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Phytoestrogen Project: Effect of Promensil on T47D Breast Cancer Cells in Regards to the Cell Cycle and Estrogen Receptor Alpha

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Phytoestrogen Project: Effect of Promensil on T47D Breast Cancer Cells in Regards to the Cell Cycle and Estrogen Receptor Alpha

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

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Abstract

Promensil is a plant-based postmenopausal medication containing phytoestrogens that mimic the structure of estrogen. Cell line treatments with Promensil were performed to study if it had any influence on the cell cycle regarding specific cell cycle proteins. Furthermore, T47D cells were administered MPP, an estrogen receptor-α specific antagonist, to verify if the Promensil antiproliferative mechanism is estrogen receptor-α mediated. Due to a variety of issues, it was not possible to make any conclusions about the effects of Promensil on the cell cycle. Promensil effects were still seen in cells treated with MPP, therefore it was determined that the antiproliferative effector pathway of Promensil is not estrogen receptor-α mediated, thus refuting the hypothesis.
Authorship

The introduction was mainly written by Madeline Levitt with input from Dan Crosby and James Velez. The methodology and appendix was developed by Dan Crosby and James Velez in full collaboration with Madeline as well. Results and discussion was written by both James and Dan.
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Introduction

1.1 Breast Cancer

Breast cancer is a disease that more commonly affects women than men. The disease occurs when breast cells rapidly grow out of control, beginning in the ducts or lobules of the breasts and possible spreading to the rest of the body (Breast Cancer). As women age, the risk of breast cancer increases. This is likely due to the change of hormones in menopausal and post-menopausal women. Breast cancer is the second most commonly diagnosed cancer among women in the United States, after skin cancer. The average age of diagnosis is 61 for women, but the death rate from the disease has decreased over time due to advances in medicine and therapies (Breast Cancer; Chalasani).

Breast cancer cells can develop in a variety of ways that must be identified to prescribe the proper treatment. Mutations in HER2, human epidermal growth factor receptor 2, or BRCA1, breast cancer type 1 susceptibility protein, may cause uncontrollable proliferation of breast cancer cells (HER2 Status; BRCA1 gene). However, about two-thirds of all breast cancers are positive for hormone receptors, either estrogen receptor (ER) or progesterone receptor (PR) positive. ER positive breast cells grow in response to estrogen, whereas PR positive cells grow in response to the hormone progesterone. Estrogen and progesterone bind to their cell receptors and signal for an increase in cell proliferation. Hormone responsive cancers are the most common forms of breast cancer, especially in older woman (Breast cancer hormone receptor status, 2018).

1.2 Estrogen and its receptors

Estrogens are a group of steroids that are responsible for key areas of growth and development in women. It is responsible for changes to a woman’s body during puberty,
including menstrual cycles, breast development, bone growth, and other metabolic processes.

The most abundant estrogen is 17 β-estradiol (E2), which is produced in the ovaries and placenta (Estrogen, 2011).

Studies have shown that ERs regulate cell proliferation and development in both men and women. There are two main ERs: ER-α and ER-β. These receptors are encoded by different genes on different chromosomes and vary in quantity, depending on the type of cell in which they are found (Lee et al. 2012). ER-α is more commonly present in breast, ovary, bone, liver, male reproductive organs, and prostate cells, whereas ER-β is more commonly found in bladder, colon, and immune system cells, with some overlap between the two. ER-α acts as a growth receptor for E2 more often than ER-β, which typically has more anti-proliferative effects (Paterni et al. 2014). ER positive breast cancer cells can have both estrogen receptors, ER-α and ER-β. Yet, ER-α is more likely responsible for increased proliferation in breast cancer cells, than ER-β (Lee et al. 2012).

1.3 Menopause

As women age, their bodies change, and their ovaries begin to stop producing estrogen, eventually causing a women’s menstrual cycle to end. During menopause, women may experience a vast number of symptoms and other health risks associated with a loss of estrogen. Some symptoms of menopause may include hot flashes, sleep problems and night sweat, and vaginal and urinary changes. Menopause also increases the risk of long-term health problems, like osteoporosis and cardiovascular disease. To alleviate the symptoms of menopause, many women resort to taking estrogen supplements, either hormone therapy with progestin or plant-based estrogen substances (The Menopause Years).
1.3.1 Hormone Replacement Therapy

The Food and Drug Administration (FDA) approved the use of estrogen as a supplement to alleviate symptoms of menopause in 1942. Since then, estrogen has become widely used as hormone replacement therapy (HRT) to relieve the side effects of menopause. Currently, studies are being conducted to look at HRT as a means of decreasing the risk of breast cancer in women. Yet, some studies have shown trends that HRT is associated with increased risk of breast cancer. Despite this, it is still commonly used to treat menopausal symptoms (Shook 2014). Despite the associations of HRT with breast cancer and other diseases, it is still relatively unknown if HRT has any effect on breast cancer or other diseases. The American College of Obstetricians and Gynecologists (ACOG) explain some of the risks of hormone replacement therapy. They say that estrogen therapy may cause uterine cancer, deep vein thrombosis, or stroke. In addition to systemic estrogen therapy, some woman may choose to take over-the-counter estrogen supplements that may be plant based to supplement the loss of estrogen (The Menopause Years).

1.4 Phytoestrogens

Phytoestrogen are compounds, structurally similarly to estrogen, that are derived from plants. These compounds can be found in commonly consumed foods, including soy products, peas, beans, flaxseeds, red wine, and tea. A review by Bilal et al. (2014) describes the role phytoestrogens have on ERs. Phytoestrogens typically possess a lower binding affinity to ERs than estrogen, but one study showed that the phytoestrogens genistein and daidzein have a higher binding affinity to ER-β than estrogen (Kuiper et al. 1998). Other studies have shown that some phytoestrogens can interact with cell growth factors and pathways (Choi and Kim et al. 2008). Particularly, one study showed that phytoestrogen genistein was able to inhibit cell cycle regulator cyclin D1 at high concentrations in breast cancer cells (Lavigne et al. 2008). Others
have shown that phytoestrogens may inhibit estrogen synthesis all together in estrogen receptor breast cancer cells (Bilal et al 2014).

Some phytoestrogens are being studied as Selective Estrogen Receptor Modulators (SERMs), which are synthetics that can bind to the ER and act as antagonists. Tamoxifen, for example, was the first approved chemopreventive by the Food and Drug Administration (FDA) and a SERM. It is metabolized by the body and broken down into smaller molecules capable of binding to ERs to prevent proliferation. Like tamoxifen, some phytoestrogens have been shown to have antiproliferative properties, whereas others act as agonists for estrogen, and are considered to possibly be natural SERMs. It is also suggested that some phytoestrogens may be able to bind to ER-β preventing stimulation of ER-α and proliferation (Oseni et al. 2008).

1.4.1 Isoflavones

Isoflavones are a class of phytoestrogens that are widely present in many food sources. Soybeans, for example, are rich in isoflavones. The four isoflavones are genistein, daidzein, biochanin A, and formononetin. Genistein and daidzein are derived from biochanin A and formononetin. Isoflavones are converted in the gastrointestinal tract into molecules that mimic estrogens (Barnes 2004).

Genistein and daidzein are the most common isoflavones and share a similar phenolic ring structure to estrogen. Both can be found in red clover and soybeans. Genistein has been extensively studied and has shown to have a high binding affinity to both ER-α and ER-β. It has been shown in many studies that genistein treatment may inhibit the growth of multiple cancer cell lines, including breast cancer. The effect of genistein is determined by the concentration metabolized by the body. Concentrations less than 1µM act as an agonist for estrogen, inducing
cell growth, whereas concentrations greater than 5µM act as an antagonist, repressing cell growth, as shown in MCF-7 breast cancer cells (Sarkar and Li 2002).

1.5 Promensil

Promensil is an over the counter woman’s phytoestrogen supplement containing isoflavones from red clover. It was created to relieve symptoms of menopause and to provide dietary support. The Promensil website says that their double strength 80mg tablets contain isoflavones genistein, daidzein, formononetin and biochanin (About Promensil).

There is limited information on the Promensil website and some studies have looked further into the effects of Promensil. One study by van de Weijer and Barentsen (2002) showed that treatments by Promensil were able to decrease hot flashes in menopausal women. In a randomized placebo-control study, the researchers gave participants either two Promensil tablets at 40 mg or two placebo tablets. Their urine was sampled to measure the total isoflavone excretion between the two groups. The researchers found that both groups of women had hot flash symptoms at baseline, but the Promensil treatment group had fewer hot flashes at the end of the study. They found that hot flashes had been reduced by 44% (van de Weijer and Barentsen 2002).

Another study by Setchell et al. (2001) investigated dietary supplements containing isoflavones, including Promensil. Their goal was to determine the content of the supplements using liquid chromatography and mass spectrometry to compare their results with the claims of the manufacturer. They found that Promensil contains 41.7 mg isoflavone per capsule, which is consistent with the manufacturer’s claim that each single strength tablet contains 40 mg (Setchell et al. 2001).
1.6 Cell Cycle

The cell cycle is a tightly regulated process that allows for growth and division. There are four stages of the cell cycle: G1, when the cell is active and stimulated by many growth factors; S, when the DNA of a cell is replicated; G2 when the rest of the cell’s organelles divide and prepare for division; and mitosis: the final division of the cell (Weinberg, R. A., 2014). Each part of the cell cycle is monitored by a series of proteins that act together to function as checkpoints. These checkpoints help to ensure that the cell is replicating without error to prevent mutated cells from accumulating in the body tissues (Weinberg, R. A., 2014).

1.6.1 Cyclin D1, p21, and PCNA

One group of proteins responsible for regulating the cell cycle are cyclins. Cyclins help to regulate the action of cyclin-dependent kinases (CDKs) (Weinberg, R. A., 2014). When CDKs bind to cyclins, the CDKs are activated and are able to then phosphorylate other protein such as retinoblastoma protein (pRB) (Weinberg, R. A., 2014). Cyclin D1 specifically binds to CDK4 and CDK6 (abbreviated as CDK4/6) (Weinberg, R. A., 2014). In particular, cyclin D1 influences the cell’s passage through the R checkpoint (Weinberg, R. A., 2014). The R checkpoint is the checkpoint that controls the cell’s entrance into S phase (Weinberg, R. A., 2014). Cyclin D1 has also been seen by Zwijsen, R. M. in 1997 to bind to ERs independently of estrogen, providing another pathway by which cyclin can influence cell proliferation (Zwijsen, R. M., 1997). They did this by looking at the expression levels of proteins whose expression is promoted by the ERs and showing that the expression levels were raised by the presence of cyclin D1 even in the absence of estrogen (Zwijsen, R. M., 1997). There is also evidence that cyclin D1 acts to promote proliferation in this way and not to halt the cell cycle (Zwijsen, R. M., 1997). Many studies have shown that an overexpression of cyclin D1 leads to an early onset of tumors.
(Elsheikh et al. 2008). It has also been shown by Takako Sakamoto et. al. in their 2010 paper that when MCF-7 cells are exposed to phytoestrogens that, except for glycitein, cyclin D1 concentration is transiently increased leading to cell proliferation (Sakamoto, T. et al., 2010). While cyclin D1 serves as an important promoter for the cell cycle, it must be kept in balance to ensure that cells don’t divide inappropriately as this could lead to cancer.

While cyclin D1 acts as an oncogene (a gene that if mutated can encourage cell proliferation and tumorigenesis), p21 is a CDK inhibitor and normally acts a tumor suppressor. p21 arrests the cell cycle in G1 by inhibiting the action of CDK2/4 when the cell is undergoing oxidative stress, when DNA damage has occurred, and when activated by extracellular signal molecules (Abbas, T., & Dutta, A, 2009). p21 may also prevent apoptosis in the cell, inhibiting with caspase-3 (Abbas, T., & Dutta, A, 2009).

Once in S phase, PCNA (proliferating cell nuclear antigen) acts as a DNA clamp (Kelman, Z, 1997). This means that it is responsible for supporting DNA replication by binding to DNA polymerase. This ensures that DNA polymerase cannot fall off the DNA molecule and that the genome is fully replicated. In cancer cells, PCNA has been seen to be overexpressed in a study run by Corinne Cayrol, Martine Knibiehler, and Bernard Ducommun in 1998. They showed that expression of wild-type p21 in P53 deficient DLD1 human colon cancer cells inhibit DNA synthesis and results in cell cycle arrest in G1 and G2 (Cayrol, C, et al., 1998). They also showed that these effects were still seen when the p21 protein was mutated such that it couldn’t interact with CDKs, but not when it was mutated in a way that prevented it from binding with PCNA (Cayrol, C, et al., 1998). This implies that the interaction of p21 with PCNA, not any of the CDKs, is what results in the cessation of DNA synthesis and the G1 and G2 cell cycle arrest (Cayrol, C, et al., 1998).
The effects of phytoestrogens on the cell cycle are still unclear. Some studies have shown that cyclin D1 is overexpressed in estrogen-responsive breast cancers. Choi et al. 2008 found that daidzein decreased the amount of MCF-7 breast cancer cells in G1 at 10uM by 31%. Additionally, they were able to show that daidzein was able to decrease the expression of Cyclin D in MCF-7 cells as well (Choi et al. 2008).

1.7 ER- Alpha and MPP

There is still no definitive answer on how exactly Promensil treatments are able to affect ER-α resulting in the repression of the transcription of certain genes, although ER-α is a major transcriptional regulator. Through microarray analysis, global gene expression profiling has shown that when estradiol (E2) is bound to ER-α it upregulates certain cell cycle proteins resulting in increased proliferation (Stossi 2009). Further research using ER-α specific antagonists has been done in an attempt to elucidate this mechanism. A study on estrogen-regulated genes revealed MPP serves as a selective antagonist that represses the inflammatory function of interleukin-6 promoter activity through ER-α, in response to local cell proliferation (Harrington et al. 2003). Increased proliferation from steroidal stimulation was fully inhibited by the addition of MPP resulting in the inhibition of ER-α mediated proliferation in the MCF-7 breast cancer cell line. This study aims to see the same effect in T47D breast cancer cells.

1.8 T47D Breast Cancer cell line

T47D cells are a breast cancer cell line derived from 54-year-old female with infiltrating ductal carcinoma breast cancer. They were isolated from mammary glands and are adherent epithelial cell with both estrogen and progesterone hormone receptors that also express the WNT7B oncogene (T47D).
1.9 Evaluating the effects of Promensil on T47D cells with and without active ER-α

This project is a continuation of the phytoestrogen project at Worcester Polytechnic Institute (WPI). One previous project has shown that the proliferation of T47D is inhibited at higher concentrations of Promensil-derived phytoestrogens (Gergel et al. 2010). Another project, using T47D cells that turn off ER-β in the presence of tetracycline, showed no difference in the number of T47D cells affected by Promensil (Wambach 2018). This indicates that Promensil does not work through ER-β. Therefore, in continuation of past projects, T47D breast cancer cells will be treated with MPP to determine if Promensil antiproliferative effects are ER-α mediated. One study was done at the Department of Molecular and Integrative Physiology of the University of Illinois that designed ER-α selective antagonists by adding basic side chains (BSC) to pyrazole complexes in seven different configurations. They found that the antagonist with the highest affinity for ER-α was methyl-piperidino-pyrazole (MPP), over 200-fold compared to ER-β (Sun 2002).

1.10 Summary

In summary, this project will investigate two aspects of Promensil effects on T47D cells. Specifically, the presence or absence of certain cell cycle proteins and if the inhibition of T47D cell proliferation is ER-α mediated. To examine cell cycle effects, the expression levels of p21, cyclin D1, and PCNA will be quantified. These proteins are involved in the regulation and activity of the cell cycle. As Promensil has been shown to reduce proliferation in these cells, it is expected that being exposed to Promensil would result in these cells expressing a higher level of p21, a lower level of cyclin D1, and a lower level of PCNA. As previously stated, in the presence of E2 ER-α upregulates several cell cycle proteins, increasing proliferation. MPP, an ER-α specific antagonist, will be used in conjunction with Promensil to verify whether or not the
antiproliferative components of Promensil act through ER-α as anticipated. Proliferation of T47D cells has been observed to be signaled through ER-α (Stossi, 2009). It is predicted that when cells are also treated with MPP the antiproliferative effects of Promensil will not be observed. This would indicate that Promensil’s antiproliferative effects are ER-α mediated.
Methods

2.1 Promensil Extraction:

Two Double strength (80mg) Promensil tablets (Lot number 18943A) were obtained over the counter. Tablets were crushed into a fine powder in a mortar and pestle and added to 80% methanol (100 mL) in a 250 mL round bottom flask. The flask was attached to a reflux condenser for one hour in a 70°C water bath. The solution was then vacuum filtered using a Whatman 3mm filter and stored at -20°C. Extract was filter-sterilized an additional time before use.

2.2 Cell Maintenance:

T47D cells were obtained from ATCC. Cells were grown in regular growth media: DMEM/F12 media with 10% Fetal Bovine Serum, and 1% penicillin/streptomycin (Pen/Strep) (100X). Insulin was added to media to a final concentration of 1.7µM directly to flask. Cells were plated in T25 or T75 flasks, depending on usage, and incubated at 37°C and 5% carbon dioxide. Cells were maintained and split when the cell’s confluence reached about 90%.

2.3 Cell Plating and Synchronization:

T47D cells were plated in 12 well plates at 200,000 cells per well in regular growth media (1mL/well + 1.7µM Insulin/well). After 24 hours, media was changed to 1mL/well + 1.7µM Insulin/well serum-free starving media: DMEM/F12 and 1% Pen/Strep. Starving media was used to remove any growth factors that stimulate proliferation of the cells so that the cells would be synchronized in terms of their cell cycles. 24 hours following the starving media, media was changed to treatment 1mL per well + 1.7µM Insulin/well media: Phenol red-free DMEM (PHRED), 10% charcoal-stripped FBS, and 1% Pen/Strep. PHRED media was used to limit the estrogenic effects on the cells caused by the phenol red structure that is similar to
estrogen. At the same time the PHRED media was added, 100nM $\beta$-estradiol (E2) and Promensil was added to the cells. Cells were treated for 48 hours and incubated at 37°C and 5% carbon dioxide before being harvested.

2.4 MPP Concentration Assay:

T47D cells were plated in 12 well plates at 200,000 cells per well in regular growth media (1mL per well + 1.7$\mu$M Insulin/well). Cell plating and synchronization protocol was followed. 10mg anhydrous MPP (Methyl-piperidino-pyrazole hydrate) dihydrochloride, a specific antagonist for ER-$\alpha$ (Sigma Aldrich) was obtained and reconstituted in DMSO. Working dilutions were made to have final in-well concentrations of 1$\mu$M, 100nM, and 1nM from a 10mM MPP stock solution. All wells received 10$\mu$L of 10$\mu$M $\beta$- Estradiol (E2) for final in well concentrations of 100nM E2. Each of the concentrations were added to the plate in triplicates, along with DMSO control wells with no MPP. Cells were treated for 48 hours and incubated at 37°C and 5% carbon dioxide before being harvested.

2.5 E2, Promensil, & MPP Treatment:

T47D cells were plated in 6 well plates (3) with a 150,000 cells/well seeding density in regular growth media (3mL per well + 1.7$\mu$M Insulin/well). The reduction in seeding density was done to reduce the cell contact inhibition that was seen in the 12 well plates. Cell plating and synchronization protocol was followed; however, cells were left in regular growth media for 48 hours instead of 24 hours. Treatments for each well respectively were as follows: no treatment, 0.8% Methanol + 1% DMSO, 100nM E2 + 1% DMSO, 1% Promensil extract + 1% DMSO, 100nM E2 + 100nMMPP, 1% Promensil extract + 100nM MPP. Cells were treated for 48 hours and incubated at 37°C and 5% carbon dioxide before being harvested.
2.6 Cell Harvesting:

After treatment, media was aspirated from cells and washed with 1 mL cold PBS on ice. For cell counting, cells were scraped off the plate into 1 mL cold PBS using a rubber spatula and samples were placed in 1.5 mL microfuge tubes. Samples were spun down at 5,000 x g for 1 minute, resuspended in 300 µL PBS and live cell concentrations were counted with the Cellometer Auto T4. For immunoblotting, cells were lysed in the -80°C freezer for 10 minutes and in the first well of each treatment, cells were scraped into 200 µL PBS then transferred to the next duplicate well to pool replicates and transfer to 1.5 mL microfuge tubes. Lysates then incubated for 10 minutes on ice. Then were vortexed and incubated for an additional 10 minutes on ice. Lysates were then spun down at 13,000 x g for 5 minutes. Supernatants were pipetted to new microfuge tubes and stored in the -20°C until ready to use.

2.7 Gel Electrophoresis Immunoblotting:

Immunoblotting was used to determine the presence of proteins of interest. Concentration of protein from lysed cells was determined using the NanoDrop Lite Spectrophotometer (Thermo Scientific). Either 10µg or 15µg of protein was combined with 6x denaturing Sample Buffer (Appendix A). Samples were vortexed and proteins were denatured on a 95°C hot plate for 5 minutes. Samples were put on ice for 2 minutes. Samples were loaded on 12% SDS 10 well Mini Protean Precast Gels in quantities normalized to total protein. 1x SDS running buffer (Appendix A) was added to gel apparatus (Bio-Rad) and gel ran at a constant 250 V for about 30 minutes or until dye had reached the black line on the bottom of the gel. Following gel electrophoresis, filter paper and Polyvinylidene difluoride (PVFD) were cut to fix approximately the size of the gel. Filter paper was soaked in transfer buffer (Appendix A), drained, and layered on Semi-Dry
Electroblotter (Owl Separation Systems). Precast gel was broken up and cut using a razor to remove well fringes and dye runoff. Gel was put in transfer buffer and placed on top of filter papers of Semi-Dry Electroblotter, protein side up. A Millipore Immobilon-P PVDF membrane (Lot number K5JN4531L; Pore size: 0.45μm) was soaked in methanol and then soaked in transfer buffer after absorbing methanol. PVDF was placed on top of gel, followed by 3 more filter papers. Gel was transferred at amperage equivalent to 0.8-1 milliamps/ cm2 of gels on apparatus for 1 hour. Following transfer, PVDF membranes were cut in half using a razor and put in blocking solution of 1% low fat instant dried milk and 1x TBS-T (Appendix A) for 30 minutes at room temperature on rocking platform. Primary antibody dilutions of 1:1000 of Cyclin D1 (A-12 sc-8396), PCNA (F-2 sc-25280), p21 (F-5 sc-6246), and caspase-3 p17 (sc-271028) (Santa-Cruz Biotechnology) were made in 1x TBS-T and kept cold. Blocking buffer was discarded and 3mL of each primary antibody were added to plastic pouches along with each replicate membrane and kept overnight at 4°C on a rocking platform. Following priming, immunoblots were washed 3 times for 5 minutes in 1x TBS-T at room temperature on rocking platform. Secondary antibody (BP-HRO sc-516102) (Santa-Cruz Biotechnology) was added to 1x TBS-T at a dilution of 1:5000. Immunoblots were covered in 3 mL of secondary solution for 1 hr. at room temperature on rocking platform. Blots were washed again 3 times for 5 minutes in 1x TBS-T at room temperature on rocking platform. Blots were covered and stained with colorimetric 1-Step Ultra TMB Blotting-Solution (Thermo Scientific) for 5-30 minutes or until bands had developed. Reaction was stopped by added 2x MilliQ water. Protocol for using Pierce 1-Step Ultra TMB Blotting-Solution was obtained from ThermoFisher Scientific. Blots were imaged using the ChemiDoc XRS Gel Photo Documentation System (BioRad).
Gels following transfer were stained with GelCode Blue (Thermo Scientific) to ensure an optimal transfer. Gels were washed 2 times for 5 minutes with MilliQ water at room temperature on rocking platform. GelCode Blue was added to cover the gel and left to stain at room temperature on rocking platform for 15-60 minutes. Gel was de-stained in three, 5-minute washes with MilliQ water.

2.8 Immunoblot Analysis:

The intensities from three trials were averaged together and the standard error was found using Excel. The intensities were then graphed.

2.9 Stripping:

Used immunoblots were stripped of their antibodies to be re-probed with other antibodies. Stripping buffer (Appendix A) was prepared to pH 2.2. PVDF membrane washed 2 times in stripping buffer for 7 minutes each. Membrane was washed 2 times in 1x PBS (phosphate-buffered saline) for 10 minutes each. Membrane was then washed 2 times in 1x TBS-T for 5 minutes each. Membrane was then processed according to immunoblotting protocol from blocking step with primary antibody of interest. Protocol was obtained from AbCam (Abcam, 2019).
Results & Discussion

3.1 MPP Concentration Assay

Synchronized T47D breast cancer cells were treated with 3 different concentrations of ER-α specific antagonist, MPP (Methyl-piperidino-pyrazole). E2 was added to all cells, left to incubate for 48 hours and then harvested for counting. See figure 1 below:

![MPP Concentration Assay](image)

Figure 1: Optimizing MPP concentrations for cell treatment. Concentrations listed are for final in-well concentrations. Error bars represent standard error (n=2)

DMSO Control wells yielded the highest average live cell counts. Concentrations of 1μM MPP showed a 38% reduction and 100nM MPP showed a 39% reduction in proliferation in comparison to DMSO control average live cell count. Therefore, 100nM MPP was deemed most effective at inhibiting estrogen-stimulated proliferation and was used for all future experiments.
The MPP concentration at 1nM was too low and lost efficacy, indicated by the comparative increase in cell proliferation to the other two concentrations with only a 14% reduction in cell proliferation.

Initially, there was some difficulty in getting the plated cells to remain viable. It was suspected that MPP alone may have had cytotoxic effects on the T47D breast cancer cell line. Upon further investigation, it was determined that MPP alone was not cytotoxic because cells remained viable in all subsequent experiments involving MPP. After cell harvesting, compare the cell counts or do the same with another unrelated/healthy cell line.

3.2 E2, Promensil, & MPP Treatments

Synchronized T47D breast cancer cells were treated with different combinations of E2, Promensil, and MPP. After 48 hours, cells were harvested and counted for treatment effects on cell proliferation. See figure 2 below:
Figure 2: Measuring the impact on proliferation of T47D cells from E2, Promensil, and MPP combinations (Averaged from 3, 6 well plates). E2 and MPP final in-well concentrations were 100nM. Error bars represent standard error (n=3)

Wells containing Promensil were expected to induce antiproliferative effects and wells with E2 would induce proliferation of T47D cells (Singh, M., & Singh, N., 2010). It was hypothesized that upon addition of ER-α specific antagonist, MPP, in Promensil wells, antiproliferative effects would be mitigated, thus providing evidence that the effector pathway of the phytoestrogen supplement is ER-α-mediated. E2 treatments with DMSO control induced the most proliferation as expected, whereas Promensil with DMSO control wells exhibited the most anti-proliferative effects with 31% inhibition of T47D cell line proliferation. MPP appeared to inhibit E2-mediated proliferation with 16% reduction in comparison to wells with E2 devoid of MPP. Wells incubated with Promensil and MPP still exhibited inhibition of T47D cell line proliferation, although not as drastic as Promensil with DMSO control, which yielded a 20% reduction in proliferation in comparison to no treatment average live cell count. An analysis of variance (ANOVA) test was done between the six conditions (P-value < 0.0000) and the data
was statistically significant. It was hypothesized that Promensil alone may have also had cytotoxic effects on the T47D breast cancer cell line similar to MPP. To certify this hypothesis, T47D cells could be plated at 1X10^5 cells/well, in similar fashion to the E2, Promensil, & MPP Treatment protocol (Methods), with and without Promensil alone. In the case that Promensil effects are not estrogen receptor dependent, the same assay could be carried out in an unrelated or healthy cell line. Additionally, in further investigations a time course study with MPP could be done to determine the optimal time it should be added to cells, either before or after Promensil treatment.

During cell treatments, micrographs of cells in representative wells for MPP, E2+MPP, and Promensil+MPP were taken. See figure 3 below:
Figure 3: Microscopy of T47D Breast cancer cell line treated with Promensil, E2 and MPP. Taken at 100X magnification.

The majority of wells presented similar cell morphology and density as normally observed prior to treatment. Cells appeared to be spread out with incomplete attachment to the monolayer. This could be due to not allowing enough time in initial growth media (Methods) for attachment, the rate of media exchange was too fast, or perhaps during cell synchronization the serum-free media (Methods) was too harsh, thus preventing cells from thriving. As seen in figure 5 above, the Promensil+MPP well exhibited much darker cells. Forty-eight hours after treatment, photographs of E2+MPP and Promensil+MPP wells revealed visual live/dead cell densities that
concur with the degree of proliferation in figure 2. It was possible that the seeding density for these treatments were too high, therefore explaining the high amount of cell debris. It is recommended to initially plate $1 \times 10^5$ cells/well for all future related treatment combinations.

3.3 Cell Cycle Immunodetection

While antibodies for 3 cell cycle proteins were used, neither p21 nor cyclin D1 resulted in visible bands when blots were developed. P21 and cyclin D1 were chosen to provide data based on both an oncogene (cyclin D1) and a tumor suppressor (p21). PCNA was chosen because it is a transcription factor that should be active if the cell is actively dividing. The lack of bands in p21 and cyclin D1 blots was possibly due to low protein concentration or low antibody affinity. The antibody to PCNA however, resolved quantifiable bands.

![PCNA Immunoblot](image)

Figure 4: A representative immunoblot for PCNA. PCNA has a molecular weight of 29kDa. (Negative=MeOH treatment, E2=Estradiol, P=Promensil). (n=3)

Figure 4 above shows the bands from the PCNA immunoblots. There are clear bands for the negative control, the E2 positive control and the Promensil experimental sample. The bands
appear between 25kDa and 37kDa. It appears that the intensity of all three bands are very similar. In order to confirm this, the intensity was calculated and is shown in figure 5 below:

![PCNA graph](image)

*Figure 5: Volume absorbance intensities measured for PCNA immunoblots (Averaged from 3 different blots). Error bars represent standard error (n=3).*

The graph above shows that there was no significant change in PCNA band intensity between Negative, Positive (E2), and Promensil treatments. The negative control was slightly higher, but the sample population was too small to know if that difference was significant or not. An ANOVA was run and the calculated p value for this data set was 0.9925. This was far higher than 0.05 and so it must be considered as showing no significant change in band intensity amongst treatments.

The data shown in Figure 5 above appears to show no change in the intensity of the bands when the cells were treated with E2 (positive control) or Promensil. It was expected that the cells treated with E2 would have a larger PCNA band intensity because an increase in PCNA
expression is associated with an increase in cell division (Singh, M., & Singh, N., 2010). The data that was collected appears to show that E2 had no effect on the volume of the bands resulting from the cells treated with E2. This discrepancy makes it difficult to come to any conclusions based on this data. It is possible that a positive change in band intensity would appear if more blots were run.

It is likely that there was no intensity change in any of the PCNA bands because the cells were plated at too high a density in the 12-well plates. The wells were very confluent even right after the wells were plated. This left very little room for the cells to grow after they were treated. They had already reached the stationary phase, or possibly even the death phase in their growth curve. There was no difference in the intensity of the PCNA bands for the E2 treated samples because there simply wasn’t enough room to proliferate anymore. The T47D cell line has been shown to exhibit contact inhibition (Kim, S, et.al., 2004). This means that when these cells grow next to each other they will stop dividing (Ribatti, D., 2017). This would have happened almost immediately when these cells were plated, thus activating apoptosis and repressing cell proliferation even when E2 was added.

In conclusion, it was known that when T47D cells were treated with Promensil, the cells would show reduced proliferation. This led to the hypothesis that when T47D cells are treated with Promensil then they would show lower levels of PCNA when analyzed through a Western blot. However, the data collected was not enough to allow for this hypothesis to be supported or rejected. The data shows underlying issues with the experimental setup, such as too high a plating density, therefore conclusions cannot be drawn from the immunodetection data presented. Promensil is similar in molecular structure to E2 and when breast cancers are positive for hormone receptor expression, E2 is known to bind to ER- α, inducing cancerous cell
proliferation. With this information, it was hypothesized that when treated with Promensil and ER-α is bound by specific antagonist, MPP, T47D cells would not exhibit antiproliferative effects. Meaning, the antiproliferative effector pathway of Promensil involves or requires ER-α to function. However, the data refutes this hypothesis as antiproliferative effects were still observed in wells containing MPP and Promensil (figure 2).
References:


T-47D. 2018. https://www.atcc.org/Products/All/HTB-133.aspx#generalinformation


Appendix A: Buffer recipes

(All buffer recipes adapted from recipes found at Abcam, 2019)

NP-40 buffer:

- 20mM Tris HCl, pH 8.0
- 137mM NaCl
- 10% glycerol
- 1% NP-40
- 2mM EDTA

Semi Dry Transfer Buffer:

- 48mM Tris-base
- 39mM Glycine
- 0.04% SDS
- 20% Methanol

1x Running Buffer:

- 25mM Tris-base
- 190mM Glycine
- 0.1% SDS

Check pH and adjust to 8.3
6x Sample Buffer:

16% β-mercaptoethanol

30% Glycerol

4% SDS

0.6% bromophenol blue

0.25M Tris-HCl

Stripping Buffer pH 2.2:

15g Glycine

1g SDS

10mL Tween-20

1x TBST:

For 1 L:

100 mL of TBS 10x

900 mL distilled water

1 mL Tween 20