Relationship between Promensil and Estrogen Receptor Alpha in Breast Cancer Cells

A Major Qualifying Project Report
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D Term Submission

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Abstract

Over the counter phytoestrogen supplements are used to treat menopausal symptoms purportedly, without the increased risk of breast cancer associated with estrogen replacement therapy. Extracts of one such supplement, Promensil, have been shown to reduce proliferation of the breast cancer cells T47D, but not to act through the estrogen receptor beta (ER-β). Our hypothesis is that Promensil acts through estrogen receptor alpha (ER-α), which normally signals breast cancer cells to proliferate in response to estrogen. To test this, T47D cells were treated with Promensil alone, or Promensil with an ER-α antagonist. The data to date rejects our hypothesis that Promensil acts through ER-α.

Acknowledgments

On behalf of our project, we would like to thank Professors Jill Rulfs and Mike Buckholt for advising this project, and allowing us to work with and treat the T47D cells in order to facilitate the experiments required for this project.

Introduction

This project researched the effects of the phytoestrogen menopause supplement, Promensil, on breast cancer cells. Breast cancer is a disease in which malignant cells begin to form and proliferate uncontrollably in the tissue of the breast. Breast cancer cells have estrogen receptors (ERs) that control cellular division in response to both estrogen and phytoestrogen. This project investigated T47D breast cancer cell estrogen receptor alpha interaction with phytoestrogens specifically, to investigate their effect on cell proliferation.

Breast cancer risk increases after menopause, which is the final menstrual period and loss of ovarian function (Menopause, 2018). At this time, the female body stops naturally producing estrogen. This hormonal decrease can lead to mood swings and hot flashes, as well as chronic issues such as osteoporosis and cardiovascular disease (Menopause, 2018). To combat these issues, hormone replacement therapy (HRT) is often used to replace estrogen in the body due to the ovaries no longer producing it. However, the estrogen administered in HRT has been found to stimulate the growth of breast cancer cells through the estrogen receptors (ChemMD, 2008).

An alternative to HRT are phytoestrogen treatments. Phytoestrogens are *xenoestrogen* compounds that naturally occur in various plants, and imitate the effects of estrogen due to their similar structure as well as their functionality, and alleviate menopausal symptoms. They are commonly used to treat postmenopausal symptoms without adding the increased risk for breast cancer, as some estrogen replacement therapy has shown (Lecomte, 2017). Phytoestrogens include well known phenolic compounds, isoflavones, which have been the potential to be used in cancer prevention as well as treatment (Patisaul, 2017).

Promensil is the brand of phytoestrogen supplement used for the experiments in this paper. Promensil is studied due to the molecular likeness of phytoestrogens to estrogen hormones. It contains four isoflavones, which consist of Genistein, Daidzein, Formononetin and

Biochanin. Independent studies have confirmed that roughly 40 mg of the four isoflavones is the lowest amount of phytoestrogens necessary to ease menopausal symptoms (Fritz, 2013). Promensil has been found to decrease cell proliferation in T47D breast cancer cells, but the mechanism is not known (Wambach, 2018).

Estrogens are a class of steroidal hormones that phytoestrogens resemble. Estrogen signals for the development of female sex traits, such as breasts. In this paper, estrogen functions as a positive control when testing the effects on cancer cell proliferation. Estrogen has been extensively studied, and when estrogen responsive breast cancer cells are exposed to estrogen, an increase in proliferation is observed. Estrogen functions as an agonist for the two classes of estrogen receptors, ER-α and ER-β. The binding of estradiol to these receptors regulates metabolic processes for cell growth gene expression, cell differentiation and proliferation (National Center for Biotechnology Information, 2018).

ERs bind to specific DNA sequences known as Estrogen Response Elements (ERE), and activate or repress gene expression when bound to estrogen, or estrogen-mimicking compounds (Klinge, 2001). The effects of estrogen related compounds are highly regulated by the ER-α and ER-β receptors. These two isoforms, alpha and beta, bind 17β-estradiol to regulate either proliferation or apoptosis. In ER-positive breast cancer, estrogen receptor alpha (ER-α) has been shown to exhibit proliferative effects, while beta (ER-β) has been shown to display anti-proliferative effects (Suba, 2013).

In previous projects studying the effects of Promensil on breast cancer cells at WPI, it was found that phytoestrogen supplements do not act through the ER-β receptor (Wambach, 2018). The decreased proliferative effects of Promensil previously demonstrated in T47D cells was unchanged when ER-β expression was turned off by adding a tetracycline to T47D-ERβ cells, which inhibit transcription of the ER-β gene (Strom *et al.,* 2004). This led to the hypothesis

tested here that Promensil acts through the alpha receptor, possibly antagonizing the effect of estrogen.

When bound to estrogen, Estrogen receptor alpha (ER-α) is known to have hyperproliferative effects on breast cancer cells (Saji et all, 2001). Differences between the structures of the alpha and beta receptors may contribute to estrogen-mimicking phytoestrogens having different effects when bound to either of the two receptors. The difference of the amino acid residues in these receptors have made creating "sub-selective" ligands difficult in the past; however, the knowledge of the binding residues and their amino acid sequences have helped in understanding the regulation of antagonistic and agonistic functions of the ER-α receptor (Paterni, 2014). Because Promensil displays a decrease in cell proliferation, and previous reports have shown that the mechanism is not through ER-β, ER-α is believed to be the receptor that Promensil binds to in order to decrease cellular proliferation.

Background

Breast Cancer

For decades, breast cancer has had a major impact on women. In the United States, a woman has about a 12% chance of developing breast cancer within her lifetime (BreastCancer.org). Breast cancer cells can be either Estrogen Receptor positive (ER+) or Estrogen Receptor negative (ER-). If the cells are ER+, there is expression of estrogen receptors in the cell. Their presence allows the binding of estrogen to the receptors, and function to control proliferation of the cells (Ali & Coombes, 2000).

T47D

The breast cancer cell line used in this paper is T47D. The T47D cells express both ER-α and ER-β, and have proved through *in vitro* experiments, to mimic the effects present in patients with ER-positive breast cancer (Yu, 2017). Here, these cells are being used to investigate estrogen receptor interaction with phytoestrogen compounds found in Promensil. This line of T47D breast cancer cells has also been transfected with a Tetracycline-dependent PBI-EGFP vector in order to control ER-β expression. Tetracycline presence in growth of these T47D-ERβ cells inhibits the transcription of the beta receptor (Evers, 2013). The T47D-ERβ cells were not used in this paper, but they have in previous experiments which set the groundwork for the hypothesis tested here (Wambach, 2018).

Menopause

Menopause is defined as the final menstrual period and loss of ovarian function in a woman (Menopause, 2018). This decrease in natural estrogen can lead to mood swings and hot flashes, as well as chronic issues, such as osteoporosis and cardiovascular disease (Menopause,

2018). Use of Hormone Replacement Therapy (HRT) is helpful to treat these side effects, but comes with additional cancer risk. HRT is performed by administering regular doses of exogenous estrogen to combat the loss of naturally produced endogenous estrogen after menopause. In a paper summarizing population based breast cancer epidemiology, studies showed significant correlation between HRT and the risk of breast cancer, demonstrating an annual breast cancer risk increase of 2.1% over years of use (Heikkenen, 2015). For this reason, alternative therapies using phytoestrogen supplements have been investigated.

Estrogen

Estrogen is a steroidal hormone that regulates growth and development of female reproductive organs, such as formation of the breasts and the uterus. On a molecular level, estrogen functions through signaling receptors on the cells of those tissues. The most common form of estrogen is 17β-estradiol, which is an 18 carbon aromatic compound with a hydroxyl group at 3β and 17β. The binding of estrogen functions as an agonist to activate the receptor.

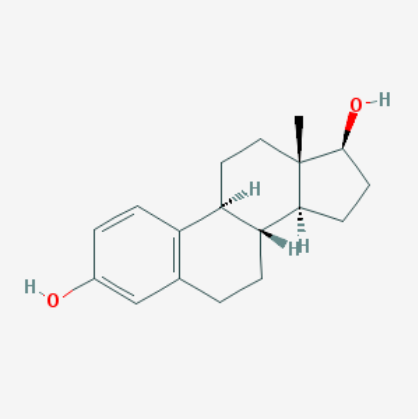

Figure 2. Structure of Estradiol ("Estradiol" NCBI, 2018)

However, complications such as osteoporosis, cardiovascular disease, and hot flashes during the postmenopausal phase of a woman may develop, due to the loss of estrogen

production. This is because estrogen also binds receptors on cells in the tissues of bones, heart, and brain among others, so HRT is used to address conditions related to these tissues.

Unfortunately, when estrogen binds these cells, it can also fulfill its developmental function of breast development by binding the receptors of breast cells, and inducing growth of neoplastic cells containing estrogen receptors. This relationship between estrogen and breast cancer cell proliferation has been researched and led to treatments involving anti-estrogen compounds (Ali & Coombes, 2000). The goal of anti-estrogenic treatments is to reduce the breast cancer proliferation by introducing estrogen receptor antagonists and reducing estrogen levels. This inhibits the receptor from being activated, and signaling of the cancer cell to divide and grow.

Phytoestrogen

Phytoestrogens are plant derived molecules that have a similar structure and functionality to estrogen, and have been used to alleviate menopausal symptoms. Phytoestrogen compounds can produce estrogenic effects in humans and animals. These effects can be successful in easing discomfort associated with menopause, with the added potential for a lower cancer risk (Ringel, 1998). Early research shows promising results on phytoestrogens and low cancer risk. An *in vitro* study tested the effect of phytoestrogens at varying concentrations on proliferation of estrogen receptor positive breast cancer cells. This study found that at low phytoestrogen doses, the proliferation of breast cancer cells increased. However, at higher concentrations, proliferation was inhibited (Zava, 1997). A study among Asian women showed a correlation with low breast cancer development risk and a diet containing soy, which is high in phytoestrogens (Seifer, 1999). These results show promising data that phytoestrogens may be a safe treatment to

ameliorate menopausal symptoms, but further research should be performed in particular to examine the observation that low concentrations may increase cancer cell proliferation.

Isoflavones are a fundamental phytoestrogen compound that have many different uses, such as cholesterol-reduction, antioxidant activity, and specific to this project, cancer treatments (Messina, 2014).

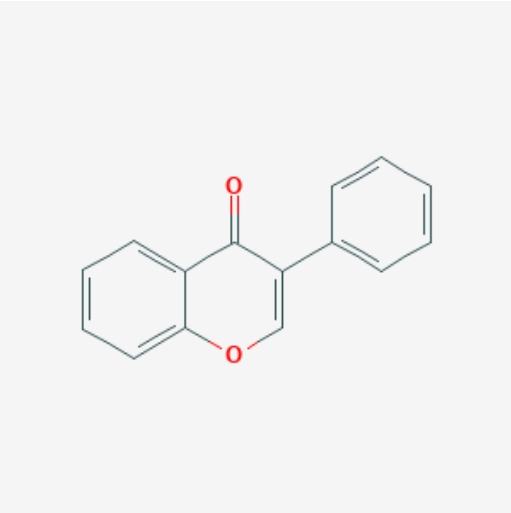

Figure 1. 2D Structure of Isoflavones ("Isoflavone" NCBI, 2018).

Isoflavones contain a phenolic ring structure, as well as hydroxyl groups that structurally and functionally imitate the human estrogen hormone 17β-estradiol (P. Basu, 2018). Due to their similar chemical and physical properties, phytoestrogens are able to exert different types of estrogenic activity, and can even compete with 17β-estradiol in binding to estrogen receptors (P. Basu, 2018).

Promensil

Promensil is the brand of phytoestrogen supplement used in this research project, and it contains four of the phytoestrogen isoflavones: Genistein, Daidzein, Formononetin and Biochanin. Genistein and Daidzein were found to be antagonists of ER-alpha (Fritz, 2013). Many experiments have concluded that approximately 40 mg of the four isoflavones is the lowest

amount of phytoestrogens necessary to ease menopausal symptoms (Fritz, 2013). An independent study confirmed that Promensil contains an average of 41.7 mg of these four phytoestrogens per tablet (Setchell et al., 2001).

In another study, bioavailability of each isoflavone was individually looked at in healthy premenopausal women to determine the differences among the four present in Promensil. It was discovered that there is a 6 hour delay until there is a substantial amount of the isoflavone present in the plasma, and of the isoflavones, Daidzein is the most abundant. The findings show that Promensil mainly increases plasma levels of Daidzein with a lesser increase in Genistein (Setchell et al., 2001).

Phytoestrogen treatments such as Promensil that have been used to ease postmenopausal symptoms also claim to lower the risks of heart disease and cancer. Although it is seen and utilized as a natural dietary supplement for women, there has been ongoing research addressing the potential adverse effects of phytoestrogen on healthy women's breasts, and the lack of understanding of their mechanism of action (S. Huser, 2018).

Estrogen Receptors

Estrogen receptors are present in cells of many different tissues, and are activated upon binding of their ligand, estrogen. Upon their binding to estrogen, the receptor is activated, and the hormone receptor complex translocates to the nucleus. The receptors bind a specific target area of the genome known as the Estrogen Response Element (ERE) region. EREs are present in the promoter region, and this binding activates transcription of genes that regulate proliferation.

The estrogen receptors have two structurally different isoforms: Estrogen receptor alpha (ER-α) and Estrogen receptor beta (ER-β), that both bind estrogen. However, the binding of estrogen to either receptor induces different proliferative functions of the cell. Estrogen receptor

alpha (ER-α) has been known to exhibit proliferative effects, while beta (ER-β) has been known to display anti-proliferative effects (Suba, 2013).

Estrogen Receptor Beta (ER-β)

Estrogen receptor beta has been known to show anti-proliferative effects in response to estrogen on T47D breast cancer cells. Upon ER-β activation in the T47D cells, reduced cyclin dependent kinase 2 (CDK2) activity was observed due to p27 inhibition. CDK2 inhibitors such as p27 help regulate cell cycle progression; additionally, with this reduced activity, the cell will divide less and have anti-proliferative effects on the cancer cell (Strom, 2015).

Due to this research, a project on the interaction of the phytoestrogen Promensil and ER-β was performed to determine if the supplement acted through binding of the beta receptor. The project tested the phytoestrogen on T47D cells that expressed the receptor and did not express the receptor. ER-β expression was controlled by a transfected vector that reduced the ratio of ER-β to ER-α in response to tetracycline. The results showed that Promensil decreased T47D cell proliferation with ER-β present or not, so it is unlikely that ER-β binding is involved in the decrease in cell proliferation (Wambach, 2018).

Estrogen Receptor Alpha

As stated previously, ER-α induces an increase in cellular proliferation when bound to estrogen. Estrogen and ER-α function by binding EREs of genes associated with cell cycle and DNA repair factors. In addition, this binding has been discovered to also attract Proliferating Cell Nuclear Antigen (PCNA), a protein that has been linked to assist the expression of the genes that the ER-α EREs bind to (Schultz-Norton, 2007). These factors lead to increased cellular proliferation in T47D cells when ER-α is activated by binding estrogen.

One study tested the binding of phytoestrogens to the human ER-alpha inserted in *Saccharomyces cervisiae* yeast cells. This study discovered two flavonoids had particularly low Kd values; Daidzein had a dissociation constant of 7-9 x 10^{-7}, and Genistein was 3-6 x 10^{-8}. These low Kd values indicate a high binding affinity of these phytoestrogen molecules to ER-α (Andres, 2013).

Another study was performed in order to assess the antagonistic effects phytoestrogens could have on ER-α. Natural substances were tested for their ability to disrupt ER-α binding to estrogen by using a luciferase assay. This study concluded that Genistein and Daidzein displayed antiestrogenic activity, and could act as antagonists (Pang, 2018). The antagonistic binding of phytoestrogens to ER-α may explain the anti-proliferative effects that have been observed in previous studies.

MPP

Methyl-Piperidino-Pyrazole (MPP) functions as a highly selective antagonist for ER-α, and is commonly used to study the function of this receptor on cellular proliferation (Hall, 2001). It was utilized in this project to bind ER-α and inactivate the receptor. MPP has a far lower substrate affinity constant (Ks) for ER-α than ER-β; the Ks value for ER-α is 5.6 nM and for ER-β is 2.3 uM. The much lower affinity constant indicates that less MPP is needed to bind to ER-α, and antagonize this receptor (Sun, 2002). Additionally, it was discovered that MPP would inactivate ER-α at a concentration of 80 nM (Zhou, 2009). With the ER-α antagonized by MPP, the receptor can be efficiently blocked in order to evaluate the effects of a compound such as Promensil with ER-α present or blocked in the cell.

Hypothesis

If Promensil does not show anti-proliferative effects through the ER-β receptor in T47D cells, then it is hypothesized that Promensil will decrease proliferation through interactions with the ER-α in the breast cancer cell line. If true, the anti-proliferative effects will decrease in the presence of an ER-α antagonist, and if not, Promensil does not act through an estrogen receptor.

Methods

Extracting Promensil

The phytoestrogen source used in this paper came from extracting three double strength Promensil tablets, as done in a previous study focusing on ER-β (Wambach, 2018). One double strength tablet contains the daily recommended dose on the package of 80 mg of isoflavone red clover extract (Promensil, n.d.). Three Promensil tablets were first ground up, and a mortar and pestle were used until the tablets were degraded into a fine powder. The powder was then mixed with 100 mL of 80% methanol, until dissolved. The mixture was then refluxed in a 70°C water bath for 1 hour using a reflux condenser. Once done, the extract was vacuum filtered to remove any remaining undissolved particles, then stored at -20°C (Setchell et al, 2001). The procedure used produced a 2.4 g/L phytoestrogen extract solution that was used for the duration of the experiments.

Cell Maintenance

T47D breast cancer cells were obtained from ATCC, and maintained in vented cell culture T75 flasks. The cells were grown in media containing DMEM with 10% Fetal Bovine Serum (FBS), 1% PenStrep, and L-Glutamine. The ATCC states that T47D cells require 0.1% insulin included in the media for proper growth, which was used in the media. Each T75 flask contained 10mL of the media. The flasks were placed in an incubator at 37°C with 5% carbon dioxide. The flasks were frequently checked and the cells were trypsinized off the plate, split, and re-plated into a new T75 as needed to maintain the culture.

Cell Plating

For each experiment, cells were trypsinized out of the flask and plated in 6 well plates at a density of either 1.5 x 10^5 or 2 x 10^5 cells per well. The cells were then plated with 3 mL/well of DMEM + 10% Fetal Bovine Serum + 1% PenStrep + 0.1% insulin, and allowed to adhere for 48 hours before continuing treatment.

Cell Synchronization

Cell synchronization was performed to bring the cells to precisely the same phase in the cell cycle. Once the cells had adhered to the plate for approximately 24 hours, the media was aspirated off and replaced with serum-free media consisting of DMEM + 1% PenStrep, without FBS. The cells were then left for another 24 hours. After this time period, the cells were given the experimental media, consisting of Phenol Red free DMEM, 10% charcoal stripped FBS, 1% PenStrep, and 0.1% insulin. The cells were then left in the incubator to adhere for another 24 hours, before beginning the cell treatments.

Experimental Conditions

The treatments were added to triplicate wells, and allowed to interact with the cells for 72 hours. A total of 6 treatments were added to the wells: no add media, methanol + dimethyl sulfoxide (DMSO), estradiol + DMSO, Promensil + DMSO, estradiol + MPP, and Promensil + MPP. Both the no add media and the methanol + DMSO were used as negative controls. 30 uL of media was added to the no add wells. Both estradiol and Promensil were solubilized in methanol, and the MPP in DMSO, so 1% (V/V) methanol and DMSO were present in all wells, other than the no add media control. 100nM estradiol + 30uL DMSO were added as a positive control. An experimental treatment of 10 uL/mL Promensil + 30 uL DMSO was added to test our

hypothesis. 100nM MPP was added to sets of wells containing either 100nM estradiol or 10uL/mL Promensil to observe the effects of the treatments with the receptor antagonized.

Cell Imaging

Before the treated cells were harvested and cell counted, they were photographed using a Zeiss Axiovert 100 inverted fluorescent microscope at 200X magnification in order to observe and analyze the morphologies produced from the different treatments added to the cells. The images were taken at the center of the well.

Cell Count

Once photos were taken, the cells were harvested and counted. This was done by aspirating the media out of each well, washing with PBS, and then trypsinizing the cells off of the plate. The wells were thoroughly washed with PBS prior to cell counting in order to wash off any dead cells, or extracellular debris excreted by the cells, that would potentially skew the results otherwise. The collected cells from each well were then sampled and counted using a Nexcelom Cellometer Auto T4 cell counter.

Results and Discussion

Images of both negative controls, as well as the cells treated with Promensil alone, and Promensil + MPP, can be found in Figure 3 below.

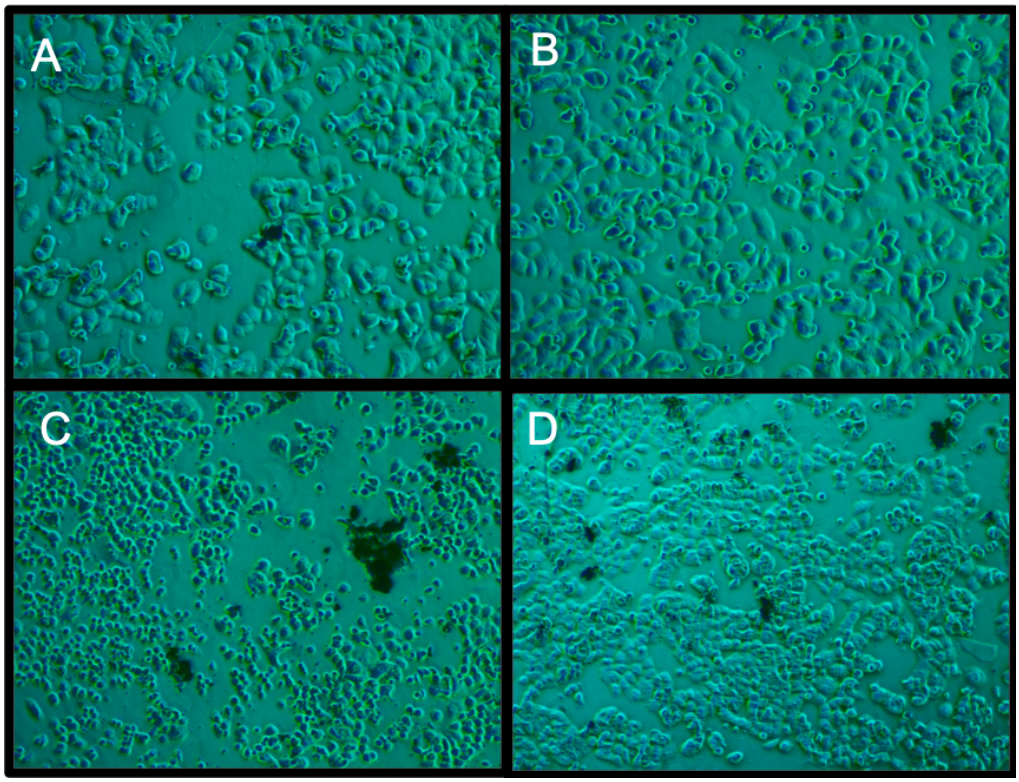

Figure 3: Comparison of T47D Cell Morphology in: **A:** No Add Media **B:** Methanol + DMSO **C:** Promensil **D:** Promensil + MPP

Looking at Figure 3 above, both the no add control in Figure 3A and the methanol + DMSO control in 3B display similar morphologies. The two negative controls show cells that display a large, round shape and appear healthy. In contrast, the Promensil + DMSO treated cells shown in Figure 3C appear to have a slightly smaller shape, and show a larger amount of dead cells floating in the well, which appear as the black "clumps" in the photograph. The inside of the cells on the well also have dark material on the inside, indicating poor cellular health as well. Figure 3D displays cells treated with Promensil + MPP in order to block ER-α. This well also displays cells that are smaller than the control cells in Figures 3A and 3B. These cells appeared

to have a less defined cellular membrane compared to the cells treated with Promensil alone in Figure 3C, which may be due to the presence of MPP in the cells. The cells in these wells also display the dead cell debris that were also observed with Promensil treatments in Figure 3C. Due to the larger amount of dead cell debris seen in Figures 3C and 3D, both of which were treated with Promensil, there is a possibility that Promensil may include an ingredient that is cytotoxic to T47D cells at a concentration of 10 uL/mL.

After the images were taken, the cells were harvested and counted. To deal with the cellular debris present, each well was washed 3 times with 1 mL of PBS, and aspirated off. This wash step removes the dead cells, or extracellular debris that have become unattached to the plate. This step ensures that a very high percentage of T47D cells remaining for the cell count are living cells. These cells were then trypsinized off and counted. Cell counts are displayed in Table 1. The value for each treatment is the average of the triplicate wells.

Table 1: Cellometer Count Values for Treated Well Plates

	Trial 1 **2 x 10^5 cells plated**	**Trial 2** **1.5 x 10^5 cells plated**	**Trial 3** **1.5 x 10^5 cells plated**
No Add Media	1.99 x10^5	2.90 x 10^5	1.58x 10^5
Methanol + DMSO	1.17 x 10^5	2.61 x 10^5	1.54x 10^5
Estrogen	2.46 x 10^5	3.78 x 10^5	1.80x 10^5
Promensil	1.22 x10^5	1.54 x 10^5	1.01 x 10^5
Estrogen MPP	1.48 x 10^5	2.43 x 10^5	1.38 x 10^5
Promensil MPP	1.85 x10^5	1.41 x 10^5	1.16 x 10^5

For each trial, n = 3 per each average cell count. The results obtained from Trial 1 displayed a large amount of T47D cell death, with the cell counts being below the initial 2 x 10^5 cells plated per well. This implies that a large amount of cells died during this run, indicating

very little cellular proliferation. The only treatment that showed cell proliferation was estrogen, which can be as expected. Promensil at 10 uL/mL in the experiment resulted in a decrease in cell number down to 1.2 x 10^5 cells per well. Interestingly, the methanol at 10 uL/mL had a similar effect, decreasing the number of cells per well to 1.17 x 10^5. These values indicate that at a plating density of 2 x 10^5 cells per well, 1% methanol can be toxic to T47D cells, altering the observations phytoestrogens may have on proliferation (Goldsmith, 2018). At higher plating densities, certain antibiotics can be known to have lesser effects on cells, which could be what is observed (Nakamura, 2018). Another possible explanation could be due to over confluence of the cells in the well. It can be assumed that 2 x 10^5 cells per cell is too many cells plated per well, which led to the over-confluency that was observed. This most likely resulted in cells competing for resources, or experiencing contact inhibition. Due to this possibility, lower cell densities were used in later trials.

In the second and third trials, the values obtained were more acceptable for 1.5 x 10^5 cells plated per well. Trial 2 displayed proliferation in all of the wells, except the ones containing Promensil. This indicates that at a plating density of 1.5 x 10^5 cells per well, T47D cells are able to grow for the duration of the experiment. The wells displayed data as expected, with the methanol + DMSO having a similar number of cells per well as the control containing no add media. In addition, estrogen had a larger number of cells than the methanol + DMSO control. When MPP inhibited binding of ER-α, the hyperprolific effects of estrogen were not seen in comparison to the methanol control. This indicates that MPP successfully antagonized ER-α. In addition, Promensil had lower cell counts than the methanol control with ER-α present and antagonized.

Trial 3 displayed a similar trend as Trial 2, but yielded lower values for the final cell counts. The average number of cells per well observed in Trial 2 was 2.9 x 10^5, and in Trial 3,

the average number of cells per well was 1.58 x 10^5. The reason for this is not known, as the same procedure was used in both trials, at the same plating density. However, the methanol + DMSO wells maintained a similar cell count as the no add control. The estrogen wells had a greater number of cells compared to the methanol control, and when in the presence of MPP with ER-α blocked, the increase of cells was not observed. As observed in Trial 2, Promensil decreased the number of cells both with ER-α open and blocked.

For each trial, the data was expressed as percent control to account for different initial number of cells plated. This was done by dividing the number of cells per well for each treatment by the average number of cells in the no add control. This allowed the data to account for the different plating densities by having each no add control being valued at 100% of the cells, and determining if each treatment raised or lowered this percentage. Table 2 displays the normalized percentage in the column next to the number of cells. This method also allowed the data to be averaged between the three treatments and the standard deviation between the percentages to be calculated.

Table 2: Cell Count Data Normalized to Percentages of Control

	Trial 1	**Trial 2**	**Trial 3**	**AVG**	**St Dev**
No Add Media	100%	100%	100%	100%	0%
Methanol + DMSO	58.60%	89.80%	97.66%	82.02%	20.66%
Estrogen	123.60%	130.07%	113.67%	122.45%	8.26%
Promensil	61.40%	53.05%	63.67%	59.37%	5.59%
Estrogen MPP	74.20%	83.70%	87.34%	87.95%	6.78%
Promensil MPP	93%	48.43%	73.63%	72%	22.35%

The normalized data allows for the trends seen in each cell count trial to be more visible. Expressing each trial as a percentage of cells compared to their own control allows for each trial to be better compared to each other. The results from the three trials as described in Trial 1 at the plating density of 2 x 10^5 cells had more variable data, which can be seen when comparing to the data obtained from trials 2 and 3 at 1.5 x 10^5 cells per well. The variability in the data is represented by the standard deviation values shown in the table. The methanol, Promensil, and MPP treatments had the highest standard deviations at 20% and 22%, respectively. Because the data for each trial was adjusted to the no add control, the percentages for those treatments was 100%, with 0% standard deviation. The estrogen treatments had percentages of 122%, which is above the baseline 100% from the no add controls. Additionally, this data had a smaller standard deviation of 8%. When the receptor was blocked by MPP, this increase was not seen, with the percentage of cells being around 88%, and a standard deviation of 7%. For Promensil, a decrease of cells in relation to the control was seen with 60% of the cells being present, with a small standard deviation of 6%. This decrease was also seen with ER-α antagonized with the percentage of cells being 72% but having a high standard deviation around 20%. The results from this table are displayed below on the bar graph in Figure 4.

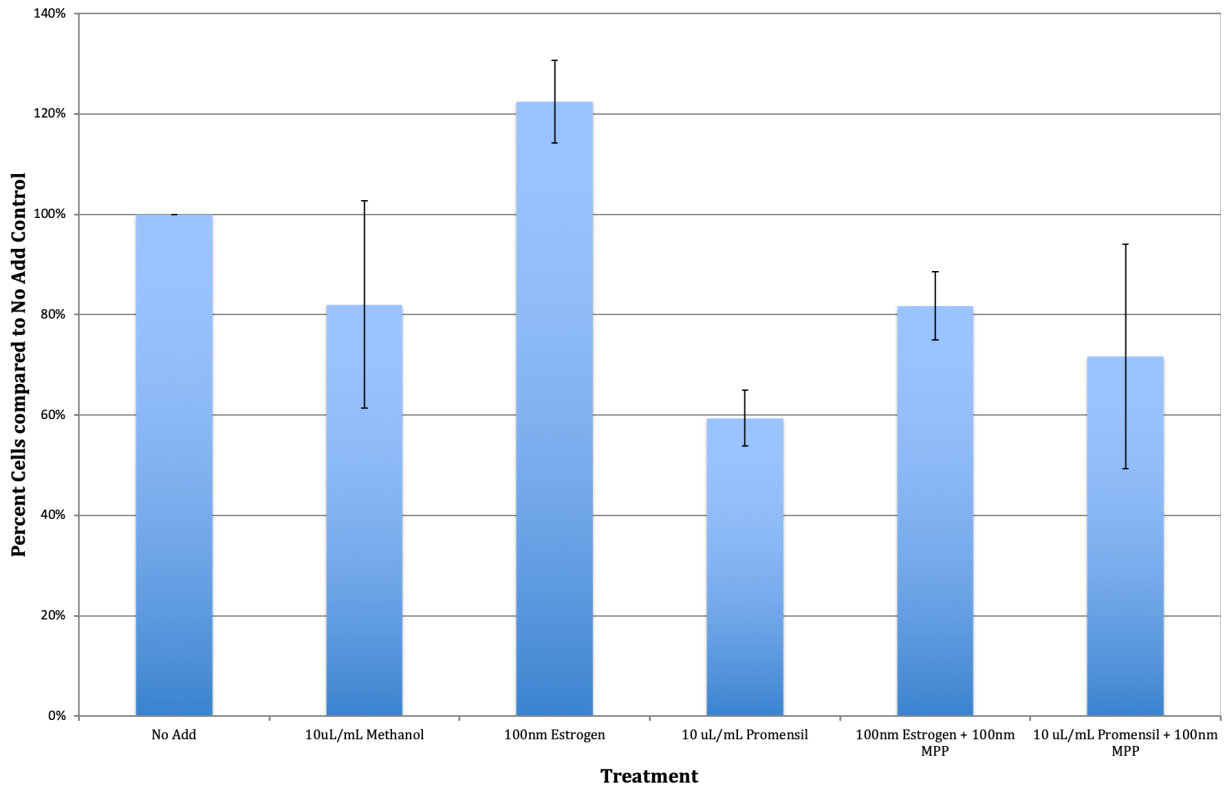

Figure 4: Effects of Estrogen and Promensil treatments on T47D cell growth with and without the presence of MPP.

Figure 4 shows the graph of each T47D cell treatment as a percent of the no add control. Based on the data, Promensil at a volume of 10 uL/mL shows the most optimal anti-proliferative effects for T47D cells. The cells treated with 100 nm β-estradiol showed a significant increase in cellular proliferation, yielding a percentage increase of nearly 125% of the control. This was expected, based on research that has shown the effects of β-estradiol on cellular proliferation (Suba, 2013). Cells treated with 100 nm β-estradiol + 100 nm MPP, showed no significant increase over methanol treated controls. This is also consistent with the activity of MPP as an ER-α antagonist. Cells treated with Promensil alone showed the anticipated decrease in proliferation compared to controls and the addition of MPP did not significantly change this effect. These results imply that although Promensil does act to decrease cellular proliferation, it does not do so working through estrogen receptor alpha. If Promensil did bind to estrogen

receptor alpha to reduce cellular proliferation, when the MPP + Promensil was added to the T47D cells, a significant increase in cellular proliferation would have been observed, yielding in a higher percent control on the graph shown in Figure 4. This would be due to the fact that when estrogen receptor alpha is blocked, Promensil would not have the same anti-proliferative effects as when the receptor is not blocked, resulting in a large increase in cellular proliferation. Therefore, there is most likely another underlying mechanism other than both ER-α and ER-β that is causing the reduction of cellular proliferation in T47D cells (Wambach, 2018).

Overall, the results found from this project led us to reject the initial hypothesis; although Promensil effectively showed reduced cellular proliferation in T47D cells, there was no change in anti-proliferative effects in the presence of an ER-α antagonist. Although the results did not correlate with the initial hypothesis, the data collected can conclude that based on previous findings combined with this project, Promensil at 10 uL/mL does not function through either the ER-α or ER-β of T47D cells, and works through another receptor, or another mechanism all together, to decrease cellular proliferation.

Recommendations

Though the results of this study inferred potentially beneficial data for projects moving forward involving Promensil and the relationship between cellular proliferation and ER-α in T47D cells, the data needs to be reproduced in order to confirm the potential findings. Based on previous MQP research projects involving Promensil and cellular proliferation regarding ER-β, and the results that were obtained from this project, the mechanism that Promensil uses to reduce cellular proliferation is most likely not through either estrogen receptor alpha or beta. For future projects, it may be appropriate to look at cells both with and without estrogen receptors, and note their interaction with phytoestrogen Promensil, which would give more insight on if Promensil acts through receptors at all to promote anti-proliferative effects. It may be interesting to assess the number of dead cells per well in addition to the alive cells by saving the wash steps before trypsinization. Quantifying the cell death associated with each treatment could help assess the cytotoxicity of a specific compound. Because Promensil is an unregulated supplement and is not yet approved by the FDA, it may be beneficial to observe the different components of Promensil that are both phytoestrogenic and non-phytoestrogenic in order to determine their impact when working through estrogen receptors in T47D breast cancer cells.

Overall, the results that have been obtained from this project have provided further insight to the ongoing Phytoestrogen Project that has been facilitated by Biology/Biotechnology department at WPI. The results that are yielded every year further progress the knowledge of what can be learned about these phytoestrogen supplements, especially their effects on a female women's body. The results from these projects help to shed light on the diversity of the components in Phytoestrogens, and may potentially lead to understanding the mechanism in which they function.

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