Effect of Promensil on Breast Cancer Cells in regard to the Estrogen Receptor Beta

A Major Qualifying Project Report
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Abstract

Promensil is an over-the-counter phytoestrogen supplement, originally intended to treat menopause, which has been shown to decrease breast cancer proliferation. This project examined this effect of Promensil, based on the estrogen receptor beta (ER-β). Using T47D cells with a tetracycline tunable ER-β, a decrease in cell proliferation was observed, but with no correlation to ER-β. Rather, Promensil appeared to compete with 17β-estradiol for ER-α. For the future, the correlation between ER-α and the phytoestrogen components of Promensil should be studied.

Acknowledgements

Thank you to Professors Jill Rulfs and Mike Buckholt for advising this project, and to Dr. Anders Strom at the University of Houston for supplying the cells necessary to carry out this project.

Background

Introduction

Breast cancer is a disease which impacts a substantial portion of society and has been a focus of scientific study for many years. Estrogen has been shown to have a strongly correlated impact on many breast cancers. Phytoestrogens are plant compounds which mimic estrogen in the human body. These compounds have been manufactured into many supplements, claiming to provide natural benefits. However, these supplements are largely unregulated and may have unknown side effects, such as an effect on breast cancer. Contradicting data have shown both positive and negative effects on breast cancer due to various phytoestrogen compounds, in varying conditions. There are many pieces to understanding fully the impact of phytoestrogens. This project aimed to discover a small piece of this picture, in relation to breast cancer specifically.

Breast Cancer

Breast cancer has a strong impact on society and its study has been a large focus of the scientific and medical communities for many years. After lung cancer, breast cancer has the highest death rate among cancers in women. It is also the second most commonly diagnosed cancer among women in the United States. It is said that about 1 in 8 women in the U.S. will develop breast cancer in their lifetime (Breastcancer.org, 2017).

Breast cancers can be ER-positive, meaning that they express estrogen receptors, while others can be ER-negative if they do not express estrogen receptors. Over half of breast cancer cells express the ER-α receptor, which has been shown to promote the growth of breast cancer upon binding of endogenous estrogen (Ali & Coombes, 2000). The presence of these receptors also

determines which treatment options are available for the cancer, such as anti-estrogen therapy. For the purpose of this project, ER-positive breast cancer was focused on in order to study the effects of estrogen mimicking phytoestrogen compounds on these cancer cells.

Estrogen

Estrogen is a term used to describe the steroidal hormones which lead to the development of the female sex characteristics. The most abundant of these hormones is estradiol, or 17β-estradiol. This hormone is an aromatic C18 compound (National Center for Biotechnology Information, 2017). The structure of estradiol can be seen in Figure 1.

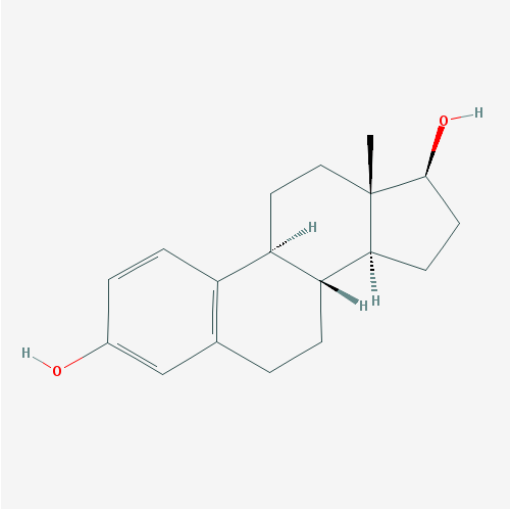

Figure 1: Estradiol structure (NCBI)

In its developmental function, estrogen serves to signal for the growth and development of the female reproductive organs. The hormone mainly affects the breasts and uterus; however it also binds receptors in the brain, bones, heart, and other parts of the body. Other functions of estrogen include bone growth, cholesterol regulation, and menstrual cycle control (Manson, 2017).

However, estrogen and progestin have also been shown to increase the size of breast cancers in postmenopausal women, suggesting that these hormones stimulate breast cancer cell proliferation (Chlebowski *et al*., 2003).

A common treatment for breast cancer is hormone therapy in which the amount of estrogen available in the body is decreased, or estrogen receptors are antagonized. When breast cancer cells have a larger quantity of estrogen receptors, a greater response to hormone therapy has been shown (Osborne *et al*., 1980). This shows the highly correlated effect between estrogen, its receptors, and breast cancer cell proliferation, therefore making the estrogen receptors an important aspect to study in regard to breast cancer.

Estrogen Receptors

Estrogen receptors (ER) are found on many cells of the body. These receptors are a part of the steroid receptor gene super-family. In response to the binding of estrogen, these ligand bound receptors translocate to the nucleus and bind to specific estrogen response elements (EREs) in the promoter regions of target genes to activate transcription (Benassayag *et al*., 2002).

There are two versions of the estrogen receptor, alpha and beta. These receptors are encoded by independent genes, on different chromosomes (Enmark, *et al*., 1997, NCBI, 2018). High affinity binding of 17β-estradiol to both receptor types has been shown (Dechering *et al*., 2000). However, these two receptors differ in their ligand binding domain, being only 53% homologous in this domain (Harris, 2005). This difference may explain why different estrogen mimicking

compounds produce different effects in regard to these receptors. The two receptors have shown varying effects on cell proliferation, specifically with breast cancer (Harris, 2005*).*

ER-α has been shown to promote cell proliferation. In one study of ER-α, transfected via plasmid into HeLa cells, transcription at a classical ERE was shown to be activated by the binding of 17β-estradiol to the receptor. In another study, ER-α was shown to be positively regulated by the MDM2 oncogene. When this oncogene was overexpressed in MCF-7 breast cancer cells, a cell growth advantage in the presence of estradiol was seen (Saji *et al*., 2001). Studies of ER-α estrogen response elements, by cDNA microarray of human MCF-7 breast cancer cells, have shown some of the target genes of ER-α to be involved in cell division, such as cyclin A1 and tryptophanyl1-tRNA synthetase (Hayashi, 2003).

ER-β, on the other hand has shown a more conflicting impact on proliferation in various studies. To begin, ER-β has been shown to have negative transactivation activity against ER-α. ER-β may play a role in inhibiting the binding of ER-α to its estrogen response element. This has been shown with the human ER-betacx isoform which forms a heterodimer with ER- α, therefore preventing the alpha receptor from binding the ERE (Ogawa, 1998). Another study looked at MCF7 breast cancer cells with an ER-β2 (beta estrogen receptor isoform) engineered to be inducible, and an endogenous ER-α. ER-β2 was shown to inhibit ER-α transactivation at the estrogen response element. ERβ2 expression also caused a degradation of ER-α by proteasomes (Zhao *et al*., 2007). This overall indicates that ER-β may play an opposite role to ER-α.

In one experiment, a complete inhibition of T47D cell proliferation was shown in response to estradiol when there were equal amounts of alpha and beta estrogen receptors present (Strom, 2004). In another study, MCF7 breast cancer cells were engineered with an adenovirus mediated ER- β along with normal ER- α expression. With only ER- α expressed, estradiol was shown to increase the breast cancer cell's proliferation in vitro. However, when ER- β was induced into the cells, this led to G_2 cell cycle arrest and therefore caused an anti-proliferative effect (Paruthiyil *et al*., 2004).

However, ER- β has also shown proliferative effects on cells. Tamoxifen is a selective estrogen receptor modulator (SERM), which mimics estrogen in order to block the binding of estrogen to the estrogen receptors. In HeLa cells transfected with ER-β and treated with tamoxifen, transcription was shown to be activated via a luciferase reporter gene under ERE control (Paech, 1997). This proves that the response of the estrogen receptor can vary and may depend on the ligand binding.

Phytoestrogens

Phytoestrogens are estrogen mimicking compounds which are derived from plants. Some common plant sources of these compounds are soy, legumes, and clover (Patisaul, 2017). The two most common classes of phytoestrogens are isoflavones and coumestans. Phytoestrogens have a polyphenolic structure similar to 17β-estradiol, as seen in Figure 2.

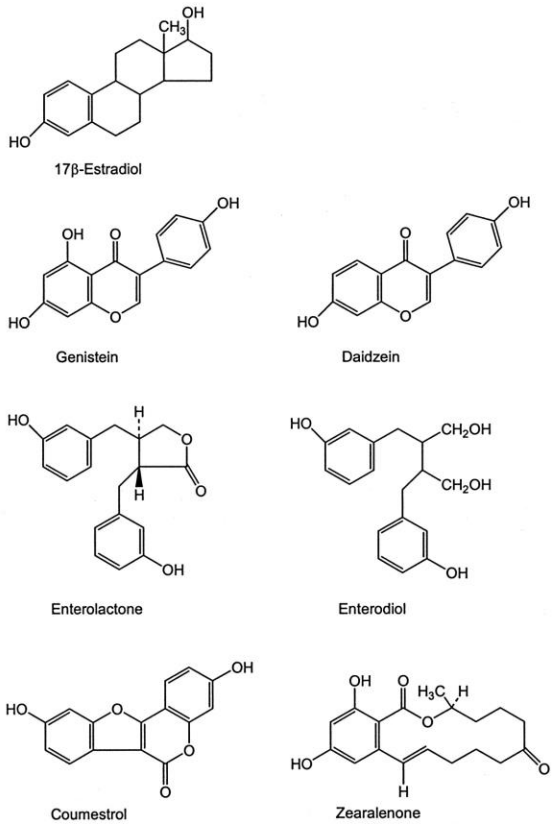

Figure 2: 17β-estradiol and some common phytoestrogen structures, covering the three major classes of isoflavones, lignans and coumestans (Belcher & Zsarnovszky, 2001)

Genistein and daidzein are two of the most common isoflavonoid phytoestrogen compounds, and are mostly found in soy and red clover. These compounds share the phenolic ring structure with estrogen, as mentioned above, but also have similarities in the distance between their hydroxyl groups, contributing to their ability to mimic estrogen. Genistein has shown both estrogenic and anti-estrogenic responses based on varying conditions, such as which receptor it binds or its concentration in the body. Genistein has been shown to bind both alpha and beta estrogen receptors, with a preferential binding to the beta receptor (Dixon, 2002). A diet high in soy, and therefore isoflavones, has been shown to produce many benefits, such as preventing various cancers, bone loss and cardiovascular diseases. Studies have proven a strong correlation between decreased rates of breast cancer in those who eat diets high in soy products (Lee *et al*., 1991).

Biochanin A is a closely related analog to genistein, simply being a methylated version of genistein (National Center for Biotechnology Information). Biochanin A is also found in red clover. This isoflavone has also been shown to be an inhibitor of cell growth in ER-positive breast cancers (Peterson *et al*., 1998)

Formononetin is another isoflavone from red clover, and is a methylated version of daidzein (Setchell *et al*., 2001) Formononetin has also been shown to induce cell cycle arrest in MCF-7 breast cancer cells both *in vitro* and *in vivo*, in mice models (Chen *et al*., 2011).

Due to the phytoestrogens' affinity to bind the estrogen receptor, these compounds are considered endocrine disruptors (Patisaul, 2017). It has been shown that most phytoestrogens bind preferably to the ER-β. Using *in vitro* synthesized rat estrogen receptors, a saturation ligand binding analysis showed genistein to have a higher binding affinity for ER-β over ER-α (Kuiper *et al*., 1997). Radioligand binding assays have shown stronger binding to ER-β for the phytoestrogens coumestrol and genistein (Kuiper *et al*., 1998).

Phytoestrogens are manufactured into many over the counter supplements, claiming various health effects. Benefits range from decreased menopausal symptoms to lowered risks of heart disease or cancer. These supplements, due to being derived from plants, are marketed as "natural" and therefore safe. However, this is not necessarily the case. Phytoestrogens have shown varying effects on breast cancer growth. It is unclear whether the phytoestrogens act as antagonists or agonists for the estrogen receptors. Different concentrations, combinations, and

other environmental factors also play a role in the effects, and therefore there is still a lot of unknowns regarding these supplements.

Menopause

Menopause occurs when a woman's ovaries no longer produce estrogen. This leads to common symptoms, such as hot flashes. There is also an increased risk of long term problems, such as osteoporosis and cardiovascular disease. To prevent these symptoms of menopause, some women turn to hormone replacement therapy (HRT) to replace the estrogen which is no longer being produced. However, these hormone replacement therapies have been shown to increase risk of breast cancer in women (Ho, 2003). Phytoestrogen supplements have been introduced as a possible alternative to hormone replacement therapy that may not have as high of a risk of breast cancer. By mimicking estrogen, these supplements claim to provide a natural method to alleviate the symptoms of menopause. Not many studies have been carried out on these unregulated, over the counter supplements. Those which have been done have shown varying effects. The actual increased or decreased risk of breast cancer due to these supplements remains relatively unknown.

Promensil

Promensil is one over the counter phytoestrogen supplement, marketed to decrease menopause symptoms. The Promensil supplement has been sold since 1997. According to the drug's website, the supplement contains four red clover isoflavones. The website claims these isoflavones only bind to beta estrogen receptors and not the alpha receptors (Promensil.com).

Label accuracy testing proved Promensil supplement to contain 40mg of phytoestrogens, consisting of genistein, daidzein, formononetin, and biochanin A. (Weijer *et al*., 2002).

An additional analysis by Setchell *et al*. used liquid chromatography along with mass spectrometry to compare the isoflavone content of various dietary phytoestrogen supplements to the manufacturers' labels. Their findings showed Promensil to contain 41.7mg of isoflavone per tablet. HPLC revealed the presence of primarily formononetin and biochanin A, along with daidzein and genistein (Setchell *et al*., 2001). This shows agreement with the manufacturer's claims.

This study by Setchell *et al*. continued with the oral administration of one Promensil tablet each to patients and the subsequent measurement of isoflavone levels in the blood plasma. Increases of genistein and daidzein concentrations in the blood were seen in much greater values than the concentrations of formononetin and biochanin A. This is most likely due to rapid de-methylation of biochanin A and formononetin by intestinal bacteria, producing genistein and daidzein, respectively, in the plasma (Setchell, *et al*., 2001)

To add to this, preliminary studies, with a limited number of trials, at Worcester Polytechnic Institute (WPI) showed Promensil to contain primarily biochanin A and formononetin. The biochanin A compound was shown to have an anti-proliferative effect on breast cancer cells at concentrations greater than 10μM, but shown to have a proliferative effect at concentrations less than this amount (Gergel *et al*., 2010)

Other preliminary projects at WPI also showed Promensil to have an anti-proliferative effect on MCF7 breast cancer cells (Ferron *et al.,* 2016). Promensil treatment of T47D-KBluc cells showed decreased PCNA, compared to estradiol treatment (Bemis & Sejour, 2013). However, in another study, T47D-KBluc cells treated with red clover extract showed increased proliferation, according to MTT assay. This red clover extract was analyzed by HPLC to contain daidzein, genistein and biochanin A.

Another limited study showed Biochanin A, from concentrations of 0.01 µM to 10 µM, to increase the proliferation of T47D cells, while at concentrations above 10 µM it inhibited proliferation of the breast cancer cells. In the same study, Formononetin was shown to have no effect on T47D cell proliferation (Gergel & O'Connell, 2010).

Based on this previous data showing generally consistent results for the anti-proliferative effect of Promensil at high enough concentrations, Promensil was chosen for this project, for further investigation into the anti-proliferative effect of phytoestrogens on breast cancer.

T47D ER-β Cell Line

Based on the research outlined above, it was decided to focus on the effect of Promensil, regarding the beta estrogen receptor, on breast cancer cells. In order to carry out this study, a T47D ER-β cell line was obtained. T47D-ER-β cells are an adherent breast cancer cell line with a tunable beta estrogen receptor. These cells are ER-α positive and transfected with a tetracycline-dependent PBI-EGFP vector for ER-β expression (Strom *et al.,* 2004). A tetracycline dose dependent increase of the ER-α to ER-β ratio has been proven. As seen in Figure 3, it was

shown that as more tetracycline was used, the amount of beta estrogen receptor decreased, in relation to a steady alpha receptor level.

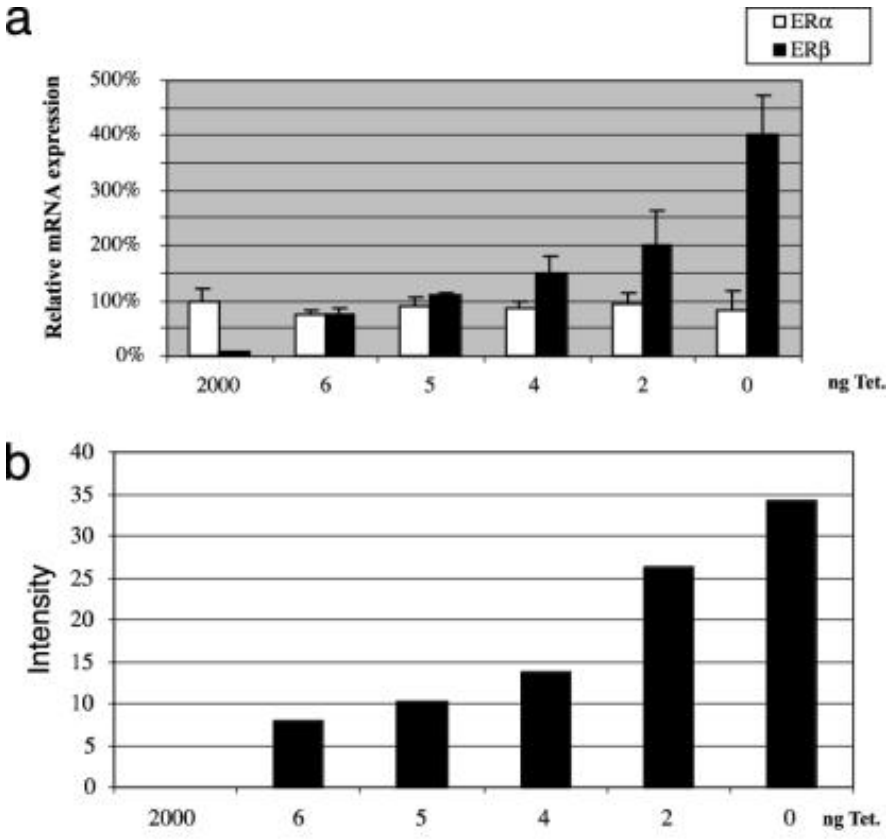

Figure 3: Tetracycline dose Dependent ER-α/ER-β ratio. a.) Results of cDNA PCR, after 12 hours of exposure. Each point represents three treatments. b.) Results of western blot of whole cell extract with 25µg protein FLAG antibody to detect ER-β (Strom *et al*., 2004)

The T47D ER-β cells display a slightly shortened form of the beta estrogen receptor, however it has shown no functional difference. Additionally, a FLAG tag has been fused to the receptor, such that FLAG antibody can be used to detect the level of ER-β present. GFP fluorescence due to co-expression on the plasmid was also an option to visualize the ER-β expression in these cells.

This cell line appeared ideal to examine the response of the beta estrogen receptor, in breast cancer cells, to phytoestrogens.

Hypothesis

It was hypothesized that Promensil supplement would show anti-proliferative results in T47D breast cancer cells when ER-β was present. The anti-proliferative effect was expected to decrease upon ER-β being turned down and/or off.

Methods

Extraction of Promensil

Double strength Promensil tablets were obtained over the counter. 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 of these tablets were used in the extraction, which represents three times the daily serving size of the supplement. The tablets were ground by mortar and pestle and then added to 100 ml methanol in a round bottom flask. This was refluxed for approximately one hour in a 70-degree Celsius water bath, using a reflux condenser. The solution was then filtered through a filter paper funnel to remove remaining solids and finally stored at -20 degrees Celsius.

Cell Maintenance

T47D cells were obtained from ATCC. T47D ER-β cells were obtained from the University of Houston Center for Nuclear Receptors and Cell Signaling, courtesy of Dr. Anders Strom. Cells were maintained in DMEM/F12 with 10% Fetal Bovine Serum and 1% PenStrep media, at 37 degrees Celsius and 5% carbon dioxide. T75 flasks were kept with 10mL media and 10µl insulin (10 mg/ml in 25mM HEPEs, pH 8.2). The T47D ER- β cells were maintained in the presence of 1µg/ml Tetracycline, except where otherwise indicated. Cell media was replaced, and cells were split, as needed.

Cell Plating

Cells were trypsinized and then counted, to plate in 12 or 24-well plates at a concentration of 2.5x10^4 cells/cm^2. For the 12-well plates a total working volume of 1ml per well was used, and

for the 24-well plates 0.5ml was used. Cells were first plated in DMEM/F12 with 10% FBS, 1% PenStrep media, with 1% insulin, and allowed to adhere for 24 hours.

Cell Synchronization

For experiments, cells were synchronized to be at the same point in the cell cycle. This was performed after allowing the cells to adhere to the plates for 24 hours. The media was replaced with the appropriate amount of DMEM and PenStrep media, without serum. Following 24 hours without serum, this media was replaced with the experimental media and the experiment continued.

Experimental Conditions

Following synchronization, the media in the wells was replaced with Phenol-Red free DMEM/F12, with 10% charcoal stripped FBS and 1% PenStrep, media along with 1% insulin. Cells were left to adjust to this media for another 24 hours, after which treatments were added in triplicate, or duplicate, as indicated in the results. For time course experiments, treatments were added at 72, 48 and 24-hour marks before the end of the experiment. For all other experiments, treatments were left for 72 hours. Methanol control treatments were used, as well as no-add, media only blanks to determine baseline cell growth.

Tetracycline

For experiments with the Tet-system off in the T47D ER-β cells, tetracycline was added at a concentration of 1µl/ml, beginning when cells were plated. Tetracycline was continually added in the same concentration as media was changed.

Cell Counts

Following experimental treatment, media was aspirated, and the cells rinsed with PBS, trypsinized off the wells and counted using a Cellometer Auto T4 cell counter from Nexcelom.

Results/Discussion

The effect of Promensil phytoestrogen extract and β-estradiol on T47D cancer cell proliferation was analyzed based on cell counts, following various treatments of plated cells. An initial time course experiment with β-estradiol at two concentrations was performed to determine the optimal conditions under which β-estradiol increases T47D cell proliferation. Figure 4 shows the results of this baseline experiment.

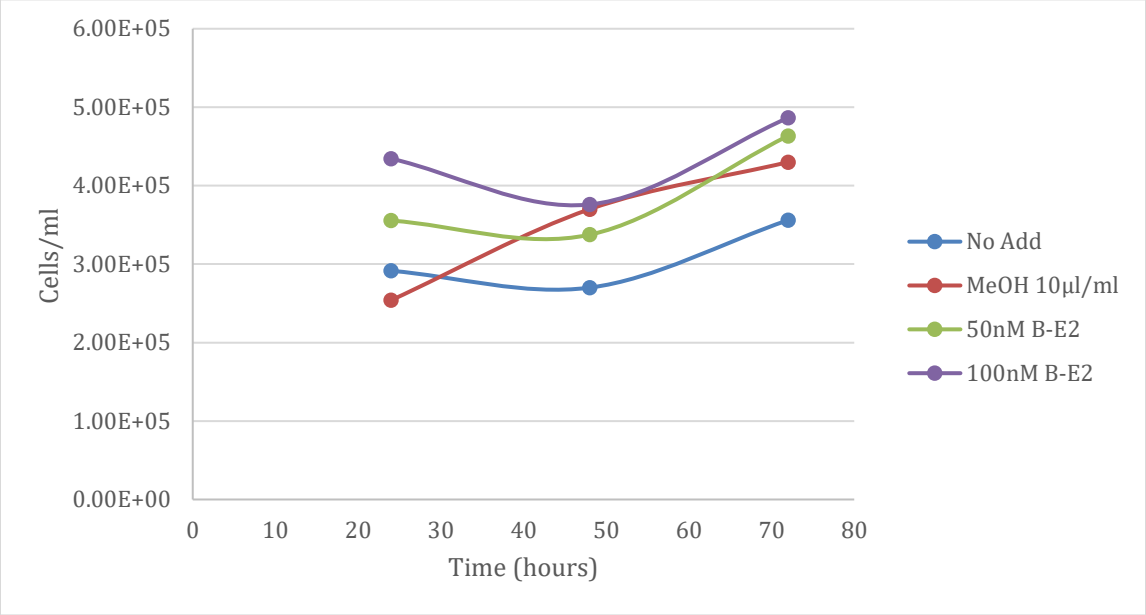

Figure 4: Estrogen dose response curve. No add (blue), methanol control at 10µl/ml working volume (red) and 17 β-estradiol treatments at 50nM (green) and 100nM (purple) were added at 24, 48, and 72 hours. N=2 for all points. Cell counts obtained by Cellometer.

As seen in Figure 4, T47D cells treated with β-estradiol at a concentration of 100nM for 72 hours showed the highest proliferation when compared to the no add and methanol controls. β-estradiol at 50nM concentration also showed an increase in cell proliferation compared to the controls, but

to a lesser extent from the 100nM concentration. These results were obtained in duplicate, so the statistical significance cannot be stated. However, these results served as preliminary findings to set the conditions for the following experiments and therefore the lack of samples could be overlooked. It was unknown why the methanol caused an increase in proliferation compared to the no add control, however this result was seen consistently throughout the project. Therefore, all treatments were compared to methanol controls throughout the course of the project to account for this unexpected increase.

Next, the phytoestrogen treatment (Promensil extract in methanol) was tested in various amounts, for 72 hours each, to determine the effect on T47D cell proliferation. Figure 5 shows the cell count results of these treatments.

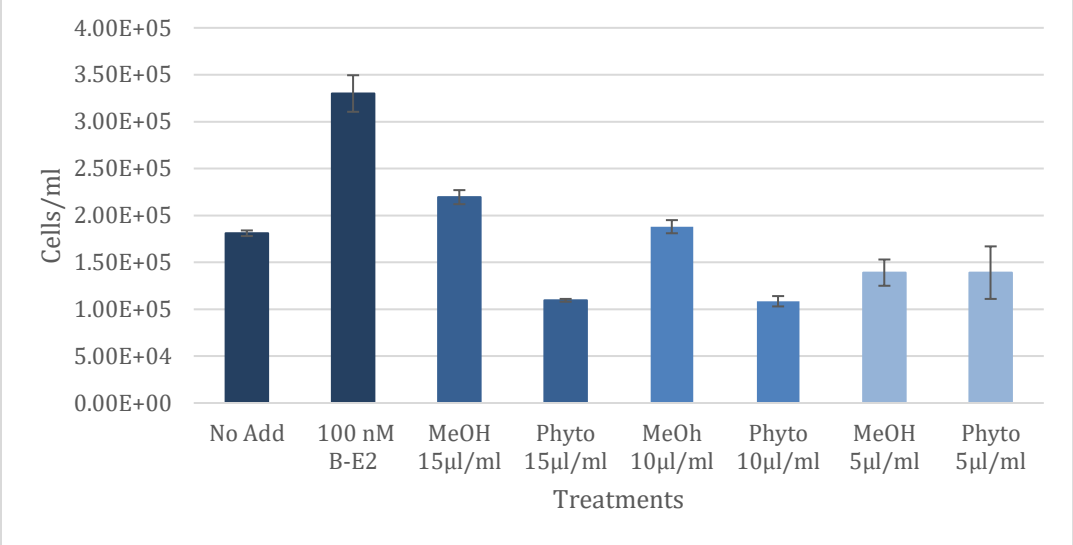

Figure 5: Phytoestrogen treatments of T47D cells. No add, β-estradiol at 100nM, methanol controls (15, 10 and 5µl/ml working volume), and phytoestrogen treatments (15, 10 and 5µl/ml working volume) were added for 72 hours before cell counts were obtained by Cellometer. N=2 for each treatment. Error bars represent standard deviation.

As seen in Figure 5, Phytoestrogen treatment at 15µl/ml working volume and at 10µl/ml working volume showed a decrease in proliferation, when compared to the methanol controls. The 15µl/ml working volume phytoestrogen treatment showed a 50.1% decrease in proliferation compared to the 15µl/ml working volume control. The 10µl/ml working volume phytoestrogen treatment induced a 42% decrease in proliferation compared to its respective control. However, phytoestrogen at 5µl/ml working volume showed no decrease in proliferation, with a 0% decrease compared to its respective control. While these results appear to be significant, it is to be noted that each data point was obtained with a sample size of two. This was still a preliminary experiment in the overall project, to show that the T47D cells responded to Promensil as expected, and therefore the small sample size could be overlooked.

Moving on from this point, the T47D ER-β cells were used. First, these cells were cultured with Tet-on and Tet-off in order to double check the ER-β expression in relation to the Tet-system. GFP fluorescence was viewed in order to confirm the ER-β receptor presence, due to co-expression with GFP off the same vector. Figure 6 shows the resultant fluorescent and regular images.

Tet-off **Tet-on**

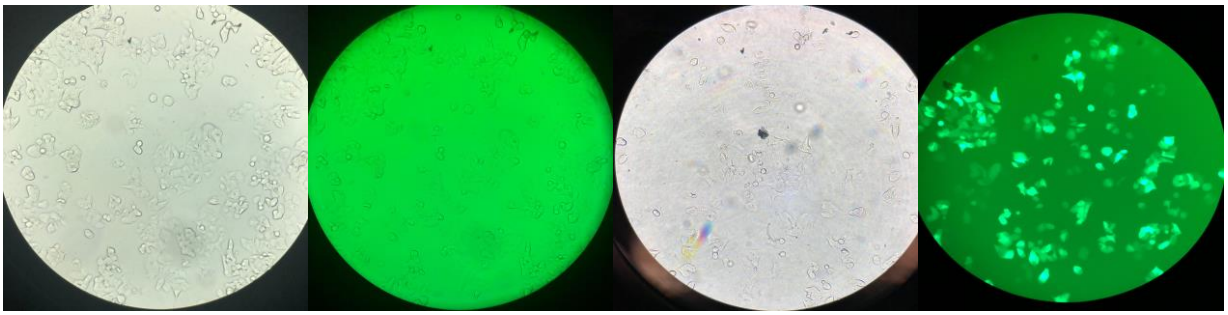

Figure 6: Regular and fluorescent microscopy of T47D ER-β cells. Two leftmost images show Tet-off with no GFP fluorescence. Two rightmost images show Tet-on with GFP fluorescence.

As seen in Figure 6, with the Tet-system on, green fluorescence protein was seen in the cells, indicating that ER-β was also expressed. This was expected according to the genetic engineering of the T47D ER-β cells (Strom *et al.*, 2004). With the Tet-system off, no green fluorescent protein was seen, indicating that ER-β was also no longer expressed in the cells. This was also expected, as the tetracycline concentration of 1µg/ml was supposed to be enough to completely turn off ER-β, according to the graph in Figure 3 of the background section (Strom *et al.*, 2004). This fluorescent imaging confirmed for the following experiments that the Tet-system was successfully working and that the ER-β could be turned off with 1µg/ml Tetracycline.

The first experiment with the T47D ER-β cells was performed with the Tet-system on; therefore the cells were expressing the ER-β receptor. Results of this 72 hour experiment with various phytoestrogen treatments are shown in Figure 7.

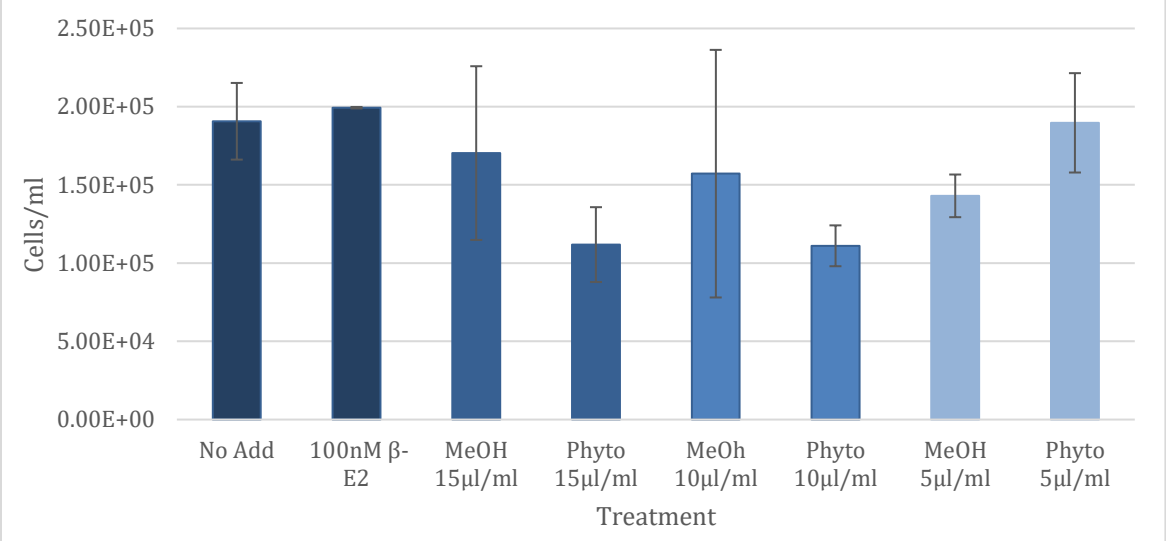

Figure 7: Treatments of T47D ER-β with ER-β expression on. No add, β-estradiol at 100nM, methanol controls (15, 10 and 5µl/ml working volume), and phytoestrogen treatments (15, 10 and 5µl/ml working volume) were added for 72 hours before cell counts were obtained by Cellometer. N=3 for each treatment. Error bars represent standard deviation.

As seen in the graph of Figure 7, the T47D ER-β cells, with the Tet-system on, showed the same response to treatments as the regular T47D cells, seen in Figure 2. The 15µl/ml working volume phytoestrogen treatment showed a 34.3% decrease in proliferation compared to the 15µl/ml working volume control (n=3, p=0.3586). 10µl/ml working volume phytoestrogen treatment induced a 29% decrease in proliferation compared to its methanol control (n=3, p=0.4928). However, phytoestrogen at 5µl/ml working volume showed a 33% increase in proliferation compared with its respective methanol control (n=3, p=0.1376). This increase in proliferation with the 5µl/ml working volume phytoestrogen treatment was unexpected because in the regular T47D cells, the Promensil treatment at this concentration still showed a decrease in proliferation when compared to the control. A possible explanation for this discrepancy could be a different ratio of ER-α to ER-β in the T47D ER-β cells compared to the regular T47D cells. If there were a lower proportion of whichever receptor the Promensil treatment is working through in the T47D ER-β cells, then it is possible that the anti-proliferative effect could be lost at a higher treatment concentration. Other unknown differences between the two cell types may also explain this discrepancy.

This experiment was then repeated with the Tet-system off, in order to suppress the ER-β expression. The results are seen in Figure 8.

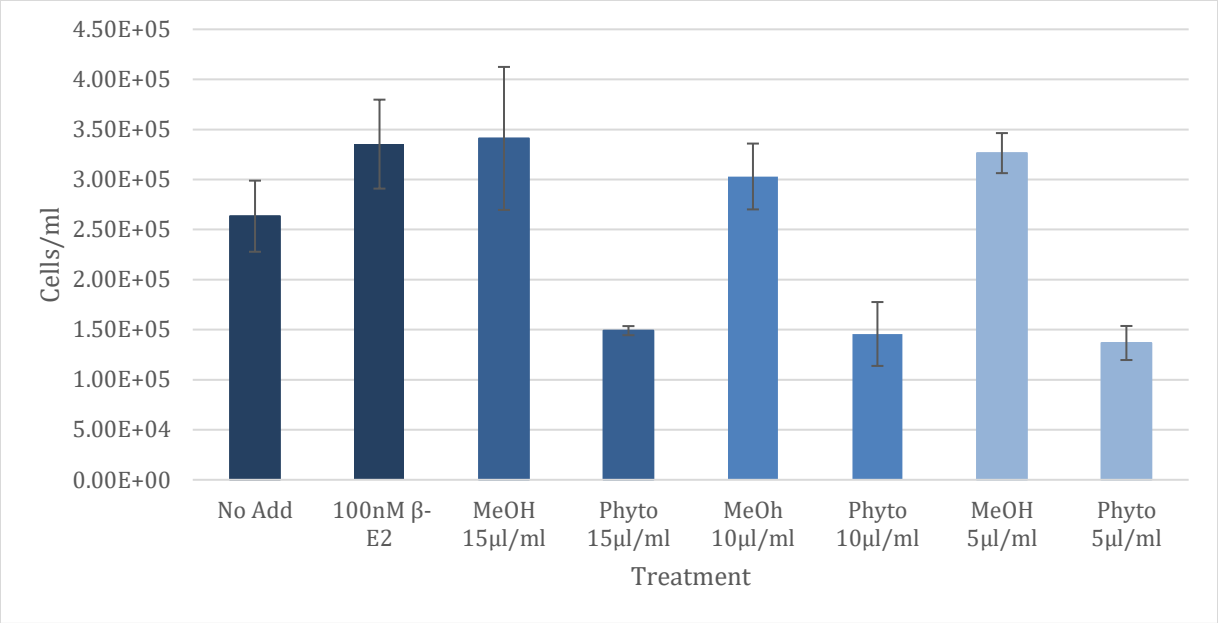

Figure 8: Treatments of T47D ER-β with ER-β expression off. No add, β-estradiol at 100nM, methanol controls (15, 10 and 5µl/ml working volume), and phytoestrogen treatments (15, 10 and 5µl/ml working volume) were added for 72 hours before cell counts were obtained by Cellometer. N=3 for each. Error bars show standard deviation.

As seen in the graph of Figure 8, the T47D ER-β cells, with the Tet-system off, showed a decrease in cell proliferation with all tested concentrations of phytoestrogen extract. The 15µl/ml working volume phytoestrogen treatment showed a 56.30% decrease in proliferation compared to the 15µl/ml working volume methanol control (n=3, p=0.0686). 10µl/ml working volume phytoestrogen treatment induced a 52% decrease in proliferation compared to its respective methanol control (n=3, p=0.0021). And, the phytoestrogen treatment at 5µl/ml working volume showed a 58% decrease in proliferation compared with its respective methanol control (n=3, p=0.0124). These results imply that the Promensil treatment is not working through the ER-β. If this were the case, then the decrease in proliferation, as seen with the Tet-system on, would have been lost when the ER-β was turned off. It is of interest that the anti-proliferative effect of the

treatment at 5µl/ml working volume was seen again with the Tet-system off. This again could be due to a different ratio of ER-β to ER-α in the cells, or due to experimental differences.

This experiment was repeated with the cells plated at half the concentration ($1.25x10^4$ cells/ml) to make sure no self-inhibition of growth was occurring. The results are seen in Figure 9.

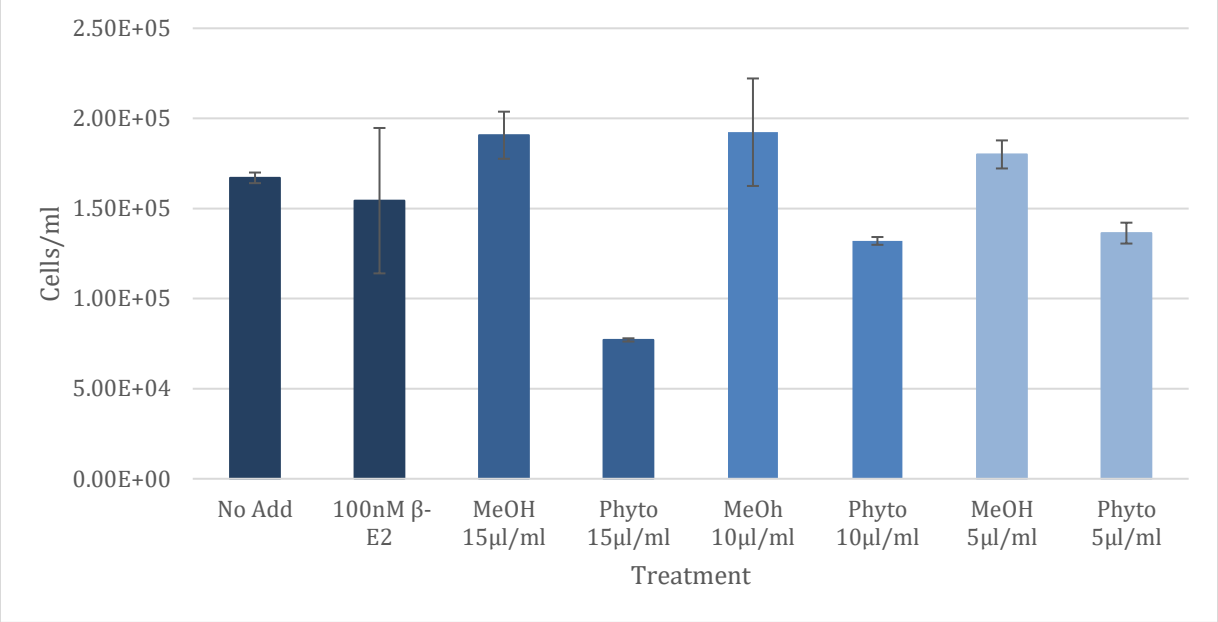

Figure 9: Treatments of T47D ER-β with ER-β expression off, plated at $12.5x10^4$ cells/ml. No add, β-estradiol at 100nM, methanol controls (15, 10 and 5µl/ml working volume), and phytoestrogen treatments (15, 10 and 5µl/ml working volume) were added for 72 hours before cell counts were obtained by Cellometer. N=3 for all treatments. Error bars show standard deviation.

The results in Figure 9 were the same as previous experiments with higher cell concentration (Fig. 8). The 15µl/ml working volume phytoestrogen treatment showed a 59.58% decrease in proliferation compared to the 15µl/ml working volume methanol control (n=3, p=0.0075). 10µl/ml working volume phytoestrogen treatment induced a 31% decrease in proliferation

compared to its methanol control (n=3, p=0.1059). And, the phytoestrogen treatment at 5µl/ml working volume showed a 24% decrease in proliferation compared with its respective control (n=3, p=0.0021). This means that no self-inhibition was occurring with the cells plated at higher concentration. The results from Figure 8 were verified and further supported by these findings as well.

Next, the phytoestrogen treatments were tested at varying concentrations of tetracycline to see if there was any difference due to a different ratio of ER-β to ER-α. The results are seen in Figure 10.

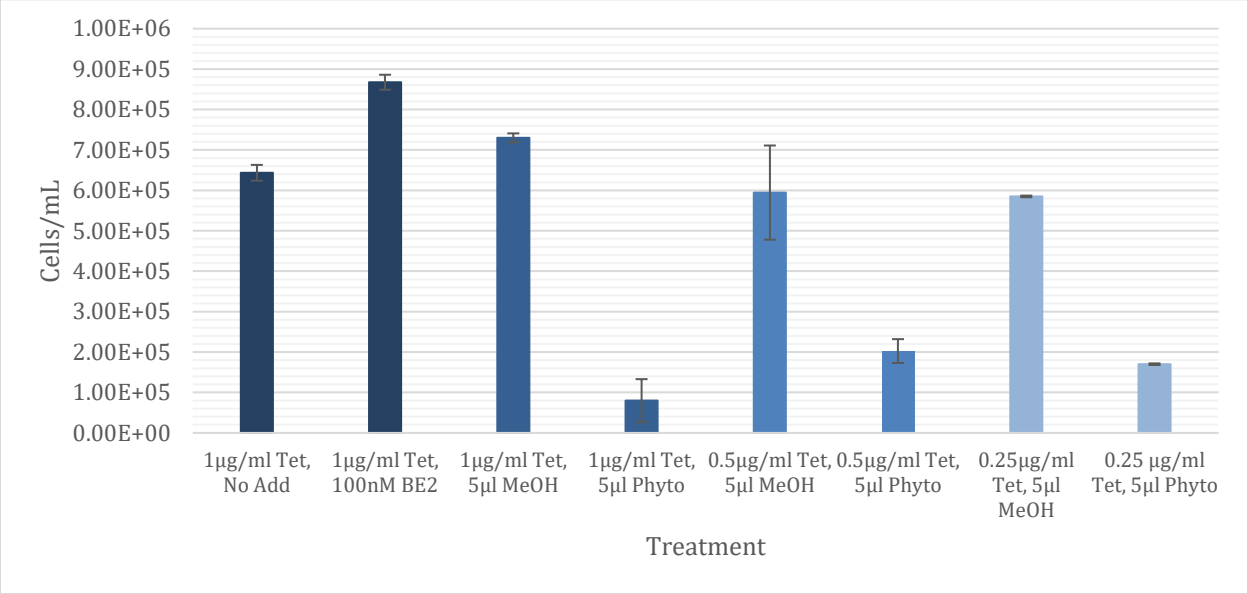

Figure 10: Treatments of T47D ER-β with Tetracycline at varying levels. No add, β-estradiol at 100nM, methanol controls of 5µl/ml working volume, and phytoestrogen treatments of 5µl/ml working volume were added to cells, with varying amounts of tetracycline in the media, for 72 hours before cell counts were obtained by Cellometer. N=2 for all treatments. Error bars show standard deviation.

As seen in Figure 10, the phytoestrogen treatments still all showed a decrease in cell proliferation at the three concentrations of tetracycline. With Tetracycline at 1µg/ml, the phytoestrogen treatment showed an 89% decrease in cell number compared to the respective methanol control (n=2, p=0.0410). With Tetracycline at 0.5 µg/ml, the phytoestrogen treatment showed a 66% decrease in cell number compared to the respective methanol control (n=2, p=0.1390). With Tetracycline at 0.25 µg/ml, the phytoestrogen treatment showed a 71% decrease in cell number compared to the respective methanol control (n=2, p=0.0061). According to Figure 3 from the background section of this paper, which was taken from Strom *et al.*, concentrations of tetracycline above 6 ng/ml will induce cells with less ER-β than ER-α. And at 2000 ng/ml tetracycline, the ER-β expression will be completely off. Based off these previously determined tetracycline concentrations for the T47D ER-β cells, it was determined that at the three Tetracycline concentrations tested in Figure 10, the ER-β expression would be undetectable in relation to ER-α. Due to this, the results were the same as in Figure 9, as expected. This shows that the 1µg/ml Tetracycline concentration was sufficiently high to turn off the ER-β expression and therefore supports that ER-β was turned off in the experiments which used this concentration of Tetracycline.

A final experiment was carried out to test the effect of the phytoestrogen treatment in combination with estradiol treatment. The results are seen in Figure 11.

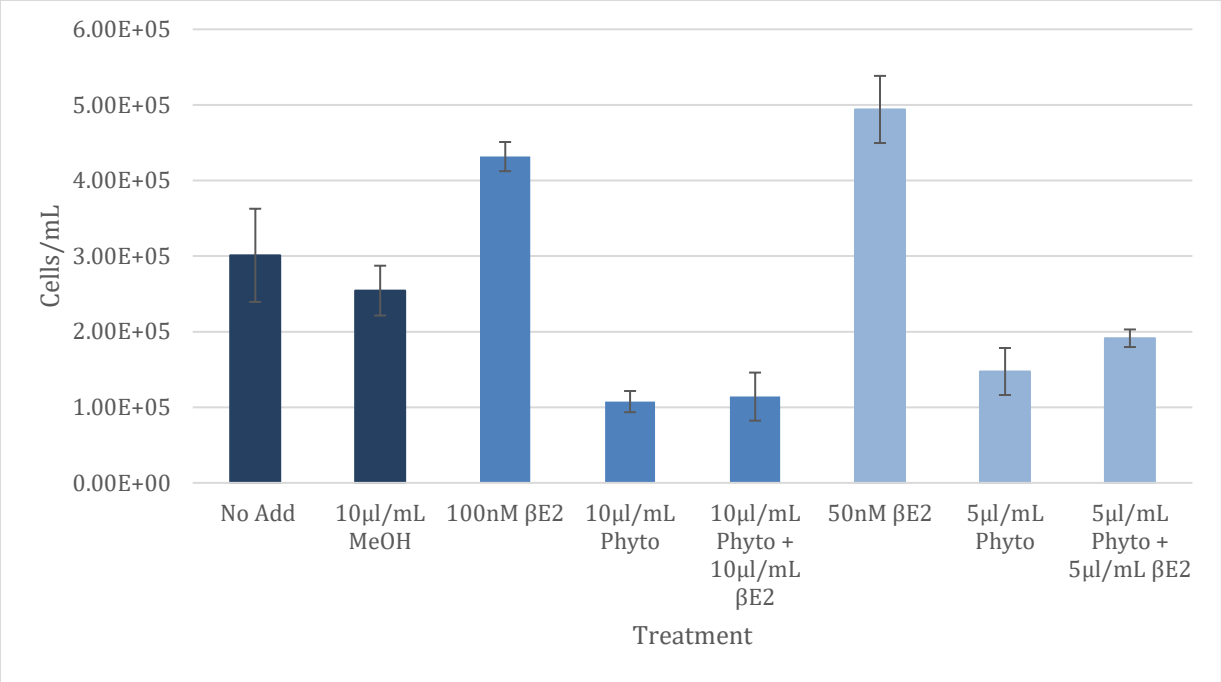

Figure 11: Treatments of T47D ER-β with ER-β expression off. No add, β-estradiol (100nM and 50nM), methanol (10 µl/ml working volume), phytoestrogen (5 and 2.5µl/mL working volume) and combination phytoestrogen/β-estradiol treatments were added for 72 hours before cell counts were obtained by Cellometer. N=3 for all treatments. Error bars show standard deviation.

As seen in Figure 11, the phytoestrogen/β-estradiol combination treatments showed approximately the same decrease in proliferation as the pure phytoestrogen treatments. When phytoestrogen and β-estradiol were added together at 10µl/mL working volume each, there was a 74% decrease in cell number seen, compared to the 100nM β-estradiol control (n=3, p=0.0011). The phytoestrogen treatment at 10µl/mL working volume showed a 75% decrease compared to the 100nM β-estradiol control (n=3, p=0.0014). Therefore, no significant difference between the phytoestrogen and combination phytoestrogen/β-estradiol treatments was seen. When phytoestrogen and β-estradiol were added together at 5µl/mL working volume each, there was a 61% decrease in cell number seen, compared to the 50nM β-estradiol control (n=3, p=0.0063).

The phytoestrogen treatment at 5µl/mL working volume showed a 70% decrease compared to the 100nM β-estradiol control (n=3, p=0.0170). Again, there was no significant difference between the phytoestrogen and phytoestrogen/β-estradiol treatments. These results imply that the Promensil phytoestrogen treatment was working through the alpha estrogen receptor, rather than beta. ER-β expression was turned off for this experiment and the anti-proliferative effect of the phytoestrogen was still seen. Additionally, β-estradiol is known to work through the ER-α (Paech, 1997). Therefore, since the combination phytoestrogen/β-estradiol treatment showed the same decrease in proliferation as the phytoestrogen-only treatment, the phytoestrogen must be somehow inhibiting the β-estradiol effect. Possibly there is some component in the Promensil extract which acts as an antagonist for ER-α.

Overall, the results found in this project did not support the initial hypothesis but instead pointed towards an opposite finding. It appears that Promensil treatment of T47D cells works through the alpha estrogen receptor to decrease cell proliferation.

Recommendations

The findings of this study were preliminary and should be confirmed through repeated trials and with larger sample sizes. If it is confirmed that Promensil decreases breast cancer cell proliferation though the estrogen receptor alpha, then further research could be performed. The ER-α specific interaction with Promensil could be examined and confirmed through cells with a tunable alpha receptor, rather than beta. Then, it could be determined which component(s) of Promensil cause this anti-proliferative effect. Promensil contains genistein, daidzein, formononetin, and biochanin A (Weijer *et al*., 2002). While formononetin and biochanin A have shown reliable data towards an anti-proliferative effect on breast cancer cells (Peterson *et al.*, 1998; Setchell *et al.*, 2001), genistein and daidzein have shown varying effects (Dixon, 2002). The antiproliferative effect seen in this study could be due to a single component of Promensil, or a combination of one or more. Additionally, the other components/phytoestrogens which make up Promensil could be studied separately to see if there is any interaction with the beta estrogen receptor, rather than the alpha receptor.

The results of this project contribute to the overall Phytoestrogen Project at WPI, progressing further the understanding of readily available phytoestrogen supplements on the human body. There is much more to be determined, as this diverse group of compounds has shown varying effects, based on different cells and different conditions.

References

"About Promensil." Promensil, Pharmacare, 2017, www.promensil.com/.

Ali, Simak; Coombes, R.C. "Estrogen Receptor Alpha in Human Breast Cancer: Occurrence and Significance." J. Mammary Gland Biol Neoplasia (2000) 5: 271. https://doi.org/10.1023/A:1009594727358

Bemis, Randal G., Sejour, Leinal A. "Phytoestrogen Project." Worcester Polytechnic Institute Electronic Projects Collection, Worcester Polytechnic Institute, 25 Apr. 2013, https://web.wpi.edu/Pubs/E-project/Available/E- project-042513-101512/unrestricted/MQP_Final.pdf

Belcher, Scott M, and Attila Zsarnovszky. "Estrogenic Actions in the Brain: Estrogen, Phytoestrogens, and Rapid Intracellular Signaling Mechanisms." Journal of Pharmacology and Experimental Therapeutics, vol. 299, no. 2, 1 Nov. 2001, pp. 408–414.

Benassayag, C., et al. "Phytoestrogens as Modulators of Steroid Action in Target Cells." Journal of Chromatography B, vol. 777, no. 1-2, 2002, pp. 233–248., doi:10.1016/s1570-0232(02)00340-9.

"Biochanin A." National Center for Biotechnology Information. PubChem Compound Database, PubChem Compound Database, pubchem.ncbi.nlm.nih.gov/compound/biochanin_A#section=Top.

Bitzas, Gabrielle J., Mathews, Tyler D., Peoples, Sherman J., Roth, Sarah A. "Content and Effects of Over-The- Counter Phytoestrogen Supplements on Breast Cancer Proliferation." Worcester Polytechnic Institute Electronic Projects Collection, Worcester Polytechnic Institute, 29 Apr. 2015, https://web.wpi.edu/Pubs/E-project/Available/E-project-042915-190713/unrestricted/Content_and_Effects_of_OTC_Phytoestrogen_Supplements_on_Breast_Cancer_Proliferation.pdf

Chen, J., et al. "Formononetin Induces Cell Cycle Arrest of Human Breast Cancer Cells via IGF1/PI3K/Akt Pathways In Vitro and In Vivo." Hormone and Metabolic Research, vol. 43, no. 10, 2011, pp. 681–686., doi:10.1055/s-0031-1286306.

Chlebowski, Rowan T., et al. "Influence of Estrogen Plus Progestin on Breast Cancer and Mammography in Healthy Postmenopausal Women: The Women's Health Initiative Randomized Trial." Obstetrical & Gynecological Survey, vol. 58, no. 11, 2003, pp. 735–737., doi:10.1097/01.ogx.0000093674.44946.d6.

Dechering, Koen, et al. “Estrogen Receptors Alpha and Beta Two Receptors of a Kind.” Current Medicinal Chemistry, vol. 7, no. 5, 2000, pp. 561–576. PubMed, doi:10.2174/0929867003375010.

Dixon, R. “Genistein.” Phytochemistry, vol. 60, no. 3, 2002, pp. 205–211., doi:10.1016/s0031-9422(02)00116-4.

Enmark, Eva, et al. “Human Estrogen Receptor β-Gene Structure, Chromosomal Localization, and Expression Pattern.” The Journal of Clinical Endocrinology & Metabolism, vol. 82, no. 12, 1 Dec. 1997, pp. 4258–4265. Oxford Academic, doi:10.1210/jcem.82.12.4470.

“ESR1 Estrogen Receptor 1 [Homo Sapiens (Human)].” National Center for Biotechnology Information, U.S. National Library of Medicine, 28 Jan. 2018, www.ncbi.nlm.nih.gov/gene/2099.

Evers, N.m., et al. “Human T47D-ERB Breast Cancer Cells with Tetracycline-Dependent ERB Expression Reflect ERa/ERB Ratios in Rat and Human Breast Tissue.” Toxicology in Vitro, vol. 27, no. 6, 2013, pp. 1753–1761, doi:10.1016/j.tiv.2013.04.014.

Ferron, Nathan A, et al. “Phytoestrogens and Their Effects on Breast Cancer Cell Proliferation.” Worcester Polytechnic Institute Electronic Projects Collection, Worcester Polytechnic Institute, 28 Apr. 2016, web.wpi.edu/Pubs/E-project/Available/E- project-042816-140259/unrestricted/Phytoestrogen_BreastCancerCells_MQP.pdf.

Gergel, Lauren E.; O’Connell, Olivia J., “The Effects of Phytoestrogens Found in Promensil on Human Ductal Carcinoma” Worcester Polytechnic Institute Electronic Projects Collection, Worcester Polytechnic Institute, 29 Apr. 2010, https://web.wpi.edu/Pubs/E-project/Available/E- project-042910-120434/unrestricted/Phytoestrogen_MQP_2010_Gergel_OConnell.pdf

Harris, D.M., et al. “Phytoestrogens Induce Differential Estrogen Receptor Alpha- or Beta-Mediated Responses in Transfected Breast Cancer Cells.” Experimental Biology and Medicine, vol. 230, no. 8, 2005, pp. 558–568., doi:https://doi.org/10.1177/153537020523000807.

Hayashi, S. “The Expression and Function of Estrogen Receptor Alpha and Beta in Human Breast Cancer and Its Clinical Application.” Endocrine Related Cancer, vol. 10, no. 2, Jan. 2003, pp. 193–202, doi:10.1677/erc.0.0100193.

Ho, Shuk-Mei. “Estrogen, Progesterone and Epithelial Ovarian Cancer.” Reproductive Biology and Endocrinology, vol. 1, no. 73, 7 Oct. 2003, p. 73. BioMed Central, doi:10.1186/1477-7827-1-73.

Kuiper, George G. J. M.; Carlsson, Bo; Grandien, Kaj; Enmark, Eva, Häggblad, Johan; Nilsson, Stefanl; Gustafsson, Jan-Åke; Comparison of the Ligand Binding Specificity and Transcript Tissue Distribution of Estrogen Receptors α and β, Endocrinology, Volume 138, Issue 3, 1 March 1997, Pages 863–870, https://doi.org/10.1210/endo.138.3.4979

Kuiper, George G. J. M.; Lemmen, Josephine G.; Carlsson, Bo; Corton, J. Christopher; Safe, Stephen H.; van der Saag, Paul T.; van der Burg, Bart; Gustafsson, Jan-Åke; Interaction of Estrogenic Chemicals and Phytoestrogens with Estrogen Receptor β, Endocrinology, Volume 139, Issue 10, 1 October 1998, Pages 4252–4263, https://doi.org/10.1210/endo.139.10.6216

Lee, H.p., et al. “Dietary Effects on Breast-Cancer Risk in Singapore.” The Lancet, vol. 337, no. 8751, 1991, pp. 1197–1200., doi:10.1016/0140-6736(91)92867-2.

Lesage, Stephanie; Manning, Taylor; Musteata, Elena; Parekh, Nirali; Renna, Theresa; Sinkewicz, Tracy. “The Effects of Over-The- Counter Phytoestrogens on Breast Cancer Cells.” Worcester Polytechnic Institute Electronic Projects Collection, Worcester Polytechnic Institute, 01 May 2014, https://web.wpi.edu/Pubs/E-project/Available/E-project-050114- 115916/unrestricted/The_Effects_of_Over-The-Counter_Phytoestrogens_on_Breast_Cancer.pdf

National Center for Biotechnology Information. PubChem Compound Database; CID=5757, https://pubchem.ncbi.nlm.nih.gov/compound/5757 (accessed Oct. 16, 2017).

Ogawa, S et al. “Molecular Cloning and Characterization of Human Estrogen Receptor Betacx: A Potential Inhibitor Of Estrogen Action in Human.” Nucleic Acids Research 26.15 (1998): 3505–3512. Print.

Paech, K. “Differential Ligand Activation of Estrogen Receptors ER and ER at AP1 Sites.” Science, vol. 277, no. 5331, May 1997, pp. 1508–1510, doi:10.1126/science.277.5331.1508.

Paruthiyil, Sreenivasan, et al. “Estrogen Receptor β Inhibits Human Breast Cancer Cell Proliferation and Tumor Formation by Causing a G2 Cell Cycle Arrest.” Cancer Research, vol. 64, no. 1, Jan. 2004, pp. 423–428. American Association for Cancer Research, doi:10.1158/0008-5472.can- 03-2446.

Patisaul, Heather B., and Wendy Jefferson. “The Pros and Cons of Phytoestrogens.” Frontiers in neuroendocrinology 31.4 (2010): 400–419. PMC. Web. 4 Oct. 2017.

Peterson, Greg T, et al. “Metabolism of the Isoflavones Genistein and Biochanin A in Human Breast Cancer Cell Lines .” Am J Clin Nutr, vol. 68, Jan. 1999, pp. 1505S–1511S. PubMed, www.ncbi.nlm.nih.gov/pubmed/9848525?report=docsum.

Lippman, Marc, et al. “The Effects of Estrogens and Antiestrogens on Hormone-Responsive Human Breast Cancer in Long-Term Tissue Culture.” Cancer Research, vol. 36, no. 12, 1 Dec. 1976, pp. 4595–4601. American Association for Cancer Research, cancerres.aacrjournals.org/content/36/12/4595.article-info.

Manson, Joann E., “Estrogen.” HealthyWomen, National Women's Health Resource Center, Inc., 2017, www.healthywomen.org/condition/estrogen.

National Center for Biotechnology Information. PubChem Compound Database; CID=5757, https://pubchem.ncbi.nlm.nih.gov/compound/5757 (accessed Nov. 28, 2017).

Osborne, C. Kent, et al. “The Value of Estrogen and Progesterone Receptors in the Treatment of Breast Cancer.” Cancer, vol. 46, no. S12, 1980, pp. 2884–2888., doi:10.1002/1097-0142(19801215)46:12 <2884::aid-cncr2820461429>3.0.co;2-u.

Saji, Shigehira, et al. “MDM2 Enhances the Function of Estrogen Receptor α in Human Breast Cancer Cells.” Biochemical and Biophysical Research Communications, vol. 281, no. 1, 2001, pp. 259–265., doi:10.1006/bbrc.2001.4339.

Setchell, Kenneth D.R., et al. “Bioavailability of Pure Isoflavones in Healthy Humans and Analysis of commercial Soy Isoflavone Supplements.” *The Journal of Nutrition,* vol. 131, no. 4, 2001, pp. 1362S-1375S. *Oxford Academic,* doi: 10.1093/jn/131.4.1362S.

Strom, A., et al. “Estrogen Receptor B Inhibits 17B-Estradiol- Stimulated Proliferation of the Breast Cancer Cell Line T47D.” Proceedings of the National Academy of Sciences, vol. 101, no. 6, 2004, pp. 1566–1571., doi:10.1073/pnas.0308319100.

“U.S. Breast Cancer Statistics.” Breastcancer.org, Breastcancer.org, 10 Mar. 2017, www.breastcancer.org/symptoms/understand_bc/statistics.

Weijer, Peter H.m. Van De, and Ronald Barentsen. “Isoflavones from Red Clover (Promensil®) Significantly Reduce Menopausal Hot Flush Symptoms Compared with

Placebo.” Maturitas, vol. 42, no. 3, 2002, pp. 187–193., doi:10.1016/s0378-5122(02)00080-4.

Zhao, C., et al. “Estrogen Receptor-B2 Negatively Regulates the Transactivation of Estrogen Receptor-a in Human Breast Cancer Cells.” Cancer Research, vol. 67, no. 8, 2007, pp. 3955–3962, doi:10.1158/0008-5472.can-06-3505.