Effect of Promensil on T47D Breast Cancer Cells with respect to Estrogen Receptors Alpha and Beta

A Major Qualifying Project Report
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Abstract

Promensil, an OTC treatment for menopausal symptoms, is known to reduce breast cancer cell number in vitro. Studies have also shown that phytoestrogens found in Promensil bind to estrogen receptors (ERs). This study investigates whether the reduction in cell number is due to phytoestrogen binding to ER. Using T47D-KBluc cells, a luciferase assay was used to see if phytoestrogens in Promensil bind to ERs. The effect of receptor antagonists on cell number was also investigated. Interestingly, at doses that decrease cell numbers, phytoestrogens did not appear to bind to ERs. Cell numbers also decreased when ER binding was inhibited. These data suggest that changes in cell number are not mediated through ERs. Further study may show whether proliferation rates decreased or apoptosis increased.

Introduction

Breast Cancer

Breast cancer is the proliferation of cancer cells in the breast tissue. It is the second most common cancer in women in the United States, and the second most common cause of cancer related deaths in women (DeSantis, 2019). About 245,000 women and 2,200 men are diagnosed with breast cancer each year in the United States, with 41,000 women and 460 men dying as a result. Alarmingly, the rate of breast cancers has risen for Black, Asian, and Pacific Islander women. There are many factors that may increase the risk of developing breast cancer, such as genetic mutations, early estrogen exposure, and late menopause. An extended menstrual cycle exposes women to estrogen for longer, which is thought to be correlated with breast cancer (Dall, 2017). This would mean that the timing of exposure to estrogen is vital in assessing the risk of cancer development. Genetic mutations in genes that produce tumor suppressor proteins, such as BRCA1 and BRCA2, may inhibit the production of such proteins. ("BRCA1 Gene," 2019).

Breast cancer cells often contain estrogen receptors (ER), making them ER-positive. Over 50% of breast cancer cells express ER-α, which is known to increase cell proliferation when bound to estrogen (Ali, 2000). The presence of estrogen receptors opens the option for anti-estrogen therapies.

Estrogen

Estrogen is a general term for the group of hormones that control the development and maintenance of female sex characteristics. There are several naturally occurring estrogens in the body, including estrone, estriol, and estradiol. Estradiol, which is also known as 17-β-estradiol is

an extremely potent steroid and is the primary female sex hormone ("Estradiol," 2005). The structure of 17-β-estradiol can be seen in Figure 1.

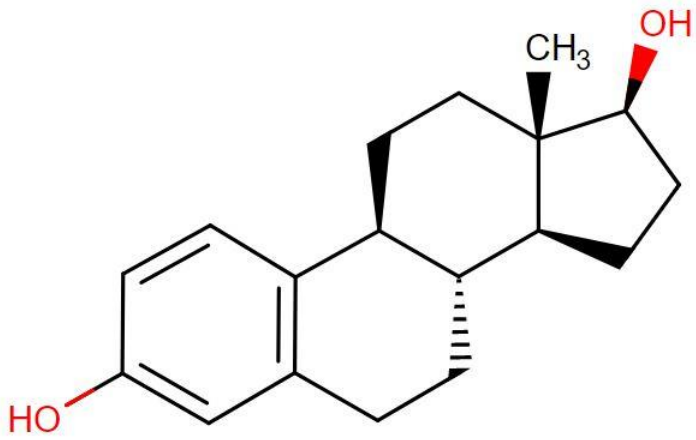


Figure 1: Structure of 17β-estradiol ("Estradiol," 2005).

17β-estradiol plays a major role in the regulation of the menstrual cycle and secondary sex characteristics ("Estradiol," 2005). This regulation is mediated by the hormone's ability to bind to estrogen receptors. It has also been found to contribute to the proliferation and apoptosis suppression of ER-positive MCF-7 human breast cancer cells through regulation of a large gene network (Frasor et al, 2003).

Estrogen Receptors

Estrogen receptors (ER) are located in the cytosol of many different cell types, including reproductive tissues, breast cells, and central nervous system cells (Jia, 2015). When an ER is bound by a ligand, it is translocated from the cytosol to the nucleus, where it binds estrogen

response elements (ERE) that then activate the transcription of specific genes. There are two different estrogen receptors, ER-α and ER-β. Both receptors are able to bind 17-β-estradiol, selective estrogen receptor modulators (SERM), and other estrogen mimicking molecules (Dutertre, 2000). These receptors can bind the same ligands but convey different effects on cell proliferation (Harris, 2005).

ER-α has been shown to increase cell proliferation and tumorigenesis in breast cancer cells (Dickson, 2004). Multiple studies with breast cancer cell lines, MCF-7 and T47D, have shown that ER-α activates the transcription of genes involved in cell proliferation through binding to an ERE (Lin, 2004) (Hayashi, 2003). While ER-α has a well-defined function, ER-β's function is not yet completely understood as it has been shown to have varying effects on cell proliferation.

Some studies have shown that ER-β decreases cell proliferation by inhibiting ER-α's ability to bind an ERE. One study looked at T47D cells with an inserted Tet-off regulated ER-β gene (Williams, 2008). Here they showed that the presence of ER-β repressed genes that were previously activated by the binding of ER-α to an ERE. In another study with MCF-7 cells, the authors found that the presence of ER-β bound to ligands caused a similar anti-proliferative effect (Harris, 2005). These results indicate that ER-β has an antagonistic function against ER-α. However, in some other cases, ER-β has also been reported to increase cell proliferation. In the same study by Hooper, when ER-β was not bound to a ligand, and was present in the cell, the authors reported an increase in transcription of estrogen-mediated genes. This could be due to ER-β allowing for the transcription of genes repressed by ER-α when active (Lu, 2017).

Menopause

Menopause is the permanent cessation of menstruation as a result of low levels of estrogen and progesterone produced by the ovaries. This condition is typically characterized by hot flashes, mood swings, and irregular to non-existent periods ("Relief for menopause symptoms," 2018). Despite being a normal part of a woman's life, occurring on average at the age of 52 for women in the United States, the condition does come with risks. These risks include an increased risk for heart disease, stroke, and osteoporosis as a result of changing estrogen levels ("Menopause basics", 2019). One possible treatment used to prevent these symptoms is hormone replacement therapy (HRT). HRT administers artificial estrogen and progesterone to the body. In 2002, HRT was shown to increase the risk of developing breast cancer, which has led to the significant reduction in use of this treatment ("Using HRT", 2017). As a result of these findings, safer alternatives to HRT were developed, with one of these alternatives being phytoestrogen supplements. The potential benefits and risks of phytoestrogen supplements have not been fully investigated; therefore, their overall effectiveness remains unknown (Moreira, 2014).

Phytoestrogens

Phytoestrogens are plant derived compounds that are structurally and functionally similar to 17-β-estradiol. There are four different classes of phytoestrogens: isoflavones, lignans, coumestans, and stilbenes. Isoflavones, found in soy, are the most common phytoestrogens with genistein and daidzein being the most studied. Lignans are the type most commonly found in plants. Coumestans and stilbenes are the least well-studied classes (Moreira et al, 2014). The structure of a phytoestrogen includes a phenolic ring with two opposing hydroxyl groups on either end of the molecule, as shown below in Figure 2.

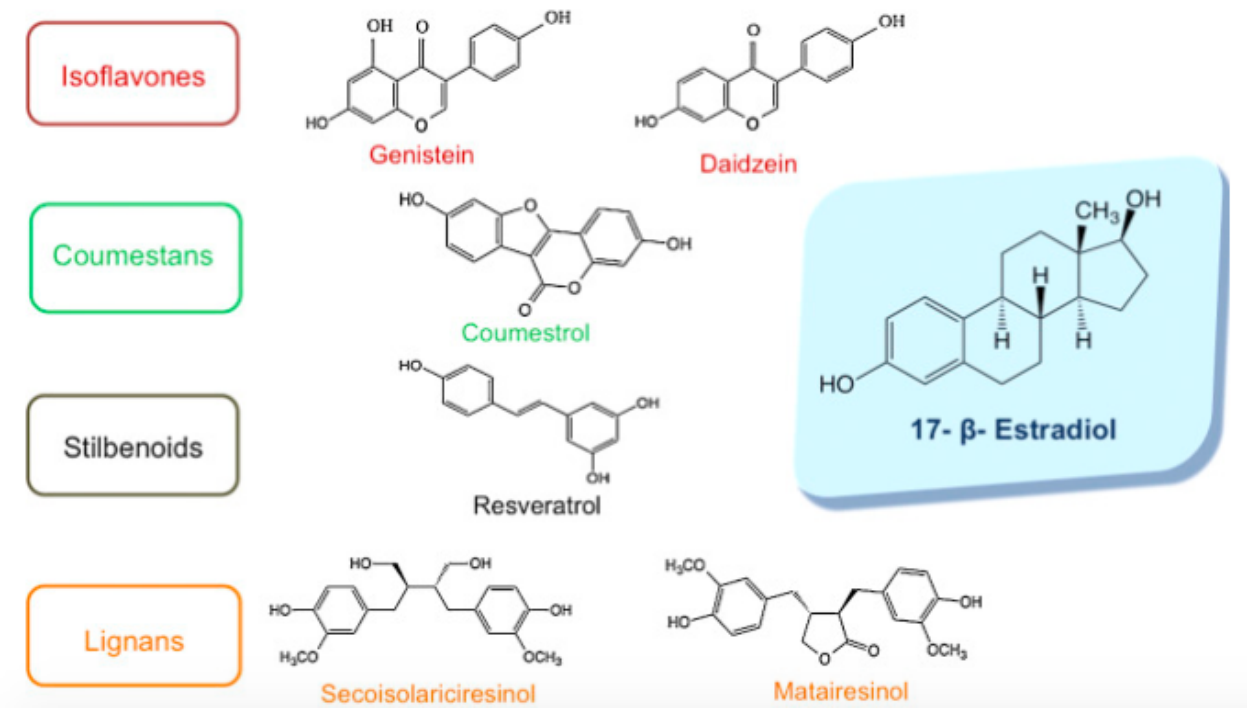


Figure 2: Structures of Common Phytoestrogens (Moreira et al, 2014)

Because of their structural similarity to 17-β-estradiol, phytoestrogens are able to bind to ERs. Of the two types of ERs, ER-α and ER-β, phytoestrogens are able to bind to both, but have a greater affinity for ER-β (Barone et al, 2008)(Kuiper, 1998). As previously stated, ER-β binding has been shown to inhibit cell growth (Moreira et al, 2014). Therefore, the replacement of estrogen with phytoestrogens could result in phytoestrogen binding to ER-β and the inhibition of cancer cell proliferation.

Promensil

An example of an alternative treatment for menopausal symptoms is the Promensil supplement. Promensil is made from the red clover plant and contains a number of chemicals including genistein, daidzein, biochanin A, and formononetin; all classified as isoflavones (“Red Clover”).

The website for Promensil claims that the isoflavones bind to the ER-β receptor as opposed to the ER-α receptor, resulting in a decrease in menopausal symptoms (Tice, 2003). In 2017, a meta-analysis was performed on several research studies that each assessed Promensil's functionality in treating menopausal hot flashes (Myers, 2017). The study found there to be a statistically significant decrease in hot flashes with Promensil when compared to a placebo.

In addition, Promensil was also tested for its effects on breast cancer cells at Worcester Polytechnic Institute (Gergel, 2010). In the study, it was found that Promensil did have an anti-proliferative effect against T47D breast cancer cells. Promensil was selected for this study to further investigate the role of phytoestrogens in Promensil's antiproliferative effect against breast cancer cells. While the effects of Promensil are known, there have not been extensive studies into how it decreases cell proliferation. Additionally, in some cases phytoestrogens have been shown to sometimes increase proliferation *in vitro*, thus adding to the difficulty in understanding the efficacy and importance of Promensil treatment in decreasing the risk for breast cancer (Bouker, 2000).

Covering Past Research

This study is a continuation of a series of studies focused on the effects of Promensil and the phytoestrogens contained within it on breast cancer cell proliferation. Previously the Project Lab at Worcester Polytechnic Institute (WPI) discovered that Promensil does have anti-proliferative effects when used to treat T47D cells (Gergel, 2010). For years, the lab has hypothesized that the estrogen imitation effect of phytoestrogens caused this anti-proliferative effect. Recently, a study using T47D cells with a tetracycline dependent ER-β receptor found that the absence of ER-β has no effect on the anti-proliferative status of Promensil (Wambach, 2018). Additionally, a similar

study suggests that the absence of ER-α also does not affect the anti-proliferative effects of Promensil (Crosby, 2019). As Promensil is made up of crushed leaves from the red clover plant, there could be another component within the plant that creates the anti-proliferative effect. Though these findings together would suggest that Promensil may not be acting exclusively through the estrogen receptors to decrease cell proliferation, the experiment performed in the Crosby paper was only done once and therefore may not be reliable.

T47D and T47D-KBluc Cell Lines

The T47D cell line was established in the 1970s from a 54-year old female patient with infiltrating ductal carcinoma breast cancer (Horwitz, 1978). T47D cells produce both ER-α and ER-β, making them ideal for this study. The T47D-KBluc cell line was created in 2004 to assess ER activity (Wilson, V. S., & Bobseine, K. 2004). This cell line has been transfected with a plasmid containing three EREs upstream of a luciferase gene. When an estrogen receptor, either ER-α or ER-β are bound by a ligand, they translocate to the nucleus and bind to the ERE DNA sequence. When this occurs in the T47D-KBluc cell line, both receptor isoforms bind to an ERE that induces transcription of the luciferase gene. Because of this, the T47D-KBluc cell line can be used to identify binding to either estrogen receptor. With the addition of luciferin to the cells, the production of the luciferase enzyme can be assessed, and therefore suggest if an ERE is being bound. This study utilizes both T47D and T47D-KBluc cell lines.

Hypothesis

While previous studies at WPI have shown that the absence of ER-β or ER-α respectively did not impact the anti-proliferative effect of Promensil, it is a known fact from other studies that phytoestrogens, a key component of Promensil, bind to both estrogen receptors. Additionally, the study done on the inhibition of ER-α was not definitive, and may be the source of Promensil's effect on breast cancer cells. Due to this, it is hypothesized that the anti-proliferative effect induced by Promensil is due to phytoestrogens binding to the ERs.

Materials and Methods

Extraction of Promensil

Double strength Promensil was obtained. The lot number of the tablets obtained was 18943A. The double strength tablets contain 80 mg of red clover isoflavones, according to the box. Three tablets, which equates to one daily dose, according to the manufacturer, were ground up with a mortar and pestle. Promensil was added to a round bottom flask filled with 100ml of 80% methanol in water. This was refluxed for an hour at 70°C with a reflux condenser. The solution was filtered through a filter paper funnel attached to a vacuum. The solution was stored at -20°C (Setchell et al., 2001).

Cell Maintenance

The T47D-KBluc cells were obtained from the American Type Culture Collection (ATCC). After thawing, the cells were kept in T75 flasks in regular growth media (89% DMEM, 10% Fetal Bovine Serum (FBS), 1% PenStrep (5000 units penicillin, 5mg/ml streptomycin, Thermo Fisher))in a humidified 37°C incubator at 5% CO_2 atmosphere. To prevent hyper-confluency cells were split every 2-4 days when the population became at least 75% confluent to dilute the population density.

Cell Counting

Prior to cell counts being performed, cells were washed with PBS and aspirated. Next they were trypsinized and removed from the flask and put into a centrifuge tube with 5ml of regular growth media. They were then pelleted by low speed centrifugation, and resuspended with 5ml of regular growth media. A sample of the cells from the tube was combined with an equal volume

of 0.4% Trypan Blue Solution (ThermoFisher Scientific) and inserted into a counting chamber and using the Cellometer Auto T4 Cell Counter from Nexcelom concentration of live cells per volume was determined.

Luciferase Assay

T47D-KBluc cells were plated in black, clear bottom 96-well plates in varying numbers of cells from $1.0x10^4$ to $5.0x10^4$ with additional regular growth media until 100ul was in each well. The plate was incubated for 24 hours at 37°C. The regular growth media was aspirated, washed with phosphate buffered saline (PBS), then the well received 100ul of stripped media. The cells were treated with Promensil and/or 0.1nM 17-β-estradiol for 24 hours at 37°C. The wells were aspirated and received 100ul of Luciferase Assay Reagent (Sigma Aldrich). The luminescence was recorded using a Victor3^{TM} plate reader with the excitation lamp blocked and the emission window wide open immediately after the addition of the Luciferase Assay Reagent.

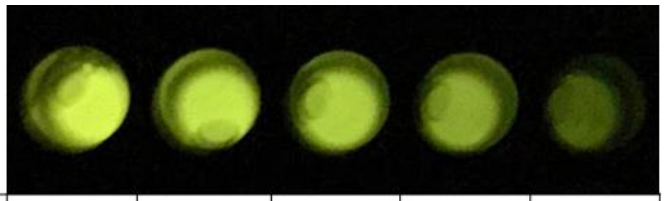
MTT Assay

T47D-KBluc or T47D cells were plated in a 96-well plate with concentrations between $2.0x10^4$ to $5.0x10^4$ cells per well with additional regular growth media until 100ul was in each well. The plate was incubated for 24 or 48 hours at 37°C. Regular growth media was switched for 100ul of stripped media (phenol red-free DMEM with 10% dextran-coated charcoal stripped FBS) then incubated for 24 hours at 37°C. Treatments of 17-β-estradiol (1.0nM), undiluted Promensil, ER-β antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo [1,5-a]-pyrimidin-3-yl]phenol (PHTPP) and/or ER-α antagonist methyl-piperidino-pyrazole (MPP), both at a concentration of 100nM, were added and incubated at 37°C for 24 hours. The cells were treated with 20ul of

CellTiter 96® Aqueous One Solution Reagent (Promega) for 4 hours at 37°C, then read using an automated plate reader at an absorbance of 595nm.

Results and Discussion

Preliminary luciferase assays were performed to confirm the functionality of the Victor3^{TM} plate reader in reading luminescence. With the luminescence confirmation, further experiments could be performed with the Promensil and 17-β-estradiol (estrogen) control treatments to provide evidence for the hypothesis that Promensil is decreasing proliferation rates through a mode other than binding either estrogen receptor (ER-α or ER-β).



Dilution	1:1	1:2	1:5	1:10	1:100
Luminescence	4.39×10^7	4.27×10^7	4.17×10^7	3.75×10^7	2.31×10^7

Figure 4: Visible luminescence from varying concentrations of diluted luciferase with the addition of the luciferin substrate.

Figure 4 displays visible luminescence. The five wells have varying dilutions of luciferase that received a luciferin substrate, from a 1:1 to 1:100 dilution. The luciferin is oxidized and catalyzed by the luciferase enzyme, producing a glow. The three highest dilutions all displayed a similar level of luminescence, which tapered off with the last two more dilute samples. As these samples were done with high concentrations of luciferase, their luminescence values are substantially higher than samples in which cells were producing luciferase, seen in Figures 5 and 6. It is seen that the change in luminescence is not linear, possibly due to the limitations of the luminometer in detecting concentrations above 1:5 dilution range. More intermediate data points would be required to determine an R value. This strong luminescence was read in the Victor3^{TM}

plate reader and produced luminescence values that ensured the device could identify and quantify luminescence that was clearly visible to the human eye.

After confirmation that the Victor3™ plate reader could read luminescence, a luciferase assay was performed with estrogen to confirm that the luminescence would differ between T47D-KBluc cells with bound EREs versus T47D-KBluc cells with unbound EREs.

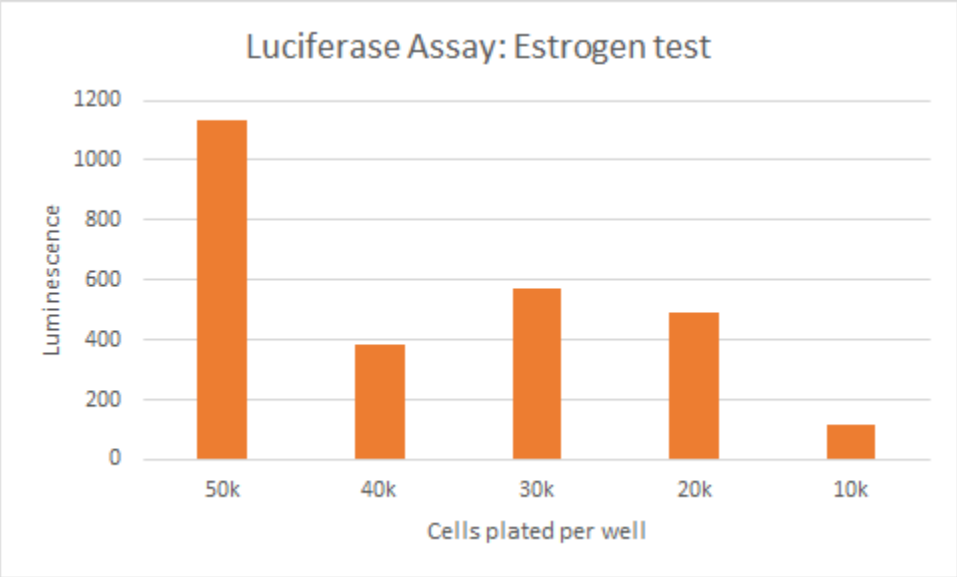


Figure 5: Luminescence readings from luciferase assay with varying cell numbers per well of T47D-KBluc cells with estrogen. Values were determined by subtracting the no treatment controls from each total.

Figure 5 displays the luminescence for varying numbers of cells treated with 0.1nM 17-β-estradiol (estrogen) and 100ul luciferin substrate. The luciferase enzyme activity produced by the luciferase assay with T47D-KBluc cells is indicative of ERE being bound. When the ERs of T47D-KBluc cells are bound by estrogen, it was found that luminescence had increased by at least 37% when compared to untreated cells, and some samples had luminescence reading

greater than 200% higher than untreated. The figure displays a trend of increasing luminescence when an increasing number of cells are plated with the exception of the luminescence reading for $4.0x10^4$ cells. This may be a result of a pipetting error as only 1ul of 0.1nM 17-β-estradiol was added to each well. It would be beneficial to dilute the 0.1nM 17-β-estradiol to ensure a greater volume is added for future experiments. Overall, this data can confirm that estrogen can be utilized to induce ERE activation and increase luminescence, thus serving as a basis comparison when performing luciferase assays with phytoestrogens from Promensil.

The luciferase assay conducted in Figure 6 used Promensil and 17-β-estradiol (estrogen) treatments separately to evaluate if phytoestrogens from Promensil are binding to an ER.

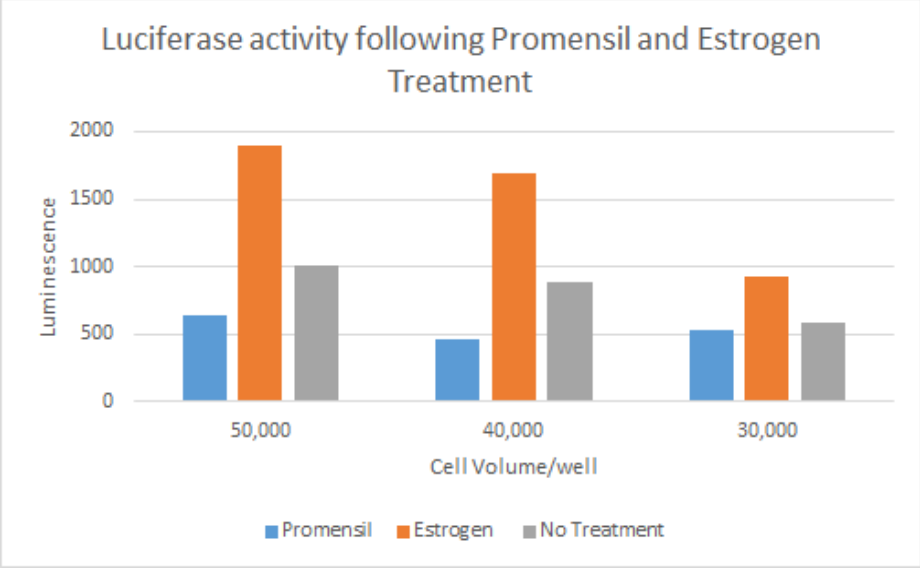


Figure 6: The luciferase assay data displayed by luminescence produced by treatments of Promensil, estrogen, and a control on T47D-KBluc cells at varying counts per well. n=3.

Figure 6 displays the average luminescence of T47D-KBluc cells with the addition of Promensil, estrogen, or no treatment. Each treatment was replicated three times and averaged. These cells naturally give off background luminescence, explaining the data seen with cells that received no

treatment. The data in Figure 6 appear to show that treatment with Promensil slightly decreased luminescence values on average compared to cells with no treatment, but this was unlikely to be a significant change, and having done the assay only once, no statistical analysis was possible. The T47D-KBluc cell line was engineered so that when a ligand binds to either estrogen receptor, an ERE controlling luciferase transcription would be activated. This suggests that when treated with a dose of Promensil, which has been shown to decrease cell numbers, neither ER in the T47D-KBluc cells are being bound at a sufficient level to activate luciferase production. By comparison, cells treated with estrogen showed luminescence values 79% higher on average than cells with no treatment, which correlated with the range seen during the estrogen test in Figure 5.

Cell metabolic activity of T47D cells with different treatments (Promensil, estrogen, or no treatment) combined with different estrogen receptor antagonists (MPP, PHTPP, neither, or both) was measured via an MTT assay. This assay was utilized to determine the proliferation rate of the T47D cells under differing conditions, and will provide evidence on whether T47D cell proliferation decreases or increases when plated with Promensil. Figure 7 graphs the absorbance produced by the MTT reagent being metabolized by the T47D cells. This can be translated to proliferation of cells plated with either Promensil, estrogen, or no treatment, in addition to being plated with MPP, PHTPP, neither, or both. This trial included three experimental replicates for each combination of treatment and antagonist.

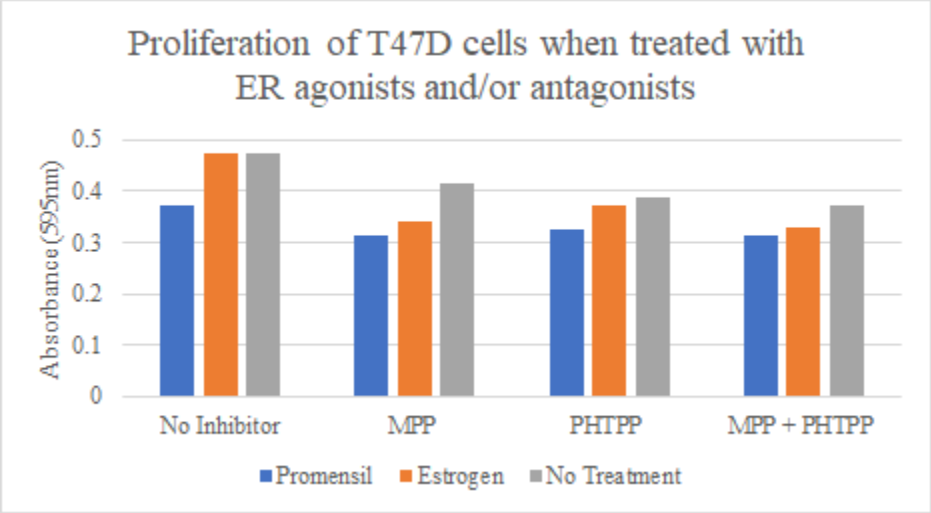


Figure 7: Absorbance (595nm) produced by the MTT reaction in T47D cells when plated with Promensil, estrogen, or no treatment, and in combination with MPP, PHTPP, or MPP and PHTPP.

T47D cells plated with Promensil and no antagonists have a much lower absorbance value than the cells plated with estrogen or no treatment and no antagonists. This may be interpreted as a decrease in cell number due to the evident decrease in cell activity. Cells treated with Promensil consistently exhibited lower cell activity than cells with no treatment across all antagonist treatments. Most notably, when both receptors are inhibited, it can be seen that Promensil still has a slight decrease in cell activity. This could suggest that Promensil may be inhibiting cell proliferation through other means besides the estrogen receptors.

However, as the MTT assay measures only cell activity, it is also possible that cell death increased with the addition of Promensil. Either way, the mechanism seems to not be ER dependent because the effect is seen when both ER receptors are antagonized. Additionally, it has been shown that estrogen binding to ER-α increases proliferation. The results seem to show,

although not it is not statistically significant, that when ER-α is antagonized, the cell activity appears to decrease as expected.

Overall, the preliminary results reject the hypothesis that the decrease in proliferation of T47D cells with Promensil was due to phytoestrogens binding to the estrogen receptors in T47D cells. However, it is vital to note these experiments were only conducted once and are therefore not statistically significant. Both luciferase binding and the MTT assay would have to be repeated across multiple experiments with consistent data to hold statistical significance to fully support our hypothesis. Additionally, the data would be more reliable if the cells that did not receive either Promensil or estrogen did not decrease in activity with the addition of the antagonists, as Figure 7 suggests that the antagonists themselves may increase cell death.

Part 2: Future Methodology and Anticipated Results

Revision of Hypothesis

Due to the preliminary results from the luminescence and MTT assays, the hypothesis was tentatively rejected in Part 1. Therefore, it is now hypothesized that the decrease in cell numbers when Promensil is added is not a result of the phytoestrogens acting through the ERs, and that the mechanism is not ER dependent.

Proposed Methods

MTT Assay

A repetition of the MTT assay with the combination of treatments (Promensil, estrogen, or no treatment) and estrogen receptor antagonists (MPP and/or PHTPP) would have been performed. This was originally performed once with three experimental replicates to account for measurement error, but additional runs would provide more data, and potentially allow calculation of statistical significance.

T47D-KBluc or T47D cells would be plated in a 96-well plate with concentrations of $3.0x10^4$ cells per well with additional regular growth media until 100ul was in each well. The plate would be incubated for 24 hours at 37°C. Regular growth media would be switched for 100ul of stripped media (phenol red-free DMEM with 10% dextran-coated charcoal stripped FBS) then incubated for 24 hours at 37°C. Treatments of 17-β-estradiol (1.0nM) or undiluted Promensil with ER-α antagonist methyl-piperidino-pyrazole (MPP) and/or ER-β antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo [1,5-a]-pyrimidin-3-yl]phenol (PHTPP), both at a concentration

of 100nM, would be added and incubated at 37°C for 24 hours. The cells would be treated with 20ul of CellTiter 96® Aqueous One Solution Reagent for at least 1 hour at 37°C, then read using an automated plate reader at an absorbance of 595nm.

Immunoblot

An immunoblot would be performed to determine the relative content of specific proteins present when T47D cells are combined with treatments of Promensil or estrogen, with MPP and/or PHTPP. This could be indicative of whether T47D cells are decreasing in proliferation or dying via apoptosis.

T47D cells would be counted with a Cellometer Auto T4 Cell Counter from Nexcelom. In a 6-well plate, $2.0x10^5$ cells would be plated and allowed to incubate at 37°C for approximately 6 hours. After incubation, treatments of Promensil or 17-β-estradiol, with MPP and/or PHTPP would be added to the appropriate wells. The plate would be incubated at 37°C for approximately 24 hours. After incubation, a rubber policeman would be used to scrape the cells off the wells after being washed twice with PBS. A protein assay would be performed to quantify the amount of total cell protein in the samples obtained from the wells. The samples would be added into separate tubes with 5ul of Blue Protein Loading Dye (New England Biolabs) and heated to denature the proteins for 10 minutes at 80-90°C. Equal amounts of total protein for each sample would be added to the wells of a Mini-PROTEAN 12% TGX Precast Gel along with the Color Prestained Protein Standard Broad Range (10-250 kDa) ladder (New England BioLabs). The gel would be run for 30 minutes at 200V to separate the proteins in each sample. A semi-dry transfer would be performed with an Immobilon membrane at 60 mA for

approximately 50 minutes. After, the membrane would be placed in a blocking solution (5% non-fat dry milk in Tris Buffered Saline) at room temperature for 30-60 minutes. The blocking solution would be replaced with primary antibody for p21, CDK4, and caspase 3 at the manufacturer's designated concentrations, then placed back in the 4°C refrigerator overnight. The primary antibody would be removed. The membrane would be washed three times with 1X phosphate buffered saline +0,1% Teen20 (PBS-T) with gentle shaking for five minutes between each wash. Then, the secondary antibody, conjugated to alkaline phosphatase, would be poured onto the membrane and gently shaken for 30-40 minutes at room temperature. The membrane would be washed three times with 1X PBST and gently shaken between each wash for five minutes. The membrane would be developed using one tablet SIGMAFAST BCIP/NBT dissolved in 10ml distilled water and then imaged with a Biorad ChemiDoc Imaging System. The results of the imaging would then be analyzed using Image J software to compare the band densities among the samples

Anticipated Results and Conclusions

The MTT activity assay performed in Part 1 provided promising results in regard to the anti-proliferative effect of Promensil. It was found that treatment with Promensil appeared to lower cell number compared to no treatment, even in the presence of receptor antagonists. Although this experiment was only conducted once, the data trends are consistent with those seen in prior MQPs (Wambach, 2018, Crosby, 2019). The first course of action would be to repeat this experiment a sufficient number of times to determine the statistical significance of those trends. In our initial experiment, it was seen that cells treated with estrogen either had the same or lower cell numbers than cells with no treatment. This was unexpected and deserves further evaluation.

Based on numerous previous reports, we anticipate that cells treated with estrogen would have increased cell numbers.

Our data and those of previous MQP teams indicated Promensil treatment resulted in a decrease of cell number. While it was initially thought that this was due to a decrease in cell proliferation, mediated through an estrogen receptor, the data shown here and in other MQP reports combined, suggest this may not be the case. There may be a decrease in cell proliferation but not mediated through an estrogen receptor or it may also be caused by the induction of apoptosis. To further investigate these possibilities, an immunoblot will be employed to determine changes in cell cycle checkpoint regulation (CDK4 and p21) and apoptosis (caspase 3) controlling proteins. The levels of each of these proteins in Promensil treated relative to differently treated or untreated cells will help to determine the cause of the examined decrease in cell number.

In examining the results of this potential immunoblot, there are two theoretical possibilities that could be seen. The first is a scenario where proliferation-controlling proteins are the cause of the decreased cell number. In this case, CDK4 would be downregulated, and p21 would be upregulated, which would be evident in band size and density compared to the control. The second scenario is that apoptosis is the cause, in which case caspase 3 would be upregulated, displaying a larger and denser band than in the control. Once these differences are identified, the impact of the ER antagonists can be tested to validate that any differences seen are not ER mediated.

The finding that antagonizing both estrogen receptors had no impact on the effects of Promensil on cell numbers was unexpected. The additional data suggesting that no ligand-ER-ERE binding was evident at doses where Promensil was effective at decreasing cell numbers further implies that the effect on cell number is not ER mediated. We regret being unable to further investigate the other possibilities suggested here to explain those data and are hopeful future MQP teams will further elucidate the mechanism of action of Promensil on reductions in breast cancer cell numbers.

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