Examining the Effects of Phytoestrogens on Prostate Cancer Cells

A Major Qualifying Project Report submitted to the faculty of
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in partial fulfillment of the requirements for the Degree of Bachelor of Science.

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Abstract

Estrogenic hormone therapy has been used to treat prostate cancer, and phytoestrogen compounds with similar structures as estrogens are similarly considered to have chemoprotective qualities that result in slower proliferation of cancer cells such as breast cancer. Preliminary studies have suggested that this quality extends to prostate cancer. This project used quantitative analysis of LNCaP and PC-3 prostate cancer cell proliferation in an attempt to confirm these findings. Phytoestrogen treatments included the isoflavones genistein, biochanin A, formononetin, and daidzein as well as Promensil, an over-the-counter supplement containing these isoflavones combined. Overall, cell counts and MTS assays following treatments with phytoestrogens, 17-β-Estradiol, and 5α-Dihydrotestosterone-D3 did not consistently show significant antiproliferative effects from phytoestrogens. However, MTS assay results showed promise of exhibiting a chemoprotective property as decreased growth for individual isoflavones and Promensil varied. Immunoblotting confirmed the presence of both estrogen receptors α and β in PC-3 cells, which likely function in the phytoestrogen pathway rather than through the androgen receptor. Due to conflicting results with proliferation assays and difficulties in LNCaP maintenance, more research should be done to investigate this relationship further and establish the true mechanism of action.

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Introduction

Prostate Cancer

Prostate cancer occurs when there is uncontrolled cell growth in the prostate gland, which regulates the production of seminal fluid and hence the transportation of sperm (American Cancer Society, 2022). It is a leading cause of cancer deaths in American men; as of 2022, 1 in 8 men will have been diagnosed and 1 in 41 men will have died of prostate cancer (Bray et al., 2018; American Cancer Society, 2022; Siegel et al., 2023). In the United States, the incidence of prostate cancer has risen over the last few years (Siegel et al., 2023). Risk factors for prostate cancer include age, race and ethnicity, obesity, prostate inflammation, and certain genetic mutations (American Cancer Society, 2022; Siegel et al., 2023). Geography and diet are also considered potential risk factors of prostate cancer. Some studies associate higher intake of dietary phytoestrogens with lower incidence of prostate cancer (Stephens, 1997; Sohel et al., 2022). This is supported by the lower rates of prostate cancer observed in Africa, Eastern Europe, and Asia, where diets are richer in soy-derived phytoestrogens and include less animal fats and dairy products than in Western countries (Stephens, 1997). However, other factors such as availability and frequency of diagnostic testing may also influence these rates (Rawla, 2019). Regardless, the potential chemoprotective effect of phytoestrogens is worth exploring.

Prostate cancer treatments can change depending on how advanced the cancer is. Tumor size, cancer growth rate, and tumor location are all factors that influence which treatments will be most feasible and effective. Surgical removal of the prostate, radiation, cryotherapy, immunotherapy, targeted drug therapy, and cancer monitoring are available active treatment options for prostate cancer patients (American Cancer Society, 2022; Mayo Clinic, 2022). Another treatment route includes hormone therapy, otherwise known as androgen suppression therapy (American Cancer Society, 2022). Androgens, most importantly testosterone and 5α-dihydrotestosterone (DHT), are hormones crucial for the development and maintenance of human male sex characteristics (Brinkmann et al., 1999). While the overall goal of hormone therapy is to reduce levels of androgens to slow cell proliferation and tumor growth, there are different mechanisms used to achieve this: (1) delivering medications that reduce or stop the body's production of testosterone, (2) delivering medications that block testosterone from interacting with cancer cells, and (3) surgically removing the testicles to permanently reduce the levels of testosterone produced in the body (Mayo Clinic, 2022).

Anti-androgens may not be effective, however, if androgen-independent tumors form. Based on this limitation, estrogen treatment is another available option to suppress the secretion of male hormones

and hence slow or inhibit cancer cell proliferation and tumor growth. Estrogen treatment in prostate cancer patients still remains investigational and is not considered standard practice, and adverse effects have been reported from such therapy (Reis et al., 2018; Lee, 2021). To avoid harmful side effects, phytoestrogens have instead been proposed as a potential preventative and therapeutic treatment for prostate cancer (Morrissey & Watson, 2003). For example, some phytoestrogens have demonstrated growth arrest and increased apoptosis in prostate cancer cells, warranting further exploration of their role in hormone therapy for prostate cancer patients (Morrissey & Watson, 2003; Feng et al., 2007; Stansbury et al., 2013). Hormone therapy is most often used to treat more advanced cases to reduce tumor growth, including patients who have relapsed or whose cancer has metastasized, leaving other treatments less effective (American Cancer Society, 2022; Mayo Clinic, 2022). Other times, hormone therapy can be used before radiation or surgery to shrink tumors and increase the efficacy of such treatments (Mayo Clinic, 2022).

Cell Lines: LNCaP and PC-3

The Lymph Node Carcinoma of the Prostate (LNCaP) is an androgen-dependent cell line isolated from a patient with metastatic tumor growth in the lymph node (Chung, 1996). LNCaP cells express both estrogen and androgen receptors (Chung, 1996). Qualities such as its rapid and reliable growth, androgen-dependency, and sensitivity to hormone treatments make this cell line ideal for *in vitro* models of prostate cancer (van Steenbrugge et al., 1989). In this study, we tested the effects of phytoestrogens on LNCaP proliferation in comparison to steroidal hormone and medium controls.

PC-3 is another prostate cancer cell line used to study prostate cancer in a slightly different context. This cell line does not express androgen receptors, making it androgen-independent and hence resistant to androgen deprivation therapies often used for prostate cancer patients (Tai et al., 2011). These cells mimic more aggressive forms of prostate cancer and, in this case, are useful for exploring possible alternatives to androgen-dependent treatments such as phytoestrogens.

Estrogen and Estrogen Receptors

Estrogens are a group of steroid hormones with four fused carbon rings synthesized in the ovaries, adrenal glands, and adipose tissue (Fuentes & Silveyra, 2019). Estrogens regulate the menstrual cycle, fetal growth, and breast tissue and sex organ development, bone density, brain function, cholesterol mobilization, inflammation control. They are generally considered a female hormone because of their function in female reproductive development; however, in males, estrogens regulate sexual behavior, maintenance of skeletal and cardio system, and maintain function of testes and prostate for sperm maturation (Pettersson & Gustafsson, 2001). The three major estrogen types in humans consist of estrone, estradiol (E2 or 17β-estradiol), and estriol (Cui et al., 2012) with E2 being the predominant form in humans.

Estrogen signaling occurs when estrogens bind to nuclear or membrane receptors after diffusing through the plasma membrane. The classic nuclear receptors, known as ERα and ERβ, are separated on highly conserved genes and function as transcription factors after binding to DNA. Ligand-receptor complexes

target genomic sequences called estrogen response elements (often referred to as EREs) for direct signaling, but they can also act indirectly via protein interactions without DNA binding (Fuentes & Silveyra, 2019). There exists anaffinity for certain ligands between the two receptors; it is known that ERβ favors binding to E2 (Pettersson & Gustafsson, 2001). Expression level of these receptors is tissue-specific with increased expression in the gonadal organs while also being found in the amygdala of the brain (Cui et al., 2012; Hua et al., 2018). The presence of estrogen receptors, especially upregulation of ERβ, have also been suggested to be critical to the inhibition of tumorigenesis in several cancer types, such as breast, ovarian, and prostate cancer (Hua et al., 2018). Hua et al. reported that in some cases of prostate cancer, ERβ transcription is deregulated and silenced (2018). Loss of estrogen receptor function is likely due to histone modifications (Mahmoud et al., 2015) or promoter hypermethylation (Zhang et al., 2007; Hua et al., 2018).

Phytoestrogens and Promensil

Phytoestrogens are plant-derived compounds chemically similar to steroids and imitate estrogen function in humans by interacting with the estrogen receptor (van de Weijer & Barentsen, 2002). They are found in edible and medicinal plants such as fruits, vegetables, and grains. The *Fabaceae* family consisting of peas, legumes, and beans produce isoflavonoids which include genistein, daidzein, biochanin A and others (Křížová et al., 2019). As mentioned previously, the lower incidence of prostate cancer in regions with higher soy consumption has led to studies assessing the relationship between phytoestrogens and carcinogenesis.

The effects of treating prostate adenocarcinoma with soy isoflavones has been tested both *in vivo* and *in vitro* with varying results, though most research has suggested an antiproliferative effect in response to isoflavones (Mitchell et al., 2000; Wang et al., 2002; Lecomte et al., 2017). A commonly proposed molecular mechanism for this is the mediation through the ERβ, as shown in Figure 1. Similar to estrogen affinity to ERβ, phytoestrogens have been shown to have up to ten times higher binding affinity to ERβ than to ERα (Pettersson & Gustafsson, 2001). In 2007, Stettner et al. used LNCaP cells treated with valproic acid, a histone deacetylase inhibitor, or the phytoestrogen tectorigenin to determine prostate cancer proliferation in relation to ERβ expression levels. Tectorigenin acted on both receptors but seemed to prefer ERβ. Both treatments resulted in reexpression of ERβ levels, decrease of ERα levels, and thus decrease of cancer cell proliferation (Stettner et al., 2007). Several studies have also reported that phytoestrogens decrease ERβ promoter region hypermethylation in prostate cancer, often in a dose dependent manner, leading to reduced growth (Mahmoud et al., 2015; Vardi et al., 2010). Vardi et al. found promoter demethylation in tumor suppressor genes after treating PC-3, DU-145, LNCaP prostate cancer cells with genistein or daidzein (2010). With either case of modification, blocking with an antagonist or knocking out ERβ eliminated any protective effect of treatments (Stettner et al., 2007; Mahmoud et al., 2015). Additionally, Bektic et al. discovered that genistein treatment downregulates the androgen receptor, a nuclear receptor to which androgen binds activating the transcription of related genes (Brinkmann et al., 1999). The authors postulate that this response is mediated via ERβ (Bektic et al., 2004). Based on these results, drug restoration of ERβ function via phytoestrogens could help with prostate cancer intervention.

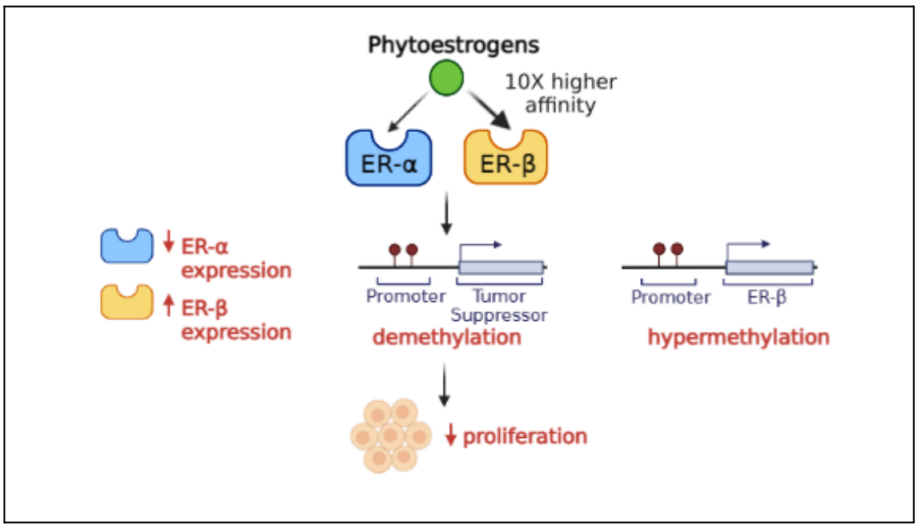

Figure 1: Proposed mechanism of phytoestrogens in reducing prostate cancer cell proliferation (Created with BioRender.com). Phytoestrogens bind both ERα and ERβ, causing downstream effects such as changes in receptor expression and promoter methylation, ultimately leading to reduced cell proliferation.

Promensil is an over-the-counter oral supplement formulated from Red clover or *Trifolium pratense*, an herbaceous plant from the legume family, reported to treat menopause symptoms. Promensil is reported to contain all four types of isoflavones: genistein, daidzein, formononetin, and biochanin A (Frequently asked questions, n.d.). Setchell et al. confirmed this composition with High-performance liquid chromatography, also showing that formononetin and biochanin A were the most prominent substances; however, plasma concentrations of genistein and daidzein increased the most after ingestion (2001). This discrepancy could be explained by the intestinal bacterial demethylation of formononetin and biochanin A, as they are precursors to daidzein and genistein, respectively (Tolleson et al., 2002). Though the medication is marketed toward patients experiencing menopause for symptom relief, the high concentration of isoflavones make it a suitable compound to test against prostate cancer cells.

Project Goal

The purpose of this study was to investigate the potential chemoprotective qualities of phytoestrogens in prostate cancer and the mechanisms through which they may operate. Based on previous research, we hypothesized that phytoestrogen treatment would decrease proliferation of both LNCaP and PC-3 cells *in vitro*. We also hypothesized that this antiproliferative mechanism would operate through ERβ, meaning that phytoestrogen treatment would show higher expression of ERβ and that both cell lines would experience highly similar patterns of proliferation based on treatment. However, we predicted a slightly greater antiproliferative effect in LNCaP cells compared to PC-3 cells based on higher methylation of ERβ and expression of AR in the LNCaP cell line.

Methods

Promensil Extraction

Over-the-counter Promensil Double Strength 80 mg tablets were obtained. Each Promensil tablet contains the isoflavones genistein, biochanin A, formononetin, and daidzein (Setchell et al., 2001; Frequently asked questions, n.d.); in order to extract the isoflavones, an extraction procedure was adapted from Wambach (2018). Two tablets were ground using a mortar and pestle. The powder was then added to a round bottom flask containing 100 mL of 80% methanol connected to a reflux condenser and water bath. Refluxing occurred for one hour in a 70℃ water bath. The resulting solution was filtered using filter paper and stored in a -20℃ freezer.

Control and Treatment Preparation

Two hormone treatments with suggested effects on prostate cancer cell division were selected as controls for these experiments. Estrogen, specifically 17-β-Estradiol (E8875-250MG), was selected as a positive control for both cell lines. 100 μg/mL of 5α-Dihydrotestosterone-D3 (DHT; Cerilliant® D-077-1ML) solution, a testosterone derivative, was also used as a positive control for the androgen-dependent cell line (LNCaP). Both controls were diluted in methanol. Isolated isoflavones (genistein, biochanin A, formononetin, and daidzein) were also diluted in methanol to create 10 mL stocks of 1×10^{-4} M. Medium with no additional treatment was used as a negative control, where no change in normal proliferation was expected.

Cell Culture Maintenance

LNCaP clone FGC (LNCaP; CRL-1740™) cells and PC-3 cells (CRL-1435™) were ordered from the American Type Culture Collection (ATCC). Both cell types were maintained in a medium containing Dulbecco's Modified Eagle Medium (Corning® 500 mL DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (PS). Cells were grown in T75 or T25 flasks at 37°C and 5% CO2 in 10 or 4 mL medium, respectively. Culture medium was replaced as needed, and cells were split once crowding was detected by microscopic visual observation at approximately 80-90% confluency.

Table 1: Example 24-well plate layout for cell counts. Treatment volumes were 1% of total well volume.

1	2	3	4	5	6
5.0 x 10^4 cells + Medium (No Add)	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Formononetin	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Daidzein	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Genistein	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Biochanin A	5.0 x 10^4 cells + 10 uL Promensil
5.0 x 10^4 cells + Medium (No Add)	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Formononetin	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Daidzein	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Genistein	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Biochanin A	5.0 x 10^4 cells + 10 uL Promensil
5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Estradiol	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Formononetin	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Daidzein	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Genistein	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Biochanin A	5.0 x 10^4 cells + 10 uL Promensil
5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Estradiol	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Formononetin	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Daidzein	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Genistein	5.0 x 10^4 cells + 10 uL 1.0 x 10^-4 M Biochanin A	5.0 x 10^4 cells + 10 uL Promensil

Cell Plating

LNCaP and PC-3 cells were trypsinized using 0.25% Trypsin-EDTA (Cat. No. 25200056) from their storage flasks, and concentrations were determined using a Nexcelom Cellometer K2 Imager Cytometer. The following formula was used to determine desired inoculation concentration:

$$[Inoculum\ Volume] = \frac{Desired\ \#\ of\ cells}{Measured\ cell\ concentration}.$$

Calculated volumes of cell solution were then plated to their designated wells in DMEM/PS and 10% FBS and were allowed to adhere for 24 hours. Total working volumes of 1 mL (12- and 24-well plates) or 3 mL (6-well plates) were used during plating. After adhering, the original medium was replaced with phenol red-free DMEM (D6046-500ML)/PS and 10% FBS and cells were allowed to adjust for 24 hours before treatment. Technical replicates of 2-4 were used to increase experimental accuracy.

Cell Counting

After 24 hours of adjustment to the new medium, cells were treated with 17-β-Estradiol, DHT, Promensil, biochanin A, genistein, formononetin, daidzein, or left untreated in the medium (see Table 1 for reference). All treatments were done in the same concentration of methanol. 24-48 hours after treatment, cells were trypsinized, pelleted, and resuspended in 2 mL DMEM/PS and 10% FBS. Cells were counted in a 1:1 ratio (cell solution : 0.2% trypan blue staining solution) using the Nexcelom Cellometer K2 Imager Cytometer (Cellometer). Total and live cell concentrations (cells/mL) and cell viability (%) were recorded.

Table 2: Example 96-well plate layout for MTS assay. Treatment volumes were 1% of total well volume.

1	2	3	4	5	6	7	8	9	10	11	12
1.0 x 10^5 cells + Medium (no add)	1.0 x 10^5 cells + Medium (no add)	1.0 x 10^5 cells + Medium (no add)	1.0 x 10^5 cells + Medium (no add)	5.0 x 10^4 cells + Medium (no add)	5.0 x 10^4 cells + Medium (no add)	5.0 x 10^4 cells + Medium (no add)	5.0 x 10^4 cells + Medium (no add)	6.0 x 10^3 cells + Medium (no add)	6.0 x 10^3 cells + Medium (no add)	6.0 x 10^3 cells + Medium (no add)	6.0 x 10^3 cells + Medium (no add)
1.0 x 10^5 cells + 1 uL 1 x 10^-4 M E2	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M E2	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M E2	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M E2	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M E2	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M E2	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M E2	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M E2	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M E2	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M E2	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M E2	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M E2
1.0 x 10^5 cells + 1 uL 10 nM DHT	1.0 x 10^5 cells + 1 uL 10 nM DHT	1.0 x 10^5 cells + 1 uL 10 nM DHT	1.0 x 10^5 cells + 1 uL 10 nM DHT	5.0 x 10^4 cells + 1 uL 10 nM DHT	5.0 x 10^4 cells + 1 uL 10 nM DHT	5.0 x 10^4 cells + 1 uL 10 nM DHT	5.0 x 10^4 cells + 1 uL 10 nM DHT	6.0 x 10^3 cells + 1 uL 10 nM DHT	6.0 x 10^3 cells + 1 uL 10 nM DHT	6.0 x 10^3 cells + 1 uL 10 nM DHT	6.0 x 10^3 cells + 1 uL 10 nM DHT
1.0 x 10^5 cells + 1 uL Promensil	1.0 x 10^5 cells + 1 uL Promensil	1.0 x 10^5 cells + 1 uL Promensil	1.0 x 10^5 cells + 1 uL Promensil	5.0 x 10^4 cells + 1 uL Promensil	5.0 x 10^4 cells + 1 uL Promensil	5.0 x 10^4 cells + 1 uL Promensil	5.0 x 10^4 cells + 1 uL Promensil	6.0 x 10^3 cells + 1 uL Promensil	6.0 x 10^3 cells + 1 uL Promensil	6.0 x 10^3 cells + 1 uL Promensil	6.0 x 10^3 cells + 1 uL Promensil
1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Daid	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Daid	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Daid	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Daid	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Daid	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Daid	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Daid	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Daid	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Daid	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Daid	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Daid	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Daid
1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Form	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Form	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Form	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Form	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Form	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Form	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Form	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Form	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Form	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Form	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Form	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Form
1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Bio A	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Bio A	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Bio A	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Bio A	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Bio A	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Bio A	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Bio A	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Bio A	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Bio A	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Bio A	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Bio A	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Bio A
1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Gen	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Gen	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Gen	1.0 x 10^5 cells + 1 uL 1 x 10^-4 M Gen	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Gen	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Gen	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Gen	5.0 x 10^4 cells + 1 uL 1 x 10^-4 M Gen	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Gen	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Gen	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Gen	6.0 x 10^3 cells + 1 uL 1 x 10^-4 M Gen

MTS Assay

To corroborate findings from the cell count experiments, MTS assays were performed following the protocol outlined by Promega Corporation for the use of CellTiter 96® AQueous One Solution (Promega, 2012). The MTS assay is a colorimetric assay that measures cellular metabolic activity and viability, where the added tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is reduced by dehydrogenases in viable cell mitochondria into a soluble formazan product and absorbance is measured through spectrophotometry (Kuet et al., 2017). Cells were plated in a 96-well plate at varying dilutions in culture medium for a total of 100 μL in each well. Three seeding densities were 1.0×10^5, 5.0×10^4, and 6.0×10^3 (see Table 2 for reference). Three technical replicates for each condition were performed and averaged. Cells were left to adhere for 24 and 48 hours for PC-3 and LNCaP cells, respectively. Afterward, wells were either left untreated in medium as a negative control or treated with 1 μL (1% of total volume) of β-estradiol as a positive control, Promensil, formononetin, daidzein, genistein, and biochanin A. Following another 24 hour incubation period, 20 μL of the CellTiter 96® AQueous One Solution Reagent was pipetted into each well using a multichannel pipette. The plate was incubated for approximately 2.5 hours and then read in a 96-well plate spectrophotometer at 450 nm to measure absorbance of each sample and gauge relative cell growth between treatments.

Immunoblotting

Immunoblotting was performed to confirm expression of ER-β, ER-α, and AR in both PC-3 and LNCaP cell lines. First, the cells were prepared to run a protein gel. Cells were trypsinized from their respective flasks and resuspended in medium in separate 15 mL conical tubes. Cells were centrifuged in the medium at 110 RCF for 3 minutes, washed in PBS, centrifuged again at 170 RCF for 3 minutes, and finally resuspended in 200 μL PBS. Protein concentrations (μg/μL) of each sample were measured using the Thermo Scientific

NanoDrop One Spectrophotometer's A280 application. The volume of cell solution to be loaded onto a Bio-Rad Mini-Protean TGX 4-20% precast polyacrylamide gel was calculated using the following equation:

$$Loading\ Volume\ (\mu L) = \frac{Desired\ Protein\ Amount\ (\mu g)}{Measured\ Protein\ Concentration\ (\mu g/\mu L)}.$$

To calculate the highest possible protein amount without overloading the wells, the loading volume was fixed at the maximum of 16 μL cell solution (leaving room for 4 μL tracking dye) and calculations were done from there. Cell solutions were combined with tracking dye in a 4:1 ratio (cell solution:tracking dye) in Eppendorf tubes and heated in a heating block at 96 degrees C for 5 minutes to denature proteins. The protein samples and 5 μL of the protein marker were loaded in wells, and the protein gel was run for 30 minutes at 200 Volts in 1x SDS Page-Buffer. Following electrophoresis, protein bands were transferred from the gel to a Millipore Immobilon transfer membrane using a semi-dry blotting method in which the membrane was wet with methanol and washed in TBS (100 mM Tris, 9% NaCl, pH 7.0). The blotting apparatus was run at 50 milliAmps for 30-45 minutes. The membrane was then blocked in 5% non-fat dry milk and TBS with continuous agitation. The membrane was incubated in primary antibodies for ER-β, ER-α, and AR at a 1:200 dilution in TBS + 0.1% Tween overnight on a rocker at 4 degrees C, then washed in TBS, TBS + 0.1% Tween, and TBS again for at least 10 minutes each. The membrane was incubated in Goat Anti-Mouse IgG secondary antibody diluted with TBS + 0.1% Tween in a 1:5000 dilution for at least one hour on a rocker at room temperature, then washed in TBS, TBS + 0.1% Tween, and TBS again for at least 10 minutes each. Bands were developed using one SigmaFast BCIP/NBT dye tablet.

Data Analysis

Quantitative results for experiments with at least 3 biological replicates were analyzed using statistical testing to determine if phytoestrogen treatment significantly changed cell growth compared to control groups. Experiments with Two-sample T tests were used to compare the live cell concentration or absorbance of each phytoestrogen treatment group to each control group. Two-sample T tests were used throughout analysis to compare the mean of two independent groups at a time. The Student's T Test was used for samples with assumed equal variance (ratio of variances < 4). The formula is seen below, where $\bar{X}$ is the sample mean, S_p is the pooled standard deviation of the samples, and n is the sample size.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{s_p\sqrt{\frac{2}{n}}}$$

.

The Welch's T test was used for samples with unequal variance (ratio of variances > 4). The formula is seen below, where $\bar{X}$ is the sample mean, S_i^2 is the estimator of the variance of each sample, and n is the sample size.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_{\bar{\Delta}}}, \text{ where } S_{\bar{\Delta}} = \sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

.

Samples with a p-value ≤ 0.05 were considered significant.

Results and Discussion

Difficulties with the LNCaP Cell Line

PC-3s and LNCaPs were chosen cell lines as models for human epithelial prostate cancer when treated with phytoestrogens. However, there were some technical difficulties in the cell lines that were encountered during the experimental process. LNCaP clone FGC cells have a doubling time between 36-60 hr (ATCC, n.d.). Because of this range, 48 hours was chosen as the standard doubling time used in experiments compared to 24 hours for PC-3 cells. The slow rate of LNCaP culture growth became a challenge as cells did not reach confluency as fast as PC-3s, making it difficult to perform technical replicates within experiments and biological replicates in a timely manner. Furthermore, LNCaP cells were weakly adhered to well and flask surfaces and had a tendency to grow on top of one another rather than become confluent, so the addition of medium or other solutions often caused cells to lift. Hence, LNCaP cell counts were suggested to be slightly incorrect despite efforts to distribute clusters when resuspending medium solutions. These complications made working with LNCaPs less than ideal, and future projects subject to this cell line must prepare for potential issues that may arise.

Immunoblotting: PC-3 cells express estrogen receptors alpha and beta.

To explore the potential antiproliferative effects of phytoestrogens in prostate cancer cells, the presence of key receptors in both LNCaP and PC-3 cell lines was examined by immunoblotting. Receptor expression was measured using the following molecular weights for ER-α, ER-β, and AR, respectively: 68 kDa, 55 kDa, and 110 kDa (Flouriot et al., 2000; Nelson et al., 2017; van Laar et al., 1989). The blot, as seen in Figure 2, suggests the likely presence of ER-α, ER-β, and AR in the PC-3 cell line, as indicated by the accompanying marker. Because the PC-3 cell line is advertised as an AR-negative cell model, it is likely that the AR is modified in a way that would inhibit function but not expression. Slow LNCaP growth limited the protein content available to load on the gel, causing less than ideal band visibility for this cell line. However, faint bands at approximately 55 and 68 kDa may suggest expression of ER-α and ER-β. Despite no observed expression of AR in this specific experiment, literature suggests that LNCaPs do in fact express this receptor (ATCC, n.d.), and it is more likely that protein concentration was too low to observe a visible band.

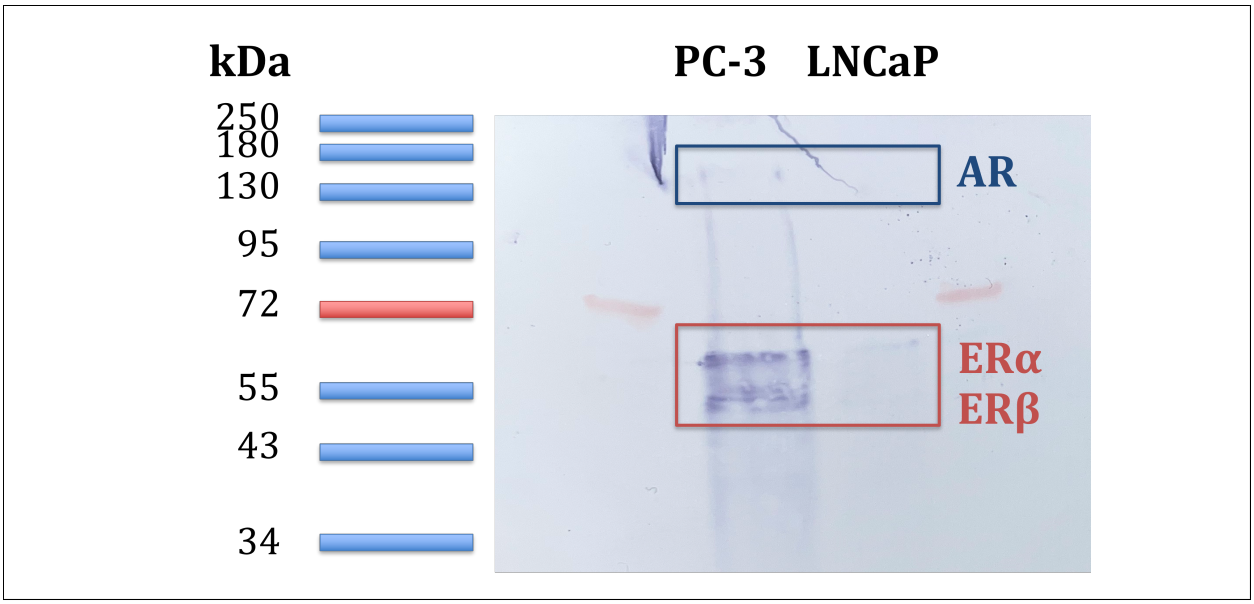

Figure 2: Western Blot results for PC-3 (second lane) and LNCaP (third lane) with markers on the end lanes. The blot shows bands for ERα and ERβ in PC-3 cells and may suggest expression of AR as well. ERα and ERβ are faintly visible for LNCaPs, but the protein concentration was too low to yield strong bands.

Cell Counts: LNCaP and PC-3 cells do not indicate decreased proliferation when treated with Promensil or isoflavones.

Cell counts of both cell lines were used to measure the proliferation of live cells when exposed to control and treatment conditions. In initial cell count experiments, both PC-3s and LNCaPs were plated and grown in medium containing phenol red. Due to the suggested weak estrogenic effects of phenol red in cell culture (Glover et al., 1988; Welshons et al., 1988), subsequent experiments were completed with phenol red-free medium. Figure 3 shows the cell proliferation of PC-3s grown in each medium, with an overall increased live cell concentration for cells grown in the medium containing phenol red. Although changes in media did not seem to alter the effects of phytoestrogen treatments, the data does show slight indication that phenol red may weakly stimulate estrogen-sensitive cell growth in general. The data represent 3 technical replicates for each condition, but the number of biological replicates for this experiment was too low to warrant statistical analysis. Subsequent cell count experiments with PC-3 cells were completed with phenol red-free media, but this was not possible for LNCaPs due to their extremely loose adherence to the wells and hence high potential for cell loss when changing the medium.

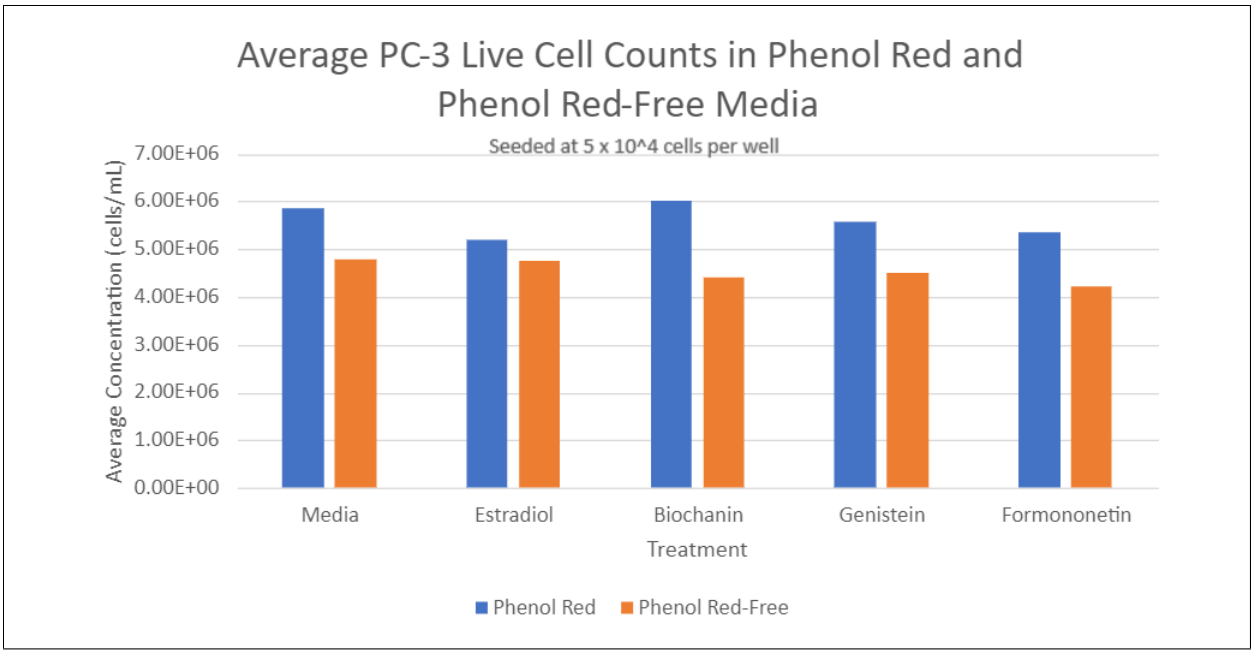

Figure 3: Average live cell concentrations (cells/mL) of PC-3 cells in phenol red-containing and phenol red-free media. After 24 hour treatments in respective media, cell counts showed overall increased live cell concentrations of PC-3s in medium containing phenol red. There was no suggested effect of phytoestrogen treatment with or without phenol red. No statistical analysis was completed due to the lack of sufficient biological replicates. Error bars denote standard deviation (SD) across 3 technical replicates in a single experiment.

Figure 4a shows the average live cell counts of PC-3 cells from 3 biological replicates using the same conditions: growth in phenol-red-free medium, seeding density of 5.0×10^4 cells, a 24-hour treatment period, and 1% phytoestrogen treatment (10 μL of 1.0×10^{-4} M isoflavones) in a total volume of 1 mL. T-tests, as described in the Data Analysis section, were used to determine statistical difference between treatment conditions given the completion of 3 biological replicates in this experiment. Compared to the media control, no treatments showed any significant difference in live cell growth. In Figure 4b, these live cell counts as a percent of the media control again appeared to have no difference in growth from the control group. These results suggest that PC-3 cells are not responsive to treatment with Promensil or any of the individual isoflavones. However, the β-Estradiol control also showed no significant difference compared to the media control. This may be due to errors in the Cellometer's ability to distinguish between live and dead cells or human errors in plating and treating cells, but some researchers also suggest that the response of prostate cancer cells to β-estradiol may be completely AR-dependent (Lafront et al., 2020), which would explain why PC-3s with non-functional, mutated ARs would show no response. This may suggest the need for a stronger, more predictable positive control for the PC-3 cell line in the future.

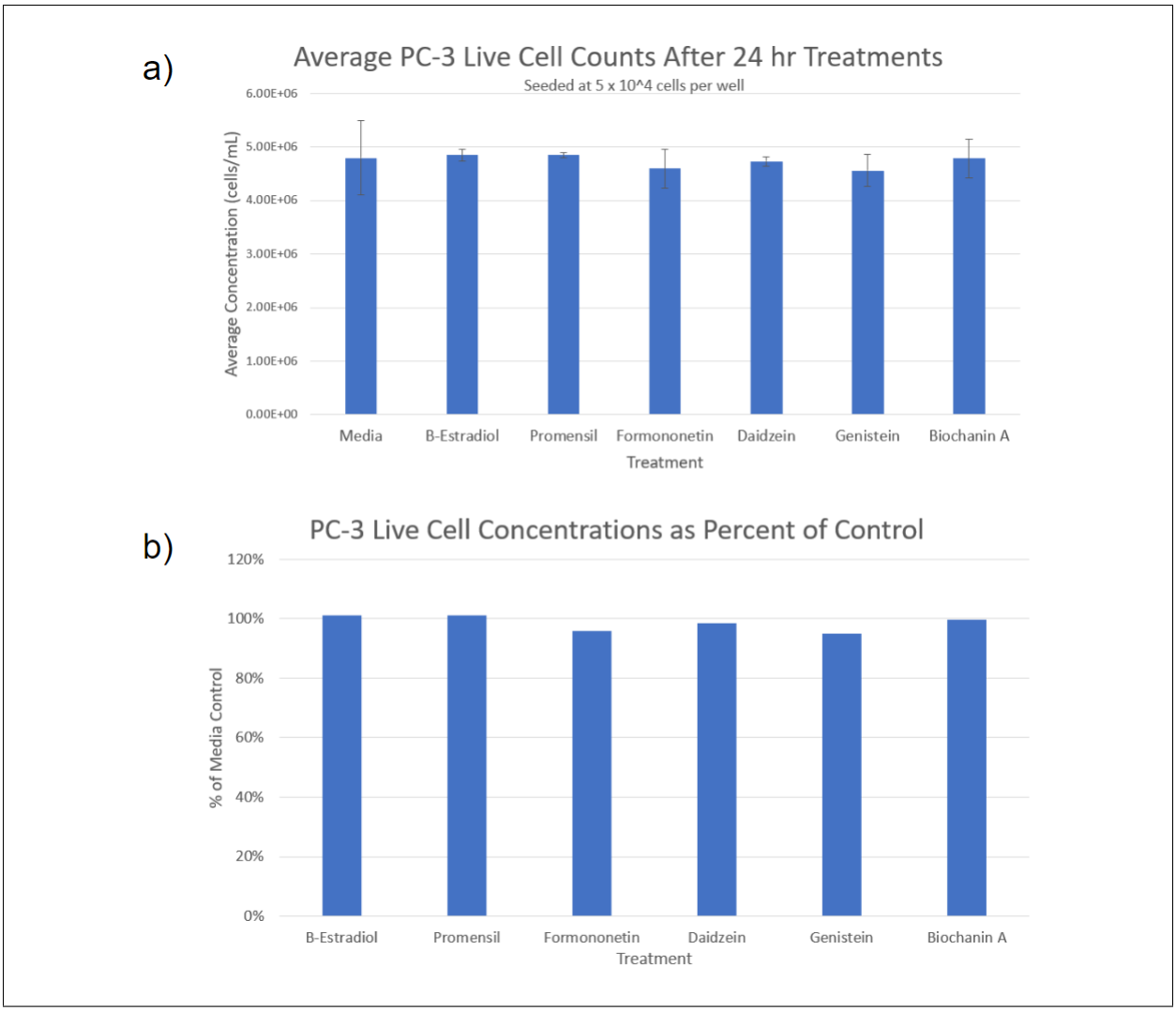

Figure 4: a) Average live cell concentrations of PC-3 cells after 24 hours of phytoestrogen treatment. Promensil and isoflavone treatment showed no significant difference in live cell proliferation compared to control groups, as determined through statistical analysis by t-tests. Error bars denote SD within the averages of 3 biological replicates (4 replicates total) for all treatment groups. b) Average live cell concentrations of PC-3 cells after 24 hours of phytoestrogen treatment as a percent of the media control. Promensil and isoflavone treatment showed no difference in live cell proliferation compared to control groups.

Live cell counts were also completed for LNCaP cells after 48 hour treatments with Promensil and isolated isoflavones. Those results are displayed in Figure 5a. Similarly, Figure 5b shows this live cell count data as a percent of the media control. For these experiments, too few biological replicates were completed to allow for statistical analysis so no significance can be claimed. However, both figures suggest that there is likely no difference in live cell proliferation of LNCaPs when treated with any of the phytoestrogen treatments. Once again, the β-estradiol and DHT controls did not behave as expected and showed no difference compared to the media control. As an androgen positive cell line, LNCaPs should be stimulated by hormonal treatments (ATCC, n.d.; Kahn et al., 2008). It is likely that the several difficulties faced with this cell line in regards to slow growth, low confluency, loose adherence, and the tendency of

cells to grow on top of one another contributed to the unexpected behavior of the positive control groups. Cellometer errors and human errors may have also played a role. Furthermore, these results are based on one experiment and it is possible that repeated experiments may have shown different proliferation patterns. Ideally, testosterone would have been used as a positive control for this cell line and may have yielded better results as a control group, but DHT was the most feasible and accessible option in the scope of this project.

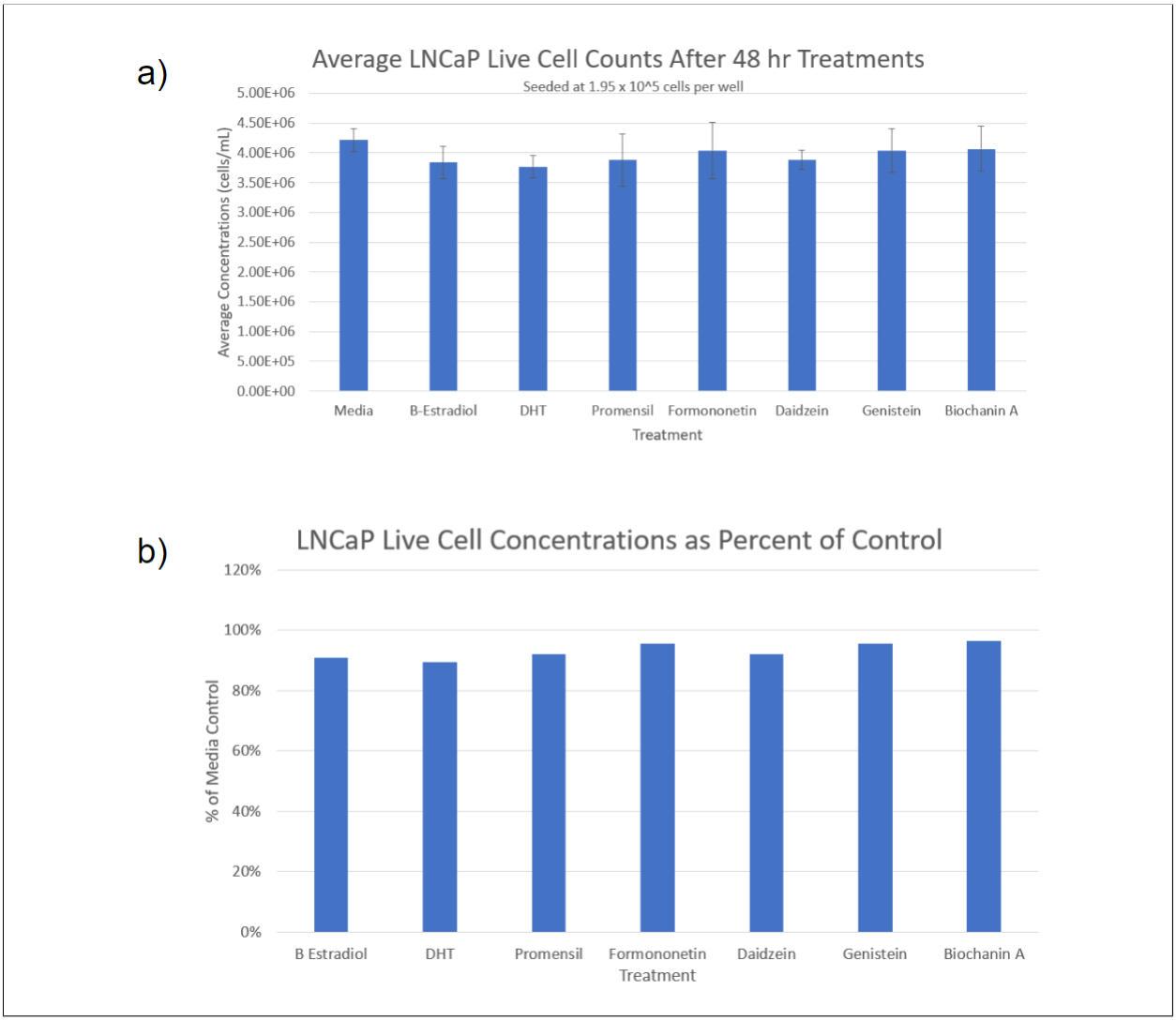

Figure 5: a) Average live cell concentrations of LNCaP cells after 48 hours of phytoestrogen treatment. Promensil and isoflavone treatment showed no indication of reduced proliferation compared to control groups. No statistical analysis was completed due to the lack of sufficient biological replicates. Error bars denote SD across 3 technical replicates in a single experiment. b) Average live cell concentrations of LNCaP cells after 48 hours of phytoestrogen treatment as a percent of the media control. Promensil and isoflavone treatment do not indicate any difference in live cell proliferation compared to control groups.

Overall, these results warrant further exploration into the relationship between prostate cancer cell proliferation and phytoestrogen treatment using a different measurement of proliferation, which would ensure that any Cellometer errors could be accounted for.

MTS Assay: Promensil components resulted in varying metabolic activity.

MTS assays were performed to measure proliferation via metabolic activity of the cells. This assay measures absorbance as a consequence of reduction reaction between the mammalian cells and the MTS reagent tetrazolium which reveals how many cells there are to engage in such activity. Due to the difficulty in obtaining nearly confluent LNCaP cells, biological replicates and therefore statistical analyses were unable to be performed for this assay. Figure 6a below shows PC-3 absorbances after one doubling period of treatments for three seeding concentrations, and averages for three replicates per concentration were calculated. There was visible reduction in absorbance for the Promensil treatments. Cells treated with Promensil had appeared to have noteworthy decreased growth in comparison to the media control for the two denser seeding dilutions, although we are unable to conclude if these data are statistically significant. Contrary to the expected outcome, the lowest seeding densities of 5×10^4 and 6×10^3 for isoflavones Biochanin A and Genistein yielded significant increases in proliferation. This disparity was suggested to be caused by components within Promensil that were not the isoflavones, as the ingredients in a tablet may contain unknown impurities. These results, nevertheless, were not consistent with our hypothesis nor the results of proceeding MTS assays.

Figure 6: Absorbance graphs for three MTS assays including percent of media control graphs. Color series represent seeding densities in three dilutions. Absorbance was measured after one doubling time: a,b) 24 hours after treatments for PC-3 cells and c) 48 hours for LNCaP cells. No statistical analysis was completed due to the lack of sufficient biological replicates. Error bars denote SD across 3 technical replicates in a single experiment.

Despite the previous outcome, MTS assays performed again on both PC-3 and LNCaP treated dilutions did not show similar results. PC-3 wells had no significant change in metabolism and proliferation compared to media controls in any concentrations (Figure 6b). Promsenil showed the greatest reduction in growth at initial cell concentration of 5×10^5 albeit not consistent with other dilutions. There appeared to be reduced proliferation for the isoflavones and Promensil as well for LNCaPs (Figure 6c). These results align with our predictions that LNCaP cells would yield a stronger antiproliferative effect, but it is not possible to conclude that these phytoestrogens inhibit proliferation due to the lack of statistical analysis.

It is interesting to note the disparities between the two proliferation assays as the tests evaluate cell growth using different methods. MTS assays are more sensitive to change due to their nature of analyzing the chemical reactions occurring from cell metabolism of tetrazolium compared to the quantitative gathering from visual characteristics in cell counting. While it is known that increased absorbance of light indicates increased metabolic activity, it is only inferred that this is attributed to the increased total number of cells. It is possible that the MTS experiments also experienced similar growth between all treatments as the cell counts did but that the treatments generated a shift in mitochondrial response to the tetrazolium reagent.

Despite seeing these distinctions in experimental treatments, the positive controls did not behave as expected in the MTS assays as well. The positive controls consisted of 17-β-Estradiol and 5α-Dihydrotestosterone-D3 (DHT) solutions in methanol. Estradiol treatments were expected to inhibit growth and viability responses for both cell lines due to tumor suppression (Carruba et al., 1994; Montgomery et al., 2010); however, the assays did not yield consistent and strong results suggesting this. As an androgen, DHT promotes prostate cell proliferation in a dose-dependent manner resulting in increased growth upon treatments with concentrations of up to 10 nM, then inhibiting growth at higher concentrations (Song & Khera, 2014; Pillai et al., 2021). LNCaPs are explicitly stated to respond to DHT (ATCC, n.d.) unlike PC-3 cells that have a much weaker response due to their mutated androgen. This sensitivity disparity in DHT response was recreated as PC-3 cell concentrations were unchanged compared to media control wells, but we expected that LNCaP cells would have an increased trend rather than slightly decreased. Again, it is important to note that statistical significance was unable to be calculated.

It is notable that biological replicates were not performed because of insufficient LNCaP growth, and some treatments were only performed in duplicates or triplicates. Even with the results from the first MTS assay, there is not strong enough evidence to claim that Promsenil has an antiproliferative effect due to any of the isoflavones. However, there is still indication that phytoestrogens may provide some chemoprotective effect that was unable to be fully presented using these assays. Due to the lack of consistency and issues in the control results, it is worth exploring the potential distinctions between over-the-counter Promensil and isoflavones further. However, several factors should first be tested, including establishing conditions with reliable and predictable responses to DHT and β-estradiol controls and determining concentrations that maximize the potential for observed phytoestrogen effects. In regards to cell line difficulties, it would be beneficial for future studies to first explore the effects of the altered AR in the PC-3 cells and determine if there is a more suitable AR-dependent prostate cancer cell line than the LNCaPs.

Conclusion

In conclusion, our results do not support the hypothesis that phytoestrogen treatment would decrease prostate cancer cell proliferation. Although the cell lines likely express the receptors of interest (ER-α, ER-β, and AR), Promensil and its individual isoflavones seem to show no consistent effects on cell growth, viability, and metabolism in LNCaP and PC-3 cell lines. One experiment following the MTS assay does suggest the potential for Promensil to decrease PC-3 cell growth, warranting the need for increased technical and biological replicates and further exploration into this assay as a measure of cell proliferation. This result may also suggest the possibility that components in Promensil other than the isoflavone components would be contributing to any antiproliferative effects observed. It can also be concluded that LNCaPs are not an ideal cell model for experiments studying prostate cancer cell proliferation due to their slow and inconsistent growth, loose adherence, and ineffectiveness in becoming confluent. Finally, it is important to note that these conclusions are made from in-vitro experiments only, and in-vivo experiments may show results with different implications for phytoestrogen treatment in actual prostate cancer patients. Hence, the potential of phytoestrogens as an alternative treatment is still valuable to explore.

Moving forward, several steps can be taken to better explore the effects of phytoestrogens in prostate cancer cells. First, more reliable and predictable controls should be established prior to investigating any phytoestrogen treatment effects. Ideally, testosterone would be available as a positive control for an androgen-dependent cell line such as LNCaPs. It would be beneficial to more thoroughly explore effective concentrations and treatment conditions for controls so that stronger conclusions can be made from experiments. Furthermore, due to difficulties in LNCaP cell maintenance, replicates of this cell line were difficult to obtain and future projects would benefit from using a different androgen-positive cell line. Additionally, completing more replicates using the MTS assay and trying other proliferation or viability assays would be beneficial here. It would also be useful to explore levels of receptor expression and methylation before and after phytoestrogen treatment since the proposed mechanism suggests these may be altered by phytoestrogens. This would also address the second part of our initial hypothesis that the antiproliferative effects of phytoestrogens would operate through ER-β and be influenced by changes in expression and methylation. Additionally, due to the contrasting results between Promensil and isoflavones in the first MTS assay, it may be worth exploring the individual chemical components within Promensil to see if any impurities may have skewed the findings. Lastly, future studies should assess difficulties with solubility of isoflavones, as incomplete dissolvement of isoflavone powders in diluent may impact their efficacy and may impact readings from certain assays. The potential for phytoestrogens to exhibit chemprotective and antiproliferative effects in prostate cancer is still worth exploring and with the necessary adjustments outlined here, more reliable conclusions can be made.

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