The Relationship between Phytoestrogens and Breast Cancer Cells

A Major Qualifying Project Report
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WPI

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Abstract

Phytoestrogens (PEs), estrogen-like plant compounds, can be used in place of traditional hormone replacement therapy (HRT) to relieve postmenopausal symptoms. HRT has been shown to increase breast cancer risk. *In vitro*, one PE product, Promensil, has been shown to decrease the number of breast cancer cells in culture. From the literature, we found the serum concentrations of four PEs, genistein, biochanin A, formononetin, and daidzein, contained in Promensil and hypothesize that these PEs are responsible for reduced cell numbers at those concentrations. We investigated two of the phytoestrogens on T-47D cells in culture and discovered that at serum concentrations, biochanin A increases cell numbers similar to estrogen while genistein maintains the same cell numbers as untreated controls.

Introduction

Menopause is a natural process women go through as they age and their estrogen levels drop. It causes physical symptoms in the body such as hot flashes and migraines as well as internal changes. To combat the physical symptoms, women can raise their estrogen levels through estrogen hormone replacement therapy (HRT). However, taking HRT for more than a year, especially with estrogen-progesterone combined therapy, has been shown to increase a woman's breast cancer risk. Decreasing the use of HRT could reduce cancer cases by 1,400 cases per year as of 2018 (*Does Hormone Replacement Therapy (HRT) Increase Cancer Risk?*, 2019). This connection between HRT and breast cancer risk has inspired the development of new treatments for the physical symptoms of menopause.

Treatments that have been developed to relieve the physical symptoms of menopause include phytoestrogens, which are estrogen-like compounds found in plant sources such as red clover, soybeans, fruits, and vegetables (Chen et al., 2015). Promensil, a phytoestrogen supplement, is derived from red clover and used to increase phytoestrogen levels in women during menopause and alleviate their symptoms. Phytoestrogen supplements do not require approval by the FDA, so research on the compounds and other effects they may have on women is scarce. Studies have looked at the overall impact of phytoestrogens on cell cultures and observed decreases in cell proliferation. In these studies, the mechanisms of action are assumed to be through estrogen receptor binding (Bilal et al., 2014). Less studied are the effects of the individual phytoestrogens found in red clover in vitro and in vivo. For this study, the phytoestrogen compounds in Promensil were obtained individually. Testing was done to determine the impact of the individual phytoestrogen components on breast cancer cells.

Chapter 1: Research

Background

Breast Cancer

Cancer is a disease caused by mutations in cells leading to abnormalities that can impact the cell's ability to regulate its growth. Breast cancer is characterized by mutations in the BRCA or HER2 genes (Maynes et al., 2010). These mutations disrupt the normal functioning or expression levels of the proteins coded by these genes, leading to uncontrolled proliferation of the cells. In addition to these mutations, the cancerous cells can also express hormone receptors for estrogen and/or progesterone. This increases their proliferation further because normal interactions with estrogen cause growth (*Breast Cancer Hormone Receptor Status | Estrogen Receptor*, n.d.). The line of breast cancer being studied in this paper is estrogen receptor-positive, but the main focus is on changes in proliferation instead of these receptors.

Estrogen

Estrogen is a category of sex hormone present at higher levels in women than in men. It promotes female development during puberty and plays a role in the menstrual cycle. Estrogen supplements have been used to help suppress the symptoms of menopause such as hot flashes and migraines. The estrogen most vital to a woman's reproductive system is estradiol, which regulates the maturation and release of an egg, as well as the thickening of the uterine lining when an egg is fertilized. As a woman ages, the egg maturation process slows and eventually stops, and this is known as menopause. Menopause is directly related to the production levels of estradiol which decrease with age, and these decreased levels are the main cause of the start of menopause. The impact of decreased estradiol levels is what causes the physical symptoms of menopause as well as decreases in bone density with prolonged low levels of estradiol (*Reproductive Hormones*, n.d.).

To combat the symptoms of menopause, women have been given hormone replacement therapy (HRT) with 17β-estradiol. Although HRT was beneficial to relieve menopause symptoms, changing the levels of estradiol in the body also increased the risk of women developing breast cancer, blood clots, and heart disease (*Reproductive Hormones*, n.d.). This led

to a push to find new treatments for the symptoms of menopause, which led to the discovery and use of phytoestrogens.

Phytoestrogens

Phytoestrogens are a class of non-steroidal compounds with estrogenic effects, produced in hundreds of plant species such as fruits, vegetables, and grains, as well as many medicinal plants from the family *Leguminosae*. These compounds were first discovered in the early 20th century and named as phytoestrogens in the 1980s. During this time, researchers soon discovered that they had not only structural similarity to estradiol but also a binding affinity for human estrogen receptors and numerous health benefits, from improved metabolism to anti-aging effects (Sirotkin & Harrath, 2014). These compounds are generally classified into two main groups, flavonoids and non-flavonoids, depending on their structural similarity to the compound flavone. Each of these groups can be further divided into subgroups, such as the non-flavonoid lignans or the flavonoid coumestans.

The non-steroidal nature of these compounds inspired the creation of Promensil, a supplement intended to reduce symptoms of menopause through agonistic effects (van de Weijer & Barentsen, 2002). The main ingredient in Promensil is red clover, which has been found to contain four different phytoestrogens: genistein, biochanin A, daidzein, and formononetin. These four compounds make up the flavonoid subgroup of phytoestrogen known as isoflavones, which are produced only by the *Papilionoideae* subfamily of *Leguminosae* plants (Dixon, 2004). Genistein and biochanin A are structurally similar, with the only difference being an additional methyl group on one of the benzyl rings. The same is true for the relationship between daidzein and formononetin, the latter being the methylated version of the former. The structures of each of these compounds are shown in Figure 1.

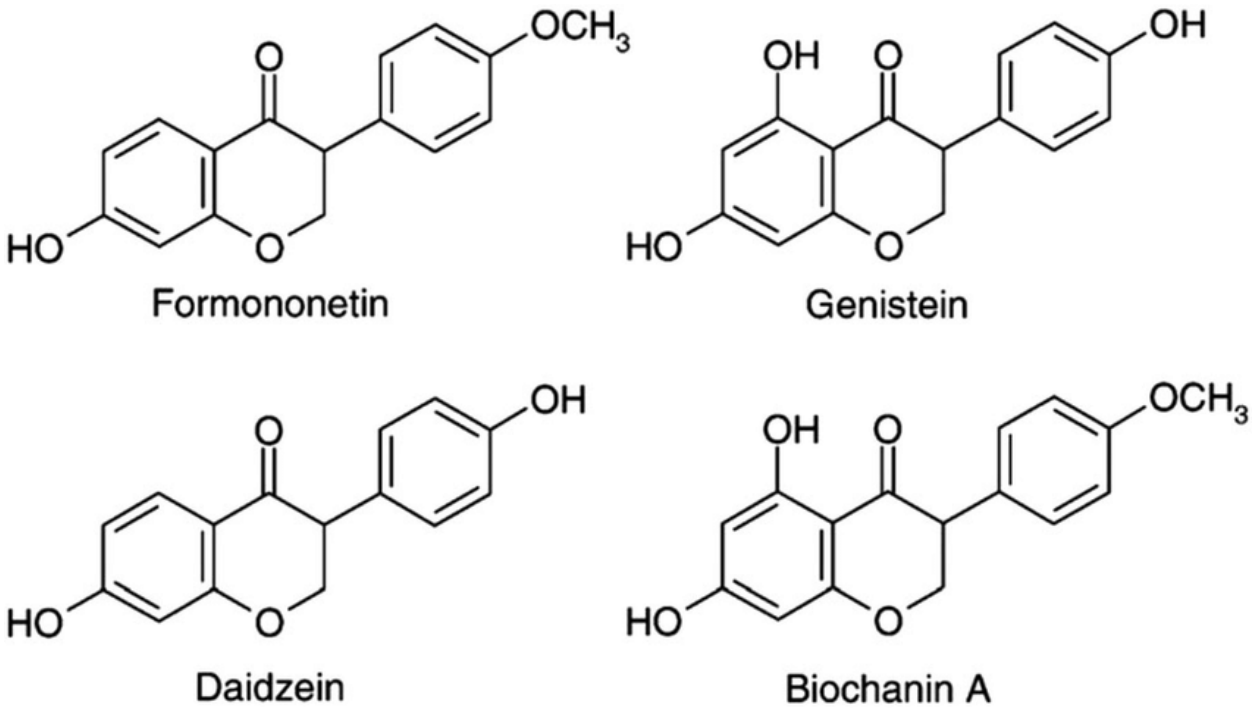

Figure 1. Chemical Structures of Formononetin, Genistein, Daidzein and Biochanin A (Figure 4 in Melchior & Myer, 2018).

Previous MQP research has shown that Promensil has an antiproliferative effect on T-47D breast cancer cells (Huber, 2021), and our study attempts to identify the cause of these effects. Based on prior research, it was determined that a single Promensil supplement produced physiological concentrations of $1.26x10^{-7}$M for genistein, $2.50x10^{-8}$ M for biochanin A, $6.29x10^{-8}$ M for daidzein, and $1.11x10^{-8}$ M for formononetin (Setchell et al., 2001). As these concentrations would be expected in a patient who has taken Promensil, these concentrations were used to construct the range at which phytoestrogen efficacy was tested. For these experiments, genistein, and biochanin A were selected as the two phytoestrogens to be tested.

Estrogen Receptors

Cell proliferation and growth in breast tissue are regulated by the estrogen receptors (ER), α and β. ERα promotes breast cell proliferation when bound by estradiol, whereas ERβ inhibits proliferation by dimerizing with ERα. ERα density is higher than the ERβ density in breast tumors, and lack of the latter is associated with an increase in the aggressiveness of tumors (Bilal et al., 2014).

Estrogen receptors are nuclear transcription factors that form the 3A member of the family of nuclear hormone receptors, and they become activated upon binding of their E/F domain to a ligand, usually the growth-promoting 17 β-estradiol, the major physiological estrogen compound in the majority of species (Heldring et al., 2007; Morito et al., 2001). Although the two ERs are similar in overall structure, they differ in amino acid sequence in the F region of the E/F domain, which may explain the differences observed in phytoestrogen binding affinity (Heldring et al., 2007; Morito et al., 2001). In general, phytoestrogens weakly bind to estrogen receptors with a relative binding affinity (RBA) between 1,000 and 10,000 times less than 17 β-estradiol's RBA to estrogen receptors, except for phytoestrogens such as genistein, whose RBA is 10 to 100 times less compared to that of 17 β-estradiol (Bilal et al., 2014). Phytoestrogens, especially isoflavones such as genistein, exhibit a higher affinity for the inhibitory ERβ than for the ERα, can bind ERs at very low concentrations, but require very high concentrations to stimulate cell proliferation (Morito et al., 2001), and inhibit proliferation at concentrations equal to or greater than 10uM (Bilal et al., 2014).

Hypothesis

Previous MQP studies at WPI have shown that a double-strength Promensil supplement when dissolved as a whole can decrease ER-positive breast cancer cell proliferation (Huber, 2021; Wambach, 2018). Looking at the supplement as a whole can cloud how each phytoestrogen interacts with breast cancer cells. The four phytoestrogens in Promensil, biochanin A, formononetin, genistein, and daidzein have each been shown to decrease proliferation, but the mechanisms by which they promote anti-proliferation effects are unclear. We propose that at physiologic concentrations, the individual phytoestrogens in Promensil decrease cell proliferation through cell cycle arrest and apoptosis.

Methods

Cell Maintenance

The T-47D cell line (ATCC Number: HTB-133) was used in vitro to study the effects of phytoestrogens on breast cancer. Distributed by ATCC (American Type Culture Collection), the T-47D cell line consists of adherent, estrogen receptor-positive epithelial cancer cells that were derived from a human female breast cancer patient 54 years of age (*T-47D | ATCC*, n.d.).

T-47D cells were maintained and grown in a prepared phenol red-containing Dulbecco's Modified Eagle Medium (DMEM) (Corning, ref # 10-013-CV) media with 1% of 1X penicillin /streptomycin (P/S) (Lonza, ref # 17-602E; 10,000 U Pen./mL and 10,000 ug Strep./mL) + 10% fetal bovine serum (FBS) in T75 culture flasks and incubated at 37.5°C with 5% CO_2. The cells were fed every 2 to 3 days by replacing the media with new media until the cells were near total confluence. At this point, they were transferred into a new flask by performing a 1:4 split.

Experimental Setup

Many of the critical steps for the experiments involved the preparation of 24-well plates prior to the addition of phytoestrogen treatments. The preparation process used three different types of media that we will refer to as standard media, serum-free media, and charcoal-stripped media. The standard media was made with phenol red-containing DMEM with 1% P/S + 10% FBS which was used to allow cells to adhere to the plates. The serum-free media was made with phenol red-containing DMEM with 1%P/S and was used to synchronize cells to the same point in the cell cycle. The charcoal-stripped media was made with phenol red-free DMEM (Sigma, ref # D4947) with 1% of 1X P/S + 1% of CD lipid concentrate (gibco, ref # 11905-031) + 10% charcoal-stripped FBS (ThermoFisher, ref # 12676029). This was used to grow cells without activating the estrogen receptors because endogenous steroids are removed in the charcoal-stripped FBS. The DMEM was free of phenol red to ensure that phytoestrogens would not have to compete with phenol red for binding to estrogen receptors. The plating density of each well was 0.1×10^6 cells in 1 mL of media.

To determine the ideal growth conditions for the T-47D cells in the well plate, a 24 well plate was set up with half the wells plated at a density of 0.05×10^6 cells and the other half with 0.1×10^6 cells. Based on the results of that plate, we determined that the ideal plating density was

0.1 x 10^6 cells and the ideal incubation period was 48 hours for the cells to adhere to the plate. Once the cells adhered, they were synchronized to the same stage of the cell cycle. This was done by aspirating the standard media and replacing it with 1mL of serum-free media. Following a 24-hour incubation period, this media was then aspirated and replaced with 1 mL of charcoal-stripped media, which lacks phenol red, as the dye has been shown to have estrogenic effects on T-47D cells (Welshons et. al., 1988). The cells were left in the charcoal-stripped media for 48 hours, at which point the media was again aspirated and replaced with phytoestrogen treatments and estradiol (positive control) dissolved in stripped media with 1% ethanol. Figure 2 shows a flow chart of the treatment conditions and incubation times for each experimental plate.

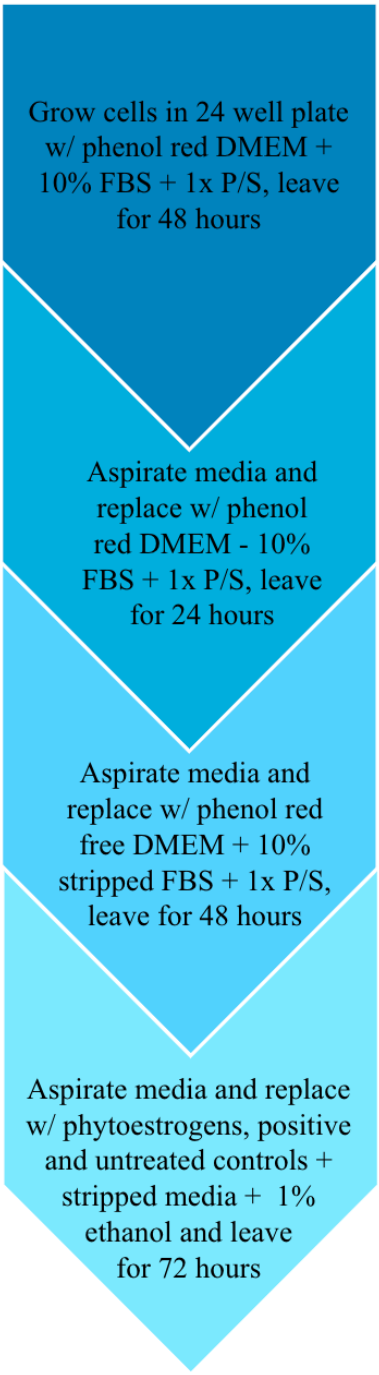

Figure 2. Treatment Methods
Flowchart of the conditions the cells were grown in, and for how long.

Treatment Conditions

The experimental plate also included positive and untreated control wells for comparison. The positive control consisted of estradiol to determine if the estrogen receptors are operating and increasing cell proliferation. The untreated control contains no phytoestrogens to determine the baseline cell growth under the media conditions. The physiologic concentration of estradiol in postmenopausal women is around 110 nM (*Tests to Determine Menopausal Status*, n.d.). To reflect this, the positive control, 100nM 17 β-estradiol in stripped media, was placed into three wells on the plate. The untreated control was three wells in the plate treated only with stripped media with 1% ethanol. The amount of ethanol was consistent in every well to control for its addition and any potential effects on the cells.

The rest of the wells in the plate were treated with a range of phytoestrogen concentrations in triplicates. The range consists of the following six final concentrations (M): $1x10^{-6}$, $5x10^{-7}$, $1x10^{-7}$, $5x10^{-8}$, $1x10^{-8}$, and $5x10^{-9}$, which were plated as shown in Figure 3. This range was chosen from the literature in which it was determined to be the physiological phytoestrogen concentration range by measuring phytoestrogen concentration in blood plasma after one male patient took Promensil as recommended (Setchell et. al., 2001). To create the experimental concentrations, the phytoestrogens were diluted from a stock to a concentration range from 100uM to 0.5uM in 100% ethanol. To reach the final concentrations, they were diluted in stripped media to a concentration range of 1uM to 0.005uM in stripped media containing 1% ethanol.

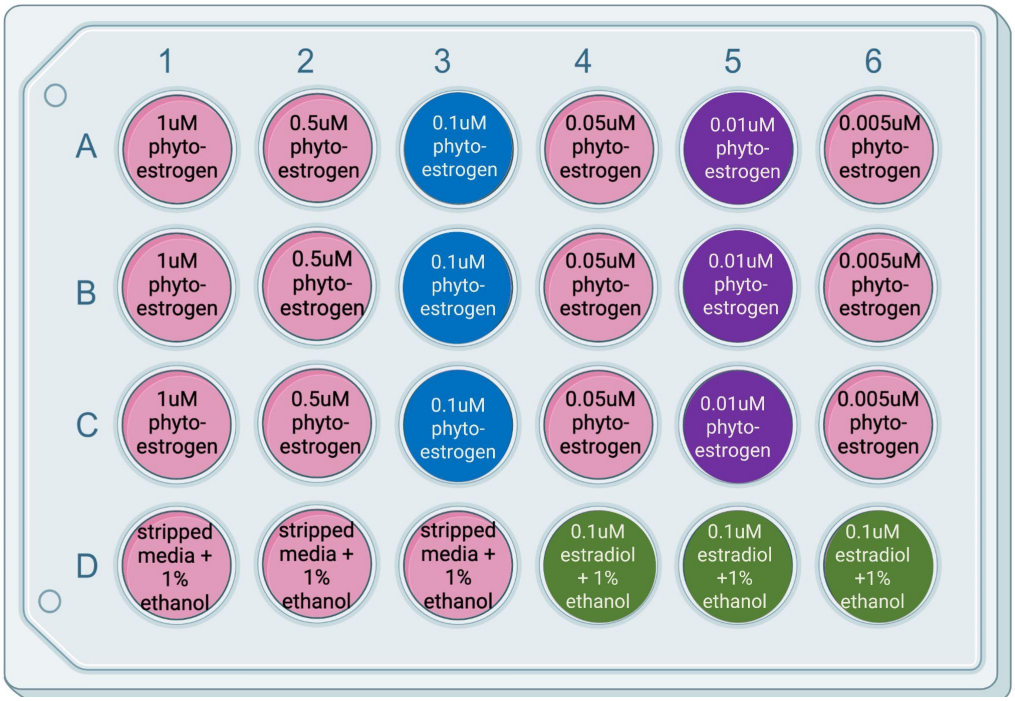

Figure 3: Plate layout for the experimental conditions.
The colors of this figure align with Figures 5 and 6. The blue highlights the exact physiologic concentration for genistein, and purple is the physiologic concentration for biochanin A.

Cell Counting

After the addition of the phytoestrogens, the plate was incubated for 72 hours at 37.5°C to allow the cells to grow in conditions with the phytoestrogen treatment. At the end of this incubation period, cell counts were performed for each well so a comparison between their growth rates could be done. Cell counts were done through the process followed for passaging in which the cells were trypsinized, resuspended, and counted in sets of three. The cells were resuspended in the mix of media and trypsin until all cell clumps disappeared before the cell suspension was placed on a slide to be counted in a Nexcelom Auto T4 Cellometer. A flow chart of these steps is shown in Figure 4.

Raw data of cell counts from triplicate wells on each plate were averaged for each treatment and control condition. The average cell count of triplicate wells on individual plates were averaged with cell counts from triplicate wells on the other plates of that experimental condition to obtain the final cell count at each concentration of phytoestrogen and at the controls. The final cell count for each phytoestrogen condition and the positive control were then normalized to the untreated condition as a percentage of the untreated control final cell count. Bar graphs with error bars were created from these normalized data points to create a visual of the change in cell counts. At each phytoestrogen concentration, the error bars are ± standard error of mean (SEM) where n (number of plates) = 3 at each phytoestrogen concentration and n = 7 for the positive control.

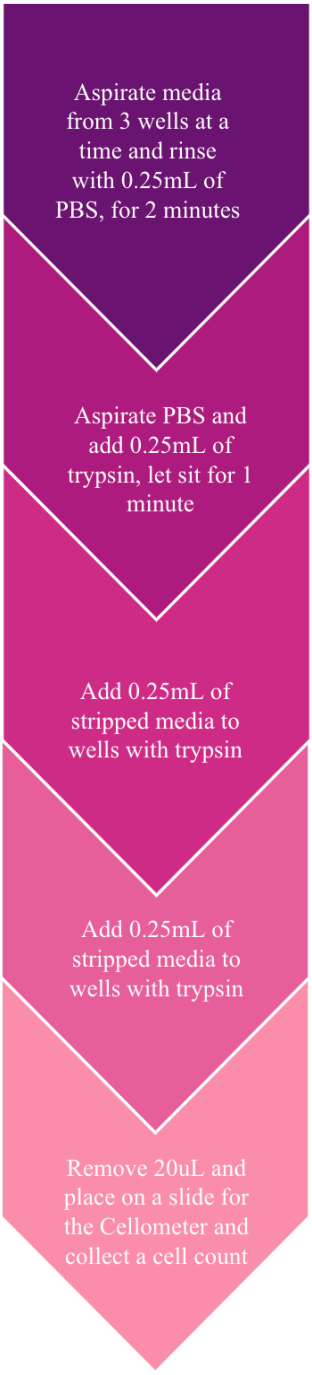

Figure 4. Cell Counting Methods
Flowchart showing the solutions and conditions used for counting the cells from each plate.

Results

The cell counts for genistein and biochanin A conditions normalized to untreated control are shown in Figure 5 and Figure 6, respectively, where the baseline level of proliferation is set by the untreated control as 100% in both figures. The positive control (estradiol), which was the same in all experiments with genistein and biochanin A conditions, increased cell numbers by 50% (150% line) compared to the untreated control (100% line). In Figure 5, genistein appears to increase cell numbers slightly only at the lowest tested concentration ($5x10^{-9}$ M) but decreases cell counts slightly at the other higher concentrations compared to the untreated control, and the error bars (based on SEM) at each genistein treatment condition indicate cell numbers within a range that is close to the baseline untreated control. Biochanin A, on the other hand, increased cell counts more than the positive control, i.e., by more than 50% (above 150% line) at every tested concentration (Figure 6). Furthermore, biochanin A also appears to increase cell numbers in a concentration-dependent manner, with higher concentrations leading to greater cell numbers than lower concentrations.

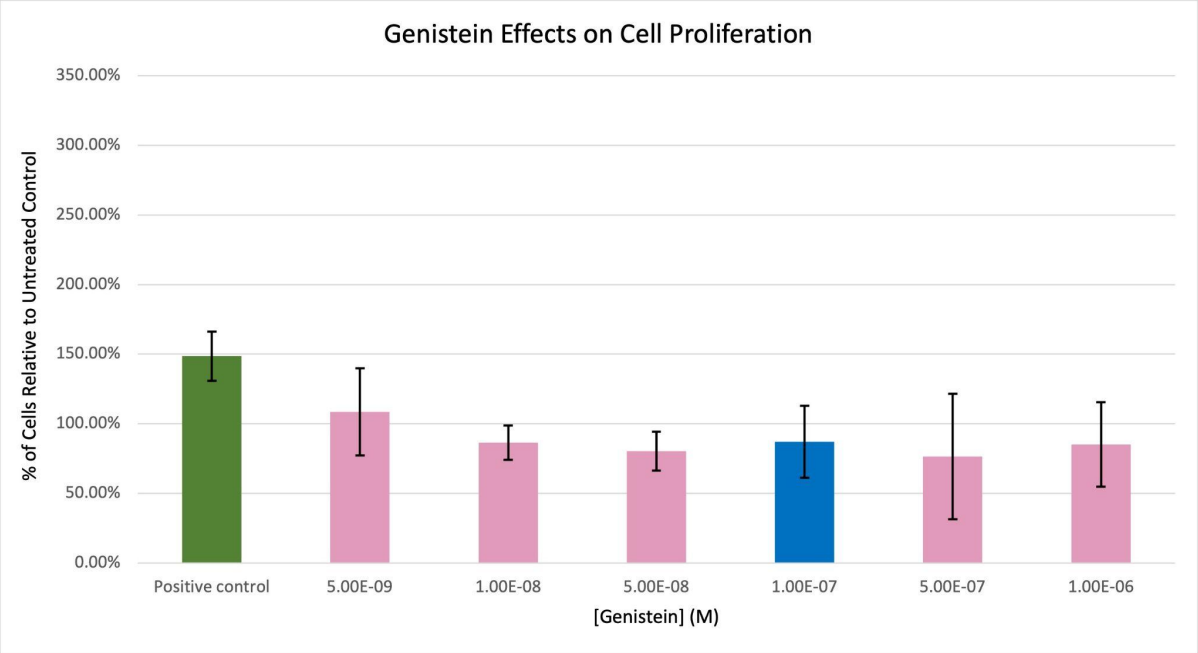

Figure 5: Cell counts for positive control and genistein conditions normalized to untreated control.

Data representing the growth of T-47D cells in genistein concentrations from $5x10^{-9}$M to $1x10^{-6}$M, including the physiologic concentration from the literature $1x10^{-7}$M (blue bar). The cell count is represented on the graph compared to the untreated control, considered 100% growth. Error bars represent

± standard error of mean (SEM) with n = 3 at each phytoestrogen concentration and n = 7 for the positive control.

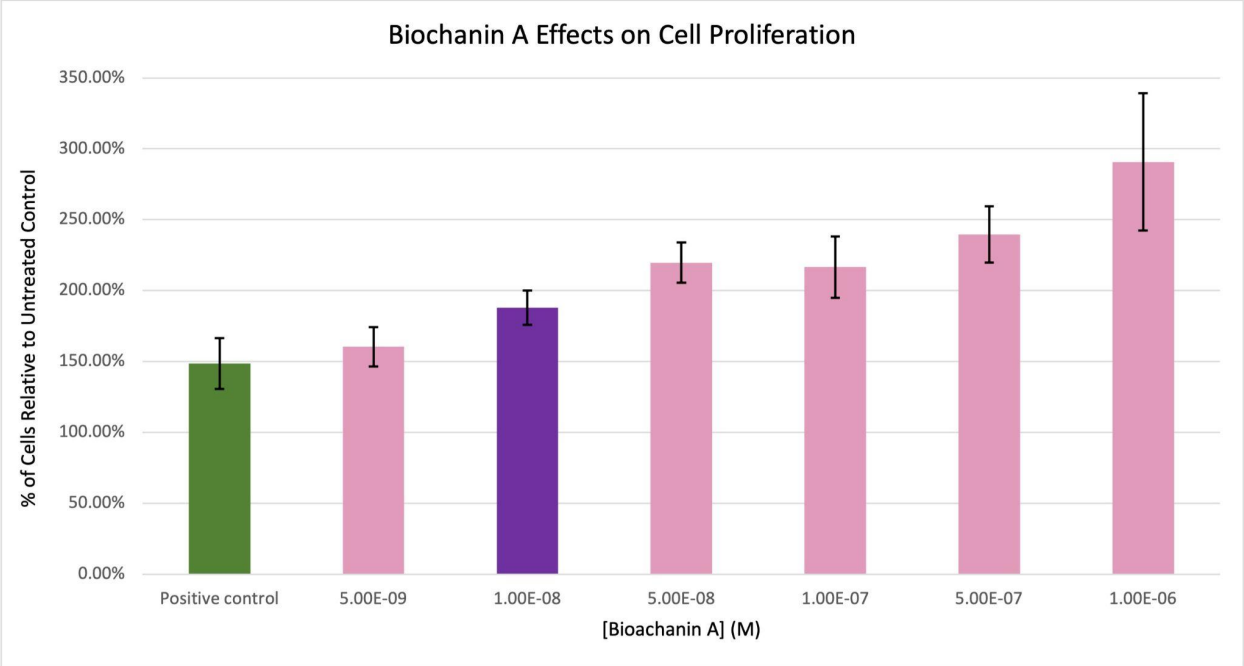

Figure 6: Cell counts normalized to untreated control for positive control and biochanin A conditions.

Data representing the growth of T-47D cells in biochanin A concentrations from $5x10^{-9}$M to $1x10^{-6}$M, including the physiologic concentration from the literature $1x10^{-8}$M (purple bar). The cell count is represented on the graph compared to the untreated control, considered 100% growth. Error bars are equal to ± SEM with n = 3 at each phytoestrogen concentration and n = 7 for the positive control.

Discussion

Genistein

Based on the cell counts displayed in Figure 5, genistein does not appear to have a significant effect on T-47D cell proliferation at the concentration range tested, as all tested concentrations of genistein showed proliferation levels similar to that of the untreated cells. These results are not consistent with the hypothesis, which predicted a significant decrease in cell proliferation relative to the untreated cells. Therefore, genistein may not alter the risk of breast cancer.

While this data does not line up with the expected result, there are some possible reasons for this. One potential factor could be the concentration range of genistein that was tested. Although this range includes the physiologic concentration of $1x10^{-7}$M (as found in Setchell et al., 2001), the hypothesis of decreased cell proliferation was based on other studies which tested significantly higher concentrations of genistein. Bilal et al. (2014) argue that antiproliferative effects of phytoestrogens in vitro are mostly found at concentrations of 10uM and greater, this would require a significantly larger dose than the physiologic concentrations from a 40mg dose of Promensil ($1x10^{-7}$M).

Additionally, the issues could arise from discrepancies between the in vitro studies, referenced by Bilal et al. (2014), and the true in vivo effects of phytoestrogen supplements. Attempts to replicate an in vivo breast cancer tumor using a cell line may fall short in generating an accurate tumor environment, which could produce a number of unforeseen changes to the effects of phytoestrogen treatments. The cell line itself, having been maintained over a long period of time, may also react differently to phytoestrogens and other compounds due to inadvertent selection and evolutionary changes that are not seen in a novel breast cancer tumor.

Biochanin A

The biochanin A cell numbers differ from genistein in that biochanin A increases cell count above the untreated control and positive control as shown in Figure 6. The cell number also increases as the biochanin A concentration increases, showing that higher concentrations of biochanin A at a physiologic range increase cell proliferation more than lower concentrations.

The mechanisms by which biochanin A increases proliferation may include inhibiting either cell cycle arrest or apoptosis.

One possible mechanism through which biochanin A may increase proliferation is by interacting with the estrogen receptors, with estrogen receptor α (ERα) increasing proliferation and estrogen receptor β (ERβ) decreasing proliferation. Since isolated biochanin A increases proliferation, it is likely that if the mechanisms of cell proliferation are connected to the estrogen receptors, then biochanin A is likely binding to the ERα. This is counterintuitive to previous studies that show that phytoestrogens have a higher affinity to ERβ than ERα (Bilal et al., 2014).

As biochanin A appears to increase cell proliferation, isolated biochanin A supplements may increase the risk of breast cancer. One possibility why Promensil decreases proliferation while biochanin A increases proliferation is that Promensil contains other compounds that may have anti-proliferative effects that overwhelm biochanin A's proliferative effect. Compounds in Promensil may also have synergistic effects in specific combinations that together create an anti-proliferation effect.

Chapter 2: Future Recommendations

Introduction

This chapter goes into detail about future steps to further understand the potential cellular mechanisms, cell cycle arrest and apoptosis, by which phytoestrogens may decrease cell proliferation. Our initial results showed an increase in proliferation so the experiments discussed in this chapter were not utilized. However, further testing could be done with formononetin and daidzein, or with different combinations of two phytoestrogen treatments at once. In the case that any of these conditions decreases cell proliferation, we recommend using an Immunoblot to analyze levels of p21 and Caspase 3. Our reasoning is explained throughout the background and methods below.

Background

The Cell Cycle and Restriction Point

The prototypical cell cycle is composed of two main phases, interphase and M phase. Interphase is the preparation phase for cell division and is composed of three sub-phases, G_1, S, and G_2. Lasting for 30% to 50% of the cell cycle, G_1 stands for growth 1 phase where cells prepare various protein synthesis for replication (Matthews et al., 2022). The synthesis or S phase involves replication of genetic material and lasts for 25% to 40% of the cell cycle (Matthews et al., 2022). In G_2 (growth phase 2), cells prepare for mitosis for 15% to 25% of the cell cycle (Matthews et al., 2022). M phase, or mitosis, is the process of cell division and lasts 5% to 10% of the cell cycle (Matthews et al., 2022).

The cell cycle is regulated by specific checkpoints with the main three being the restriction point at the end of G_1, the G_2-M checkpoint at the end of G_2, and the spindle checkpoint during the transition from metaphase to anaphase (Matthews et al., 2022). Progression through each checkpoint depends on the activation of cell cycle regulating proteins known as cyclin-dependent kinases (CDKs) which are regulated by other protein subunits known as cyclins (Pennycook & Barr, 2020). These Cyclin/CDK complexes can be inhibited by cyclin-dependent kinase inhibitors (CKIs) which prevent cell cycle progression and cause cell cycle arrest (Pennycook & Barr, 2020).

The restriction point is regulated by the Cyclin D:CDK4/6 complexes and the Cyclin E:CDK2 complex (Pennycook & Barr, 2020). Cyclin D levels start rising at the beginning of G_1, and the binding of Cyclin D to CDK4 and CDK6 phosphorylates the Rb protein also known as pRb (Pennycook & Barr, 2020). pRb blocks entry into S phase by inhibiting cell proliferating transcription factors known as E2F transcription factors, and the inhibition of pRb allows E2F transcription factors to begin DNA replication (Pennycook & Barr, 2020). At the end of G_1, Cyclin E is produced and binds with CDK2. As Cyclin E:CDK2 accumulates, pRb is further phosphorylated which completely inactivates Rb protein function, committing the cell to enter S phase and begin replication (Pennycook & Barr, 2020).

The commitment process from G_1 to S is inhibited by two CKIs, p27 and p21 (Pennycook & Barr, 2020). p27 binds to both Cyclin D:CDK4/6 and Cyclin E:CDK2 complexes and inhibits the ability for the CDK complexes to phosphorylate (Pennycook & Barr, 2020). As Cyclin D:CDK4/6 levels increase, p27 is more likely to bind with Cyclin D:CDK4/6 instead of Cyclin E:CDK2 (Pennycook & Barr, 2020). The uninhibited Cyclin E:CDK2 complex would then phosphorylate and inhibit p27, creating a feedback loop that further inactivates p27 activity (Pennycook & Barr, 2020). If DNA damage is detected, p21 is produced which also inhibits CDK activity by binding to both Cyclin D:CDK4/6 and Cyclin E:CDK2 complexes (Pennycook & Barr, 2020). This binding disturbs the normal feedback loop between p27, Cyclin D:CDK4/6 and Cyclin E:CDK2 and fully prevents all CDK activity until p21 levels are reduced (Pennycook & Barr, 2020).

Apoptosis

Apoptosis is the programmed cell death in eukaryotes which comprises two main pathways of activation, the intrinsic pathway of apoptosis and the extrinsic pathway of apoptosis (Fulda & Debatin, 2006). The intrinsic pathway is caused by internal cellular stress whereas the extrinsic pathway is caused by extracellular cell signaling, typically by immune cells (Fulda & Debatin, 2006). Both pathways lead to the same execution and phagocytic mechanisms which terminate DNA and cell function (Fulda & Debatin, 2006).

The intrinsic pathway begins with the detection of cellular stress, such as DNA lesions, which upregulates the TP53 gene to produce p53 protein. p53 within the cell undergoes conformational change through phosphorylation which increases half-life and allows p53 to act

as a transcription factor for repair genes (Hafner et al., 2019). If the cellular repair is untenable and the cellular stress remains after TP53 activation, p53 begins regulating genes to initiate apoptosis by upregulating the p53 upregulated modulator of apoptosis (PUMA) and downregulating Bcl-2 (Yu & Zhang, 2008). The initial p53 regulation allows for the activation and upregulation of the Bcl-2-associated X-protein (BAX) and the Bcl-2-antagonist/killer protein (BAK) which attach to and decrease the electric potential of the mitochondria (Youle & Strasser, 2008). The change in electric potential causes a conformational change in mitochondrial pores known as voltage-dependent anion channels (VDAC) which adopt the open conformation (Youle & Strasser, 2008). With the channels open, mitochondrial proteins such as Cytochrome C escape into the cytosol and complexes with APAF-1 and dATP to form the apoptosome. Apoptosomes function by recruiting and cleaving Procaspase-9 into Caspase 9 (Riedl & Salvesen, 2007). The apoptosome and Caspase 9 then activate Caspase 3 from Procaspase-3. Finally, Caspase 3 acts as an endonuclease for DNA and cleaves both cytosolic and nuclear proteins, causing phagocytosis (Li & Yuan, 2008).

Hypothesis

Phytoestrogens inhibit cell proliferation through cell cycle arrest by upregulation of p21 and apoptosis by upregulation of Caspase 3.

Recommended Methods

Phytoestrogen Treatments

To further determine the effects of Promensil, the other two phytoestrogens within Promensil, daidzein and formononetin, can be studied using the same methods as for genistein and biochanin A in Chapter 1. Cell counts can be collected after treatment with daidzein and formononetin instead of genistein and biochanin A. The phytoestrogen concentrations should be prepared with a similar range around the physiologic concentrations of daidzein and formononetin as calculated from Setchell et al. (2001).

In addition, combinations of the four phytoestrogens can be tested to determine synergistic or antagonistic effects due to the biochemistry of each phytoestrogen. Combinations of two, three, or all four phytoestrogens are possible for a total of 11 combinations. Combinations can be created with equimolar concentrations or based on the physiologic mole fraction of the phytoestrogens.

Immunoblot

After identifying phytoestrogens, or combinations of phytoestrogens, that decrease cell proliferation relative to the untreated control, determine the mechanisms of action with gel electrophoresis and immunoblot using the cell lysates. Since the decrease in cell proliferation is assumed to be impacted by the cell cycle, stain for the cell cycle regulator p21, and proapoptotic marker caspase 3 on the immunoblot. After immunoblotting, the density of the bands for each marker can be analyzed using a program such as ImageJ to quantify changes in levels of either protein, based on specific treatments. If the decrease in cell proliferation is due to cell cycle arrest or apoptosis, then the protein bands for p21 and caspase 3 would be denser for the cells treated with phytoestrogens than for the positive and untreated controls.

References

Bilal, I., Chowdhury, A., Davidson, J., & Whitehead, S. (2014). Phytoestrogens and prevention of breast cancer: The contentious debate. *World Journal of Clinical Oncology*, *5*(4), 705–712. https://doi.org/10.5306/wjco.v5.i4.705

Breast Cancer Hormone Receptor Status | Estrogen Receptor. (n.d.). Retrieved April 25, 2022, from https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/breast-cancer-hormone-receptor-status.html

Chen, M.-N., Lin, C.-C., & Liu, C.-F. (2015). Efficacy of phytoestrogens for menopausal symptoms: A meta-analysis and systematic review. *Climacteric*, *18*(2), 260–269. https://doi.org/10.3109/13697137.2014.966241

Dixon, R. A. (2004). Phytoestrogens. *Annual Review of Plant Biology*, *55*, 225–261.

Does hormone replacement therapy (HRT) increase cancer risk? (2019, February 27). Cancer Research UK. https://www.cancerresearchuk.org/about-cancer/causes-of-cancer/hormones-and-cancer/does-hormone-replacement-therapy-increase-cancer-risk

Fulda, S., & Debatin, K.-M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*, *25*(34), 4798–4811. https://doi.org/10.1038/sj.onc.1209608

Hafner, A., Bulyk, M. L., Jambhekar, A., & Lahav, G. (2019). The multiple mechanisms that regulate p53 activity and cell fate. *Nature Reviews Molecular Cell Biology*, *20*(4), 199–210. https://doi.org/10.1038/s41580-019-0110-x

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Ström, A., Treuter, E., Warner, M., & Gustafsson, J.-Å. (2007). Estrogen Receptors: How Do They Signal and What Are Their Targets. *Physiological Reviews*, *87*(3), 905–931. https://doi.org/10.1152/physrev.00026.2006

Huber, S. (2021, May 2). *Student Work | Mechanism of Action for Promensil in Regard to T47D Cell Proliferation | ID: js956j54d | Digital WPI*. https://digital.wpi.edu/concern/student_works/js956j54d?locale=en

Li, J., & Yuan, J. (2008). Caspases in apoptosis and beyond. *Oncogene*, *27*(48), 6194–6206. https://doi.org/10.1038/onc.2008.297

Matthews, H. K., Bertoli, C., & de Bruin, R. A. M. (2022). Cell cycle control in cancer. *Nature*

Reviews Molecular Cell Biology, *23*(1), 74–88. https://doi.org/10.1038/s41580-021-00404-3

Maynes, D. L., Hunt, K. S., Pockaj, B. A., Gray, R. J., Tong, W. P., Bothe, M. R., Dueck, A. C., & Northfelt, D. W. (2010). Are HER2-positive breast cancer and BRCA mutation-associated breast cancer mutually exclusive diseases? Evidence from the Mayo Clinic Arizona Cohort. *Journal of Clinical Oncology*, *28*(15_suppl), e21075–e21075. https://doi.org/10.1200/jco.2010.28.15_suppl.e21075

Melchior, E. A., & Myer, P. R. (2018). Fescue toxicosis and its influence on the rumen microbiome: Mitigation of production losses through clover isoflavones. *Journal of Applied Animal Research*, *46*(1), 1280–1288. https://doi.org/10.1080/09712119.2018.1496920

Morito, K., Hirose, T., Kinjo, J., Hirakawa, T., Okawa, M., Nohara, T., Ogawa, S., Inoue, S., Muramatsu, M., & Masamune, Y. (2001). *Interaction of Phytoestrogens with Estrogen Receptors a and b*. *24*(4), 6.

Pennycook, B. R., & Barr, A. R. (2020). Restriction point regulation at the crossroads between quiescence and cell proliferation. *FEBS Letters*, *594*(13), 2046–2060. https://doi.org/10.1002/1873-3468.13867

Reproductive Hormones. (n.d.). Retrieved April 25, 2022, from https://www.endocrine.org/patient-engagement/endocrine-library/hormones-and-endocrine-function/reproductive-hormones

Riedl, S. J., & Salvesen, G. S. (2007). The apoptosome: Signalling platform of cell death. *Nature Reviews Molecular Cell Biology*, *8*(5), 405–413. https://doi.org/10.1038/nrm2153

Setchell, K. D., Brown, N. M., Desai, P., Zimmer-Nechemias, L., Wolfe, B. E., Brashear, W. T., Kirschner, A. S., Cassidy, A., & Heubi, J. E. (2001). Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *The Journal of Nutrition*, *131*(4 Suppl), 1362S-75S. https://doi.org/10.1093/jn/131.4.1362S

Sirotkin, A. V., & Harrath, A. H. (2014). Phytoestrogens and their effects. *European Journal of Pharmacology*, *741*, 230–236. https://doi.org/10.1016/j.ejphar.2014.07.057

T-47D | ATCC. (n.d.). Retrieved April 25, 2022, from https://www.atcc.org/products/htb-133

Tests to Determine Menopausal Status. (n.d.). Retrieved April 25, 2022, from https://www.breastcancer.org/treatment-side-effects/menopause/types/testing

van de Weijer, P. H. M., & Barentsen, R. (2002). Isoflavones from red clover (Promensil) significantly reduce menopausal hot flush symptoms compared with placebo. *Maturitas*, *42*(3), 187–193. https://doi.org/10.1016/s0378-5122(02)00080-4

Wambach, R. (2018, April 24). *Student Work | Effect of Promensil on Breast Cancer Cells in regard to the Estrogen Receptor Beta | ID: 79407z79f | Digital WPI*. https://digital.wpi.edu/concern/student_works/79407z79f?locale=en

Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: Opposing activities that mediate cell death. *Nature Reviews Molecular Cell Biology*, *9*(1), 47–59. https://doi.org/10.1038/nrm2308

Yu, J., & Zhang, L. (2008). PUMA, a potent killer with or without p53. *Oncogene*, *27*(Suppl 1), S71–S83. https://doi.org/10.1038/onc.2009.45