and E (tocopherol) are derived [15, 16]. Being that

these are both strong antioxidants this is one way triptolide can act as an anti-cancer agent. Furthermore, triptolide is known to reduce proliferation by sequestering production of T and B-cells, which are known to increase inflammation [16]. While most cancer drugs work to activate tumor necrosis factor-alpha (TNF- α) which is known to induce apoptosis in cancer cells, they can also be responsible for activating nuclear factor-kappa-beta (NF- $\kappa\beta$). NF- $\kappa\beta$ is responsible for tumor promotion [17], the result is having these two processes cancel each other out. Triptolide is solely responsible for activating TNF- α while preventing the activation of NF- $\kappa\beta$ [17, 18].

Triptolide is known to induce apoptosis, and impede tumor growth in a variety of cancer cell lines [19, 20, 21]. Apoptosis, or programmed cell death, is vital during embryonic development and throughout the life of all eukaryotic organisms because it removes damaged cells before they become cancerous [22]. Apoptosis, can be compared as an opposite of mitosis, is a very structured and regimented process that occurs naturally in somatic cells and is carried out by various enzymes that degrade cytoskeletal components via biochemical reactions [23].

Programmed cell death is a very clean and efficient way of destroying damaged cells. This differs greatly from another form of cell death, necrosis. Necrosis is usually the result of cell injury, which causes rupture of the plasma membrane, thus allowing the contents of the cell to leak out and affect neighboring cells, this leaking cellular debris can actually cause more damage to the organism [24]. In the case of cancer, cells that die via necrosis, or cells that lyse can cause the cancer to spread to neighboring cells. Therefore, the most efficient way of killing cancer cells is through

apoptosis. It is the goal of chemotherapeutic, radiation, and hormonal treatments [25, 26] often to induce apoptosis in cancer cells, however cancer cells often acquire resistance to these treatment modalities.

While many pathways can trigger apoptosis, one of the two most common is when cytochrome *c* is released from the mitochondria into the cytosol. The role of cytochrome *c* is to form a structure called the apoptosome [27] that promotes apoptosis through a cascade of reactions. The first step in the mitochondrial pathway is when cytochrome *c* itself binds to a protein known as apoptosis activating factor 1 (APAF-1) and to procaspase-9, forming the aforementioned apoptosome. Once the structure has formed, caspase-9 (known as an initiator caspase) becomes activated which causes cleavage of procaspase 7 to cleaved caspase 7 (executioner caspase) which begins to degrade cell components [27, 28]. The role of the initiator caspases is to carry a message to the executioner caspases to begin degrading cellular components. It is best thought of as a 'passing the baton' where the initiator caspases pass the signal to the executioner caspases to begin apoptosis. After the cell has activated the executioner caspases (3, 6, and 7) the cell undergoes apoptosis.

Once the cell has undergone apoptosis its cellular components must be disposed of, this process is known as phagocytosis. During the process of apoptosis a phospholipid that is normally found in the inner leaflet of the plasma membrane, phosphatidylserine, migrates to the outer leaflet. This migration marks the cell and signals nearby phagocytes that the cell is ready to be engulfed [29]. During this process, the phagocyte engulfs the apoptotic bodies and degrades them with the help of

various enzymes found in lysosomes. Phagocytes can be macrophages, which are part of the immune system, or a neighboring cell that helps to remove the degrading apoptotic bodies [30].

Apoptosis can also be induced in a lysosomal-mediated manner [31].

Lysosomes act as “cell dumping site” where they digest damaged cellular components, remove waste products from the cell, and are known to be a part of the apoptotic process. This organelle contains an array of enzymes that are capable of breaking down proteins, carbohydrates, and lipids [32]. Lysosomal-mediated apoptosis is still largely under investigation and not fully understood, however what is known is that the membrane of the lysosome becomes permeable. Originally, this was thought to only be a part of necrosis [33], the release of acidic hydrolases from the lysosome into the cytosol, now research suggests specific lysosomal proteases play an active role in apoptosis [33, 34].

While the data on lysosomal-mediated apoptosis is still accumulating, of the roughly 50 hydrolases present in lysosomes, cathepsins are best understood in terms of their role in triggering apoptosis [33, 34]. What appears to occur is depending upon how permeable the membrane of the lysosomes becomes, the cell dies via necrosis or apoptosis. If there are large amounts of hydrolases released (as is the case in cell trauma) necrosis will occur, however if there are lower levels present this can trigger apoptotic signaling. Triptolide treatments create an oxidative stress [34, 35] within the cancer cells; this can be the triggering mechanism that causes lysosomal membrane permeabilization. In the hopes of illustrating that lysosomal pathways are also involved

in triptolide-induced apoptosis, we examined the treated cells with LysoTracker and acridine orange to assess the functionality of this organelle.

Triptolide has been thought to induce apoptosis exclusively via the p53 pathway [36]. However in our studies, evidence is provided to show that there are other factors involved, starting with the activation of caspases. As previously discussed, the mitochondrion plays a large role in apoptosis with the release of cytochrome *c* into the cytosol. If there is an increase in executioner caspases this suggests that cytochrome *c* was released, and also supports the notion that the mitochondria is involved in triptolide induced apoptosis. Furthermore, the mitochondria could induce apoptosis via a non-caspase dependent mechanism by stimulating PARP, which can trigger an apoptosis-inducing factor. Also with the membrane of the lysosome becoming permeable and the release of lysosomal enzymes, apoptosis can be achieved through a lysosomal mediated manner. Seen in Figure 2 is our proposed model for triptolide-induced apoptosis through lysosomal and mitochondrial mediated pathways.

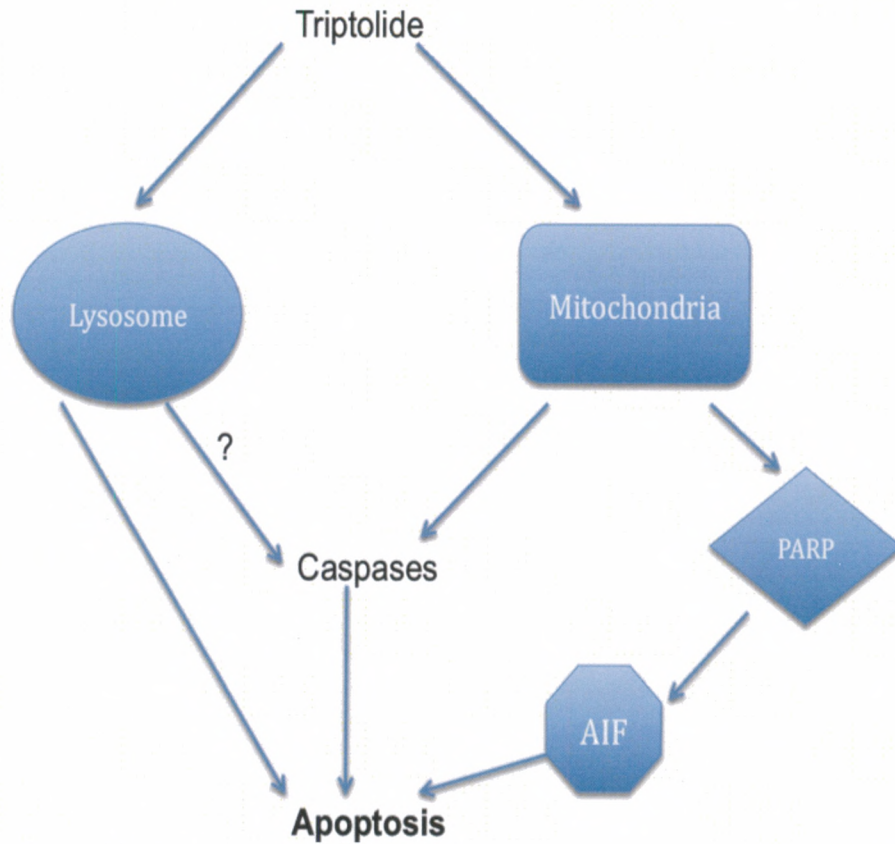


Figure 2: Proposed model of triptolide-induced apoptosis.

This research will illustrate other mechanics at work that provide evidence there are multiple pathways contributing to the apoptosis of MCF-7 and SK-BR-3 cells when treated with triptolide.

2. Materials and Methods:

2.1 Cell Culture:

MCF-7 and SKBR-3 were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in α -Minimally Essential Media (α MEM; BioWhittaker, Walkersville, MD) and supplemented with 0.01mg/mL insulin, 100

parts/mL Streptomycin/Penicillin (ATCC), and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). All cells were grown in T-25 flasks (Gibco, Grand Island, NY) and incubated at 37°C in a humidified chamber at 95%O₂/5%CO₂. Cells were subcultured by washing with a 1X phosphate buffered solution (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH adjusted to 7.4) and using Trypsin (Invitrogen, Eugene, OR, 0.25% (w/v) Trypsin - 0.53 mM EDTA solution).

2.2 Hemotoxylin and Eosin Staining:

Cells were allowed to grow on coverslips until confluent in six-well plates. Upon reaching a confluent state cells were treated in the presence or absence of triptolide (10ng/mL) and incubated at 37°C for 24 h. After treatment, coverslips were rinsed with 1X PBS and cells were fixed to the coverslips using a 4% paraformaldehyde solution and incubating for 20 min. After cells had adhered to the coverslips they were dipped in hematoxylin for 60 sec, and rinsed in dH₂O for 2 min. Next, cells were dipped into eosin for 30 sec and placed in solutions of 95% ethanol and 100% ethanol for 2 min and 5 min, respectively. Slides were then fully dehydrated in a solution of 100% ethanol for 2 min and then equilibrated in xylene by dipping for 2 min three separate times. Finally, coverslips were mounted onto slides using Crystal Mount (Sigma, St. Louis, MO) and examined by light microscopy to examine cell morphology.

2.3 MTT Cell Viability Assay:

To test for apoptosis the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Sigma, St. Louis, MO) was used. Stock solutions of MTT were prepared in α MEM at a concentration of 5 mg/mL. Cells were allowed to grow until confluent in 6-well plates and then treated with varying concentrations of triptolide (1 ng/mL, 5 ng/mL, 10 ng/mL, and 15 ng/mL) for 24 h. After the treatment period, the medium was removed and replaced with 2mL of fresh medium that contained the MTT and incubated at 37°C for 4 h. After the incubation with the MTT, the medium was removed and acidic isopropanol was added. The acidic isopropanol (0.1 N HCl in 100% isopropanol) was needed in order to stop the reaction and dissolve the tetrazolium crystal that formed. Finally, the isopropanol was removed and absorbance (which relates to cell viability) was measured at 570 nm. All readings were done in triplicates.

2.4 LysoTracker™:

Lysosomes play a crucial role in apoptosis by degrading cellular components. Using the LysoTracker™ (Invitrogen) we can visualize lysosomes in cells. Stock solutions of LysoTracker™ were diluted in α MEM to 5 mg/mL. Because the probe is known to stain other cellular structures at a higher concentration, we choose to go with 50 nM as our concentration for treatment. Cells were grown in 6-well plates until confluent and then treated for 24 h with varying concentrations of triptolide (1 ng/mL, 5 ng/mL, 10 ng/mL, and 15 ng/mL). After the incubation period, the cell culture medium was removed and 1mL of the LysoTracker™ solution and 1mL of fresh

medium was added to the wells and allowed to incubate for 45 min. After the staining period, the probe solution was removed and replaced with regular growth medium. Cells were immediately analyzed using fluorescence microscopy (EVOS FL, Advanced Microscopy Group, Bothell, WA).

2.5 MitoTracker™:

Mitochondria play a role in apoptosis by starting a cascade of caspase activity. To look for a change in mitochondrial permilibration as well as to localize mitochondrion, the MitoTracker™ kit (Invitrogen) was used. Stock solutions (1 mM) of MitoTracker™ were prepared by adding 1 mL of dH₂O to the vial of MitoTracker™ dye supplied. Solutions for the assay were then diluted to 2 µg/mL in αMEM. MCF-7 cells grown in 6-well plates until confluent and were then treated with triptolide for 24 h at varying concentrations (1 ng/mL, 5 ng/mL, 10 ng/mL, and 15 ng/mL). Afterwards, the medium was removed and 1 mL of MitoTracker™-containing medium was added in conjunction with 1 mL of normal medium (to give a final concentration of 1 µg/mL of MitoTracker™). Cells were then incubated for 45 min at 37°C; fresh pre-warmed media was added; and images were captured via fluorescence microscopy using the EVOS FL.

2.6 Hoescht Staining:

Cells were treated in the presence or absence of 10 ng/mL of triptolide for 24 h at 37°C and then with 1 µg/mL of Hoechst for 15 min at 37°C in the dark. Images were captured using the EVOS FL fluorescence microscope.

2.7 Acridine Orange:

Cells were grown on coverslips and incubated for 24 h in the presence or absence of triptolide. Afterwards a 5 mg/mL stock solution of acridine orange was prepared by dissolving 5.0 mg of acridine orange in 5mL α MEM. The medium was removed from the wells and 1 mL of acridine orange-containing medium as well as 1 mL of fresh pre-warmed medium was added and allowed to incubate for 15 minutes. Cells were rinsed with 1X PBS and coverslips were mounted using Crystal Mount. Cells were examined using fluorescence microscopy.

2.8 Protein Isolation

Cells were treated at varying concentrations of triptolide (1 ng/mL, 5 ng/mL, 10 ng/mL, and 15 ng/mL) for 24 h in 6-well plates. After incubation, the medium was removed and cells were rinsed once with 1X PBS. Two hundred μ l of SoluLyse-M (Genlantis San Diego, CA) were added to each well and incubation occurred at room temperature for 10 min. Cell lysates were then collected and transferred to Eppendorf tubes, which were centrifuged for 5 min at 14,000 g. Supernatants were transferred into clean Eppendorf tubes for further analysis.

2.9 BCA Protein Concentration Determination

Once proteins were extracted from the cells, the BCA Protein Assay Kit (Pierce Rockford, IL) was used to determine protein concentration. A preparation of diluted bovine serum albumin (BSA) was made in order to generate a standard curve in which

the unknown samples could be compared. As provided by the kit each ampule contained 2.0mg/mL of the albumin standard, enough to perform three replications of each diluted sample. The range prepared, as suggested by the manufacturer, was from 2,000 μ g/mL to 0 μ g/mL (blank).

After preparation of the standards, the BCA working solution was prepared using the following formula: (# of standards + # of unknowns) x (# of replicates) x (volume of working reagent per sample, 2.0mL) = total volume of working reagent needed (Pierce). The working reagent was prepared by adding 50 parts of BCA reagent A to 1 part of BCA reagent B (50mL Reagent A with 1mL Reagent B).

To begin the protein analysis, 10 μ l of each standard and unknown were added to labeled test tubes. Next, 200 μ l of the working reagent was added and the tubes were vortexed for 5 sec. Tubes were then incubated for 30 min at 37°C to allow the reaction to proceed. Samples were then analyzed at 562nm using the Nano-Drop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). A standard curve was generated and the protein concentrations of the unknown samples were determined.

2.10 SDS-Page

SDS-Page was performed using 10% EZ-Run protein gel solution (Fisher Scientific, Pittsburg, PA) and the E-Run running buffer. First 130 μ l of freshly prepared 10% ammonium persulfate (500mg in 5mL dH₂O) and 12 μ l of tetramethylethylenediamine (TEMED) were added to 10mL of the EZ-Run protein gel solution. The solution was added to the gel chamber with a comb present and allowed to polymerize for 20 min. While waiting for polymerization to occur, samples were

prepared by the addition of Laemmli's SDS-sample buffer (Tris-HCl [250 mM, pH, 6.8], SDS [8%], glycerol [40%], β ME [8%], and bromophenol [0.02%]), and boiling for 5 min. A stock solution of 1X EZ-Run running buffer was prepared from the 20X EZ-Run running buffer. Upon polymerization, the comb was removed and the wells were rinsed with the SDS-PAGE buffer and samples were loaded to each well. Standard 1X loading dye was added to each empty well to prevent the proteins from shifting during electrophoresis. Gels were run at 150 V for 60-90 min, or until the samples reached the bottom of the gels.

2.11 Coomassie Staining

After SDS-PAGE, gels were incubated in a Coomassie blue solution (2g in 100mL) for 1 h and then destained overnight in the destaining solution (100 mL acetic acid, 450 mL methanol, 450 mL dH₂O). Both staining and destaining were done with gentle shaking to allow for the gel to be completely exposed to both solutions.

2.12 Colorimetric Western Blot Analysis:

Following the SDS-PAGE, proteins were transferred to PVDF membranes. Transfer buffer (25mM Tris, 192mM glycine, 20% Methanol, 775mL dH₂O, and pH adjusted to pH of 8.3) was made in order to transfer the proteins. To make the trans-membrane blot sandwich 6 pieces of Whatman paper, the PVDF membrane (pre-treated for a few sec in 100% methanol), and the sponges were all soaked in the transfer buffer. Then the trans-blot sandwich was built using the cassette with a sponge, three pieces of Whatman paper, the membrane, the gel, three more pieces of Whatman paper, and the last sponge. Together this was placed into the transfer

chamber and filled with the transfer buffer. The apparatus was run for 2 h at 130V. After the transfer was complete, the membranes were removed from the apparatus and rinsed in 1X PBS for 5 min.

To visualize the proteins, membranes were first blocked for 1 h at room temperature in the blocking buffer (1X TBS) and then rinsed for 5 min in a 1X PBS solution. Primary antibodies were purchased from Cell Signaling (Danvers, MA) and were raised in rabbits. Primary antibody incubation occurred at a dilution of 1:500 (Dilution Buffer 1X PBS-T) for 24 h at 4°C. Following the incubation period membranes were washed in wash buffer (1X PBS) for 1 h at room temperature. Subsequently, secondary antibodies (Goat-anti Rabbit) were incubated with membranes for 1 h at room temperature, membranes were then washed for 30 min. Finally, to visualize bands then membranes were incubated in a solution of Blue Liquid Substrate (Sigma- BCIP/NBT) for 30 min. Once the bands began to develop the solution was decanted and dH₂O was added to terminate the reaction and minimize high background. Bands were then compared to the molecular weight standards to confirm the presence of the protein-of-interest.

2.13 Acid Phosphatase:

Cells were treated in the presence or absence of triptolide for 24 h at 37°C and were then transferred into a 0.9% NaCl solution and centrifuged for 5 minutes at 10,000 g. Cells were then placed in the reaction mixture (0.5 mL p-nitrophenol phosphate, 0.5 mL 90mM citrate buffer [pH=4.8], and 0.1 mL of centrifuged cells) and allowed to incubate for 30 min at room temperature. The reaction was terminated with

the addition of 0.5mL 0.1 N NaOH. Samples were then analyzed spectrophotometrically at 410 nm.

2.14 Statistical Analyses:

Student *t*-tests were performed to check for statistical significance. MTT absorbance values for triptolide treated cells were considered statistically significant with a p-value <0.05.

3. Results

3.1 Hemotoxylin and Eosin Staining

Cancer cells exhibit morphology quite different from that of normal somatic cells, such as hypertrophic nuclei, irregularity in cell membrane, and loss of anchorage-dependence [37]. We examined the affect of triptolide treatment on the morphology of MCF-7 cells by using hematoxylin and eosin staining. Control cells displayed normal breast cancer cell morphology – cells formed outgrowths and domes (Figure 3, left panel). Cell shrinkage, blebbing [19] of the plasma membrane, and fragmented cells were detected in response to triptolide treatment (Figure 3, right panel). These data suggest that triptolide induces morphological changes in the cells that are consistent with apoptotic cell death.

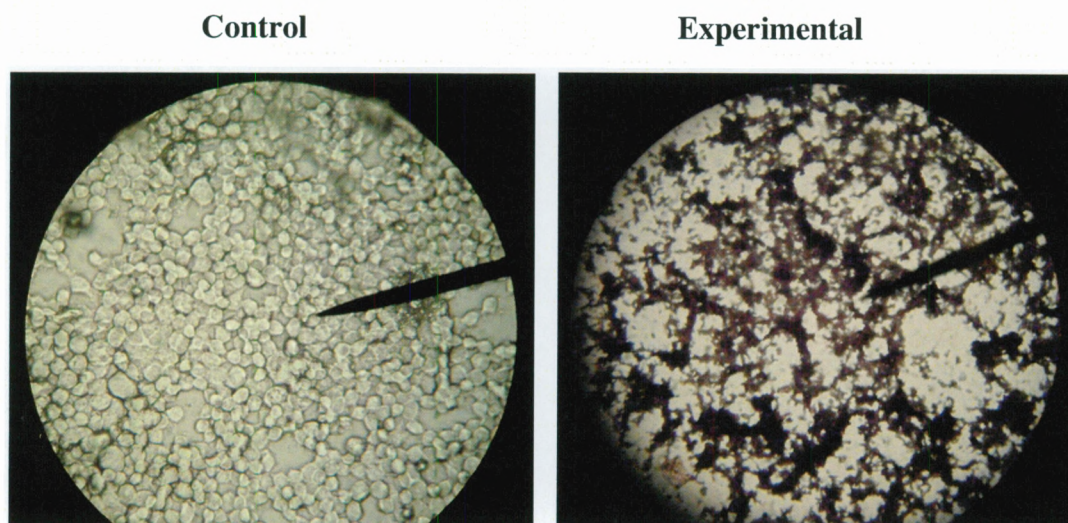


Figure 3: Hematoxylin and Eosin Staining. Control cells are nearly confluent and show normal morphology (left panel). However in cells treated with 10ng/mL triptolide cells have shrunk and become fragmented (right panel). Shown are representative images from three independent experiments. Magnification at 400x.

3.2 MTT Cell Viability Assay

The MTT cell viability assay is a quantitative way of examining cell proliferation by spectrophotometric means. Mitochondrial function in viable cells reduces the tetrazolium salts through metabolic pathways into insoluble formazan crystals [38]. Therefore, the more cells that are undergoing apoptosis the fewer purple formazan crystals will be present, thus suggesting apoptosis is occurring in these cells. For our purposes both MCF-7 and SK-BR-3 cells were treated in the presence or absence of triptolide at varying concentrations and for prolonged time periods to determine if there was a dose-dependent and/or time-dependent relationship in conjunction with triptolide treatments.

As seen in Figure 4A, MCF-7 cells exhibited an increase in cell death with increasing concentrations of triptolide. All OD readings were done in triplicates and an average was taken. Student *t*-tests were performed on the absorbances to check for statistical significance and all *p*-values were < 0.05. The results represent percentage of cell death in comparison to control cells. SK-BR-3 cells also demonstrated the same behavior with an increase in triptolide concentration causing an increase in cell death (Figure 4B).

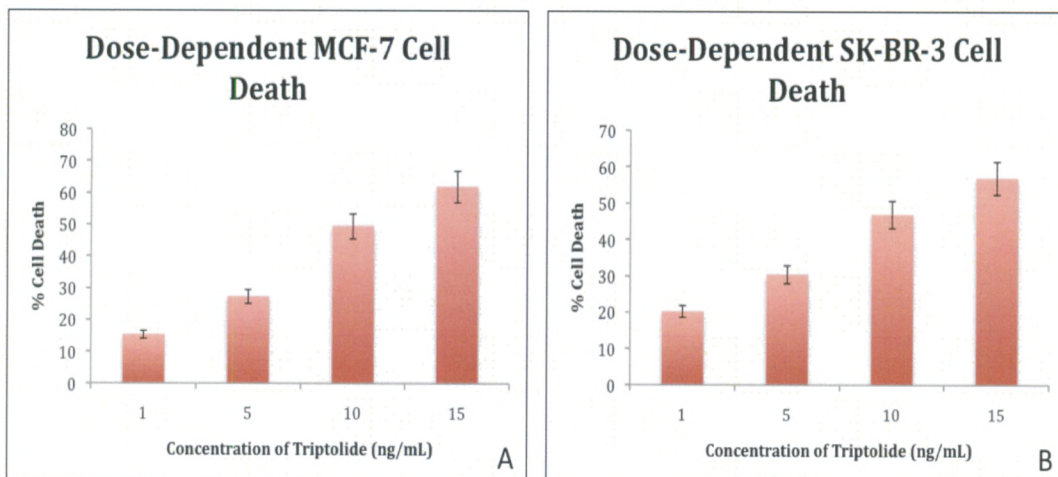
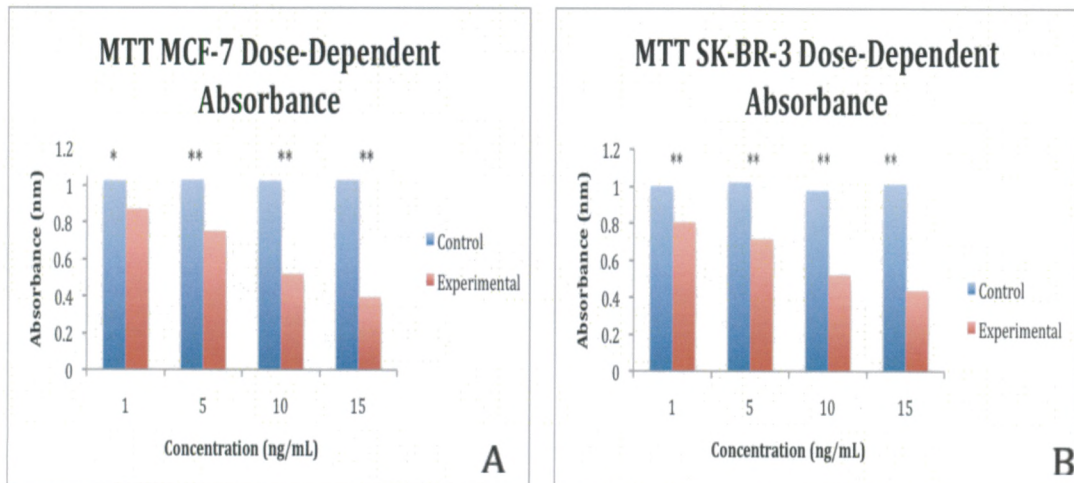


Figure 4: Dose-Dependent MTT results for MCF-7 and SK-BR-3 cells. Shown here both cell lines show an increase in cell death when treated with a higher concentration of triptolide. Student *t*-tests were performed and yielded *p*-values <0.05. Data shown below (* indicates *p*< .05, ** indicates *p*< .001). Studies were conducted in triplicates and the values are from three independent experiments.



To examine whether triptolide had an effect over an extended period of time, a time-series study was conducted as well. Cells were treated with 10ng/mL (LD_{50}) for 24 h, 48 h, and 72 h and again the cell viability was measured. As seen in Figure 5A and 5B in both MCF-7 and SK-BR-3 there was an increase in cell death the longer the cells were exposed to triptolide.

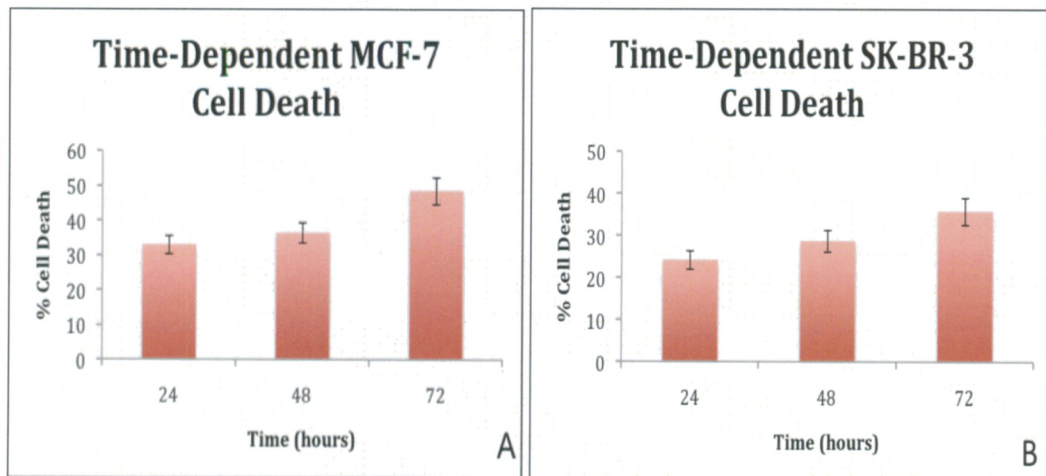
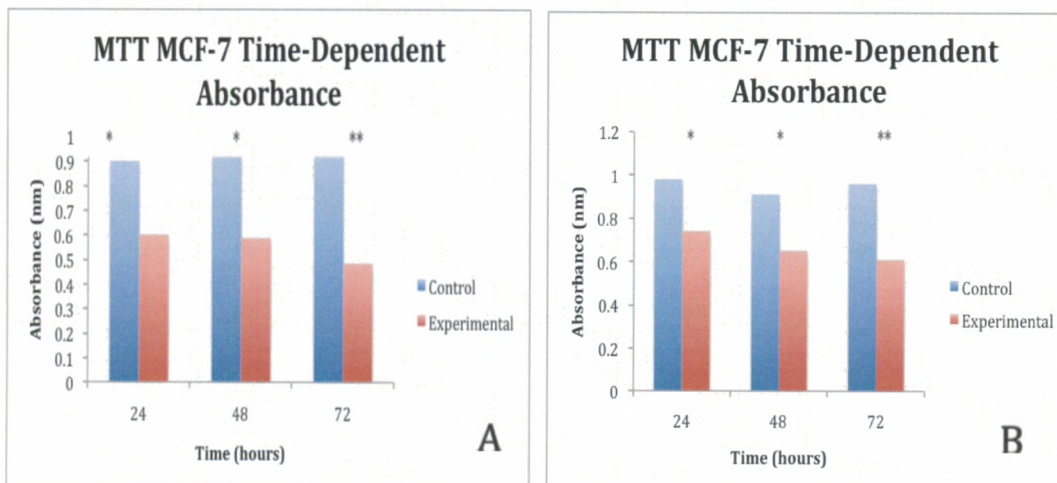


Figure 5: Time-dependent MTT results for MCF-7 and SK-BR-3 cells. Cell lines also showed that with longer incubation periods of triptolide cell viability decreased. However, it is worth noting that between 24 and 48 h the change in cell death was only 3.5%. Again student *t*-tests were performed and all *p*-values were <.05. Data shown on next page (* indicates *p* < .05, ** indicates *p* < .001). Studies

were conducted in triplicates and the values are from three independent experiments.



3.3 Immunofluorescence Staining

The beauty of immunofluorescence assays is that they show localization of the target (whether it be a protein, an organelle, or DNA) within the cell. In these studies all three are tested to exhibit different ways that triptolide can cause cancer cells to undergo apoptosis. Through the use of the MitoTrackerTM and LysoTrackerTM the organelles are directly stained to show their functionality before and after treatment. Hoescht staining is done to check for chromatin condensation, a definitive characteristic of apoptotic cells. Acridine orange, which collects in acidic environments, is also done to test for lysosomal-mediated apoptosis. Together with the LysoTrackerTM staining we hoped to show that there was a link between triptolide treatments and lysosomal mediated apoptosis in cancer cells.

As previously mentioned there are two organelles that participate in pathways that are crucial steps of apoptosis: the mitochondria, and lysosomes. Mitochondria,

with the release of cytochrome *c*, play a vital role in triggering a cascade of caspases resulting in the cleavage of various cellular substrates. Using the MitoTracker and LysoTracker immunofluorescence assays, we demonstrated how these organelles are affected by triptolide. Apoptosis is an endergonic reaction requiring a large amount of energy to breakdown the cellular components [39]. By using MitoTracker™ we can look for an increase in mitochondrial activity. In Figure 6, we compared the control cells with experimental cells (those treated with 10ng/mL of triptolide) for mitochondrial activity.

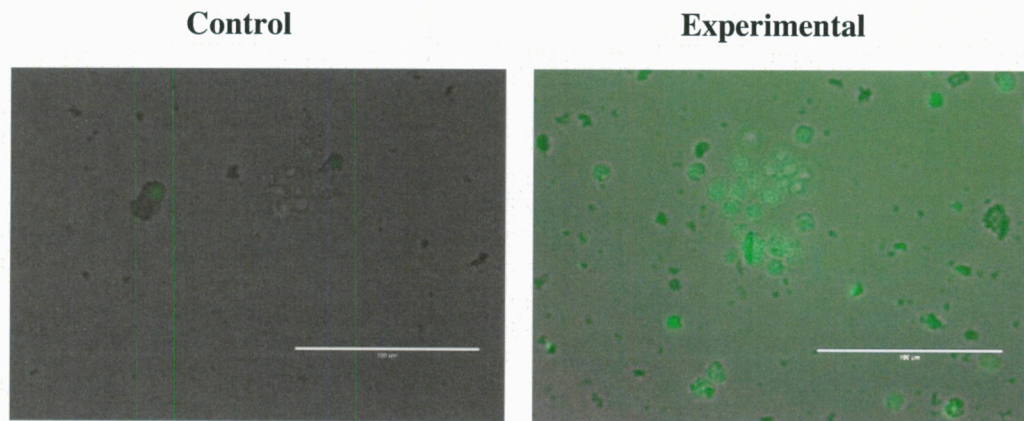


Figure 6: Results of MCF-7 MitoTracker when treated with triptolide. We detect an increase in mitochondrial activity in experimental cells, possibly suggesting a membrane permeability change. Shown are representative images from three independent experiments.

Experimental cells were more active and had a change in membrane permeability suggesting that cytochrome *c* may have been released into the cytosol. This was detected because the dye was able to collect within the mitochondria. If this is the case, we can also expect to detect the activation of caspases through Western. These results lead us to believe that triptolide is successful at causing various pathways, other than p53, to become active and aid in the process of apoptosis. Now in

figure 7, lysosomal function was examined using the LysoTracker™ assay. Here again we notice that in the experimental cells they are almost completely fluorescing suggesting that lysosome are beginning to degrade the cellular components [40]. This is a hallmark of apoptosis, with cellular machinery needing to be broken down.

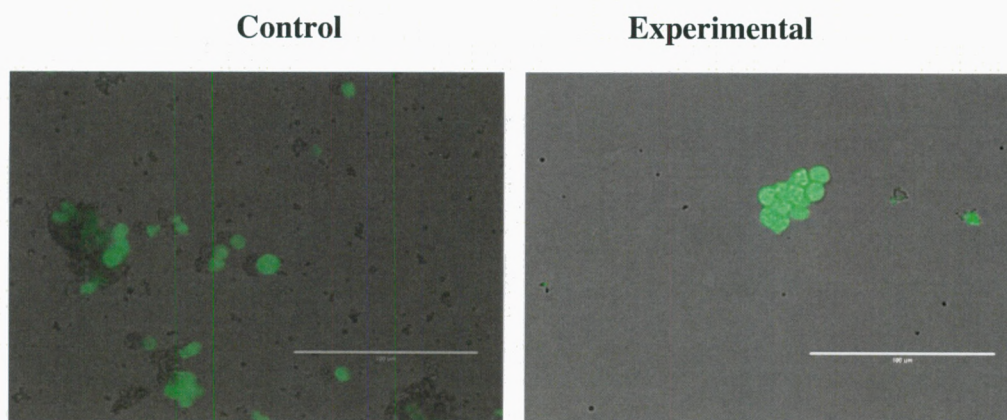


Figure 7: Results of MCF-7 LysoTracker in the presence and absence of Triptolide. As seen in the experimental cells (right panel) lysosomal activity was much higher than in the control cells. This could be due to triptolide activating lysosomal mediated apoptosis. Shown are representative images from three independent experiments.

For further support that apoptosis is mediated using lysosomes when treated with triptolide we used the acridine orange assay which is a fluorescent dye that accumulates in acidic environments. Because lysosomal-mediated apoptosis is still not widely accepted, we used multiple assays to assert our claim. As seen below in Figure 8, cells were stained with acridine orange in the presence and absence of triptolide. What we illustrate is that within the control cells, the signal detected is mostly red. This indicates that the hydrolases that acrdine orange recognizes are primarily still within the intact lysosome. However in the right panel, showing experimental cells, the green color represents a change in lysosomal membrane permeability suggesting

that the contents of lysosomes have now spilled out into the cytosol of the cells. This release of hydrolases can trigger apoptosis to occur in triptolide treated cells.

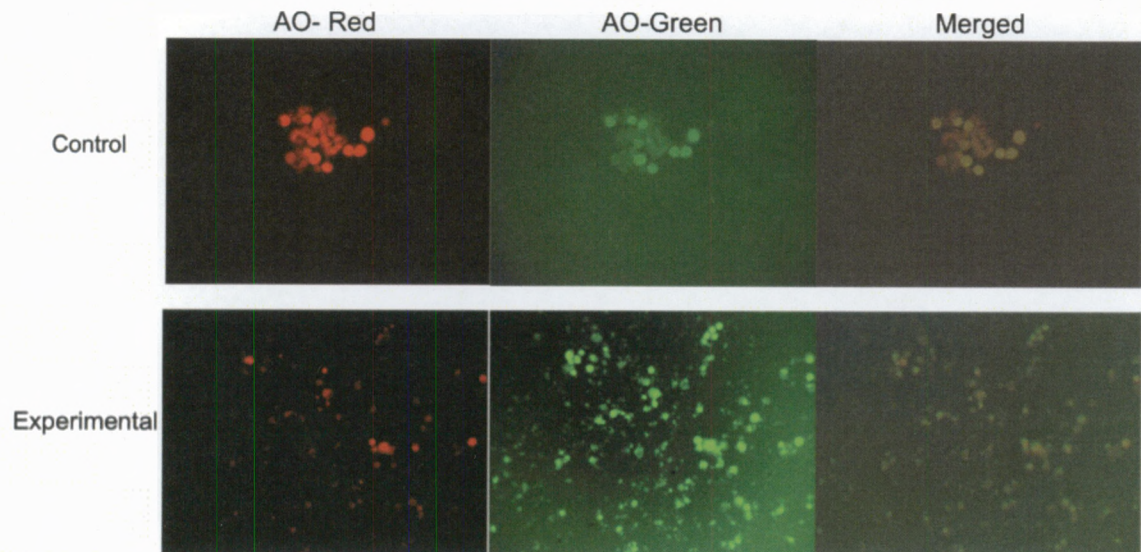


Figure 8: Control cells (top panel) show primarily red staining, which signifies that most of the hydrolases are located within the lysosomes. Seen in the experimental cells (lower panel) the green color indicates a change in lysosomal membrane permeability resulting in the hydrolases collecting in the cytosol.

DNA fragmentation and eventually degradation is the final step in the process of apoptosis. The destruction of the nuclear envelope thus exposing the DNA to various proteases and caspases allows for the genetic information of the cell to be disposed of [41]. This is of particular importance in cancer because if the genome of the cell is mutated, the DNA is the most crucial part to destroy so no other cells can take on that cancerous mutation. Through the use of the Hoescht stain we display the effects of triptolide on cancer cells in detecting chromatin condensation. In Figure 9 we can detect chromatin condensation, a hallmark of apoptosis within the experimentally treated cells. This was not detected in the control cells.

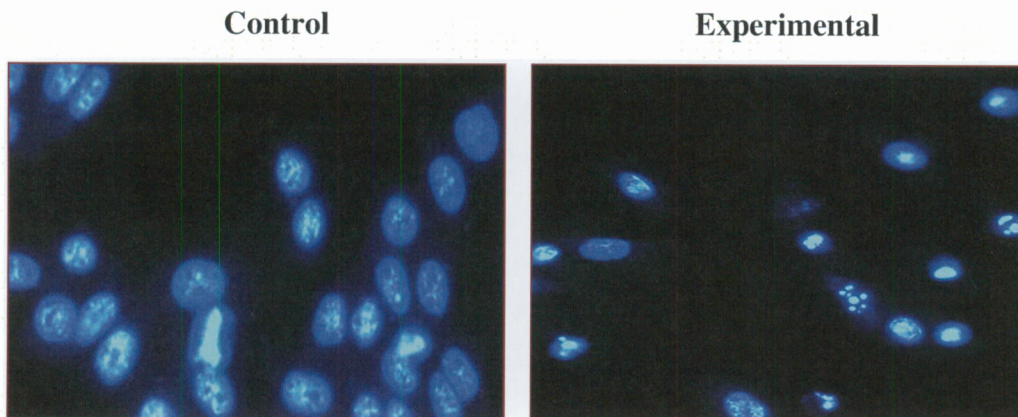


Figure 9: Seen here MCF-7 cells treated with triptolide exhibit chromatin condensation detected with Hoechst stain (right panel). Control cells (left panel) which do not exhibit this condensation. Shown are representative images from three independent experiments.

3.4 Western Blotting

Apoptosis is regulated by the cyclic patterns of various proteins, when certain proteins are expressed the next phase of apoptosis can occur. Continuing with this idea, it is the proteins themselves that are responsible for the cellular breakdown, with different proteolytic reactions that take place. It was our intent to see how triptolide regulated various proteins, including those that are pro- and anti-apoptotic, proteins that are the result of tumor suppressor genes, as well as different caspases, which are part of a cascade resulting in cleavage of many cellular components leading to the formation of apoptotic bodies.

Western blot analyses were done using colorimetric reagents on samples that came from triptolide treated cells, as well as control cells. In Figure 10, we are looking at the way triptolide affects pro-apoptotic and anti-apoptotic proteins. Because cancer

cells are looking to proliferate and colonize at distant sites, cell death avoided by causing an up-regulation of anti-apoptotic proteins. One such protein is Bcl-2, which is responsible for maintaining cell homeostasis and sequestering apoptosis from occurring [36]. Conversely, p53 is an example of a pro-apoptotic protein which is known to play a large role in causing apoptosis [11, 24, 42].

The p53 protein is known to play many roles as an anticancer agent by inducing DNA repair enzymes, stopping angiogenesis, causing arrest in the cell cycle at the G₁/S gap to allow for DNA repair to occur, or if the DNA is too badly damaged initiating apoptosis [42]. Therefore this protein is essential to maintaining genome stability and without it, cells are able to proliferate unchecked. Normal cytosolic concentrations of p53 are quite low [42, 43], however in apoptotic cells this concentration is increased. We tested for the up-regulation of p53 and the down-regulation of Bcl-2 in cells that were treated with triptolide through Western blotting using antibodies against each of the proteins of interest. Our rationale was that if apoptosis is occurring, we should find higher levels of p53 and steadily decreasing levels of Bcl-2. According to Figure 10 our assumptions were supported, in the experimental cells we find that both p53 and Bcl-2 are differentially regulated where p53 was up-regulated to promote apoptosis, and Bcl-2 concentrations fell.

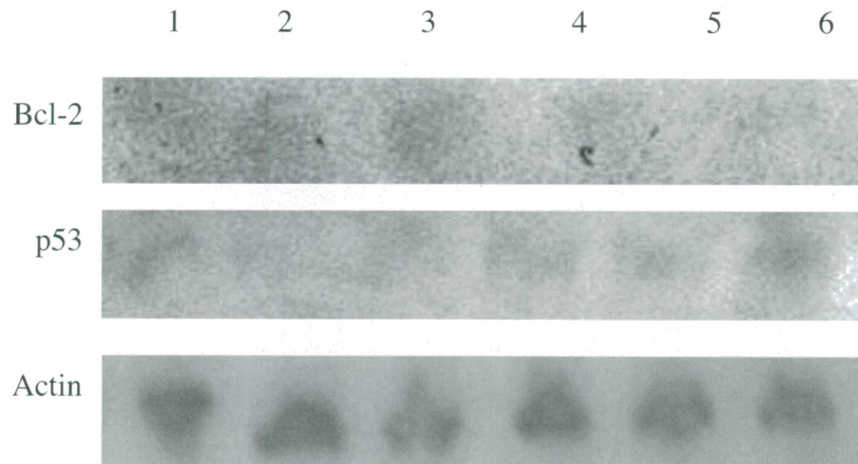


Figure 10: Western Blot analysis for p53 and Bcl-2. Triptolide treated cells expressed a decrease in Bcl-2 and an increase in p53 protein expression. Lane 1- MW standard, Lane 2- Control, Lane 3- 1 ng/mL, Lane 4- 5 ng/mL, Lane 5- 10 ng/mL, Lane 6- 15 ng/mL.

Caspases themselves are part of a cascade system which work to carry messages (initiator caspases) and induce apoptosis (executioner caspases). As previously mentioned, caspases play vital roles in apoptosis and because this system depends on the formation of an apoptosome, high cytosolic levels of caspases could signify a mitochondrial mediated pathway at work. In our study, we treated cells with varying concentrations of triptolide and then measured caspase concentration through Western blot analysis; Figure 11 shows the results of these tests. We first looked for the presence of cleaved caspase-9, an initiator caspase. Cleaved caspases are ones that are active, when they received a signal to start functioning part of the caspase that is not necessary is cleaved off.

As seen in the figure, this initiator caspase showed an increase in band size upon treatment with higher concentrations of triptolide. Also, seen in Figure 11 are the results for cleaved caspase-7, an executioner caspase. Similar to the results found in

cleaved caspase-9 are shown below, with the overall concentration increasing with more triptolide. Lastly, caspase-3 another initiator caspase was not detected (data not show) this is because caspase-3 does not exist in MCF-7 cells, as suggested by Janicke *et al.* (1998) [44]. Caspase-3 does not exist in these cells because of a 47 basepair deletion on exon 5 resulting in caspase-3 not being expressed.

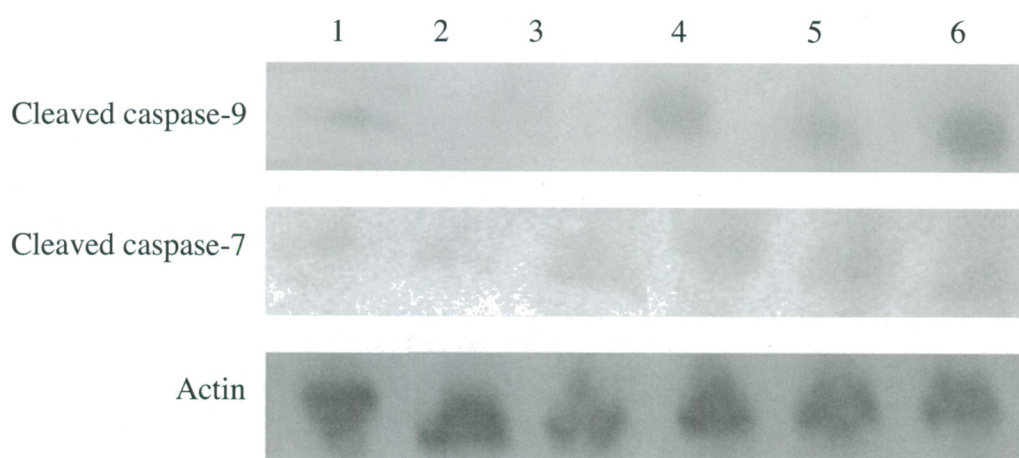


Figure 11: Western blot analyses show that with increasing concentrations of triptolide treatments there is an increase in both initiator and executioner caspases. Lane 1- MW standard, Lane 2- Control, Lane 3- 1 ng/mL, Lane 4- 5 ng/mL, Lane 5- 10 ng/mL, Lane 6- 15 ng/mL.

Poly (ADP-ribose) polymerase, or PARP, is a unique protein in that it can repair DNA damage, mediate post-translation modifications to proteins, and can directly induce apoptosis [45]. When DNA damage is detected PARP can induce apoptosis by producing a protein called PAR which causes the mitochondria to release apoptosis inducing factor (AIF) [46]. This pathway is thought to be independent of the

caspace cascade because it is first triggered by PARP detecting DNA damage. PARP expression was tested for in the same way that the other proteins were and the results showed results similar to those from the above figures. Figure 12 shows that PARP expression increased in response to increasing concentrations of triptolide treatment. This could suggest that triptolide successfully activates PARP to detect DNA damage and induce apoptosis. Through Western blotting analyses we were able to demonstrate how triptolide is able to differentially regulate various pro- and anti- apoptotic proteins.

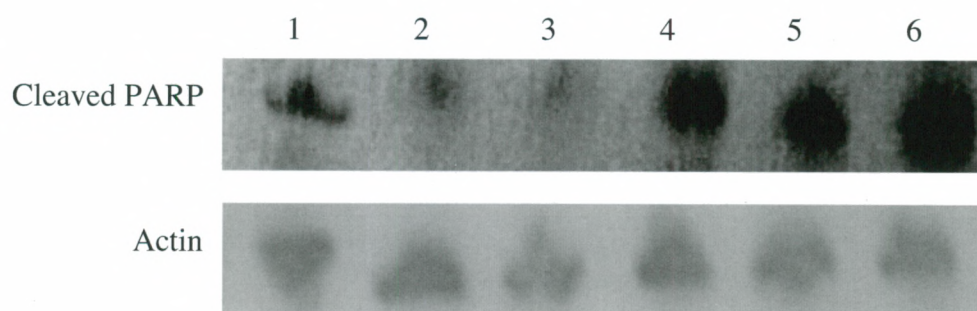


Figure 12: Expression of cleaved PARP was shown to have increased in response to higher dosages of triptolide treatments. Lane 1- MW standard, Lane 2- Control, Lane 3- 1 ng/mL, Lane 4- 5 ng/mL, Lane 5- 10 ng/mL, Lane 6- 15 ng/mL.

3.5 Acid Phosphatase

To further confirm that apoptosis is mediated in a lysosomal-mediated manner upon treatment with triptolide, an acid phosphatase assay was conducted. Acid phosphatase, as previously mentioned, is a marker enzyme for lysosomes and its increase is used to monitor apoptosis [47, 48]. It works on the presence that acid phosphatases work to remove phosphate groups from phosphoric acids in acidic

environments; therefore if lysosomes are more active we expect to find higher levels of acid phosphatase [49]. This rationale holds true as seen in Figure 13, the activity of lysosomal enzymes were much higher after a 24 h treatment with triptolide compared with the control cells, suggesting a role for lysosomes in apoptosis.

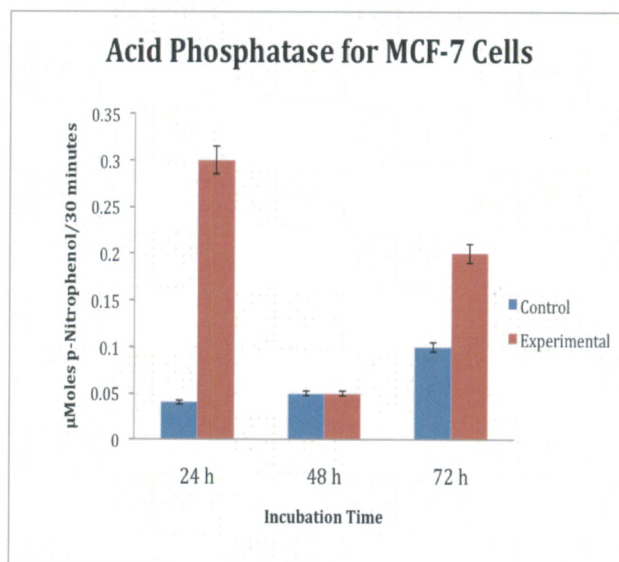


Figure 13: Acid phosphatase, which measures lysosomal activity, shows that lysosomes are much more active in the 24 and 72 h treatments in comparison with the control. Studies were conducted in triplicates and the values are from three independent experiments.

4. Discussion

It was the goal of this research to investigate possible mechanisms by which triptolide, an extract from the Chinese herb *Tripterygium wilfordii* Hook F, induces apoptosis in human breast cancer cells. Current research suggests that triptolide is responsible for activating the p53 pathway and causing cell death, however our research offers additional alternatives. By triggering apoptosis in breast cancer cells we are able to suggest a clean and efficient manner by which these cells can be killed

off without harming or infecting neighboring cells. The nature of triptolide to act as an anti-inflammatory, anti-cancer, and anti-proliferative agent makes it a great alternative to current methods of treatment. The side effects of chemotherapy and radiation can be debilitating to the patients health [50, 51] and in some cases actually make the cancer worse or bring on a new type of cancer [50], for these reasons new and safer alternative treatment methods need to be investigated.

Apoptosis is a complicated process by which damaged cells are degraded in a very clean and efficient way. Many types of death stimuli such as DNA damage, loss of control over the cell cycle, protein interactions, as well as a host of other implications also control this process. Because apoptosis is so complex, we believe that when cancer cells are treated with triptolide there are multiple pathways activated to bring about this cell suicide. It has already been widely accepted that triptolide is responsible for activating the p53 pathway [36, 52] however we sought to investigate how mitochondrial and lysosomal involvement could aid in the cells destruction.

First, we examined exactly what triptolide did upon treatment and looked for morphological changes as well as its affect on cell viability. The results were evident after hematoxylin and eosin staining that the cell morphology changed greatly, displaying cell shrinkage and fragmentation within the triptolide-treated cells, as would be expected in apoptotic cells. Next, we wanted to assess overall cell viability to see how and if triptolide worked at increasing cell death. Both MCF-7 and SK-BR-3 cells exhibited dose- and time-dependent relationships in response to their exposure to triptolide. Cell death (which was compared to the control) increased at higher

concentrations of triptolide and an LD₅₀ was established around 10ng/mL. This concentration was then used in time-series studies and we showed that cells that were exposed to triptolide longer also experienced more cell death. These correlations did not come as a big surprise as triptolide is known to induce apoptosis in other cancer cell lines [53, 54].

To determine if mitochondrial function played a role, we first looked for activity of the mitochondria using MitoTracker™. With an increase in fluorescence signal from triptolide treated cells compared to control cells, this suggested a change in the membrane permeability of the mitochondria occurred allowing them to become more permeable. If there was a change in the permeability of the membrane, this could allow for the release of cytochrome *c*, one of the first steps in triggering the caspase cascade. Because caspases are known to play a vital role in apoptosis it would be expected that they are involved in triptolide-mediated apoptosis. To test for this, Western blot analyses were conducted to examine if the expression levels of different initiator and executioner caspases. Our results indicate there is an increase in expression of caspases-7 and -9 both of which are part of the caspase cascade responsible for triggering apoptosis [43, 44]. Caspase 3, another caspase that is vital for the caspase cascade was not detected, however this is due to the fact that MCF-7 cells do not code for caspase-3 [44]. MCF-7 cells have a 47-base pair deletion on exon three within the *CASP-3* gene which is why caspase-3 protein is not synthesized in these cells.

We then sought to elucidate a lysosomal-mediated pathway by which triptolide induces apoptosis. This process is known to involve several lysosomal enzymes, however acid phosphatase is known to exclusively be active within the acidic conditions of the lysosomes. To test for lysosomal functionality we tested for the activity of acid phosphatase and found that its activity was increased in 24 and 72 hour treated cells relative to control cells. Also, using LysoTracker™ we localized lysosomes within cells that were treated in the presence or absence of triptolide. Our results were similar to the MitoTracker data in that there was an increase the fluorescent intensity of the lysosomes in the experimental cells. Because of the role of lysosomes in degrading cellular components and aiding in phagocytosis [29, 55], establishing a link between triptolide and their activation will help to better explain how apoptosis is achieved in these cells.

Lastly, we investigated the status of the chromatin in response to triptolide. Through Western blot analysis we noticed that in triptolide treated cells, Bcl-2 was down-regulated while p53 was up-regulated. This is in accordance with several other studies [36, 52, 55] showing that the anti-apoptotic proteins had decreased in expression and pro-apoptotic ones increased when treated with anti-cancer agents. PARP expression was also examined because of its roles in inducing apoptosis as well as in repairing DNA damage. In the experimental cells, we noticed that PARP expression was much higher than that of the control. And finally, to see if chromatin condensation (one of the last stages of apoptosis) [56] was occurring, cells were stained with Hoechst, and our experimental cells did show cells that had undergone chromatin condensation. All of these studies collectively lead us to believe that triptolide-

associated apoptosis is mediated through multiple pathways including the increase of p53, lysosomal-mediated apoptosis, and the mitochondrial role in the triggering of the caspase cascade.

5. Conclusion

In summary, our results demonstrate mechanisms, besides the p53 pathway, by which triptolide-treated breast cancer cells undergo apoptosis. Through the use of homeopathic and natural treatments we may be able to find safer, non-invasive ways of treating cancer. Future studies can investigate the effects of triptolide *in vivo* looking at tumor volumes as well as protein expression from xenograft tumors in nude mice, as well as through TUNEL analysis to determine if DNA fragmentation is occurring suggesting that apoptosis is achieved in an animal model.

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