Phytoestrogen Project:

Effect of Phytoestrogens on Apoptosis of T47D Cells

A Major Qualifying Project Report, Submitted to the faculty of
Worcester Polytechnic Institute in partial fulfillment of the requirements for the
Degree of Bachelor of Science

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Abstract

Phytoestrogen supplements are commonly utilized by women for relief from menopausal symptoms. Some research suggests that certain phytoestrogens may cause cellular apoptosis, indicating significance as an anti-cancer treatment in addition to menopause treatment. T47D cells, a cell line acquired from cancerous breast ductal cells, were cultured and then treated with biochanin A and resveratrol. Results suggest these phytoestrogens cause apoptosis of T47D cells.

Acknowledgements

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1. Introduction

Hormone replacement therapy (HRT) is an evolving science, and has particular significance in the treatment of menopausal women. Traditional HRT for these women includes the prescription of estrogen and/or progestin supplements (Feigelson 1996); however due to the potential health risks surrounding the use of estrogen, such as cancer and stroke (Minelli 2004), there is a high demand for finding a safer option. One promising alternative may be the use of phytoestrogens, plant-derived compounds that are similar in structure to estrogen, which may thereby have similar effects in humans. Phytoestrogens are a popular treatment method, but their reliability and effectiveness is questionable, as minimal research has been conducted to date. Research indicates that the use of phytoestrogens by menopausal women may result in fewer incidences of HRT-related cancers. Furthermore, some research has suggested that phytoestrogens may decrease growth of cancerous cells. In a study by Sakamoto (2008), the phytoestrogens genestein, glycitein, and resveratrol were shown to cause apoptosis in MCF7 cells. Likewise, in a study by Caron (2007), the over-the-counter phytoestrogen supplement Promensil was shown to reduce proliferation of MCF7 cells.

Based on these findings, this project was initiated with the purpose of determining the apoptotic effect of resveratrol and biochanin A, two phytoestrogens, on the breast cell line T47D. Cells were cultured and treated with either phytoestrogen or estrogen, and tested for apoptosis. Specifically, the analysis of caspase 3 and mitochondrial transmembrane potential was used to determine apoptosis. Results suggest that high concentrations of resveratrol are toxic to cells, while lower concentrations seem to induce apoptosis in cells. Likewise, high and low concentrations of biochanin A have a noticeable apoptotic effect on T47D cells relative to controls.

1.1 Hormone Replacement Therapy

HRT is a general term for the use of externally provided hormones for medicinal or other beneficial purposes. Treatment of hypothyroidism, growth hormone deficiency (Frasier 1983), and Androgen Deficiency Syndrome in men (Bhasin 2006), are common applications for HRT. However, the prescription of external hormones is a complex science with a high potential for

health risks, and the use of HRT is still being studied extensively. A safe method of HRT for treating the irregular hormone levels in menopausal women has yet to be discovered since the traditional application of estrogen as a supplement has shown a high incidence of associated health problems. While benefits of using estrogen HRT include reduced coronary heart disease, osteoporotic fractures, and colorectal cancer, in addition to reduction of menopausal symptoms, strong evidence points to a heightened incidence of breast cancer and stroke. Stevenson (2002) described the following HRT trial, which was halted after 5 years of its intended 8.5-year course due to a significant occurrence of breast cancer in individuals participating. A quote from the author describes the astonishing prevalence of health risks in the study:

> *"For 10, 000 women taking hormone replacement therapy each year, compared with those not taking it, there would be an additional eight cases of invasive breast cancer, seven heart attacks, eight strokes, and eight pulmonary embolisms. However, there would also be six fewer bowel cancers and five fewer hip fractures."* (Stevenson 2002)

Historically, two approaches to HRT were used for menopause treatment. The first involves the use of only estrogen, also known as estrogen replacement therapy (ERT) (Feigelson 1996). In this approach, a minimal daily dose of estrogen is prescribed to relieve menopause symptoms and aid in the prevention of osteoporosis. The use of ERT peaked in the 1970s (Benderly 1997); however, an increase in endometrial cancer was observed and attributed to ERT. In a 1993 study by Levi on hospitalized patients (158 cancerous and 468 non-cancerous), 38% of the total patients who had used ERT were diagnosed with endometrial cancer. Furthermore, 95% of individuals who reported using ERT for more than five years had confirmed cases of endometrial cancer. This indicated that in general, the use of ERT increased the risk of cancer to around 40%, while the use of ERT for an extended period of time (greater than five years) caused a drastic increase in the risk of cancer. These results, among many similar findings, encouraged researchers to find another form of HRT.

This initiated the second approach to HRT, known as "combined therapy", which uses both progestin and estrogen. It was initially believed that the addition of progestin would combat the formation of cancer; however, research indicates that combination HRT may actually increase the risk of cancer. In a study by Ross (2000), analysis of patient history with ERT and CHRT (combined hormone replacement therapy) indicated a 6% increased risk of breast cancer for every five years of using ERT, and a striking 24% increase in risk after using CHRT for five

years. In general, results show the increased risk of cancer occurring only after prolonged HRT; thus, in an attempt to minimize this risk, the current approach for HRT involves careful prescription of small dosages for short periods of time (Benderly 1997).

In an attempt to reduce menopause symptoms while avoiding the dangerous side effects associated with ERT, research focus has shifted to the use of phytoestrogens. Phytoestrogens are estrogens found in plants such as soy, red clover, and flax seed oil (Sakamoto 2008). While little conclusive data has been presented to confirm an anticancer effect, some studies have suggested that certain phytoestrogens, such as genestein, resveratrol, and glycitein may cause apoptosis of cancerous cells (Sakamoto 2009).

1.2 Phytoestrogens

Resveratrol and biochanin A are two phytoestrogen compounds that are common components of many over the counter supplements. Resveratrol is most acknowledged for its presence in grapes and other fruit. It is thought to be a phytoalexin with significance in preventing pathogen infection, and is present in grape skin at about 50-100μg per gram of skin (Jang 1997). Research has suggested that resveratrol has activity in the inhibition of cyclooxygenases. These enzymes are known to cause cancer by catalyzing the synthesis of pro-inflammatory substances, which may inhibit immune surveillance and induce tumor cell growth (Jang 1997). Furthermore, resveratrol is known to be preventative in the formation of free-radicals, an event that is associated with the initiation of cancerous cells (Jang 1997).

Biochanin A is a primary component of the popular over-the-counter phytoestrogen supplement, Promensil, which is derived from red clover. Biochanin A has been the focus of a number of studies on prostate cancer (Sun 1998), and has been shown to be a potent antioxidant, a quality that enables it to scavenge free-radicals, thereby preventing tumor growth (Ruiz-Larrea 1997).

Because of their potential for anti-cancer effects, studies have been conducted on phytoestrogens, some of which support the hypothesis that certain phytoestrogens induce cancer cell apoptosis. In a study by Sakamoto (2008), the phytoestrogens daidzein, genestein, coumestrol, resveratrol, and glycitein were analyzed for various effects on MCF7 breast cells. Observations indicated estrogenic effects as well as significant anti-tumor activity. Genestein, glycitein, and resveratrol were all shown to have caused apoptosis, and resveratrol greatly

increased the activity of p53, a tumor-suppressing gene. In an additional study by Caron (2007), the effect of genestein, a component of Promensil, on MCF7 cells was analyzed. Results showed equivalent growth curves for both genestein and cells given 17β estradiol, but significantly reduced proliferation in cells given 100% Promensil. This data was complemented by results indicating that Promensil, but not genestein, induced significant upregulation of procaspase-3, a cellular precursor for apoptosis. This suggests that some component of the supplement, other than genestein, may have an apoptotic effect on breast cancer cells. Taken together, these data provide convincing arguments for the potential of phytoestrogens as a safe form of HRT that not only may reduce the risk of breast cancer, but may also terminate the initiation of cancer cell growth.

1.3 Apoptosis

Apoptosis, in its most simple definition, is the process of programmed cell death, also known as "cellular suicide". It is a naturally occurring event in all cells that is induced by a variety of stimuli, and is necessary for maintaining cellular homeostasis, as well as preventing the spread of viral infection. In addition, it has significance in the prevention of cancer progression by inhibiting the continued proliferation of cells with damaged DNA, thereby halting potentially cancerous cell growth (Mooney 2002). Because it is a highly regulated process, apoptosis does not evoke the inflammatory responses that typically accompany necrotic cell death (Dash).

A variety of cellular events occur that are characteristic to apoptosis. These include "blebbing" or bulging of the cell membrane, cell shrinkage due to cytoskeletal cleavage, and degradation and condensation of chromatin (Dash 2005). Of principle significance to this process are the cysteine proteases known as caspases, which contribute to the majority of the morphological changes to the cell. Additionally, the regulation of mitochondrial transmembrane potential is highly significant in the process of apoptosis. During apoptosis, it is believed that the mitochondrial permeability transition pore opens, allowing for a reduction in membrane potential and the release of cytochrome c. Cytochrome c is known to activate caspases, and thereby may initiate the apoptotic pathway (Mooney 2002). A general outline for the events occurring in apoptosis can be seen below in figure 1.

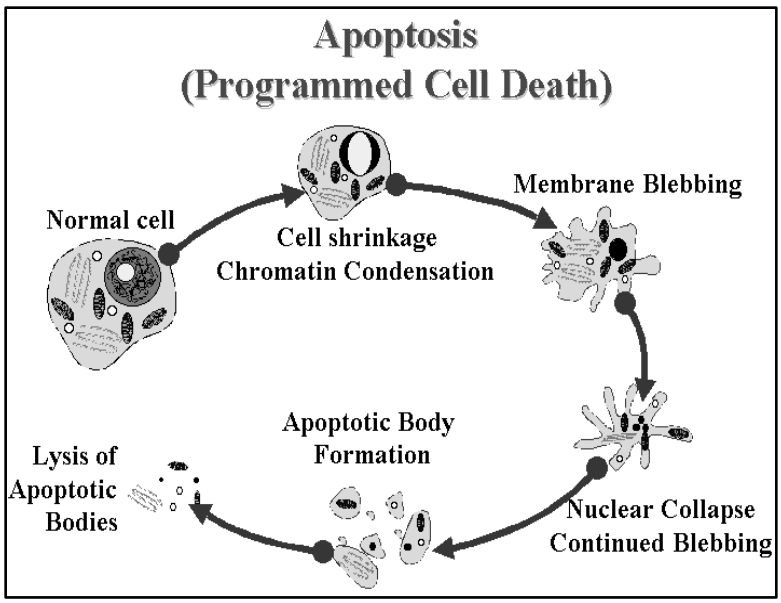

Figure 1. Process of apoptosis (Kalmakoff, 2007).

1.4 Caspases

Caspases are a family of cysteinyl aspartate-specific proteases that mediate inflammation and apoptosis through cascade processes. There are an estimated 11 human caspases (Los 2002), although the exact number is still debated. Caspase structure consists of a prodomain whose length varies between specific caspases, and a large p20 subunit and a smaller p10 subunit which are identical in all caspases (Los 2002). Caspases are activated by cytochrome c (Waterhouse 2001), and activation occurs by proteolysis at aspartate residues between the prodomain, p20 subunit and p10 subunit. This cleavage forms tetrameric caspases containing two p20/p10 heterodimers in a head-to-tail configuration, leaving the active sites at opposite ends of the molecule. The availability of active sites then gives rise to the initiation of caspase cascades (Duncan 2010), and ultimately, the cellular events of apoptosis.

Caspase cascades are either intrinsic or extrinsic pathways composed of initiator and effector caspases. Initiator caspases are located upstream of effector caspases which they activate, allowing for the cleavage of other proteins in the cell to initiate apoptosis. Loss of function of caspase pathways can lead to tumorigenesis (Duncan 2010).

In this project, caspase 3 was used as a marker for apoptosis in T47D cells. Caspase 3 is an effector caspase which, when activated, may induce a number of apoptotic effects, such as fragmentation of DNA and membrane blebbing, both of which will ultimately lead to

programmed cell death (Porter 1999). Caspase 3 is activated by the initiator caspase 9, which is activated by cytochrome c. The various actions induced by caspase 3 can be observed in the figure below, taken from an article by Porter (1999).

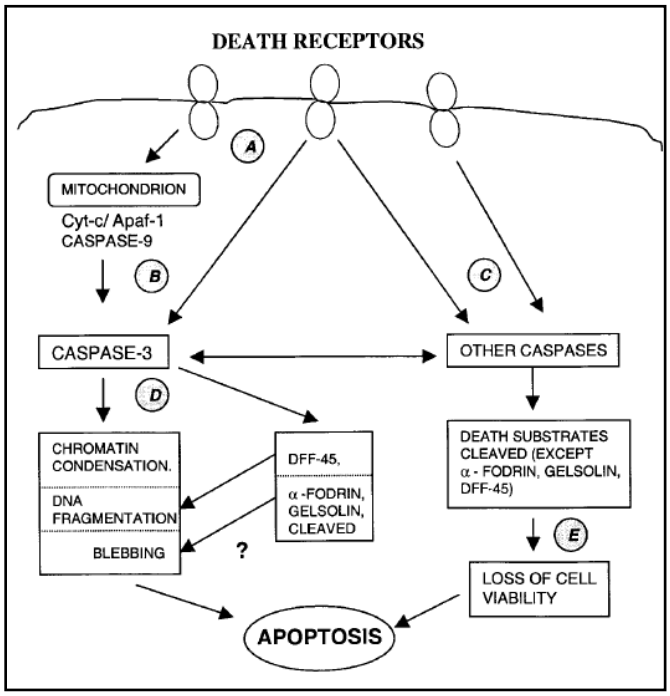

Figure 2. The role of caspase 3 in apoptosis (Porter, 1999, Figure 1).

1.5 Mitochondrial Transmembrane Potential

One of the first occurrences characteristic to apoptosis is a reduction in transmembrane potential of cellular mitochondria. This event is catastrophic to cells because, in addition to ceasing mitochondrial protein production and gene transcription, it halts oxidative phosphorylation, thereby preventing further energy-dependent cellular processes from continuing (Zamzami 1996). In a functional mitochondrion, the inner membrane is impermeable to charged molecules and thus accumulates transmembrane potential, a characteristic that is essential for the establishment of a proton gradient and the electron transport chain. Research suggests that, when signaled to initiate apoptosis, permeability transition pores emerge in the mitochondria, allowing for a dissipation of transmembrane potential (Zamzami 1996). In addition to the reduction of this charge gradient, increased permeability of the mitochondrial membrane allows the release of cytochrome c into the cytoplasm, thereby initiating caspase activation (Waterhouse 2001). This

series of events surrounding the change in transmembrane potential of mitochondria at the start of apoptosis leads to the striking characteristics that define apoptotic cells (Porter 1999).

1.6 T47D Cell Line

The cell model system on which experiments were performed is the T47D cell line from the American Type Culture Collection (ATCC.org). These cells are from the ductal carcinoma of the mammary gland from a pleural effusion of a 54 year-old adult female *Homo sapiens* (ATCC.org). This cell line was selected for use in this project because of its origination in female breast tissue, which is of specific interest in the study of anti-cancer effects of phytoestrogens on post-menopausal women. T47D cells are reported to be positive for the estrogen receptor α (Strom 2003), a necessity for the comparison of the effects of estrogen and phytoestrogens. Furthermore, studies have verified that the cell line is highly responsive to minimal quantities of estrogen (concentrations greater than 1.0 pM) (Faber 2001), which is an important factor in facilitating experimental observations by providing measurable output. The use of this cell line for this project to study the effects of phytoestrogens on breast tissue is advantageous because it is reported to be estrogen responsive (Strom 2003), and thus provides a hopeful substitute for the widely used MCF-7 cell line. Traditionally, MCF7 cells were used for estrogen studies; however, recent research indicates that the certain strains of the cell line are becoming non-responsive to estrogen, such as the MCF-7S strain analyzed by Hamelers (2003).

2. Methodology

2.1 Cell Culture

T47D cells were cultured for use in these experiments using sterile cell-culture techniques. Cells were grown in vented cell culture flasks stored horizontally to enable cell adhesion, and incubated at 37°C and 5.0% $C0_2$, and humidified. Media in which cells were grown consisted of RPMI containing L-glutamine and sodium bicarbonate, with 10% Fetal Bovine Serum (FBS), 1% PenStrep, and 0.2 units/mL insulin. The ATCC reports T47D cells to require insulin for proper growth (ATCC.org), and PenStrep was used with the purpose of preventing bacterial contamination of cultures. All cell counts were made using a hemocytometer. During certain procedures discussed below, in which cells were grown in the presence of hormones, they were provided with specific media components in order to optimize results. When given estradiol, resveratrol, or biochanin A, cells were grown in phenol red (PHRED) – free DMEM, a Phenol red has been shown to bind to the estrogen receptor of MCF-7 cells due to structural similarities in the molecules, and has even led to "estrogen" induced proliferation of these cells (Berthois 1986). Additionally, FBS was reduced from 10% to 1% in order to minimize exogenous hormone effects.

2.2 MTT Cell Proliferation Assay

The MTT Cell Proliferation Assay was used according to the manufacturer's (Promega) instructions for determination of estrogen responsiveness. This assay measures proliferation of cells via colorimetric quantitation of metabolic activity. The chief component of the assay is a tetrazolium dye, which is converted to formazan by metabolically active cells. The formazan product is easily detected by absorbance in a plate reader (Promega Technical Bulletin 2009). Cells were grown in a 24-well plate at varying densities, and were provided with 0.1μM of of 17-β estradiol (E2), as shown below in Table 1. The estradiol concentration was derived from similar experimental studies, including the methods employed by the previous group working on this project (DeVault 2009).

200K cells 0.1 μM E2	200K cells 0.1 μM E2	200K cells 0.1 μM E2	200K cells No E2	200K cells No E2	Culture media
150K cells 0.1 μM E2	150K cells 0.1 μM E2	150K cells 0.1 μM E2	150K cells No E2	150K cells No E2	Culture media
100K cells 0.1 μM E2	100K cells 0.1 μM E2	100K cells 0.1 μM E2	100K cells No E2	100K cells No E2	Culture media
50K cells 0.1 μM E2	50K cells 0.1 μM E2	50K cells 0.1 μM E2	50 K cells No E2	50 K cells No E2	Culture media

Table 1. Layout and cell density of a 24-well plate used for the MTT proliferation assay .

This was a time course experiment that lasted a total of 100 hours. Cells were plated at time 0 hours in the original growth media of RPMI, 10% FBS, 1% PenStrep, and insulin; cells were suspended in 1000μL of combined cell stock solution and growing media. At 48 hours, the media was replaced with PHRED-free, DMEM media containing 1% FBS, 0.2 units/mL insulin, and 1% PenStrep. At 72 hours the following reagents were added to the designated wells in the following concentrations: 0.1 μM of estradiol, 0.65% ethanol to dissolve estradiol, 0.2 units/mL insulin, and 1% FBS by volume. At time 96 hours, the MTT assay was run according to the Promega protocol. At time 100 hours, plate absorbance was read at 450nm in a BioTek EL800 plate reader.

2.3 Caspase Immunoblot

In order to detect the presence of caspase 3 as a measure of apoptosis, immunoblotting was performed. In this technique, proteins from a cell lysate were first separated by size using the Laemmli method for one-dimensional discontinuous denaturing gel electrophoresis, and subsequently transferred to a membrane for identification. Mini-gels, with final dimensions of 8 x 10 x 0.1 cm, were created with 12% acrylamide.

Table 2 below shows the plate layout for cell preparation prior to electrophoresis. Cells were plated at a density of 300,000 cells per well in a 24 well plate in RPMI with 10% fetal bovine serum, 1% PenStrep, and insulin for 48 hours. Media were removed by aspiration and PHRED-free DMEM media containing 1% FBS, 1% PenStrep, and insulin was added biochanin A, resveratrol, or estradiol. Biochanin A and resveratrol were added at either a high concentration (10uM), or a low concentration (0.1uM). Estradiol was added at 0.1uM, based on the data shown in figure 5 of the results section, which suggests T47D cells are proliferative at this concentration. Cells were grown for 24 hours in this new media, and after removing the media, were then flash frozen in liquid nitrogen to induce cell lysis, and stored at -80°C.

300K Cells 10uM Biochanin A	300K Cells 0.1uM Biochanin A	300K Cells 10uM Resveratrol	300K Cells 0.1uM Resveratrol	300K Cells 0.1uM Estradiol	300K Cells No hormone
300K Cells 10uM Biochanin A	300K Cells 0.1uM Biochanin A	300K Cells 10uM Resveratrol	300K Cells 0.1uM Resveratrol	300K Cells 0.1uM Estradiol	300K Cells No hormone
300K Cells 10uM Biochanin A	300K Cells 0.1uM Biochanin A	300K Cells 10uM Resveratrol	300K Cells 0.1uM Resveratrol	300K Cells 0.1uM Estradiol	300K Cells No hormone
300K Cells 10uM Biochanin A	300K Cells 0.1uM Biochanin A	300K Cells 10uM Resveratrol	300K Cells 0.1uM Resveratrol	300K Cells 0.1uM Estradiol	300K Cells No hormone

Table 2. Layout of the 24-well plate used for immunoblot analysis for detection of caspase 3.

Protein samples were resuspended in a minimal volume of 1X SDS-PAGE non-reducing sample buffer, taken from a pool of two duplicate wells, and 30μLof each sample was loaded onto the gel. SDS, or sodium dodecyl sulfate, binds to and denatures proteins while providing them with a negative charge proportional to their mass (Schagger 1987). A constant voltage of 150 V was applied to the gel in order to separate the proteins present in the samples.

After the gel was run, proteins were transferred to a hydrophobic, polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer unit for 90 minutes at a constant current density of 60 mA, for an 8 x 10 x 0.1cm membrane. Before transfer, the hydrophobic membrane was wetted briefly in methanol, and then it, as well filter paper, was equilibrated in transfer buffer. The gel was sandwiched between two sheets of whatman 3mm filter paper and the membrane, and placed on top of the cathode of the transfer unit. The gel and membrane were placed in the proper orientation to ensure that the negatively charged proteins would travel from the gel up to the membrane toward the positive anode (Gallagher, 2001).

After being transferred, the membrane underwent a series of room temperature incubations with gentle shaking to detect the presence of caspase 3 in the cell lysates. The membrane was first incubated for 60 minutes with blocking buffer consisting of 0.1% tween-80 and 1% non-fat dry milk (NFDM), in order to prevent non-specific binding of antibodies in subsequent steps. Next, the membrane was incubated overnight with a mouse monoclonal primary antibody for caspase 3, catalog number sc-56052 from Santa Cruz Biotechnology Inc. This antibody recognizes full-length procaspase 3, and was diluted 1:200 in 1X PBS with 0.1% Tween-80 (PBS-T). After this incubation step, the membrane was washed with PBS-T three times for five minutes, and then incubated with secondary antibody for 90 minutes. The

secondary antibody was a goat-anti-mouse antibody conjugated to alkaline phosphatase, catalog number sc-2008 from Santa Cruz Biotechnology, Inc., and diluted 1:2000 in PBS-T. After one hour, the membrane was washed three times with PBS and incubated for 30 minutes with the alkaline phosphatase substrate, SIGMA *FAST*™ BCIP/NBT from Sigma-Aldrich, which was dissolved in 10μLof distilled water as directed by the manufacturer (Gallagher, 2001).

The caspase signal was normalized using two methods. The first included performing a second blotting of the same membrane for the housekeeper protein, β-Tubulin, following the general outline described above. First, the membrane was washed with TBS-T to remove any excess precipitate. After incubation with blocking buffer, the membrane was placed overnight in a solution of rabbit monoclonal anti-β-Tubulin antibody from Imgenex Corp. catalog number IMG-5810A, diluted 1:500 in PBS-T. The secondary antibody used was a horse radish peroxidase (HRP) conjugated goat-anti-rabbit antibody from Pierce, catalog number 31480, diluted 1:1000 in PBS-T. TMB peroxidase substrate was used to react with the HRP. Due to operator error, after incubation with secondary antibody, the membrane was initially incubated with NBT/BCIP, the incorrect substrate, for 20 minutes, at which point the error was realized, the membrane was washed three times with 1X PBS, and the correct substrate was added. After immunoblotting, the membrane was photographed and analyzed by densitometry using ImageJ, a Java-based image processing and analysis program downloaded from the National Institute of Health (NIH) website.

The second method for normalization included performing a Bradford protein assay using Coomassie Plus Dye from Pierce on pooled duplicate wells from the original plate. However, these pools were taken from different wells than were run through the gel, as the SDS in those samples would have interfered with protein absorbencies. Therefore, these results may not have provided conclusive data on their own, but were used to support the housekeeping blot data. The Bradford protein assay works by an interaction between the anionic Coomassie Blue dye and arginine and lysine residues of proteins (Kruger 2002). The dye has an absorbance at 595nm, allowing for measurement based on optical density of protein samples.

The caspase signals were normalized to protein absorbencies and β-tubulin signals by the following equations:

$$Normalized_{Tubulin} = \left(\frac{Area\ caspase}{Area\ \beta\ Tubulin}\right) \times Area\ \beta\ Tubulin_{min}$$

Equation 1. β-tubulin normalization.

$$Normalized_{Bradford} = \left(\frac{Area\ caspase}{Absorbance}\right) \times Absorbance_{min}$$

Equation 2. Bradford Normalization.

In order to conserve sample, each was diluted 1:5 in dH_2O by mixing 10µLprotein with 40µLwater. This was then added to 1.5mL of the Coomassie Blue dye and incubated for five minutes at room temperature at which point the absorbance at 595nm was taken.

2.4 MitoCapture Apoptosis Detection Kit

As a further measure of apoptosis in T47D cells, the MitoCapture Apoptosis Detection kit was used according to manufacturer's instructions (CalBiochem 2006). This assay works by performing fluorescence microscopy on cells after being incubated with the MitoCapture reagent. In healthy cells, the reagent is able to aggregate in the mitochondria and fluoresces red, while in apoptotic cells, characterized by decreased mitochondrial transmembrane potential, the reagent remains monomeric in the cytoplasm of cells and fluoresces green.

Cells were plated at a density of 500,000 cells per well in a twelve-well culture plate in the standard culture media used in previous experiments, and grown at 37°C for 24 hours. Next, the media was aspirated, and cells were provided with phenol red free DMEM, 1% FBS, 1% PenStrep, 0.2 units/mL insulin, and varying hormones to match the experimental plan used for the immunoblotting procedure, as described in table 3 below. However, deviations from the immunoblot setup include using biochanin A in a different solvent. After originally storing this hormone dissolved in acetone, the solution had evaporated, making it necessary to use biochanin A from another group, which was solubized in DMSO.

500K Cells 10uM Resveratrol	500K Cells 0.1uM Resveratrol	500K Cells 10uM Biochanin A	500K Cells 0.1uM Biochanin A
500K Cells 10uM Resveratrol	500K Cells 0.1uM Resveratrol	500K Cells 10uM Biochanin A	500K Cells 0.1uM Biochanin A
500K Cells 0.1uM Estradiol	500K Cells 0.1uM Estradiol	500K Cells No Hormone	500K Cells No Hormone

Table 3. 12-well plate layout for growth of T47D cells for MitoCapture assay.

Cells were grown in hormones for a 24-hour period, and then analyzed for change in transmembrane mitochondrial potential using the dye provided in the MitoCapture assay kit. Cells were first visualized at 400X magnification under a fluorescent microscope using a fluorescin and rhodamine filter to view green and red fluorescences, respectively. Sections of the well plate bottom on which cells had been grown were then physically removed from the plate using a dremel, and one field of view of each sample was counted for normal and apoptotic cells. Photography was accomplished by observing cells under the 40X objective and using a Canon camera at 4X zoom giving a total magnification of 160X for photographs. A random field of view for each sample was counted for total and non-apoptotic cells based on fluorescence.

3. Results

3.1 MTT Cell Proliferation Assay

T47D cells were successfully cultured for use in the MTT proliferation assay and tested for estrogen responsiveness. Standard morphology of the cells can be observed in figure 3 below. Cells are in culture media composed of RPMI, 10% FBS, 1% PenStrep, and insulin. Cells were viewed on an inverted microscope at 200X magnification and photographed using a digital camera at 4X magnification.

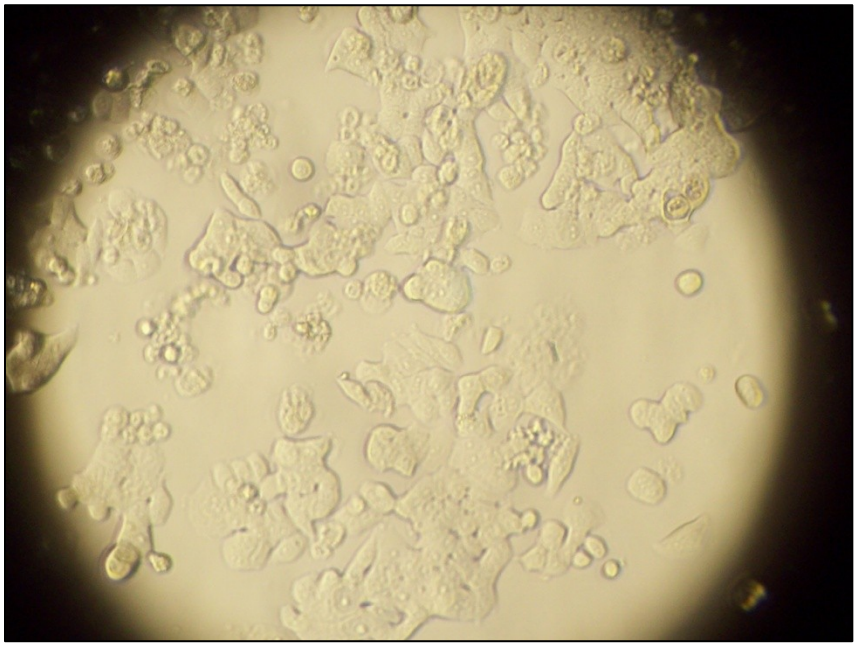

Figure 3. T47D cells at a density of 200,000 cells per well in a standard 24-well plate.

Table 4 below shows the absorbance of the MTT reagent provided to cells at a wavelength of 450nm. Cells were plated ranging from 50,000 to 200,000 cells per well in a 24-well plate. The right-most column was kept free of cells to determine the baseline absorbance of assay media which served as a negative control. Cells were either grown in 1uM estradiol or no additional hormone, and all wells were given the MTT assay reagent to quantitatively determine metabolic activity in each well via optical density of the metabolized product.

Cells/well	1uM estradiol			No hormone		Media
200K	0.365	0.351	0.376	0.398	0.411	0.208
150K	0.331	0.327	0.350	0.338	0.355	0.213
100K	0.270	0.290	0.295	0.302	0.320	0.210
50K	0.210	0.241	0.195	0.259	0.259	0.219

Table 4. Optical density at 450nm.

Figure 4 shows a plot of absorbance at 450nm versus cell density based on the values seen in table 4. The green line labeled "No Cells" represents the baseline absorbance of the cell culture media. The blue line labeled "E2" represents cells grown in 17-β estradiol, and the red line labeled "No E2" represents cells grown without the added hormone. Linear fits of these data were performed, and the best-fit equation can be seen underneath the respective labels in the legend.

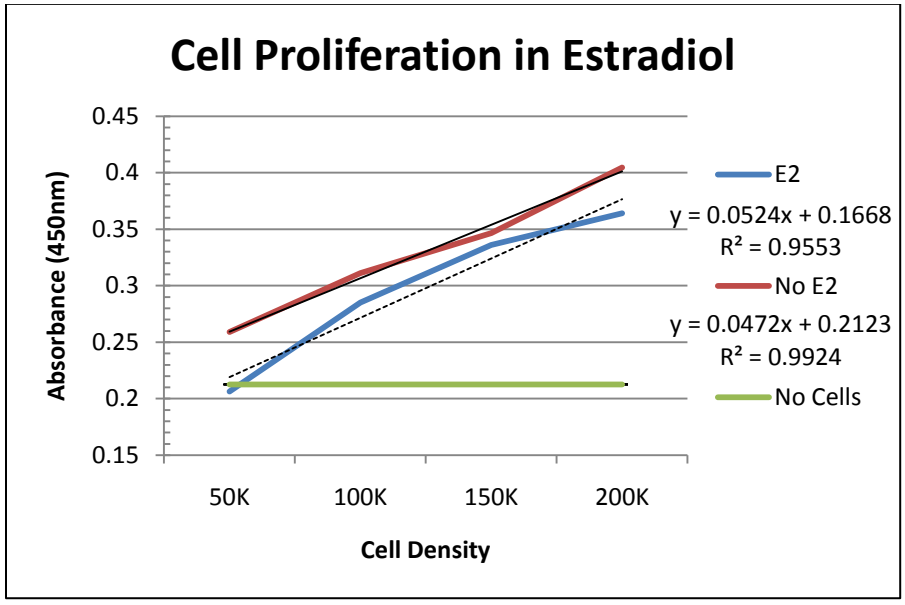

Figure 4. Plot of absorbance vs. cell density of T47D cells grown with and without estradiol.

Figure 5 below shows cell proliferation in the presence of estradiol, taken from another group working on the same project (Gergel et. O'Connell 2010). Cells were grown to 80% confluence in growing media consisting of RPMI with 10% FBS, 1% PenStrep and 0.2 units/mL insulin. Media was then removed by aspiration and replaced with PHRED-free DMEM media containing 1% dextran coated charcoal (DCC) –stripped serum, 1% PenStrep, and 0.2 units/mL insulin. Cells were grown for 24 hours and provided with the reagent from the MTS proliferation kit and measured for absorbance. In the figure below provided by Gergel and O'Connell,

proliferation is plotted as a percent difference from control cells grown in the absence of estradiol.

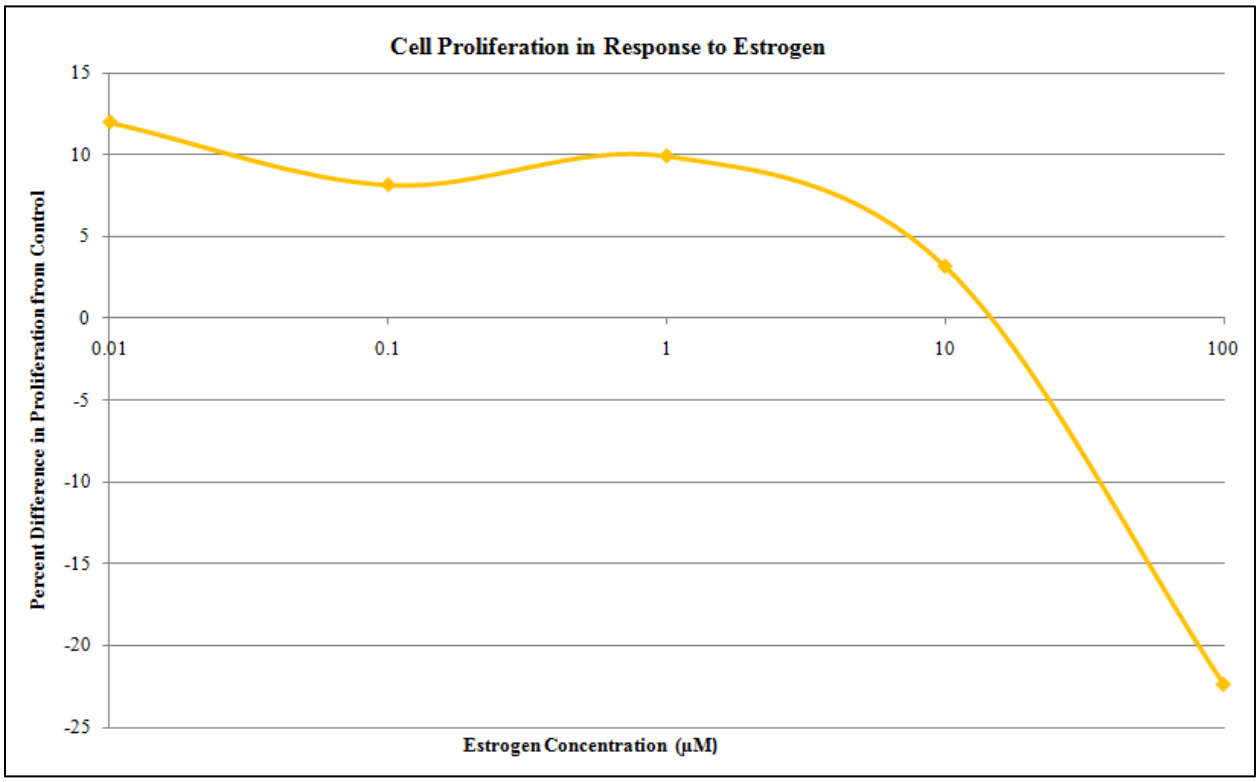

Figure 5. Estrogen responsiveness curve (Gergel and O'Connell, 2010).

3.2 Caspase Immunoblot

Figure 6 below shows the immunoblot for caspase 3 and β-tubulin for T47D cells grown in different hormone conditions. Cells were grown in a high and low concentration (10uM and 0.1uM, respectively) of either resveratrol or biochanin A, in 0.1uM 17-β estradiol, or without any additional hormones. The large bands seen at the bottom of the blot indicate the presence of caspase 3 in the cell lysate of the respective sample, while the small double bands at the top of the blot represent β-tubulin.

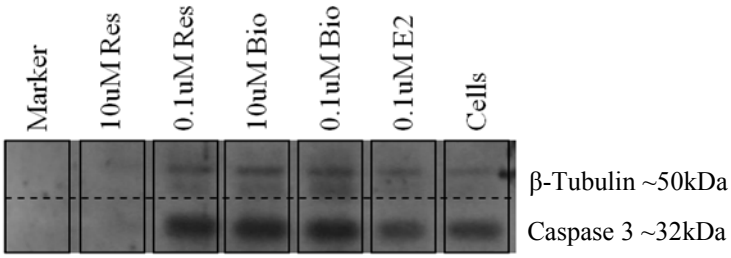

Figure 6. Immunoblot with caspase 3 and β-tubulin bands.

Densitometry analysis of the above bands was completed using ImageJ, which calculated the area of each peak representing the intensity of the signal. Furthermore, the Bradford protein assay was used to determine absorbance of duplicates of the samples used in the blot seen above. Values for densitometry and protein absorbance can be seen below in table 5. Densitometry for the caspase bands was normalized by comparison to the β-tubulin and absorbance values. This was conducted using the equations below. Normalized values following the below equations can be seen in table 5.

Lane	Marker	10μM Resveratrol	0.1μM Resveratrol	10μM Biochanin A	0.1μM Biochanin A	Estradiol	No Hormone
Area Caspase	-	408	7474	6738	8789	2996	3657
Area β-Tubulin	-	686	2499	2212	4320	1118	1995
Normalized to Tubulin	-	**408**	**2052**	**2090**	**1396**	**1838**	**1257**
Bradford Absorbance	-	0.202	0.307	0.328	0.304	0.279	0.250
Normalized to Bradford	-	**408**	**4918**	**4150**	**5840**	**2169**	**2955**

Table 5. Normalization results.

These values were then plotted for analysis of changes in caspase 3 quantities between samples. The histogram can be seen below in figure 7, which shows normalized values versus cell conditions. The blue columns represent the normalized values with respect to the Bradford absorbance, while the red columns indicate values normalized to the area of β-tubulin bands.

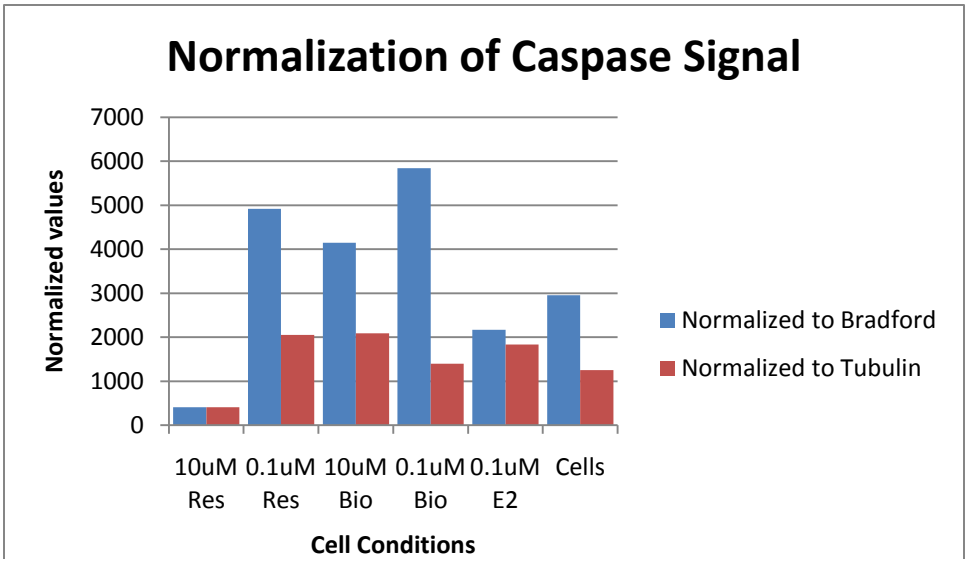

Figure 7. Normalization of caspase signal from immunoblot.

3.3 MitoCapture Apoptosis Detection Kit

Figure 8 below shows cell morphologies at densities of 500,000 cells per well 24 hours after being exposed to varying hormone conditions and prior to being subjected to the MitoCapture dye. Cells were viewed at 200X magnification and photographed 100X using a Canon camera at 4X magnification in AV mode. Picture descriptions are as follows: A. 10uM resveratrol; B. 0.1uM resveratrol; C. 10uM biochanin A; D. 0.1uM biochanin A; E. 0.1uM estradiol; F. No hormone.

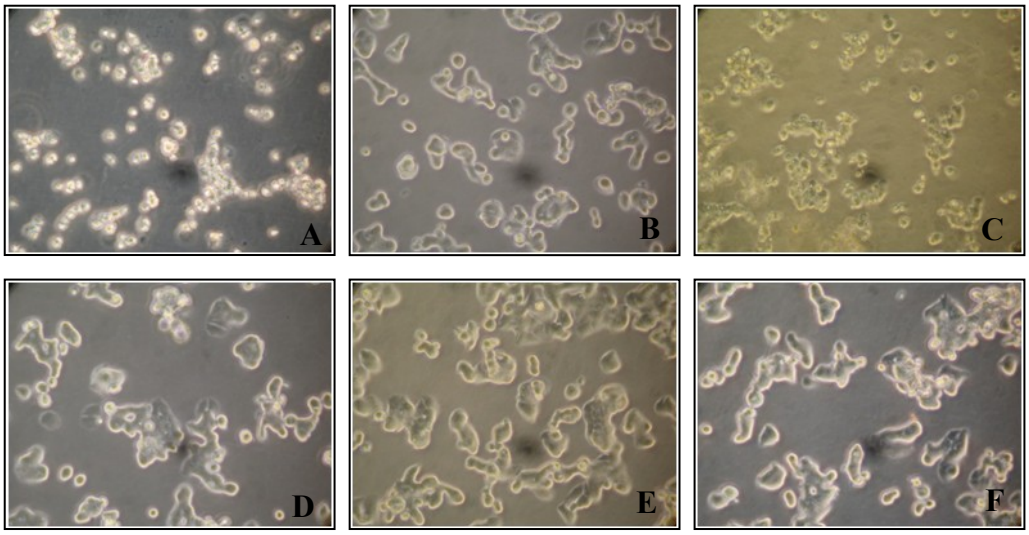

Figure 8. Cell morphologies 24 hours post-hormone exposure.

Figure 9 below shows an enlarged version of photograph F seen in figure 8 above, containing cells with no additional hormone. Cells appeared healthy in morphology, large, and were clearly adhered to the plate bottom.

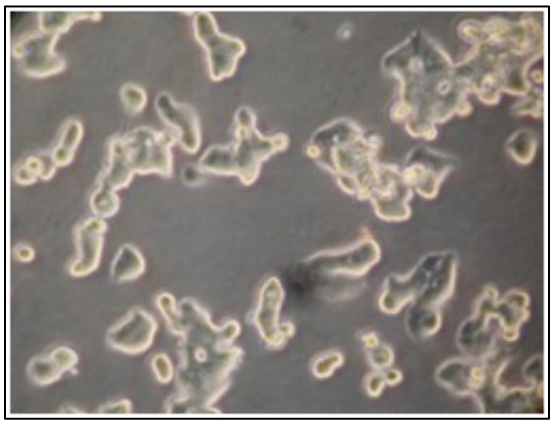

Figure 9. Cells with no added hormone.

As a comparison with figure 9 above, figure 10 below shows an enlarged version of photograph A taken from figure 8. In this picture, cells have been grown in 10uM resveratrol. Cells appeared small, rounded, granular, and very few were still plated down, while many were floating in solution.

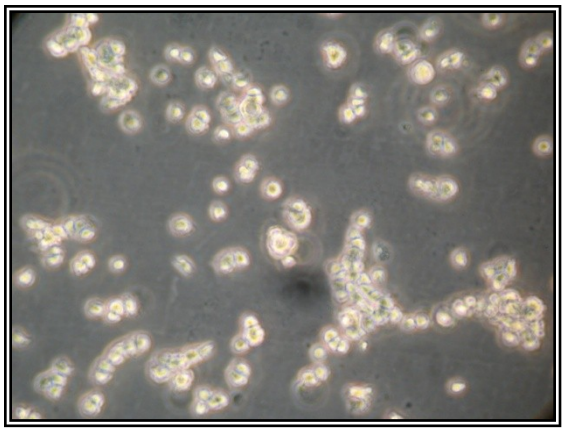

Figure 10. Cells with 10µM resveratrol.

Figure 8 shows that cells grown in 0.1µM resveratrol, 0.1µM biochanin A, and 0.1µM estradiol appear to be healthy in morphology and adherence. Cells grown in 10µM biochanin A appear to be small and granular and generally less healthy than control cells.

Figure 11 below shows photographs taken of cells using fluorescent microscopy. Cells were viewed at 400X magnification and photographed at a total of 160X magnification with a Canon camera at 4X magnification in AV mode. The column on the left, showing panels A, B, and C represents cells grown in 10uM resveratrol. The column on the right, showing panels D, E, and F, represents cells grown in 0.1uM resveratrol. Panels A and D show the cells viewed under normal light (Note that photo D has a reddish appearance due to accidentally leaving the rhodamine filter on while viewing under normal light). Panels B and E show the cells viewed under just the fluorescein filter. Panels C and F show the cells viewed under just the rhodamine filter. Spots that appear to fluoresce red in both fluorescein and rhodamine panels should be discounted, as they are simply dye particulates resulting from poor solubility in aqueous solutions (Calbiochem 2006). Cells grown in 10µM resveratrol do not appear to fluoresce green or red. Cells grown in 0.1µM resveratrol appear to fluoresce faintly green, but not red.

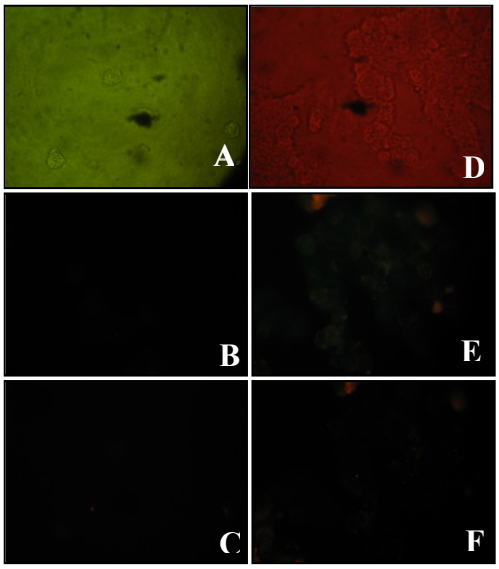

Figure 11. 10uM and 0.1uM resveratrol cell fluorescences.

Figure 12 below shows photographs taken of cells under a fluorescent microscope, viewed and photographed in the same conditions as above. The column on the left, showing panels A, B, and C represents cells grown in 10uM biochanin A. The column on the right, showing panels D, E, and F, represents cells grown in 0.1uM biochanin A. Panels A and D show the cells viewed under normal light, panels B and E show the cells viewed under just the fluorescein filter, and panels C and F show the cells viewed under just the rhodamine filter. Cells grown in 10μM biochanin A do not appear to fluoresce green or red. Cells grown in 0.1μM biochanin A appear to fluoresce green, but not red.

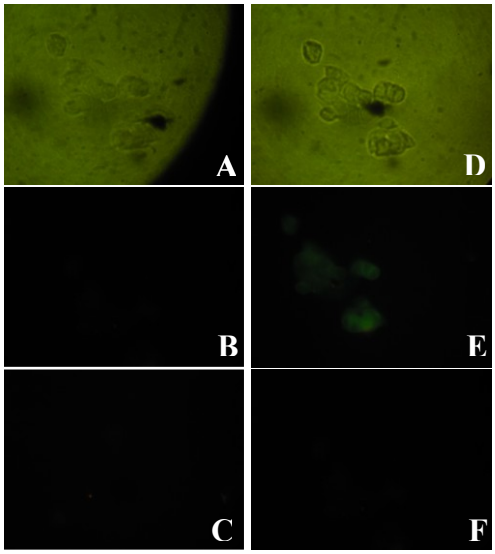

Figure 12. 10uM and 0.1uM biochanin A cell fluorescences.

Figure 13 below shows photographs taken of cells under a fluorescent microscope with the same conditions as used above. The column on the left, showing panels A, B, and C represents cells grown in 0.1uM 17β-estradiol in the absence of phytoestrogens. The column on the right, showing panels D, E, and F represents cells grown with no additional hormone. Panels A and D show the cells viewed under normal light, panels B and E show the cells viewed under just the fluorescein filter, and panels C and F show the cells viewed under just the rhodamine filter. Cells grown in 0.1μM estradiol appear to fluoresce green with just a few cells fluorescing red. Cells grown with no additional hormone fluoresce brightly green as well as red.

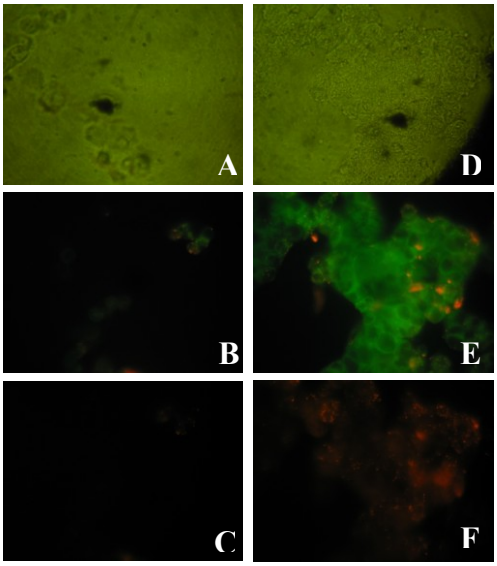

Figure 13. 0.1uM estradiol and no hormone cell fluorescences.

Table 6 shows the cell count of a random field of view at 400X magnification, taken from each of the sample conditions. Total cells were determined by the number of cells fluorescing green under the fluorescein filter. Non-apoptotic cells were those which also fluoresced red under the rhodamine filter. The percentage of total cells that were non-apoptotic was calculated from these counts. Due to difficulties in performing cell counts, only one field of view was counted for each sample; therefore these counts cannot be counted as statistically significant, but instead provide a rough representation of cell condition that was visually verified. Figure 14 shows a histogram representing percent non-apoptotic cells for each sample condition.

Treatment	Total cells	Non-apoptotic	% Non-apoptotic
10uM Resveratrol	18	0	0
0.1uM Resveratrol	68	36	53
10uM Biochanin A	11	1	9
0.1uM Biochanin A	40	16	40
0.1uM Estradiol	17	6	35
No Hormone	5	5	100

Table 6. Number and percentage of non-apoptotic cells.

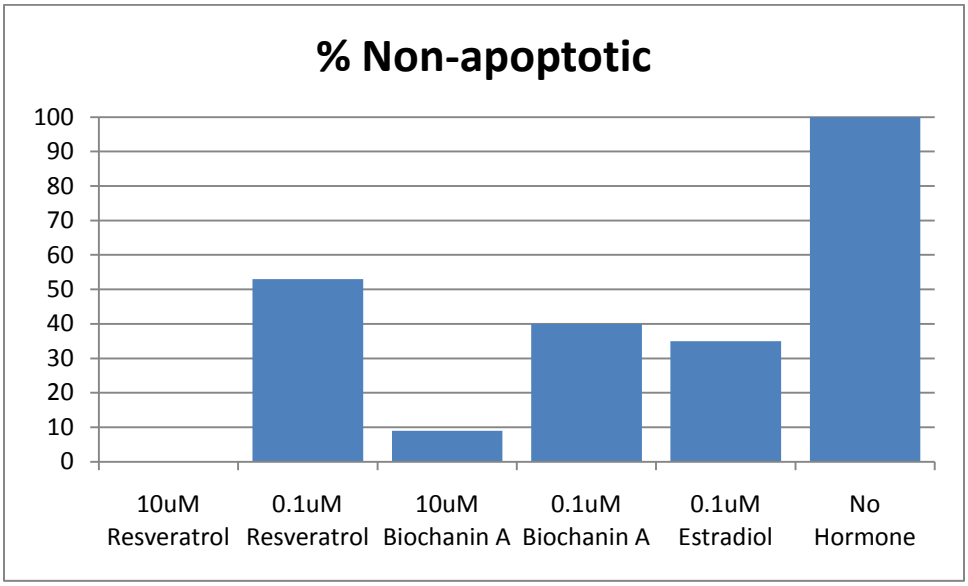

Figure 14. Percentage of non-apoptotic cells based on fluorescence.

4. Discussion

4.1 MTT Cell Proliferation Assay

Figure 4 shows the plot of absorbance versus cell density derived from the MTT proliferation assay results. The control line, containing no cells, shows a baseline absorbance at around 0.2 absorbance units (AU) which stays constant in all control wells. This value is clearly below the absorbencies of samples containing high densities of cells, with or without hormones, which increase linearly with respect to cell density. Cells at a density of 50,000 cells per well grown with estradiol gave the same absorbance as assay media. As can be seen in the figure, however, cells grown for 24 hours in 17-β estradiol did not demonstrate a difference in proliferation when compared to cells cultured without added hormone. Nonetheless, another group working on the same project was able to observe an increase in proliferation of T47D cells in 17-β estradiol, as can be seen in figure 5. This figure suggests proliferative effects of estradiol on cells in concentrations between 0.01uM and 10uM (Gergel et O'Connell 2010). This data provided the necessary conclusion that cells were estrogen responsive, permitting the initiation of tests on the effects of phytoestrogens on apoptosis.

4.2 Caspase Immunoblot

Figure 6 shows an immunoblot containing bands for the housekeeping protein β-tubulin and caspase 3. In addition to serving as a size marker, lane 1, containing the His-tagged marker, acts as a negative control for the blot, as it is not expected to contain either β-tubulin or caspase 3. Any bands appearing in this lane would then be the result of non-specific binding by either the primary or secondary antibodies. This absence of such binding is confirmed by the lack of bands in lane 1.

Lane 2, representing cells grown in 10uM resveratrol, was expected to contain both caspase 3 and β-tubulin. However, only very minimal quantities of protein were detected, as can be observed by the faintness of the bands. The samples used for this blot were used on additional gels and blots, and all results (data not shown), support that very little, if any, protein was present in this particular sample.

These results, in conjunction with the observations of cell morphology immediately prior to the MitoCapture assay, seen in figures 8, 9, and 10, suggest that 10uM resveratrol is either highly toxic to cells, and causes them to lose their adherent properties, or is highly proliferative to cells. It is well known that adhesive cells commonly lose the ability to attach to the surface on which they are grown, during proliferation (Hunter, 1995). As can be seen by figure 10, cells grown in 10uM resveratrol were small and granular in appearance, and seemed to be unsuccessful in adhering to the plate. During the procedures required to run a gel electrophoresis for subsequent Western blotting, it is necessary at many steps to aspirate the media off of the cells, a step which assumes that cells will be properly adhered to the plate. If cell adhesion is disrupted, however, cells will be aspirated off the plate. This is supported by the absence of a band in the respective lanes of the gel and membrane, as well as the low protein absorbance seen by the Bradford assay.

Besides the lack of bands in the 10uM resveratrol lane, all other lanes contain pronounced bands at ~ 32kDa, consistent with the presence of caspase 3. Comparison of the normalized intensities of these bands to the β-tubulin bands can be seen in table 5, and is plotted in figure 7, as can be seen by the red bars. This figure suggests that 0.1uM resveratrol and 10uM biochanin A may induce production of slightly more caspase 3 than 0.1uM estradiol, which induces more caspase 3 than control cells. However, 0.1uM biochanin A causes production of less caspase 3 than estradiol, but slightly more than control cells. Assuming that 10uM resveratrol is highly apoptotic to cells, these data suggest that resveratrol at low and high concentrations, as well as a high concentration of biochanin A, may induce a slight increase in apoptosis in T47D cells relative to control cells. However, because the values are not largely different from one another, one might also conclude that there is no significant increase in apoptosis. Due to time limitations, only one successful membrane was obtained, and statistical analysis was therefore omitted for these data.

It is notable to point out that the bands seen in figure 6 show large lower bands which appeared after probing with anti-caspase antibodies, and small upper double bands appearing only after probing with anti-β-tubulin antibodies. By these observations, it is evident that the upper bands correspond to β-tubulin; however, the reason behind the presence of a double band as opposed to a single band is still unknown. It is possible that the anti-β-tubulin antibody may be picking up signal from the slightly smaller α-tubulin protein; it may also be possible that some

of the β-tubulin in the samples is physically altered, which might account for the presence of double bands.

In an attempt to validate these results, the Bradford assay was used as another method of normalization. Protein absorbances were taken from duplicate samples to those used for the electrophoresis and immunoblot, values of which can be seen in table 5. The intensities of the caspase bands were normalized to these values, producing the blue bars seen in figure 7. Unlike normalization by β-tubulin, these produced much more striking results when compared to control cells. These data suggest that 0.1uM biochanin A causes the most apoptosis of T47D cells, closely followed by 0.1uM resveratrol and 10uM biochanin A. These values are clearly much higher than those represented by control cells, which are slightly higher than cells grown in estradiol. This observation is supported by the data in figure 5 suggesting 0.1uM estradiol is proliferative to cells. No statistical analysis of these data can be performed because the data are from a single experiment. However, the results support repeating this study in the future.

4.3 MitoCapture Apoptosis Detection Kit

Figures 11, 12, and 13 show photographs of cells viewed using fluorescence microscopy. The top picture in each column shows cells viewed under normal light, the middle shows cells viewed using a fluorescein filter, and the bottom shows cells viewed using a rhodamine filter. Healthy cells are represented by the control cells seen in figure 13, panels D, E, and F. The three panels clearly show the same cells, as is indicated by the identical arrangement of cells. The middle panel shows cells which fluoresce green, and the bottom panel shows the same cells clearly fluorescing red, except in more condensed locations. The green fluorescence seen in the middle panel indicates a cell with an intact outer membrane, through which the MitoCapture dye was able to penetrate to accumulate in the cytoplasm. However, because of the large size of the cytoplasm, the dye is too dilute to dimerize and fluoresce red, and thus stays green. Likewise, the red fluorescence in the bottom panel indicates that the mitochondrial membrane is intact as well, since the dye was able to enter and aggregate, causing dense spots of red fluorescence throughout the cell. This is in contrast to cells grown in 0.1uM estradiol, seen in panels A, B, and C of the same figure, which show cells fluorescing faintly green, only a few of which appear to fluoresce red in the bottom panel. These observations suggest that few cells contain intact mitochondria, and thus that they may be apoptotic. Furthermore, because the intensity of the green fluorescence

is not comparable to that which is seen in the control cells, it might be suggested that the outer cell membrane is compromised in these cells, preventing the dye from properly accumulating even in the cytoplasm.

Using these observations, figure 12 suggests that 10uM biochanin A causes cells to become apoptotic, with possible damage to the outer membrane, resulting in no green or red fluorescence; and that 0.1uM biochanin A may induce apoptosis but is less damaging to cells than 10uM biochanin A, since no red fluorescence is observed but they are able to fluoresce green. Likewise, figure 11 suggests the same trends for 10uM resveratrol and 0.1uM resveratrol, as no green or red fluorescence is seen in 10uM resveratrol cells, but some green and no red is seen in 0.1uM resveratrol cells.

After photographic observation, random fields of view were counted for green and red fluorescence. These values can be seen in table 6. Cells that fluoresce green were marked in the category "total cells", while cells that fluoresced red from the same field of view were marked in the category "non-apoptotic cells". These percentages were plotted in the histogram seen in figure 14. These results suggest that 10uM resveratrol yields the smallest percentage of non-apoptotic cells, reporting no healthy cells out of the 18 total that were counted. The next highest inducer of apoptosis was 10uM biochanin A, which only yielded 9% of the total cells being healthy. Estradiol and 0.1uM biochanin A yielded the next two smallest percentages of healthy cells, respectively, while 0.1uM resveratrol indicated approximately 50% of cells counted being healthy. 100% of control cells counted were reported to be healthy. The result for estradiol is somewhat unexpected, although it is possible that it is a result of the vehicle, ethanol, having an adverse effect on the cells. Repetition of this assay with a vehicle only control should be performed to confirm this.

4.4 Conclusions

Taken in conjunction, these data suggest slightly conflicting, but in general consistent, results. Immunoblotting results suggest that 10uM resveratrol may be highly toxic, and possibly apoptotic, to cells, or highly proliferative to cells. Results also suggest that low concentrations (0.1uM) of resveratrol and biochanin A are more apoptotic than high concentrations of biochanin A. Furthermore, immunoblot results suggests that cells grown in estradiol experience less

apoptosis than control cells, supporting the data suggesting that estradiol at a concentration of 0.1uM may be proliferative to cells.

MitoCapture results also suggest that 10uM resveratrol is highly toxic and possibly apoptotic to cells. However, these results suggest that 10uM biochanin A is highly apoptotic to cells, whereas 0.1uM biochanin A and estradiol are less apoptotic to cells, and 0.1uM resveratrol yields about 50% apoptosis. Thus, there are discrepancies in the apparent apoptotic effect of estradiol and 10uM biochanin A. Immunoblot results suggest that estradiol is non-apoptotic to cells with comparison to control, while MitoCapture results suggest that it does cause some apoptosis, slightly more than low concentrations of resveratrol and biochanin A. Immunoblot results suggest that 0.1uM biochanin A is more apoptotic to cells, while MitoCapture results suggest that 10uM biochanin A is more apoptotic. In general, these results suggest apoptosis of T47D cells is induced by addition of resveratrol and biochanin A compared to control cells; however, the relative amounts of apoptosis between different concentrations of hormones are debatable.

Future experiments include conducting the above tests in many more replicates and variations in order to give statistical significance to the results. Furthermore, it would be beneficial to test medial concentrations of resveratrol between 0.1 and 10μM to see at which concentration cells begin to lose their adherent properties. To determine whether these cells are apoptotic or proliferative they could be stained with DAPI to identify the presence of mitotic nuclei. Additionally, future use of the MitoCapture assay may call for a more effective method of observing cells under a fluorescent microscope, perhaps by using an inverted scope, as well as a better method of counting cells than by physically removing sections of the plate using a dremel. This might be accomplished by growing cells directly on a microscope slide or coverslip. A suggestion for a more detailed method of observing apoptosis was visualization of various cell structures using electron microscopy. It has also been suggested that non-immortalized breast cells be cultured as a negative control for these assays to determine the effect of phytoestrogens on non-cancerous cells.

It is possible that the current use of phytoestrogens by menopausal women may provide more beneficial effects than have been recognized. However, due to the inconsistent amounts of specific phytoestogens in over-the-counter supplements, it is difficult to determine whether women are receiving the necessary concentrations of those phytoestrogens which may have the

most potent effects. Overall, these results provide promise for the future study of the phytoestrogens resveratrol and biochanin A as a safe form of treatment for menopausal women, which, in addition to providing symptomatic relief, may also potentially induce apoptosis of neoplastic or developing cancer cells.

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