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The Effects of Over-the-counter Phytoestrogen Product on Prostate Cancer Cells

Yiling Bao

Professor Michael Buckholt, Major Advisor

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Abstract

Purified phytoestrogens have been shown to have anti-proliferative effects on prostate cancer cells. The plant based over-the-counter phytoestrogen product Promensil has previously been demonstrated to have similar effects on breast epithelial cells. I have investigated the effects of the OTC product on LNCaP prostate cancer cells to determine whether it also has anti-proliferative effects on prostate cancer. Future studies will isolate the individual components of Promensil and examine their individual and synergistic potential to reduce prostate cancer cell proliferation.

Acknowledgments

I would like to thank my advisors, Michael Buckholt and Jill Rulfs for their guidance and expertise in working with cell culture projects. Their knowledge of troubleshooting problems in the experiment was incredibly helpful and allowed me to try almost everything before the project was over. I would also like to thank Abbie White for her help in ordering supplies for my project. Without her help, I would not have been able to complete this project.

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Introduction

Prostate Cancer & Dietary Factors:

Prostate cancer is the second leading cause of death due to cancer in men. However, the incidence varies more than twenty-fold across the regions of the world. Incidence rates are highest in Australia/New Zealand and Western Europe (104 and 93 per 100,000 person in 2008, respectively) and lowest in South-Central Asia (4 per 100,000 person) (Ferlay et al. 2008). Therefore, demographic dissimilarities in lifestyle factors have been implicated as probable regulators of prostate cancer. Dietary factors have been considered as the most important factors. High intake of vegetables and low intake of animal fat could explain the low incidence of prostate cancer in Asia (Jemal et al. 2011).

Phytoestrogens:

Several studies suggested that the risk of prostate cancer is reduced due to the increased consumption of phytoestrogen (Yan and Spitznagel 2009). Phytoestrogens are naturally occurring chemicals in plants that induce weak estrogenic and antiestrogenic responses in mammalian tissue by binding to estrogen receptors (ER) (Setchell et al. 1984). Isoflavones and lignans are two main classes of phytoestrogens which are abundant in soy and flaxseed products. The isoflavone genistein has anti-angiogenic effects and blocks uncontrolled cancer cell growth, and the cytotoxic activity is based on tyrosine kinase inhibition and DNA topoisomerase II inhibition

(Morito et al. 2011). The other important isoflavone is biochanin-A, a methoxylated isoflavone in red clover, which induces delay of the S phase into the G2/M phase progression, and is a powerful agonist of the human aryl-hydrocarbon (ArH) receptor (Medjakovix and Jungbauer, 2008). A recent study showed that genistein, biochanin-A treatment affects inhibition of prostate cancer cell proliferation after induction of apoptosis through promoter element p21 for transcriptional inhibition of Polo-like kinase-1 (Young et al. 2011).

Over-the-counter Phytoestrogen Product Used:

Promensil, a commonly marketed phytoestrogen product will be tested for its effects on LNCaP cell proliferation. Each tablet of Promensil contains a total of 40 to 43.5 mg of isoflavones: genistein, daidzein, formononetin and biochanin as well as number of unidentified compounds sourced from red clover (Trifolium pretense) (Lowdog, 2005). Promensil has been reported to be able to relieve hot flashes from baseline in menopausal women.

LNCaP:

The cell line LNCaP is an epithelial adenocarcinoma cell line isolated from a metastatic lesion of human prostatic cancer. LNCaP cells are hormonally responsive. High-affinity specific androgen and estrogen receptors are present in the cytosol and nuclear fractions (Horoszewicz et al. 1980).

PCNA:

Proliferating Cell Nuclear Antigen is an intranuclear 36 kD polypeptide whose expression and synthesis is linked with cell proliferation (Takasaki et al. 1984). PCNA interacts with many DNA replication-related proteins by binding to them. Some of these proteins include DNA ligase, topoisomerase, protein kinases such as p21 (Moldovan et al. 2007). Because PCNA is a proliferation marker, it was used in this project to measure the proliferation of LNCaP cells in response to phytoestrogen treatment and controls.

MTT:

The MTT assay is a colorimetric assay for measuring the number of living cells. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is originally yellow. It will be reduced to purple crystal in living cells. Isopropanol will be added to dissolve to insoluble purple crystal into a colored solution. The absorbance of this colored solution can be quantified by measuring at the wavelength of 595 nm by a spectrophotometer (Mosmann, 1983).

In this study, I used phytoestrogens extracted from Promensil tablets to treat LNCaP cells to investigate its potential for growth inhibition. Methanol was used as a solvent control and methyltestosterone and estradiol treatments were also performed to verify the response of the cells to androgenic and estrogenic compounds.

Materials and Methods

Over-the-counter Phytoestrogen Products & Preparations:

The phytoestrogen product Promensil ®(Novogen Ltd., Australia) was purchased at local retail outlets. Four 40mg tablets were grounded to a fine powder. The powder was suspended in 80ml 80% (V/V) methanol in a 250ml round bottom flask equipped with a water jacketed reflux condenser. The solution was then refluxed for 1 hour at 65 ℃. The resultant greenish brown liquid was bought to a final volume of 100ml in 80% (V/V) methanol and store at 4 ℃ in the dark.

Media:

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (Pen/Strep) supplemented with 1% glutamine at 37 ℃ and 5% CO_2. Assays were performed in phenol red free DMEM with 10% dextran-coated charcoal stripped FBS, Pen/Strep and glutamine as above.

LNCaP Proliferation Assay #1 Immunoblot

LNCaP cells were maintained in T25 and T75 flasks. Cells were harvested using trypsin-versene mixture and plated in 12-well plates at a density of 1.5*10^5 cells /well for 24 hours. Media were replaced with phenol red free DMEM with 10% dextran-coated charcoal stripped FBS for another 24 hours and then cells were treated with 1% methanol, 1 μM methyltestosterone, 1 μM estrodial and 1%(V/V) Promensil

extracts.

A Bradford Assay was performed to determine the total protein concentrations which were used to normalize protein loading for gel electrophoresis. To separate the proteins by size, SDS-PAGE electrophoresis was run with 4-20% Mini-PROTEIN TGX precast gels (BIO-RAD, Hercules, CA, USA) for approximately 20 minutes at 240V in SDS-PAGE buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). The separated proteins were then transferred onto an Immobilon-P membrane in CAPS transfer buffer (10 mM CAPS, 10% methanol, pH 11) using semi-dry blotting technique run at 60mA for 30 minutes. The membrane was then blocked in 5% non-fat dry milk Tris Buffered Saline (10mM Tris, 150 mM NaCl, pH 7.4) for 10 minutes, washed in TBS, then incubated in anti-PCNA mouse antibody (Santa Cruz Biotechnology, Inc.) at 1:500 dilution in TBS/Tween (TBS with 0.1% Tween 20) for 2 hours at room temperature. The membrane was washed again in TBS followed by TBS/Tween and incubated in phosphate labeled goat anti-mouse IgG-AP (Kirkegaard & Perry Laboratories, Inc.) at 1:1500 dilution for 30 minutes. After washing with TBS and TBS/Tween, the membrane was incubated for 15 minutes with NBT/BCIP reagent until bands appeared, then stopped reaction with water.

LNCaP Proliferation Assay #2 MTT

LNCaP cells were harvested using trypsin and plated in 96-well plates containing normal media (DMEM and 10% FBS) at a density of 1*10^4 cells /well for 24 hours. The cells were then incubated in phenol red free DMEM with 10% dextran-coated

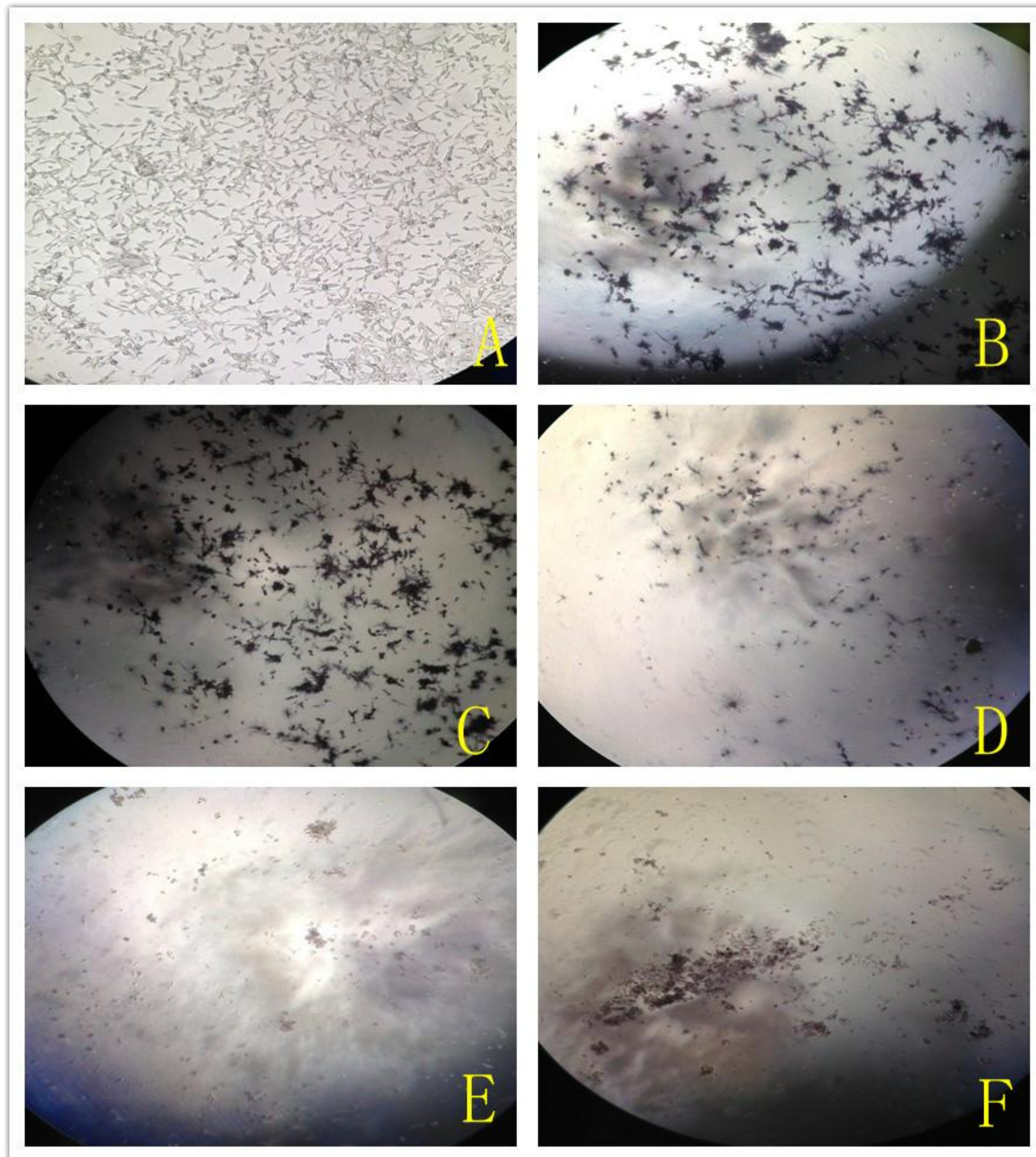
charcoal stripped FBS for another 24 hours and then treated with methanol, testosterone, estrogen and Promensil extracts at the final concentration reported above. MTT substrate was added after another 24 hours and isopropanol was added two hours later. The absorbance was read at the wavelength of 595 nm by a spectrophotometer within one hour.

Results & Discussion

Previous research showed that purified phytoestrogens have anti-proliferative effects on prostate cancer cells (Young et al. 2011). A previous project done by other students has also demonstrated that the plant based over-the-counter phytoestrogen product Promensil has similar effects on breast epithelial cells (Park and Patchchel, 2011). My project is to investigate whether or not Promensil has the anti-proliferative effects on prostate cancer cells. I used the androgen-responsive prostate cancer cell line LNCaP.

LNCaP Observation

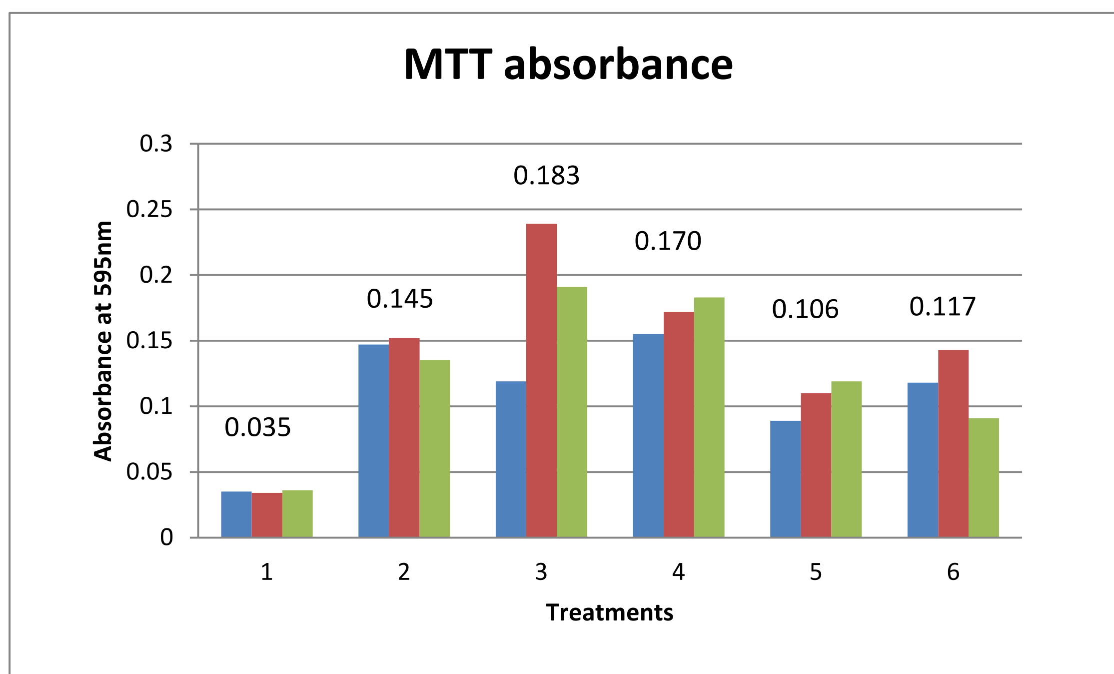
LNCaP cells are adherent epithelial cells that have spindle shape when adhered to the surface of a plate or dish. Panel A of Figure 1 is a representative photo showing the amount of confluence that was reached in normal media (DMEM, 10% FBS) before beginning proliferation assays. Panels B-F show cells treated with various steroidal compounds. Formation of purple crystals is indicative of viable cells and varies directly with viable cell number. As is evident, many fewer crystals were formed in cultures treated with estrogen or the phytoestrogen extract as compared to both methanol control and testosterone treated cells.



[Figure 1] LNCaP Morphology **[A]** LNCaP cells plated at 70% confluence in normal media (DMEM,10%FBS), 50X microscope magnification **[B]** LNCaP cells treated with methanol after added MTT reagent **[C]** LNCaP cells treated with testosterone after added MTT reagent **[D]** LNCaP cells treated with estrogen after added MTT reagent **[E]** LNCaP cells treated with 1% Promensil extract after added MTT reagent **[F]** LNCaP cells treated with 0.5% Promensil extract after added MTT reagent. Concentrations of steroids are as previously reported.

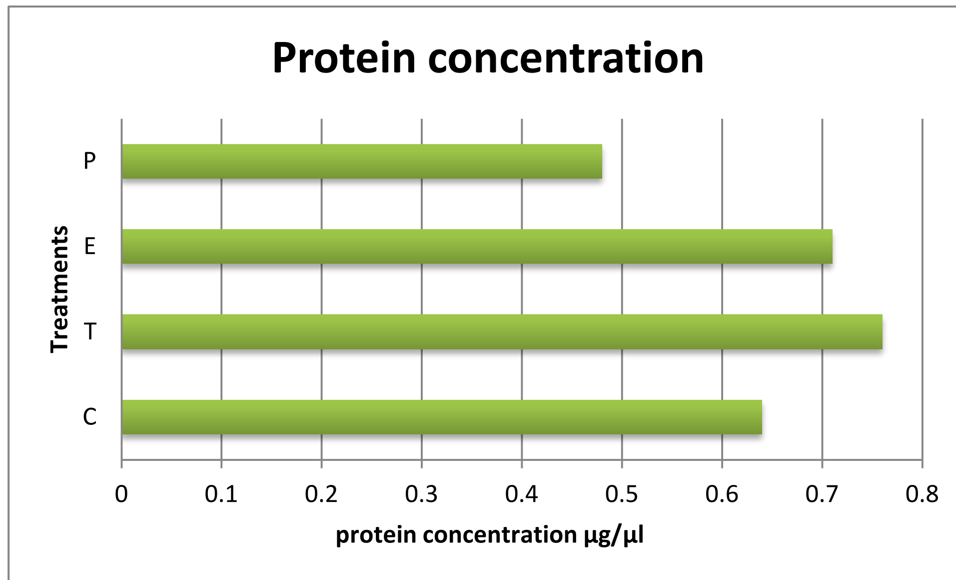
LNCaP Cell Proliferation Assay #2 MTT

When crystals were solubilized in methanol and samples subjected to spectrophotometric quantitation at 595 nm, the results shown in Figure 2 were obtained. Since the assay was only performed once, the data from triplicate wells is a simple average and no statistical analysis is possible. However it appears that cell numbers increased somewhat in the presence of testosterone and decreased with phytoestrogen treatment when compared to solvent control.



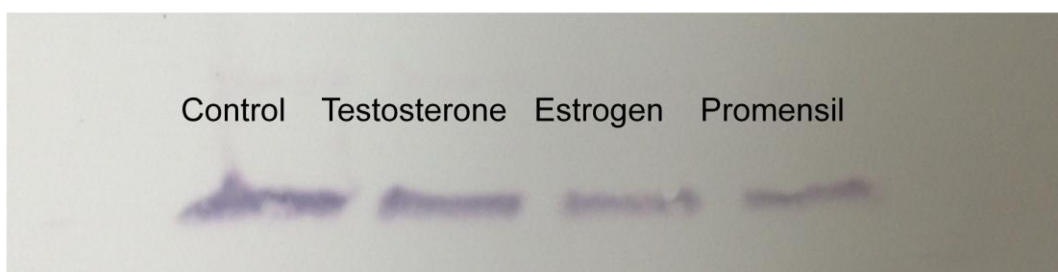
[Figure 2]: Absorbance of MTT assay. Treatment groups:**[1]** no cells; **[2]** 10^4 cells+2 µl 1% methanol; **[3]** 10^4 cells+2 µl 1 µM testosterone; **[4]** 10^4 cells+2 µl µM estrogen; **[5]** 10^4 cells+1% Promensil extract; **[6]** 10^4 cells+1 µl 1% methanol+0.5% Promensil extract.

LNCaP Cell Proliferation Assay #1 Immunoblot



[Figure 3] Protein concentration after 24 hrs of treatments

Figure 3 shows the protein concentration for each sample after 24hrs of treatments. Since all samples were harvested identically and assayed using the standardized Bradford assay, these numbers provide another indication of cell proliferation as compared to control. Final protein concentrations for each sample are as follows: cells treated with methanol 0.64 µg/µl; cells treated with testosterone 0.76 µg/µl; cells treated with estrogen 0.71 µg/µl and cells treated with Promensil extract 0.48 µg/µl. From the bar chart shown in Figure 3, the testosterone sample has the greatest amount of protein/well and the Promensil treated cells has least.



[Figure 4] Immunoblot using antibodies against PCNA: 1.18 μg protein was loaded in each well and the samples were treated as detailed in methods.

The results of an immunoblot for PCNA in cells treated with the various steroidal compounds are shown in Figure 4. While no quantification by densitometry was conducted. It appears as though the bands in the estrogen and Promensil lanes are lighter than the bands in the control and testosterone lanes. This suggests that both estrogen and Promensil extract treated cells expressed lower amounts of PCNA compared to the control. Although we anticipated testosterone would show increased PCNA expression; it is not indicated in this assay.

Although each assay was only performed once and no statistical analysis can be performed on any of these data, there are some trends that are evident. Some of the results are less consistent. The MTT assay and the total protein analysis suggest that testosterone increases the proliferation of LNCaP cells. However, the immunoblot data doesn't indicate this. While these cells are identified as being testosterone responsive, we were not able to obtain pure testosterone in the market. So the effects of testosterone are difficult to interpret. Likewise the effects of estradiol are also difficult to interpret.

Clearly there is more work to be done, but these preliminary experiments indicate that the over-the-counter product Promensil has anti-proliferative effects on LNCaP cells. Previous study has proved that genistein, biochanin-A and apigenin treatments induce up-regulation of p21 expression, and p21 inhibits transcription of PLK-1, which promotes apoptosis of cancer cells (Young et al. 2011). In the future study, we will step into separating the components of Promensil to look for individual and synergistic potential to reduce prostate cancer cell proliferation. These are confirmed isoflavone contents in Promensil by HPLC and ESI-MS: biochanin-A, formononetin, BA-glyc, daidzein, F-glc, genistin and daidzin (Setchell et al. 2001).

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