The Effect of Commercial Phytoestrogen Products on MCF-7 Cells

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Abstract

With the high risk of cancer presented by hormone treatments, over the counter phytoestrogen products have become an alternative method of menopause treatment for women. In this experiment, cell proliferation assays were performed to test the \textit{in vitro} effect of phytoestrogen products on MCF-7 breast cancer cells and an immunoblot was run to detect the expression of DNA processivity factor PCNA. Results indicate the product Promensil exerts significant anti-proliferative effects on the MCF-7 breast cancer cell line.
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Introduction

**Menopause & Estrogen**  Menopause is a state of hormone depletion which naturally results from reduced levels of estrogen production in women (Bodinet, 2004). Estrogen, a steroid hormone, is responsible for the differentiation and growth of female reproductive organs. The principal form of estrogen found in humans, 17- β estradiol, acts by binding to ligand-induced transcription factors known as estrogen receptors. The two known estrogen receptors are ER α and ER β (Morito, 2001). Estrogen functions by binding to estrogen receptors that are localized in the nucleus (Cornwell, 2004). The dimer that forms when estrogen binds to an estrogen receptor regulates the transcription of estrogen-responsive genes by interacting with the estrogen response element (ERE). Modern hormone replacement therapy for menopause involves the use of progestin, a synthetic steroid hormone, in addition to estrogen.

**Hormone Therapy**  Studies show that such combined hormone therapy increases the risk of breast cancer (Schairer, 2000). The U.S. Preventive Services Task Force has published a recommendation against the use of estrogen and progestin in relieving menopausal symptoms (Bodinet, 2004). Recently, a randomized trial of combined hormone therapy (estrogen and progestin), after a total mean follow-up of 11.0 years, has shown an increase in “breast cancer incidence and mortality” among a total of 16,608 postmenopausal women (age 50 to 79 years) from 40 different clinical centers in the United States. Results of this study show that combined hormone therapy, compared with placebo, is associated with more invasive breast cancers, more deaths directly attributed to breast cancer, and “more death from all causes occurring after breast cancer” (Chlebowski, 2010). Such results
indicate that an alternative to hormone replacement therapy for menopausal symptoms is necessary.

**Phytoestrogens**  Alternative approaches to menopause symptom relief are currently sought using phytoestrogen supplements. Phytoestrogens are plant-derived estrogenic compounds whose molecular conformations resemble that of estrogen. This similarity allows phytoestrogens to bind estrogen receptors with an affinity approximately thousandfold weaker than that of 17-β estradiol (Albertazzi, 2008). [Figure-1] shows the structures of 17-β estradiol and three different isoflavones (daidzein, genistein, and glycine).

![Figure - 1] Structure of 17 – β estradiol (far left) and Isoflavones (Daidzein, Genistein, Glycine) (17)

Phytoestrogens are divided amongst three major classes: isoflavones, coumestrans, and lignans (Peeters, 2003). Isoflavones such as genistein and daidzein, predominantly found in soy products, are the most extensively studied of the three. When taken orally, phytoestrogens vary greatly in their metabolism depending on gut microflora, transit time within the intestines, and genetic polymorphisms (Peeters, 2003). Many individuals are
incapable of producing the metabolites of phytoestrogens such as daidzein, whose metabolite (equol) is only excreted by 30 to 50% of adults (Peeters, 2003). Since individuals differ in phytoestrogen metabolism, and none of the phytoestrogens used in this experiment were metabolized (they were simply extracted with methanol, as explained in the methods section), it is important to note that the results from this project may not apply to all individuals.

**Commercial Phytoestrogen Products Used**

Promensil, a commonly marketed phytoestrogen product, and two other commercial herbal extracts of black cohosh and soy will be tested for their ability to promote or inhibit MCF-7 cell proliferation. Promensil contains red clover extract (*Trifolium pretense*) and various isoflavones. Research has found that a single tablet of Promensil contains 40 to 43.5 mg of isoflavones and possesses a 1.9 : 1.0 ratio of genistein to daidzein (Lowdog, 2005). Promensil has been reported to be able to significantly reduce hot flashes from baseline in menopausal women. A significant 44% decrease in hot flushes was observed between active and placebo group in a study by Weijer & Barentsen, 2001. Another commercial product, Natrol Soy, whose main ingredient is soy extract, contains isoflavones such as genistein, daidzein, and glycitein. The specific isoflavone contents of the third herbal product, black cohosh, remain unknown (*Cimicfuga racemosa*).

**MCF-7**

A common model in phytoestrogen research, the MCF-7 cell line is an epithelial adenocarcinoma cell line derived from human breast tumor. This cell line can be maintained for years under ideal conditions *in vitro*. The ability of MCF-7 cells to process estrogen in the form of 17-β estradiol makes them an estrogen responsive positive control.
This has proven useful for breast cancer studies since the cell line was first isolated in 1970 (Levenson, 1997).

**PCNA**  Proliferating Cell Nuclear Antigen is a cofactor of DNA polymerases that is responsible for recruiting crucial factors of the replication fork during DNA replication. The protein belongs to a family of DNA sliding clamps which form ring-like complexes and are able to encircle DNA and slide freely in both directions (Moldovan, Pfander, and Jentsch, 2007). It is an essential cofactor in DNA synthesis as it tethers DNA polymerases firmly onto DNA and increases the processivity of DNA polymerases. PCNA also interacts with many DNA replication-related proteins by binding to them. Some of these proteins include DNA ligase, topoisomerase, protein kinases such as p21, and PARP (Poly ADP-ribose polymerase) – 1. PCNA also plays a role in preventing mutations by activating bypass replication and bypassing any DNA lesions that were not repaired during the S phase. Since most DNA lesions cannot fit into the active sites of replicative DNA polymerases, without PCNA and without bypass replication, prolonged stalling of replication forks would occur, leading to cell-cycle arrest (Moldovan, Pfander, and Jentsch, 2007). Because PCNA is a proliferation marker, detection levels of this protein were used in this project to measure the proliferation of MCF-7 cells in response to phytoestrogen treatment.

**Previous Studies**  The effect that phytoestrogens have on breast cancer seems to be different depending on the concentration of the isoflavone and the variety. For example, in a study by Hsieh et.al., genistein, an isoflavone found in soy, was tested to determine the minimum concentration required to either stimulate or inhibit MCF-7 proliferation by monitoring MCF-7 cell growth in response to various concentrations of genistein. Results
showed that genistein increased MCF-7 cell growth in the 0.01 - 1µM range, but produced decrease in cell growth at higher concentrations (25 - 100µM). In the same study, the \textit{in vivo} effect of genistein was tested by implanting MCF-7 cells in mice and monitoring the growth of the tumor when given a genistein diet (genistein added to an AIN semi purified diet that satisfies all then nutritional requirements of mice), and the study reports that larger tumors were observed in the genistein-treated group than the negative control group (Hsieh, et.al. 1998). The biphasic effect of genistein is reported in another study in which the isoflavone genistein was found to stimulate MCF-7 cell growth at low concentrations and inhibit cell growth at high concentrations. Also, phytoestrogens are known to have agonistic properties in low levels of endogenous estrogen but antagonistic properties at high levels of endogenous estrogen (Matsumura, 2005). On the other hand, scientific research also shows that some phytoestrogens may prevent breast cancer cell growth. One study reports that Sprague-Dawley rats given dietary genistein had reduced susceptibility to mammary cancer by having fewer terminal buds, which are undifferentiated terminal ductal structures that are “most susceptible to carcinogenesis” (Fritz et. Al, 1998). Ethanol extracts of Chinese licorice (\textit{Glycyrrhiza uralensis}) have also been shown to induce apoptosis in cancerous cells. A study shows that, when exposed to licorice root extract for 72 hours, MCF-7 cell numbers decrease while there is a corresponding increase in the expression of pro-apoptotic protein Bax occurs (Jo, 2005). In controlled experiments, herbs such as black cohosh (\textit{Cimicifuga racemosa}) have also shown to reduce proliferation of MCF-7 breast cancer cells. When introduced to cells grown with charcoal-stripped FCS in phenol red media, black cohosh was found to exhibit no cytotoxic effects within the tested dilution range but significantly inhibited MCF-7 cell proliferation.
at certain dilutions (Bodinet, 2004). In the same experiment, soy induced stimulation of MCF-7 cells and strong proliferation increase was observed at certain concentrations.

Resulting from a lack of estrogen production, the symptoms of menopause may be treated with phytoestrogens. These compounds are theorized to compensate for estrogen deficiencies and effectively prohibit the proliferation of breast cancer cells. By exhibiting properties similar to those of the estrogen hormone 17-β estradiol, phytoestrogens can bind the estrogen receptors of human breast cells. In this study, using the MCF-7 breast cancer cell line as a model, the proposed anti-proliferative effects expressed by phytoestrogens will be measured to assess the efficiency of commercial phytoestrogen products.
Materials & Methods

Commercially Available Products & Preparations  The phytoestrogen products Promensil® (Novogen Ltd., Australia), Black Cohosh®, and Natrol Soy® (Natrol, Chatsworth CA, USA) were purchased at local retail outlets. Each product was prepared by using a mortar and pestle to grind one daily recommended dose (as defined by the manufacturer) into a fine powder before being diluted in 80mL of absolute methanol. A reflux condenser was then used to reflux the solutions at approximately 65°C for one hour. The resulting solutions were then stored at -20°C until use.

MCF-7 Proliferation Assay #1  MCF-7 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in T25 and T75 flasks in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Manassas, VA, USA) supplemented with 10% fetal bovine serum (HyClone, South Logan, Utah, USA) and 1% penicillin-streptomycin solution (Mediatech, Manassas, VA, USA). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂. To conduct a proliferation assay, cells were harvested and plated in 12-well plates containing normal media (DMEM, 10%FBS, 1% penicillin-streptomycin) until visibly confluent (70%). The cells were then incubated in phytoestrogen media (2% methanol extract of phytoestrogen product, 10% charcoal dextran-treated FBS, phenol red-free DMEM) or methanol media (2% methanol, 10% charcoal dextran-treated FBS, phenol red-free DMEM). Proliferation was assessed through cell counts performed every 24 hours for 72 hours. To prepare the assays cells were transferred using trypsin and centrifuged at 500rpm. The resulting pellets were suspended in 300µL normal media at 24 and 48 hours
and 100µL at 72 hours. 10 µL Trypan Blue was added to the cell suspension before loading 10 µL of each sample into a hemocytometer for observance under a compound microscope.

**MCF-7 Proliferation Assay #2** MCF-7 cells were maintained as described above, plated on 12-well plates and allowed to adhere and grow until visibly 70% confluent under the microscope. Cells were then incubated in media containing predetermined concentrations (0.5%, 1%, 2%) of phytoestrogen extracts or methanol. Proliferation was assessed by performing cell counts as described in Assay#1 after 24 hours of incubation.

**Immunoblot** MCF-7 cell samples that had been collected but not used for the proliferation assays were pooled together by category. A Bradford Assay was performed to normalize the starting protein concentration of the samples. To separate the proteins in the samples by size, an SDS-PAGE was run with 12% Mini-PROTEAN TGX precast gels (BIO-RAD, Hercules, CA, USA) for approximately 30 minutes at 150 V in 5X running buffer. The separated proteins were then transferred onto an Immobilon-P membrane (Millipore, Billerica, MA, USA) using semi-dry blotting technique run at 32mA for 60 minutes. The membrane was then blocked in 5% non-fat dry milk in distilled water for 10 minutes, washed in PBS, then incubated in PCNA mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200 dilution for 2 hours at room temperature. The membrane was again washed in PBS and incubated in secondary antibody (goat-anti mouse IgG-AP) at 1:2000 dilution for 30 minutes. After washing with PBS again, the membrane was stained in ( ) for 30 minutes to highlight the protein residues. The membrane was then dried at room temperature for 15 minutes and photographed for analysis.
Results & Discussion

Previous studies pertaining to alcohol extracts of plants such as Chinese licorice (Jo, 2005) and black cohosh (Bodinet, 2004) report anti-proliferative or inhibitive effects on breast cancer cell lines. Such reports led us to investigate the effect of methanol extracts of commercial phytoestrogen products on a breast cancer cell line. MCF-7 breast cancer cells were treated with low concentration (0.5~2%) methanol extractions of various commercial phytoestrogen products. Cell counts taken at different time points were used as indicators of cell proliferation. The goal of the cell proliferation assays was to test the *in vitro* effect of phytoestrogen products on the proliferation of breast cancer cells. An immunoblot assay was also performed using antibodies for the protein PCNA (proliferating cell nuclear antigen), a cell proliferation marker, to identify possible reasons behind the anti-proliferative properties of these phytoestrogen product extracts. It was hypothesized that all phytoestrogen products would have anti-proliferative effects on MCF-7 cells, and that lower levels of PCNA would be observed in cells that were treated with phytoestrogen. Our hypothesis regarding the effect of phytoestrogens on MCF-7 cell proliferation was supported by observation of significantly lower cell counts in samples of cells that had been treated with methanol extracts of commercial phytoestrogen products. The hypothesis that low PCNA levels would be observed in cells treated with phytoestrogen was confirmed in those cells treated with Promensil and Black Cohosh extracts. The extract of Natrol Soy, however, was found to express higher levels of the protein PCNA.
**MCF-7 Cell Observation**  MCF-7 cells are adherent cells that have triangular morphology when adhered to the surface of a plate or dish. Panel A of [Figure-2] is a representative photo showing the amount of confluence that was reached in normal media (DMEM, 10%FBS, 1%antibiotics) before beginning proliferation assays. Picture B of [Figure-2] shows a very rare occasion that occurred in an assay not included in this report. Here, MCF-7 cells were incubated in 2% Black Cohosh extraction media on a T25 plate for 48 hours and virtually all the cells on the plate were found to be unattached to the surface. Though effects were not as severe as seen in picture B of [Figure-2] in any of the other assays, failure to attach was observed in all samples for all assays including the control samples (methanol), which means that the cell’s failure to attach may have been caused by the presence of methanol. However, since failure to adhere to the surface was observed at much higher severity in cells that had been incubated in phytoestrogen product extraction media, it is much likelier that the presence of phytoestrogens in the media has an effect on MCF-7 cells either by directly causing failure to adhere to the surface, or by somehow enhancing methanol’s effect on the MCF-7 cells’ ability to adhere to the surface.
**[Figure - 2] MCF-7 Morphology**  

[A] MCF-7 cells plated at 70% confluence in normal media (DMEM, 10%FBS, 1%antibiotics), 50X microscope magnification, 4X digital camera optical magnification  

[B] MCF-7 cell after being incubated for 48 hours in 2% Black Cohosh media (Phenol Red-free DMEM, 2% Black Cohosh methanol extract), 50X microscope, 4X digital camera  

[C] MCF-7 cell morphology in normal media, 400X microscope, 4X digital camera  

[D] MCF-7 cell morphology after being incubated for 24 hours in 2% Promensil media (Phenol Red-free DMEM, 2% Promensil methanol extract)

**MCF-7 Cell Proliferation Assay#1**  
Proliferation Assay#1 tested the effect of a set concentration of phytoestrogen product methanol extract media (2%) on MCF-7 cells over time. Results show that anti-proliferative effects occur after only 24-hours of exposure to the phytoestrogen extracts.
[Figure - 3] MCF-7 Cell Proliferation Assay #1 Cell Counts from Phytoestrogen Media—
Containing Wells as Percent of Cell Counts from Control (Methanol) Well

[Figure-3] depicts the results of proliferation assay#1 by showing the cell counts from phytoestrogen methanol extract-containing wells as percent of control (2% methanol). After 24 hours, the cells incubated in 2% phytoestrogen extract media produced cell counts totaling less than 45 percent of the cell counts from the control well (2% methanol). The effect is even more dramatic after 48 hours with the cultures incubated in phytoestrogen extract media yielding less than 15 percent of the cell counts from the control well. Results also show that extracts of Promensil and Black Cohosh exert stronger anti-proliferative effects on MCF-7 cells than extracts of Natrol Soy. As described in the MCF-7 cell observation section, an abundance of cells were found to be unable to attach to the surface of their plates. All of these unattached cells were discarded in the process of washing the
wells with PBS before trypsinizing. Thus, the anti-proliferative effect observed may not entirely be produced by the phytoestrogen extracts, but rather occur as a result of the cells inability to adhere to their surroundings. However, as seen in [Figure-3], wells containing phytoestrogen media yielded less than 50% cell number of that observed in the well with 2% methanol (control). For example, the phytoestrogen product that seemed to have the weakest anti-proliferative effect on MCF-7 cells was Natrol Soy, and yet after 48 hours the well containing 2% Natrol Soy extract had only 15% of the cell counts observed in the control well (2% methanol). Thus it is clear, regardless of the observation that methanol may have been contributing to the MCF-7 cell’s inability to attach to the plate (resulting in loss of live cells during the process of washing away serum containing-media before trypsinization, and contributing to the decrease in cell counts), the anti-proliferative effect of phytoestrogens on MCF-7 cells is still evident.

**MCF-7 Cell Proliferation Assay#2** From the previous proliferation assay it was determined that 24 hour incubation was sufficient for phytoestrogens to exhibit anti-proliferative effects on MCF-7 cells. Therefore, Proliferation Assay #2 tested the effect of different concentrations (% by volume) of phytoestrogen product methanol extracts on MCF-7 cells over 24 hours.
[Figure - 4] MCF-7 Cell Proliferation Assay #2 Cell Counts from Phytoestrogen Media—Containing Wells as Percent of Cell Counts from Control (Methanol) Well

[Figure-4] shows that results of the second proliferation assay. As in [Figure-3], only cell counts from flasks that had phytoestrogen-containing media are shown as a percent of the cell counts from the control flask. Results from this assay are not as dramatic as those observed in assay #1. The lowest cell count observed from the flasks containing phytoestrogens was for the flask containing Promensil extract which yielded approximately 45% the number of cells the control flask did. Worth noting is that in [Figure-3] the highest cell count observed from the phytoestrogen-containing well was 45% in the first assay. The cell counts from assay#1 (2% phytoestrogen extract) for all three commercial products are lower than the cell counts from the 2% phytoestrogen extract data in assay#2. Thus the anti-proliferative effect of phytoestrogens on MCF-7 cells was not
as prominent in the second assay. However, the same trend is observed, as cell counts from flasks that had extracts of any of the three commercial products are lower than cell counts from the control flask. Also, cell counts from the flask treated with Promensil extracts are lowest, and cell counts from the flask treated with Natrol Soy extracts are highest. Lowering the concentration of phytoestrogen extracts down to 0.5% (by volume) did not result in the biphasic effect that some studies (Hsieh, et.al. 1998) report, as cell counts from the flask of cells treated with 0.5% phytoestrogen extracts were all lower than control.

**Immunoblot**  The result of an immunoblot against PCNA (proliferating cell nuclear antigen) performed for cells treated with methanol, Promensil extract, Natrol Soy extract, and Black Cohosh extract is shown in [Figure-5]. The band intensity is strongest for the lane that contained cells that had been exposed to Natrol Soy extracts, and weakest for Promensil extract-treated cells. Since initial protein concentrations were normalized with a standard curve generated with known concentrations of BSA, stronger band intensity indicates that higher amounts of the protein PCNA were produced by the cells. However, weak bands such as that seen in the Promensil lane indicate that, for whatever reason, the cells were producing very low amounts of PCNA. Since PCNA is responsible for recruiting crucial factors of the replication fork during DNA replication, and is an essential cofactor in DNA synthesis, (Moldovan, Pfander, and Jentsch, 2007) it is a cell proliferation marker. Thus, PCNA expression is directly related to cell proliferation. Bands in the Promensil and Black Cohosh lanes have lower intensities than the band in the control lane, which agrees with the observations from the proliferation assays (that extracts of commercial phytoestrogen extracts have anti-proliferative properties against MCF-7 cells); but the
band in the Natrol Soy lane has higher intensity than the band in the control lane, which indicates that higher levels of PCNA (a cell proliferation marker) was being produced in cells treated with Natrol Soy extracts. Since this result disagrees with the observations made in the proliferation assays, an immunoblot against BAX, an apoptosis-signaling protein, is suggested for future studies to determine the mechanism behind the observation that methanol extracts of Natrol Soy have anti-proliferative effects on MCF_7 cells.

[Figure - 5] Immunoblot using Antibodies against PCNA (Proliferating Cell Nuclear Antigen)

**Conclusion** In summary, the results of multiple cell proliferation assays on MCF-7 breast cancer cells exposed to media containing phytoestrogens suggest phytoestrogens may induce anti-proliferative effects. The commercially available products Promensil, Black Cohosh and Natrol Soy were used in this experiment as practical and medicinally relevant sources of phytoestrogens. In accordance with performed cell counts, all products were observed to induce anti-proliferative effects on MCF-7 cells, albeit to varying degrees. Immunoblotting for the processivity factor PCNA produced further evidence suggesting the products, especially Promensil, inhibit the replication of cancerous cells.
**Future Studies**  We suggest that future studies explore the specific phytoestrogen composition of the methanol extracts of commercial phytoestrogen products used in this project, especially Promensil (since it was observed to have the highest anti-proliferative properties on MCF-7 cells). Then, each specific compound may be tested at different concentrations to test if any single compound has significantly high anti-proliferative properties on MCF-7 cells. Other breast cancer cell lines should also be tested to see if the same effect is present. Specifically, breast cancer cell lines with estrogen receptors because phytoestrogens mimic estrogen activity by binding to estrogen receptors. We also suggest that the digested derivatives of each specific compound be tested for anti-proliferative effects. Phytoestrogens may or may not be metabolized by gut-living microorganisms after ingestion (Morton, Michael S. et al., 2002), and since phytoestrogens were extracted and directly applied to MCF-7 cells in this project, observations from this project may not accurately represent what might be observed in vivo. We also suggest that immunoblots for any other proteins that may be involved in the anti-proliferative effect be used to see detect for increase in apoptotic signals or decrease in proliferation factors.
References


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