The Effects of Genistein on Breast Epithelial Cancer Cells

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

Mohammed Suhail Akhtar

Christina Ferrari

Date of Submission: 29th April, 2010

Advisors:

Professor Mike Buckholt

Professor Jill Rulfs

Table of Contents

List of Figures

List of Tables

Abstract

Phytoestrogens are popular substances used in hormone replacement therapy due to their similar structure to estrogen and ability to interact like estrogens in the body. One particular phytoestrogen compound, genistein, has been observed to have an antiproliferative effect on breast cancer cells. The experiments investigated the effects of estrogen and genistein on the cells through MTS assays and PCNA immunoblots. The cell line demonstrated estrogen responsiveness through both assays, but results as to genistein's effect on the T47D breast epithelial cells were inconclusive.

Acknowledgments

We would like to acknowledge Dr. Jill Rulfs and Dr. Mike Buckholt for their support and guidance as our major advisors throughout this project. We also extent a special thanks to Ms. Abbie White for her support with laboratory supplies and for imparting some of her wisdom to us. We would like to thank Dr. JoAnn Whitefleet-Smith for extending her laboratory space and knowledge to us. We thank the biology department and our fellow phytoestrogen project partners for the constant support.

Introduction

The effects of hormone replacement therapy (HRT) and its association with increased cancer risks has recently been examined (Ross et al., 2000). Research supports that HRT does increase the risk for developing cancer (Holmberg et al., 2008). Therefore, many women have chosen to take over-the-counter phytoestrogens to treat the symptoms of menopause. It is suggested that since phytoestrogens have a similar structure to estrogen, they may act through the same pathways in the body to relieve the menopausal symptoms caused by the steep decrease in circulating estrogens. Phytoestrogens are often favored over HRT, because they are plant derived compounds viewed as healthier, natural alternatives that are widely available but unregulated.

The aim of this project was to observe the possible estrogenic or anti-estrogenic effects of the phytoestrogen, genistein, on a breast epithelial cancer cell line. It was hypothesized that the addition of genistein in a high estrogen environment would result in antiproliferative effects while the addition of the phytoestrogen at a low estrogen concentration would cause an increase in cell proliferation.

An estrogen responsive cell line, T47D, was purchased from the ATCC with expressed estrogen receptors in order to test the effects of the addition of genistein to the cells. Several experimental conditions with different concentrations of genistein and estrogen provided inconclusive data, which did not support the hypothesis.

Background

Breast cancer is the most common form of cancer affecting woman in the United States (Centers for Disease Control and Prevention, 2010). This has lead researchers to examine the major risk factors associated with the disease, as well as the differences between breast cancer rates of women in the United States as compared with women living in other countries. In particular, the rising risk of breast cancer is linked to the participation of premenopausal and postmenopausal women in hormone replacement therapies (HRT).

The physical symptoms of menopause in premenopausal and postmenopausal women are often treated with HRTs, usually containing doses of estrogen and progesterone. The severe drop in the estrogen hormone due to menopause can produce a range of symptoms, including hot flashes, cold sweats, difficulty sleeping, and irritability (Dennerstein et al., 2000). These changes in the body can lead many women to make the choice to participate in HRT.

However, studies have shown an increased risk of breast cancer as an HRT side effect (Holmberg, et al., 2008). A randomized study by L. Holmberg and H. Anderson was performed in 2002 with 442 Scandinavian, female, breast cancer survivors with follow-ups over a median of 4 years. The subjects were either administered hormone therapy or symptomatic management of menopausal symptoms without hormone addition. The women in the hormone therapy group developed twice as many new cancer events as the group receiving symptomatic management. The study concluded a statistical significance of increased risk of new breast cancer events in the hormone therapy group (Holmberg, et al. 2008).

The rates of American women developing breast cancer are much higher than those of Asian women, with the risk for an American woman having been recorded as nearly seven times greater than the risk for an Asian woman (Bouker and Hilakivi-Clarke, 2000). However, this discrepancy is mitigated as Asian women immigrate to the United States, which suggests that the increased risk factors are not based on genetic factors but are environmental (Wu, et al. 1996). A case study performed in 1986 compared the estrogen plasma levels of premenopausal Caucasian woman living in Boston with plasma concentrations from woman born in various Southeast Asian countries and residing in Hawaii. The results found a thirty-two percent increase in estrogen plasma levels in premenopausal, Caucasian females living in the United States

(Goldin, et al., 1986). It can be proposed that the cancer rate discrepancy between American and Asian women may be caused by the difference in circulating estrogen levels in the body.

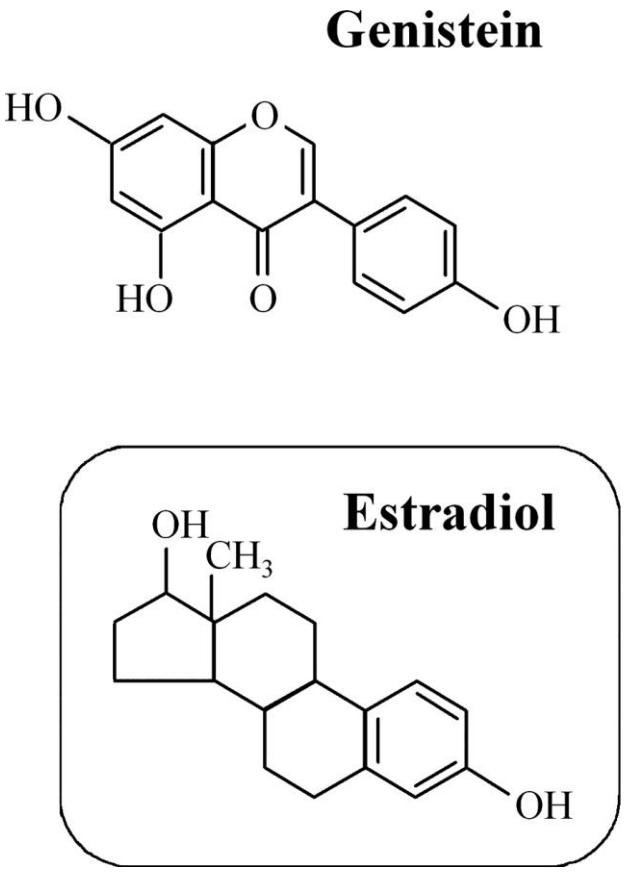
Research has also found that Asian woman born in America have a sixty percent greater risk than Asian woman living in Asian countries of developing breast cancer, which again strengthens the argument of environmental factors contributing to the varied estrogen levels and breast cancer rates (Bouker and Hilakivi-Clarke, 2000). These environmental factors can be further narrowed down to the role of diet, as researchers have attributed dietary factors in contributing to half of recent breast cancer diagnoses (Bouker and Hilakivi -Clark, 2000). The consumption of soy is comparatively higher in Asian countries than the United States and is believed to be responsible for the cancer rates.

Soy is often attributed as the dietary factor that reduces breast cancer rates among Asian females due to its high concentrations of many naturally occurring phytoestrogens, such as the isoflavones, genistein and daidzein (Ososki and Kennelly, 2003). Although research on daidzein is limited, the effect of genistein on cell proliferation has been examined.

Phytoestrogens have a similar structure to estrogen, as shown in Figure 1, but are seen as safer, natural alternatives to estrogen therapies that originate from plants and non processed food products. In response to increasing criticism of HRTs and the rising rates of breast cancer, many women opt to pursue more natural remedies and choose over-the-counter phytoestrogen supplements to find relief for their menopause symptoms. Genistein is one such phytoestrogen, and is an active component in many over-the-counter products and supplements that aim to lessen the symptoms of menopause. These phytoestrogens are usually marketed as the 'natural' form of hormone replacement therapy.

The exact pathway, in which phytoestrogens interact in the body, whether by mimicking estrogens or competitively blocking the estrogenic response, is not known (Warren and Devine, 2002). It is hypothesized that phytoestrogens act as estrogens at low concentrations and act to block estrogenic activity at high concentrations (Ososki and Kennelly, 2003).

Figure 1: Phytoestrogens, genistein, and 17β-estradiol structures (Wood et al., 2006)



Estrogens are steroid hormones produced from cholesterol in the ovary, placenta, and adrenal cortex in the female body. Prior to menopause, the predominant estrogen, 17β-estradiol, is found in female circulation at a concentration of 40-400pg/mL. Post-menopause, estradiol is found at a concentration of 10-20 pg/mL (*Anti Aging Guide*, 2010). This hormone functions at the higher, premenopausal levels to produce the secondary sex characteristics in women. Estrogen molecules diffuse into cells and bind to the domains of estrogen receptors (ERs), which are transcription factors activated by the estrogen hormone. 17β-estradiol binds specifically to either the ER α or ER β, which activates the transcription factors to modify gene transcription and possibly activate specific, mutated oncogenes to express tumor producing cells.

Oncogenes are a family of genes responsible for normal development and tumor suppression in the body, but when mutated or overexpressed, can cause cancerous cell growth (Creighton, 1999). Estrogen is known to activate certain oncogenes, such as the inherited BRCA-1 and BRCA-2 genes, which are suspected to mutate and cause breast

cancer cell proliferation (Hilakivi-Clarke, 2000). This mode of ligand binding would not typically disrupt normal cell growth, but can cause cancerous cell growth when the genes activated are overexpressed, such as in the case of when women are exposed to high estrogen levels for long periods of time. It is believed that the longer the exposure to estrogen in the body, the greater the risk of developing cancer due to the hormone increasing expression and mutations in oncogenes. An early menarche and late menopause both foster environments of high estrogenic levels and activity for long periods of time to contribute towards an increased breast cancer risk (Clemons and Goss, 2001). The addition of the hormone post menopause, when natural estrogen levels have decreased, may therefore result in the activation or mutation of oncogenes to produce breast cancer (Kennemans and Bosman, 2003).

One of the first major experiments relating estrogen production with breast cancer was performed by Beatson in 1896, in which the ovaries, a major source of estrogen synthesis, were removed from the bodies of patients suffering from breast cancer. This procedure yielded significant results and patients with the ovariectomies exhibited regression of the disease (Ali and Coombes, 2002). These results have also been supported by recent experiments where the removal of ovarian estrogens in postmenopausal women produces a significant decrease in breast cancer development (Hilakivi-Clarke, 2000). Therefore, it can be concluded that exposure to estrogen can affect development and progression of breast cancer. However, not all breast cancers are responsive to estrogen. In a study by McGuire et al. in 2003, the level of estrogen responsive tumors was determined by measuring the final level of progesterone receptors (PgRs), which are indicated to be under the direct control of estrogen. PgRs were found in about 59% of metastatic breast cancer tumors with expressed estrogen receptors (McGuire et al., 2003). This signified that approximately 59% of estrogen receptors in metastatic tumors are responsive to estrogen and can respond to hormone therapy.

Phytoestrogens are supplemented as an alternative to HRT and exhibit a high binding affinity for ER β. They are able to bind in a similar manner as estradiol and exhibit estrogenic effects, such as the increase in cellular transcription and proliferation (Bouker and Hilakivi-Clarke, 2000). Genistein is believed to exhibit a 20 to 30 fold greater binding affinity for ER β than ER α, but an overall 1,000 to 10,000 fold lesser binding affinity to either estrogen receptor when compared to the binding affinity of estradiol molecules (Kuiper et al., 1998). Studies have also found that the ER β may

counteract the activity of ER α and act in an anti-proliferative manner (Strom et al., 2003).Therefore, it is hypothesized that at higher estrogen concentrations, genistein may compete with 17β-estradiol for binding sites at the ER to prevent estrogenic activity and prevent overexpression or stimulation of mutated oncogenes. If the ER β does have anti-proliferative effects as the research suggests, then the addition of phytoestrogens in a high estrogen environment will only decrease cell growth by altering the ratio of bound receptors in favor of the ER β. However, at low estrogen concentrations, when the ER is not saturated, genistein may only have an additive proliferative effect by introducing more bound phytoestrogens into the cell. Once again, this could be dangerous in terms of mutated oncogenes or overexpression of the genes it activates to possibly cause cancer.

Genistein is also theorized to exhibit antiproliferative effects through other non-receptor mediated pathways, such as the inhibition of tyrosine kinase or topoisomerise II activity to possibly arrest cellular replication. These inhibitory mechanisms may result in increased cell apoptosis or decreased cell proliferation, with both mechanisms acting to reduce the growth of cancerous cells (Trock, et. al, 2006).

In order to examine the affect of genistein at high and low estrogen conditions, it is necessary to find a breast epithelial cell line that exhibits estrogen responsiveness. This has proven a difficult task in previous Major Qualifying Projects (MQPs) where both the 2007 and 2008 studies of phytoestrogens on the MCF-7 cell line found the cell line to be unresponsive to estradiol (Raasumaa, 2008; DeVault, Kosmaczewski, and Tracy, 2009). This cell line was known to contain expressed estrogen receptors but the increased number of cell passages could have resulted in decreased sensitivity to estrogen (Pratt and Pollak, 1993). Due to the MCF-7 cells' lack of estrogen responsiveness in previous MQPs, a new cell line was explored. The T47D breast epithelial line was originally obtained from the duct of the mammary glands in a 54 year old female diagnosed with infiltrating ductal carcinoma. The cells were found to have expressed receptors for 17-β estradiol as well as several other steroids (ATCC, 2010).

These adherent cells of the T47D line were examined in our investigation under experimental conditions. It was necessary to demonstrate proof of concept by validating estrogen responsiveness in the new cell line. The cancerous breast cells were predicted to show increased proliferation in higher concentrations of estrogen, since the hormone when bound to the ER acts as a transcription factor to regulate cell growth. It was also

hypothesized that the phytoestrogen, genistein, would competitively inhibit estradiol binding to its receptor at high estrogen concentrations, but would exhibit estrogenic effects by binding to the ER receptor at low estrogen concentrations. These data could have significant implications in dietary recommendations and its associated breast cancer rates, as well as the regulation of over-the-counter phytoestrogen products.

Methodology

Cell Cultures

The T-47D breast epithelial cancer cell line was used throughout this project. This cell line was obtained from the American Type Culture Collection (ATCC) (Product # HTB-133). The ATCC is a private, nonprofit biological resource center and research organization that aims to provide biological products and services that further the specific objectives of research organizations, both public and private (ATCC, 2009). The T47D cell line has expressed receptors for estrogen, progesterone, and glucocorticoid. The doubling time for the cells was listed as 32 hours. However, handling of these cells in the lab showed the doubling time to be approximately between 28 hours to 32 hours. The cells were maintained in T75 flasks and in situations where there were too few cells to cultivate, the culture was initiated in a T25 flask. The cells were always incubated at 37°C and 5% CO_2. Based on the ATCC recommendations, the cells were cultured in RPMI-1640 growth medium (Sigma Aldrich, Catalog No. R8758), modified by adding 10% Fetal Bovine Serum (FBS) (Thermo Scientific, Catalog No. SH303396.03), 1% Penicillin-Streptomycin Solution (Cellgro, Catalog No. 30-001-CI) and 0.2units/mg of Bovine Insulin (Sigma Aldrich, Catalog No. 11070-73-8). The cells were passaged upon reaching a confluency of >80%, and were split at different ratios based on the experiments being performed.

Cell Passaging

Cells were passaged according to the following protocol. The following steps are directed to a T75 flask of cells. The volumes of different solutions used for these steps were scaled down when cells were being passaged in T25 flasks.

1. After removing the media from the flask, the cells were rinsed in 5mL Hank's Balanced Salt Solution (Life Technologies, Catalog No. 14170-112) for 30 seconds.
2. This was aspirated off and the cells trypsinized using 0.25% Trypsin (Gibco, Catalog No. 15050-065). About 2-3 mL of 0.25% trypsin was added and the flask incubated at room temperature for 2 to 3 minutes.

3. Upon getting 50% of the cells off the flask by visual inspection, 9mL of the culture media was added to the cells and after completely rinsing the flask bottom with the media; the cells were transferred into a 15mL centrifuge tube using a suitable pipette.
4. The cells were centrifuged at 1000xg for 3 minutes to pellet them.
5. After aspirating off the media, the cells were re-suspended in 4mL of fresh culture media.
6. Using a hemocytometer, the cells were counted and further sub-cultivation ratios were decided.

Assays

MTS Assay

In order to test for estrogen responsiveness, a cell proliferation assay was done. The Cell Titer 96®AQueous Non-Radioactive Cell Proliferation Assay was obtained from Promega (cat# G5421) to determine the cell proliferation of T47D cells in the presence of estrogen. The assay is composed of solutions of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent, phenazine ethosulfate (PMS).The MTS is reduced into a formazan product by living cells and can be measured directly at 490nm. The MTS gets converted into formazan due to the dehydrogenase enzymes present in metabolically active cells. The higher the absorbance at 490nm, the higher the number of living cells in the culture.

Cells were plated at 150,000 cells/well and 75,000 cells/wells in 24-well plates, in the RPMI growth media as described previously, for a time period of 48 hours. The RPMI media was then aspirated off and fresh media was added to each well. This fresh media consists of Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Catalog No. 12-917F), which is absent of L-Glutamine or Phenol Red. To this media, 0.2 units/mg of insulin and Dextran Charcoal Treated (DCT) 10% Fetal Bovine Serum (Hyclone, Catalog No. SH 30068.03) were also added. The cells were incubated in this modified media for an additional 24 hours. The DMEM media, which lacked Phenol Red, was used to minimize any false positive readings for estrogen receptors since Phenol Red,

which is a pH indicator, is found to bind the estrogen receptors in human breast cancer cells MCF7. Even though a different cell line was used, this step was taken as a precautionary measure so as to be sure of observing actual results and not false positives, due to the reaction with Phenol Red. A MTS Cell proliferation assay was started after a total growth time of 72 hours, which was run for 12 hours to measure cell proliferation. The cell proliferation was measured by adding 200 µl of the MTS assay solution to each well of 1ml cells, and running the assay for 12 hours before reading the absorbance values at 450nm. Several slight variations of this assay were run over the timeline of this project, to determine if the cells were responsive to different concentrations of estrogen which included 100µM, 10 µM, 1 µM, 0.1 µM and 0.01 µM. Some of these variations included plating a different set of cell densities, or switching from 24 well plates to 96 well plates and vice versa.

One particular variation of this assay is the addition of 1% FBS instead of the 10% DCT FBS to the modified DMEM media, which is used to change the media of the cells after their initial growth period of 48 hours. This modification was performed to help cells stick to the bottom of the plate as opposed to the large clumps of cells floating in the cell media, and was hence incorporated into the assay method for future assays.

The assays in the 96-well plates seemed to be failing consistently for no apparent reason but they could be attributed to the following reasons. There was very little working volume in 96-well plates (100 µl to 200 µl), which made it hard to maintain consistency of the number of cells added. Additionally, after performing a few experiments with different cell densities, it was still unclear what the best possible cell densities were to plate per well in a 96-well plate In order to overcome these issues, it was decided to switch to 24 well plates, since their working volumes are larger (1ml) and cell densities of 75,000 cells/well and 150,000 cells/well were plated.

Immunoblotting

A second approach, which consisted of immunoblotting, was chosen to demonstrate estrogen responsiveness in the cells and a few experiments were carried out using this approach. The immunoblot was performed using the PCNA antibody. PCNA or Proliferative Cell Nuclear Antigen is a protein that is mainly synthesized in the G1 and S phases of the cell cycle. It is involved in the DNA replication and aids the leading strand productivity during transcription (Santa Cruz Biotechnology, 2010).

Therefore, this protein was chosen as the target protein for the immunoblot process since cell proliferation is directly related to the amount of PCNA protein present in the cells.

The cells were plated onto a 24-well plate and the following steps, similar to the MTS assay procedure were performed. The cells were plated at 150,000 cells/well and 75,000 cells/wells in 24-well plates, and grown for a period of 48 hours in the modified RPMI growth media as described previously. Five different concentrations of 17-β-estradiol were prepared in the modified DMEM media with 1% FBS as described previously. These concentrations were 100 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM and 0 μM. When 48 hours of cell growth in the RPMI media elapsed, the RMPI media was aspirated off and 1ml of each estrogen concentration was added to the respective well of cells. After completing this step, the plate was left to incubate for an additional 24 to 30 hours and then the plate was frozen down at -80°C, after aspirating off the DMEM media and rinsing the cells with 1% PBS.

In order to perform the immunoblot, 12% acrylamide gels were cast. The protocols for this were taken from Current Protocols in Molecular Biology Vol.2, pages 10.2.5 to 10.2.9 and 10.8.4 to 10.8.6 (Ausubel, et al.). There were a few changes made to the protocol as follows:

For the separating and stacking gels, 40% Acrylamide was used instead of a 30% Acrylamide, mentioned in the protocol. Therefore, all the volumes of the other ingredients were scaled accordingly. Once the gels were prepared, the frozen cells were thawed and resuspended in 1%PBS, making sure as many of the cells were scraped off the bottom of the wells in the plate as possible, and resuspended in the solution.

Immuno-analysis of the Protein Membrane

The membrane was then processed through the immunodetection technique for PCNA antibody. The mouse monoclonal antibody PCNA (Santa Cruz Biotechnology Inc, Cat. No. sc 25280) was used as the primary antibody for the immuno-detection. This was diluted at 1:1500 in the blocking buffer. A goat anti-mouse IgG (Santa Cruz Biotechnology, Cat. No. sc 2008) was used as the secondary antibody in the detection.

Bradford Assay

After the immunoblot, a Bradford assay was carried out to test for actual protein concentrations of the cell samples. A Coomassie (Bradford) Protein Assay Kit was used to perform the Bradford test (Thermo Scientific, Catalog No. 23200). The Bradford assay consisted of diluting each cell sample 50 fold in the provided Bradford Coomassie Reagent and reading absorbance values at 595nm in a spectrophotometer (Thermo Scientific, 2010). A standard curve was created using an Albumin BSA Standard (Piece, Catalog No. 1856269). The instructions for creating the different concentrations of the BSA standard were taken from the Instructions of Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, 2010). The absorbance for the standard curve was also measured at 595nm and then a graph was plotted using the blank-corrected absorbance value of each BSA standard with its concentration in μg/ml. The standard curve was then used to determine the protein concentrations of the different cell samples.

Results

MTS Assay Validation

In order to validate the Cell Titer 96®AQueous Non-Radioactive Cell Proliferation Assay (MTS), cells were plated at 100,000, 50,000 and 25,000 cells/mL concentrations in 12 well plates. The cells were incubated in the modified RPMI media described in Methods for 12 hours. This RPMI media was aspirated off and changed to the modified DMEM media detailed in the methods and the cells were incubated for another 12 hours. After a total of 24 hours incubation period, the MTS assay solution was added to the cells and the assay was run for 4 hours and the absorbencies of the cells were read on a spectrophotometer at 450nm. The microphotographs at 0, 12, and 24 hours after plating the cell concentrations are shown in Figure 2. The cell growth was visibly noticeable by different concentrations plated and increased with time incubated.

Figure 2: Cell Growth Microphotographs

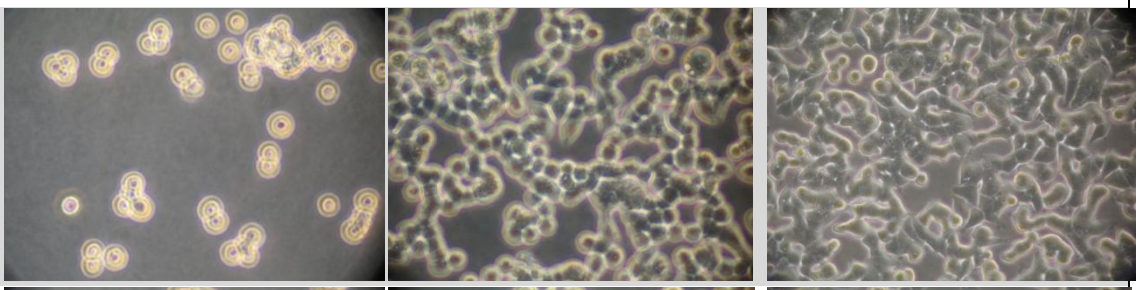
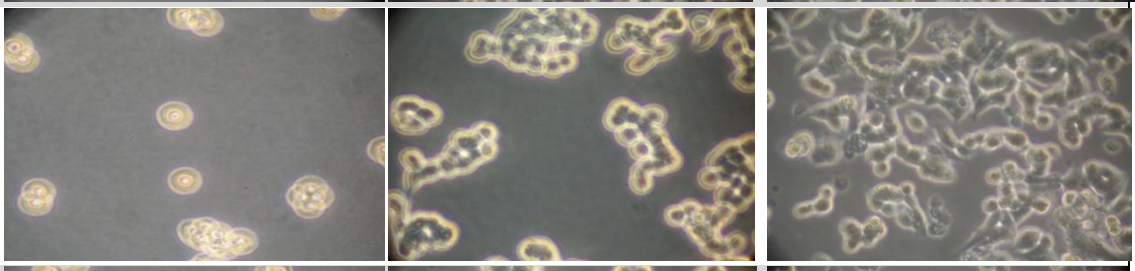
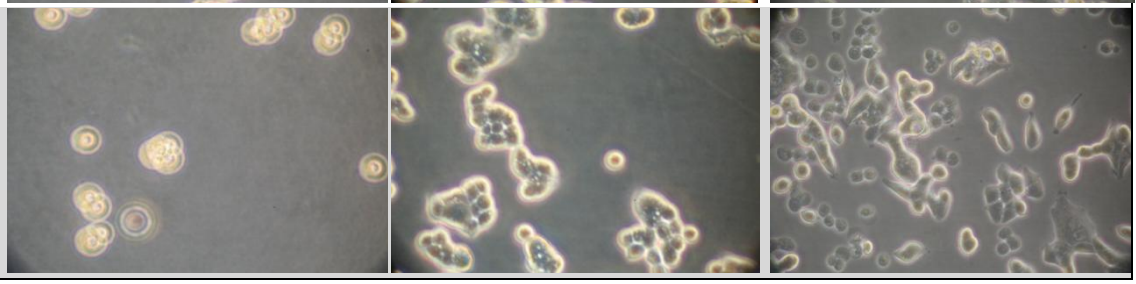
	Time after plating T47D cells		
Cells/ mL	0 hours	12 hours with media change	24 hours with media change
100,000			
50,0000			
25,000			

Table 1: MTS Assay Validation Data

		Cell Concentration Plated (cells/mL)			
		100,000	50,000	25,000	0 (Assay Reagent Blank)
Absorbance (450 nm)	Assay Kit #1	0.842	0.762	0.717	0.174
	Assay Kit #2	0.775	0.727	0.671	0.196

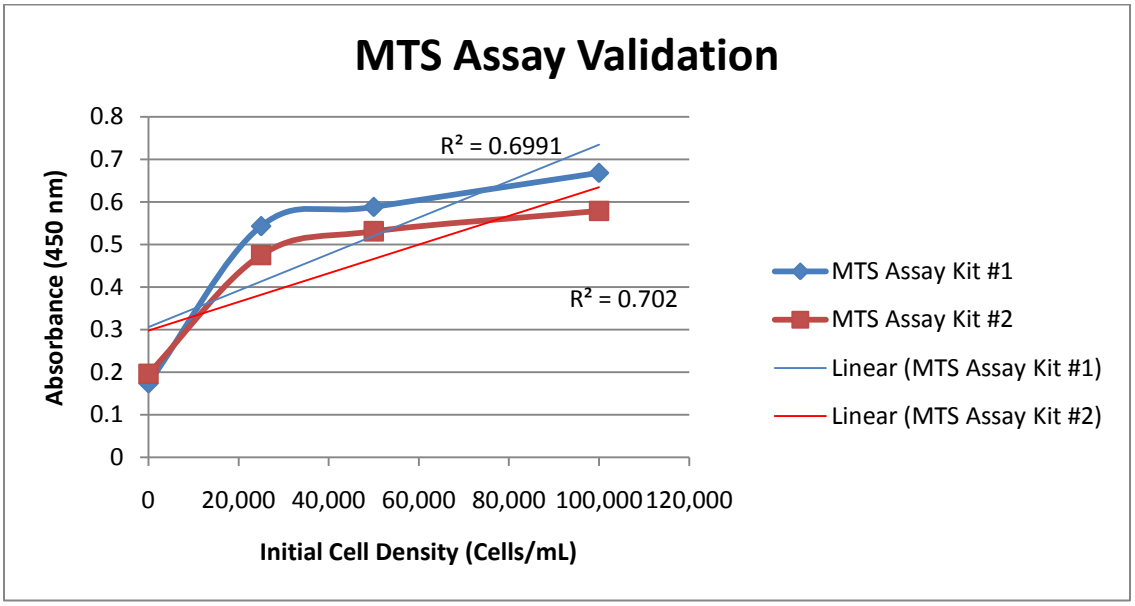
Assay Kit #1 = Promega CellTiter 96® AQueous One Solution Cell Proliferation - DOE = June 2010 (Cat # G3580)

Assay Kit #2 = Promega CellTiter 96® Aqueous Non-Radioactive Cell Proliferation - DOE = March 2012 (Cat # G5421)

The spectrophotometer readings from the plate are shown in Table 1. The data exhibits a trend in increasing absorbance with increasing cell densities. However, these absorbance values are not for the specific cells numbers listed in Table 1. Almost 28 hours had passed since the cells were first plated and this accounts for approximately one doubling time, which essentially means the absorbencies noted were for the cell densities of around 200,000 cells/well, 100,000 cells/well and 50,000 cells/well.

The data in Table 1 was plotted in Figure 3.The curves show a positive relationship between absorbance and cell concentration for both assay kits. The R^2 values are displayed on the graph and support the positive correlation.

Figure 3: MTS Assay Validation Plots



The Figure 3 shows the two different MTS assay kits used to demonstrate the MTS cell proliferation assay validation. Each plot shows a constant increase in absorbance values with an increase in the cell densities and is almost saturated since the cells are almost confluent.

Estrogen Responsiveness Assay using the MTS Kit

Table 2 below shows the absorbance values of the formazan dye emitted from the cells during the MTS assay. These are results obtained 36 hours after the estrogen hormone was added to the cells.

Table 2: Absorbance Values from MTS Assay for Different Cell Densities Plated at Different Estrogen Concentrations

Cell Densities	10 µM	1 µM	0.1 µM	0.01 µM	0 µM	Controls	Remarks
150K	2.821	2.469	2.521	2.614	2.593	2.303	Cells in media
						0.794	No Cells
75K	1.906	1.796	1.406	1.14	1.321		
						0.346	Assay Control Blank

Table 2 shows the absorbance values measured at 450nm of the formazan dye in the cells for each of the estrogen concentrations. It is seen that for both the cell densities, the 10 µM concentration of estrogen showed the highest absorbance values, which relates to a higher cellular proliferation rate. This data is plotted in Figure 4, in order to better visualize and draw conclusions.

Figure 4: Estrogen Responsiveness Plot: Absorbance versus Estrogen Concentrations

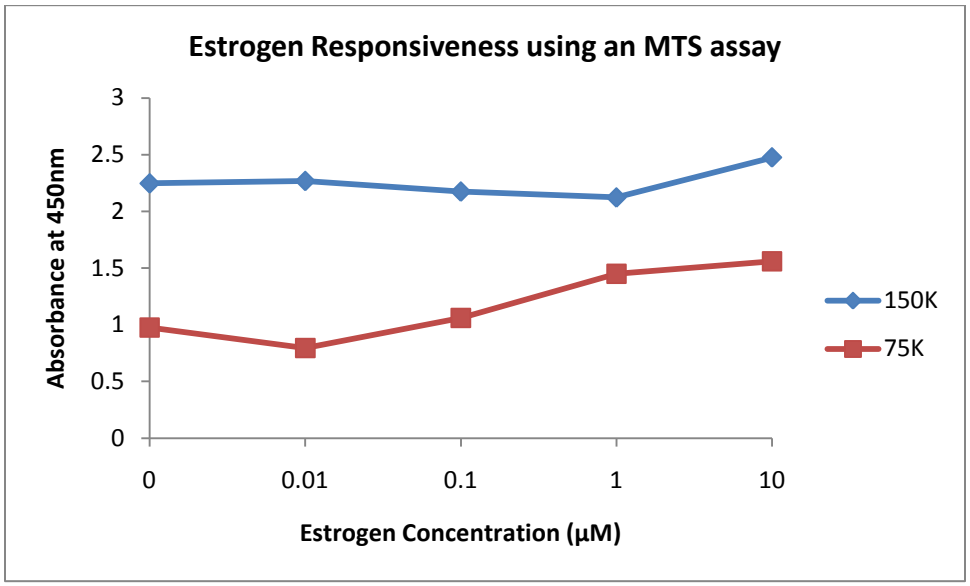


Figure 4 above shows the two cell densities plotted at different estrogen concentrations. It is seen that the cell densities of 75,000 cells/well demonstrates estrogen responsiveness whereas the cell densities of 150,000 cells/well seems to decline with increasing concentrations of estrogen. This could be due to the fact that the cells at densities of 150,000cells/well reached confluence in the 72 hours of growth time plus 12 hours of assay time and hence proliferation was inhibited.

Immunoblotting

After having obtained results from the MTS assay for estrogen responsiveness, a PCNA protein immunoblot was performed in order to both validate the results obtained with the MTS assay as well as show consistency in the results obtained.

Table 3: Absorbance Values of Cells at the Different Estrogen Concentrations

	Estrogen Concentrations				
Cell Densities	**10 µM**	**1 µM**	**0.1 µM**	**0.01 µM**	**0 µM**
75K cells/well	0.712	0.754	0.628	0.773	0.702

Table 3 above shows the different absorbance values emitted by the formazan dye in the MTS assay at different estrogen concentrations for 75,000 cells/well. This data is plotted in Figure 5 to get a visual image, which shows inconclusive data since all the data points are very close to each other. Another duplicate set of cell samples taken from another plate as the samples in Table 3 were run on a SDS-PAGE to quantify cellular proliferation at different estrogen concentrations. A picture of the transferred membrane is seen in Figure 6.

Figure 5: Absorbance Values for Cells at each Estrogen Concentration

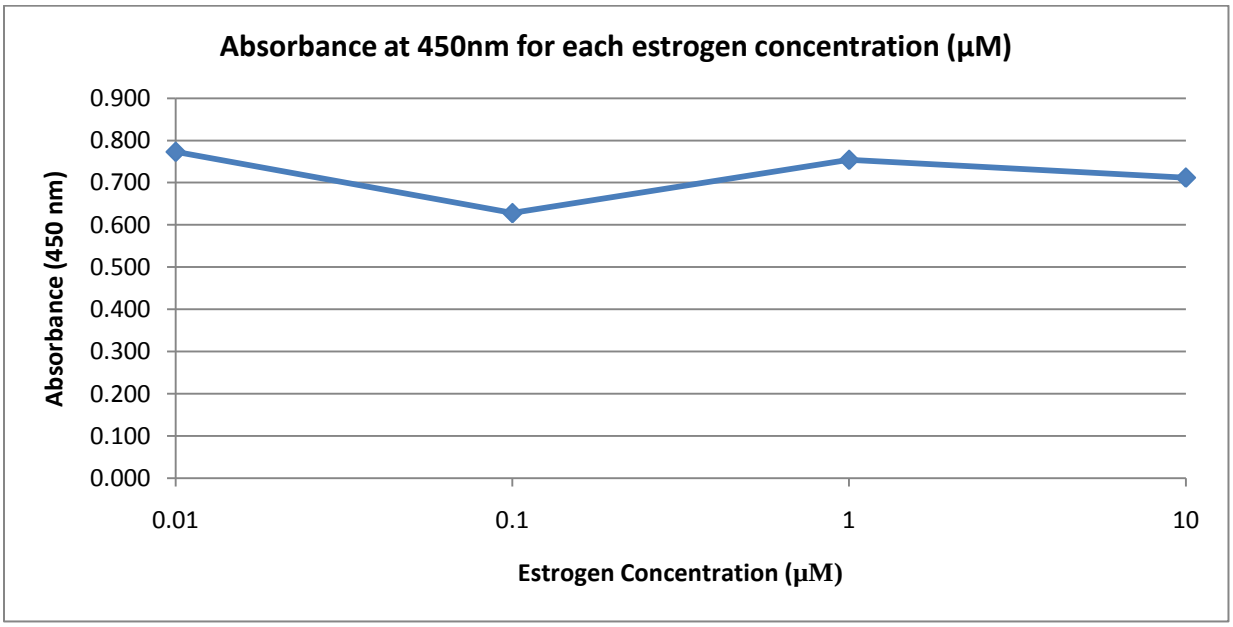


Figure 6: Immunoblot Bands for Concentrations of Estrogen

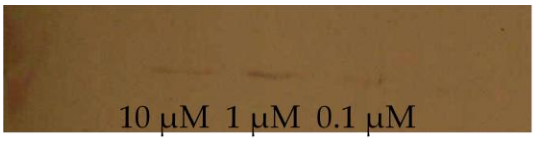
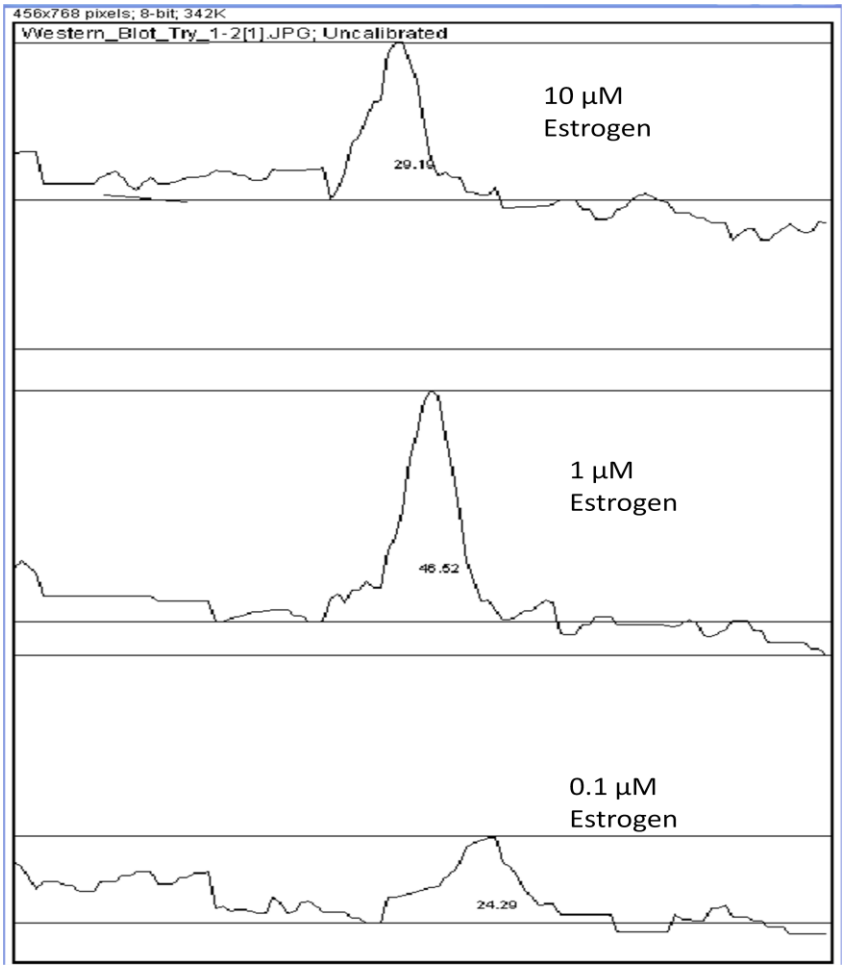


Figure 6 shows the immunoblot obtained after processing it with the PCNA antibody. Faint bands appear that correspond to the different estrogen concentrations. These faint bands can be associated with too little protein having been loaded onto the gel. Figure 7 below shows a peak analysis of the immunoblot in Figure 6 which shows that the 1 µM concentration of estrogen has the brightest band although all of the visible bands are

very faint. Therefore nothing can be substantially concluded from the immunoblot and the data should be regarded as is observed.

Figure 7: Different Immunoblot Band Intensities as Individual Peaks



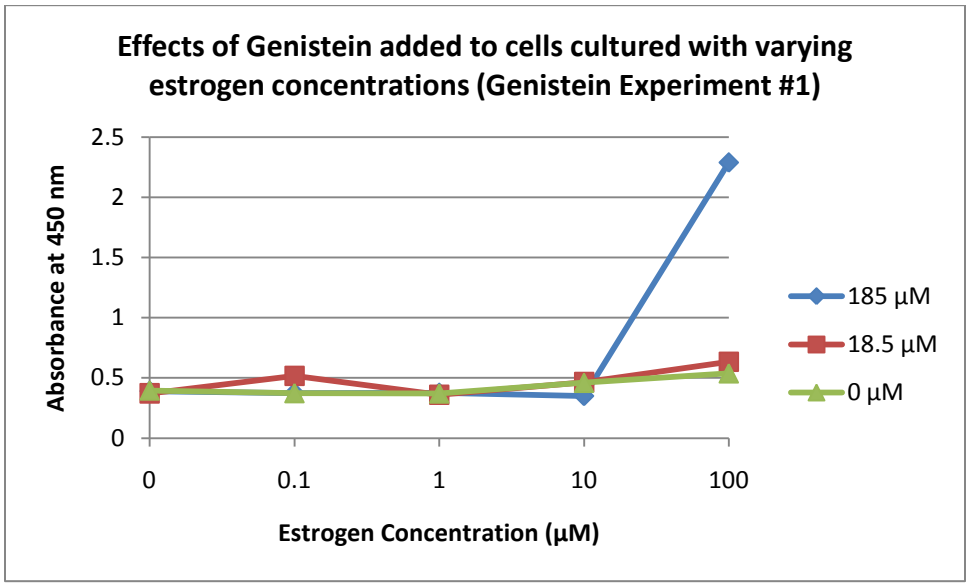
Genistein Experiments

Table 4 displays the proliferation effects of genistein added to cells cultured with varying estrogen concentrations, as measured by the MTS assay. From Table 4, the only effect of genistein is seen at 100 µM concentration of estrogen, which is an order of magnitude higher than the other values. Apart from this single data point, there appears to be no effect of either genistein or estrogen at lower concentrations of estrogen. Similarly, there is only a slight increase in absorbance values at 18.5 µM of genistein, at both 100 µM and 10 µM of estrogen. However, this data is again inconclusive since the effects of 100 µM estrogen have not been investigated previously.

Table 4: Effects of Genistein at Varying Estrogen Concentrations (Genistein Experiment 1)

	Estrogen Concentration (µM)				
Genistein Concentration (µM)	**100**	**10**	**1.0**	**0.1**	**0.0**
185.05	2.289	0.349	0.375	0.374	0.39
18.5	0.634	0.465	0.361	0.517	0.372
0	0.539	0.46	0.371	0.374	0.396

Figure 8: Absorbance Values of Genistein Treated Cells at Varying Estrogen Concentrations



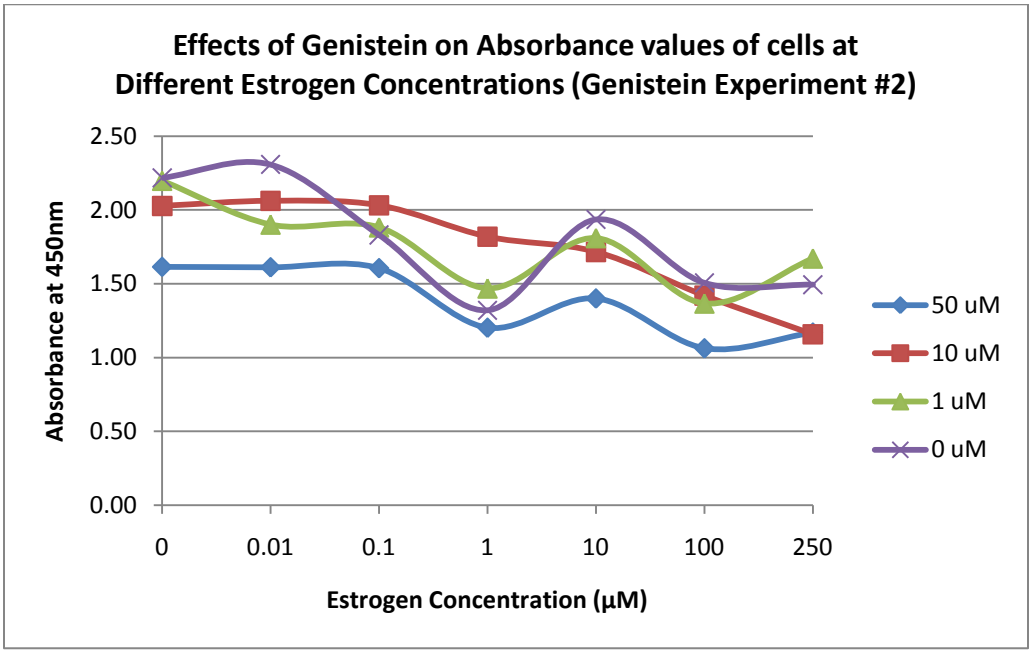
The genistein experiment described in Table 4 and Figure 8 was slightly modified and repeated, as can be seen in Table 5 and Figure 9. Additional estrogen concentrations were added and the individual genistein concentrations used were decreased, as shown in Table 5.

Table 5: Effect of Genistein at Varying Estrogen Concentrations (Genistein Experiment 2)

	Estrogen Concentration (µM)						
Genistein Concentration (µM)	250	100	10	1	0.1	0.01	0
50	1.169	1.063	1.400	1.202	1.605	1.611	1.614
10	1.158	1.420	1.716	1.819	2.031	2.061	2.027
1	1.671	1.366	1.809	1.468	1.882	1.901	2.198
0	1.494	1.506	1.936	1.320	1.831	2.308	2.218

From Table 5, it is seen that at 50 µM concentration of Genistein, there is a slight anti-proliferative effect at every concentration of estrogen. However, it is not significant enough to conclude an anti-proliferative effect of genistein. Figure 9 shows the data in Table 5 as plotted on a logarithmic x-axis. In Figure 9, there is a steady decline of absorbance values for each genistein level, as the estrogen concentrations rise; however there is an unexpected spike at 10 µM concentration of estrogen that cannot be explained. Hence no trends can be drawn from this data and the interpretation of this data is difficult.

Figure 9: Logarithmic Plot of Absorbance Values of Genistein Treated Cells at Varying Estrogen Concentrations



Bradford Assays for Protein Concentrations

For the above Genistein experiments (Table 4), an immunoblot was also performed. However, at the end of the entire immunoblotting process (gel electrophoresis, transfer, primary antibody incubation, secondary antibody incubation, substrate detection), there were absolutely no bands visible on the membranes. In order to troubleshoot this incident, Bradford Assays were performed on the failed westerns for the different Genistein concentration samples to see if there was any protein in the actual samples themselves. The Bradford Assay Standards are displayed in Table 6 and the calculated protein concentrations for the different cell samples are in Table 7.

Table 6: Albumin BSA Standards with Absorbance Values

Absorbance at 595nm	Protein Concentrations (µg/ml)
1.487	2000
1.355	1500
1.161	1000
0.998	750
0.847	500
0.615	250
0.556	125
0.474	25
0.464	0

Table 6 above shows the concentration of the albumin standards used for creating a standard curve for the Bradford Assay. The absorbance values obtained were then used for creating a standard curve which is shown in Figure 10 below. The equation of the line was obtained using a linear trend line and then the protein concentration values of each sample were obtained as seen in Table 7.

Figure 10: Absorbance versus Protein Concentration

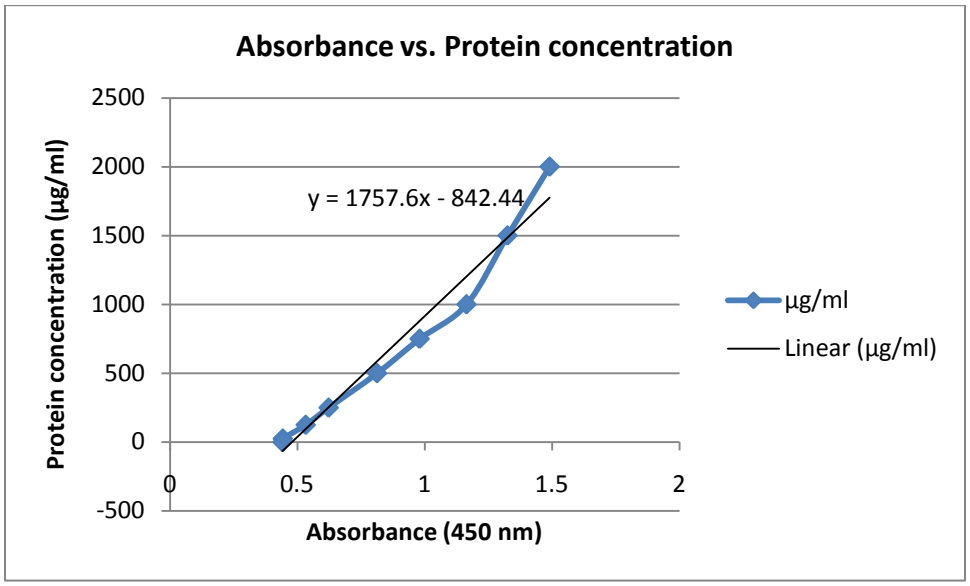


Table 7: Calculated Protein Concentrations for Samples at Different Estrogen and Genistein Levels

	100µM	10µM	1µM	0.1µM	0µM
185.05 µM	284	51.1	60.9	57.1	64.7
18.50 µM	41	56.3	66.3	44.5	55.6
0 µM	291.4	65.5	163.2	60.9	67.9

From Table 7, it is seen that there is a considerable amount of protein only in the samples where the estrogen concentration is at 100 µM and when the genistein concentration is either 185.05 µM or 0 µM. The middle concentration of 18.5 µM genistein does not show any significant protein concentration. Additionally, there is a spike in the protein concentration for 0 µM genistein and 1 µM estrogen concentration. This spike cannot be associated to any factors. These absorbance values translate into the amount of protein that was loaded onto each gel.

Discussion

The MTS assay was used as the main technique for measuring cell proliferation throughout the experiment, apart from the immunoblotting technique that was performed on two occasions and in addition to the MTS assay. The MTS assay measures absorbance values at 450nm for the different samples, which are directly proportional to the amount of cells present. In order to use this assay to determine the proliferation of breast epithelial cancer cells in the presence of estrogen and genistein, the assay had to be validated. Table 1 contains the raw data obtained from the validation experiment, where the three cell densities plated were measured with the spectrophotometer. Figure 3 shows the plot where the increase in absorbance readings is seen as directly proportional to the higher cell concentrations. The microphotographs in Figure 2 provide visual evidence that the higher cell concentrations did actually contain a greater amount of cells, which the MTS assay in Figure 3 validated with readings of higher absorbance values. The microphotographs taken after 24 hours depict the confluency of the cells where the 100,000 cells/mL and 50,000 cells/mL concentrations of cells are observed as nearly confluent. This growth in the wells would have limited space and media available to cells to cause contact inhibition and prevent further cell proliferation. This would prevent the MTS assay from detecting any significant growth after 12 hours and could explain why the absorbance values measured appear to level between the 12 hour and 24 hour time points. This may also explain why the validation of the MTS assay did not produce an ideal, linear proliferation curve.

In addition, the initial cell densities plotted against the absorbance readings in Figure 3 do not reflect the final cell densities. The time elapsed for the experiment was 24 hours and the doubling time for the cell line is approximately 28 hours to 32 hours, so the final concentrations would be closer to double the initial concentration. The experiment could have been repeated with lower cell concentrations to avoid confluence at 24 hours, but a positive correlation was still observed between absorbance readings and cell densities, thus validating the MTS assay. The two assay kits used were also compared. Both kits produced similar ranges of absorbance readings, supporting the validation of the MTS assay, but the assay kit #1, the one step MTS assay, was continued with for the rest of the experiments.

The T47D cell line was found to be estrogen responsive during this experiment. Initially, there were issues with proving estrogen responsiveness in the T47D cell line.

This could have been due to several reasons. One reason is that there could potentially be problems with the estradiol stock solution being used. Halfway through the project, a new vial of 17-β-estradiol was bought and a new estrogen stock solution (100 mM) was prepared to carry out our experiments. The former estradiol stock was not labeled accurately and it was possible that solution was actually of a lesser concentration. If the concentration was 100 to 1000 fold less diluted than we believed, the cells would not have exhibited a strong estrogen response. Assuming that the estrogen stock was a reason behind the absence of measured estrogen sensitivity, it could also be suggested that the old stock was not as potent as a newly obtained stock solution, since the year it was purchased or opened was not labeled and the bottle was not stored appropriately. The new stock solution may have been relatively much more potent, which could explain the differences in measurements obtained. The purchase of a new estrogen stock solution may be the reason for the successful estrogen responsiveness experiments that were seen later during the project.

Another problem could have been the addition of the DCT stripped FBS to the initial DMEM growth media. This was observed to be problematic to cell adhesion in the plates since replacing it with just FBS proved useful. DCT FBS helps to reduce serum concentration of endogenous hormones and growth factors such as estradiol that may interfere with the experimental process (Gemini Bio-Products, 2010). However, the DCT FBS could also reduce the lipids in the media that help the cells to plate down into the wells, which could have been a reason for no estrogen responsiveness.

A final problem could have been that after several preliminary experiments, the ideal cell densities to be used were still not determined. The different cell densities used were 150,000 cells/well and 75,000 cells/well for a 24 well plate; and 75,000 cells/well, 50,000 cells/well, 25,000 cells/well and 10,000 cells/well for a 96 well plate. These cell densities could have either reached confluence to shown no responsiveness beyond a certain cell population, or could have had very few cells in each well that they couldn't survive and died off when transferred from the T75 flasks to the new environment of the plate wells.

The Promega protocol recommended performing MTS assays with a starting cell concentration as low as 5,000 cells/well for a 96 well plate and 20,000 cells/well, for a 24-well plate (Celltiter 96® aqueous, 2009). Although a much lower cell concentration was recommended for the MTS assay, we observed significant growth in Figure 4 in 24 wells plates with a concentration of 75,000 cells/well and chose to continue with this

concentration for the rest of the project. In addition, the microphotographs in Figure 2 show that a concentration between 25,000 cells/well and 50,000 cells/well in a 12 well plate produced an appropriate amount of growth after 24 hours. In a 24 well plate, these ideal concentrations would translate to concentrations between 50,000 cells/well and 100,000 cells/well. Therefore, the visual evidence of cell growth per well over a 24 hour period in Figure 2 supports the decision to continue experiments in a 24 well plate with a concentration of 75,000 cells/well.

The experiments performed demonstrated estrogen responsiveness in the cells. This can be observed in Table 2 and Figure 4. However, this was not consistent with different cell densities, mostly due to the fact that at higher cell densities of 150,000 cells/well in a 24 well plate, the cells reached confluence during the growth period and were not able to grow properly in the wells due to contact inhibition. Hence, a cell density of 75,000 cells/well was chosen for the 24-well plates as the ideal number of cells since estrogen responsiveness was seen at that cell density and supported by the conclusions regarding confluence in Figure 1.

After the T47D cell line was demonstrated as an estrogen responsive cell line, the phytoestrogen, genistein, was introduced as an experimental variable. Genistein was tested in both high and low estrogen concentrations to determine its effect on proliferation in the presence of estrogen. The inclusion of estrogen with the addition of genistein in the following experiments corresponded to the physiological conditions, since the human body has estrogen flowing at all times, albeit at different concentrations, depending on age, gender, and other factors. The first genistein experiment recorded in Table 4 found genistein to have an additive proliferative effect on cells. The curve in Figure 8 where no genistein was added had a final absorbance value of 0.539, while the curve with the greatest concentration of genistein, 185.05 μM, had a final absorbance of 2.289, both at a corresponding estrogen concentration of 100μM. These results did not support our hypothesis that genistein would act as an inhibitory molecule at higher estrogen concentrations and exhibit estrogenic activity at lower estrogen concentrations.

The unexpected results may be the result of incomplete saturation. If the estrogen receptors were not fully saturated, then the addition of phytoestrogens would cause the genistein molecules to bind to the ERs in a non competitive manner and may cause a proliferative effect (Ososki & Kennelly, 2003). However, the additive proliferative effect was not observed at the highest estrogen concentration, 100 μM, for

the 18.5 μM and 0 μM concentrations of genistein. This would suggest that the very concentrated 185.05 μM addition of genistein was the only factor differing from the other experimental groups and was the cause for the increased absorbance reading. If the ERs had not been fully saturated, then the control group with only estrogen would have measured increased proliferation, as well, for the higher estrogen concentrations.

Another possibility for these results is that the genistein and estrogen bound only to the ER α and that the ER β may not have been expressed. It is hypothesized that the ER α stimulates proliferation while the ER β may act antagonistically and demonstrate anti-proliferative effects. If the ER β receptor was no longer expressed, which can occur in malignant cells of cancers, the hormone estradiol and genistein would only bind to the ER α to cause a binding preference of estradiol over genistein and an additive proliferative effect. This proliferation would be greatest at the highest concentration of estrogen and genistein, which was observed in Figure 8. However, it would be expected that the absorbencies for the estrogen control would also increase in a linear manner as the estrogen concentration increased, which was not shown in the absorbance values in Table 5.

The reason for the high absorbance reading for the185.05 μM genistein is most likely due to the crystallization of the genistein. The phytoestrogen was not dissolved properly and crystallized in the media due to a high concentration After observing the inability for genistein to fully dissolve at that concentration, the remaining additions were heated and vortexed to ensure solubilization. The first addition at 100 μM estradiol was the exception and most likely absorbed the formazan dye from the MTS assay to read a much higher absorbance than the actual cell population would have measured.

Research shows that genistein has anti-proliferative effects when in concentrations greater than 10 μM and proliferative at concentrations less than 10 μM (Bouker and Hilakivi-Clarke, 2000). It was decided to repeat the experiment and decrease the genistein concentration significantly so that it can dissolve in media. The initial genistein concentration of 185.05 μM was changed to 50 μM while the other concentrations were 10 μM, 1 μM, and 0 μM.

The additive affects of genistein could also be linked to non estrogen mediated pathways. The aim of this project was to observe the effect of genistein based on the concentration of the estrogen environment, so any non ER mediated effects of genistein could not be studied thoroughly.

The second genistein experiment produced data that differed greatly from the initial findings and supported the original hypothesis. The data in Table 5 supports the anti-proliferative effect of genistein at high estrogen concentrations and the estrogenic activity at low estrogen concentrations. Research suggests that genistein may be antiproliferative when in concentrations greater than 10 μM and proliferative at concentrations less than 10 μM (Bouker and Hilakivi-Clarke, 2000). This could be due the 1,000 to 10,000 fold greater affinity of 17β-estradiol compared to genistein in binding to an estrogen receptor (Kuiper et al.,1998). Higher concentrations of genistein may be necessary in order to competitively inhibit estradiol when estradiol is preferred by the receptor. This concept was supported by the data for the different genistein concentrations. The 50μM concentration of genistein showed a decrease in cell proliferation with higher estrogen levels, while the 10 μM genistein concentration began to increase at around a 250 μM estrogen concentration. The lesser concentrated genistein values, such as the 1.0 μM curve, demonstrated the greatest increase in slope at the highest estrogen concentration. The control curve of 0 μM genistein leveled off at 250μM estrogen, which could indicate that the cells' estrogen receptors were completely saturated. However, the control value for the 0 μM estrogen concentration did not exhibit normal activity and demonstrated a similar curve to the data sets containing genistein. This control set should have shown estrogenic activity directly correlating cellular proliferation with estrogen concentration. It is possible that some form of contamination with genistein occurred which might have distorted the results or that similarly to the previous genistein experiment, only the ER α was expressed at such high estrogen concentrations.

Overall, the results were not conclusive as to the exact activity or mechanism through which genistein acts. The preliminary genistein results also did not support our hypothesis, while the final experimental results did indicate a trend more supportive of genistein inhibition of estrogenic activity at high estrogen concentrations and showing estrogenic activity to increase cell proliferation at low estrogen concentrations. The wide-ranging results could indicate that genistein may be working through more than just the estrogen receptor mediated pathway as mentioned previously (Bouker and Hilakivi-Clarke, 2000). It is known that genistein also works through inhibiting tyrosine kinase activity and topoisomerase II activity (Bouker & Hilakivi-Clarke, 2000). This could possibly be the reason for our inconsistent findings throughout the course of this

project. Our findings necessitate further research and experiments to determine the action of the phytoestrogen, specifically genistein, on breast epithelial cancer cells.

All the variables in these experiments could be optimized and standardized in further projects. Starting with the cell densities, the MTS protocol recommended using lower, initial cell numbers. Closer adherence to this suggestion may provide more conclusive data, since the possibility of the MTS assay measuring lower absorbance values than expected could have been attributed to confluent cells inhibiting further growth. Additionally, a set range of estrogen concentrations need to be established, where it is proven that cells show a specific trend with increasing estrogen concentrations. A third variable that needs to be handled is the incubation times for cells between experimental conditions and also during them. Certain time course experiments could be performed to determine exact durations for experimental procedures. Furthermore, after the role of genistein is established in being antiproliferative to breast cancer cells, further investigations should be carried out with the other phytoestrogen compounds present in the different over-the-counter phytoestrogen supplements.

References

Ali, Simak, & Coombes, Charles. (2002). Endocrine-responsive breast cancer and strategies for combining resistance. *Nature Reviews Cancer*, *2*.

ATCC. (2008). Catalog Entry for T47D, Number HTB-133. [Online]. <http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=HTB-133&Template=cellBiology>.

ATCC. (2009). *ATCC: About*. Retrieved February 12, 2010, from ATCC The Global Bioresource Center. [Online] Retrieved from < http://www.atcc.org/About/tabid/138/Default.aspx>.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J., Smith, J. A., et al. *Current Protocols in Molecular Biology.* Wiley.

Bouker, Kerrie, and Leena Hilakivi-Clarke (2000). Genistein: Does It Prevent or Promote Breast Cancer? *Brogan & Partners,* 108(8), Retrieved from <http://www.jstor.org/stable/3434722?cookieSet=1>.

Celltiter 96® aqueous non-radioactive cell proliferation assay. (2009, May). Retrieved from http://www.promega.com/tbs/tb169/tb169.pdf

Centers for Disease Control and Prevention (2010, March 11). *Breast cancer statistics.* (2010). Retrieved from http://www.cdc.gov/cancer/breast/statistics/

Clemons, Mark, and Paul Goss (2001). Estrogen and the risk of breast cancer. *New England Journal of Medicine,* 344(4) Retrieved from <http://www.endo.gr/cgi/reprint/344/4/276.pdf>.

Creighton, Thomas (1999). Estrogen. *The Encyclopedia of molecular biology*. Retrieved from http://www.knovel.com/web/portal/basic_search/display?_EXT_KNOVEL_DISPLAY_bookid=737

De Vault, Victoria, Kosmaczewski, Sara, & Tracy, Sarah. (2009). *The Effects of phytoestrogens on breast epithelial cells*. Worcester Polytechnic Institute, Worcester, MA.

Dennerstein, Lorraine, Dudley, Emma, Hopper, John, Guthrie, Janet, & Burger, Henry. (2000). A Prospective population-based study of menopausal symptoms. *Obstetrics and Gynecology*, *96*(3).

Dickson, Robert, Bates, Susan, McManaway, Mary, & Lippman, Marc. (1986). Characterization of estrogen responsive transforming activity in human breast cancer cell lines. *Cancer Research*, *46*(41), Retrieved from http://cancerres.aacrjournals.org/cgi/reprint/46/4_Part_1/1707

Fritz, Wayne, Lori Coward, Jun Wang, and Coral Lamartiniere (1998). Dietary genistein: perinatal mammary cancer prevention, bioavailability, and toxicity testing in the rat. *Carcinogenesis,* 19(12), Retrieved from <http://carcin.oxfordjournals.org/cgi/reprint/19/12/2151>.

Gemini Bio-Products. (2010). *Charcoal: Dextran Stripped Fetal Bovine Serum*. Retrieved April 25, 2010, from Gemini Bio-Products: <http://www.gembio.com/index.cfm?page=1180993>.

Goldin, BR (1986). "The relationship between estrogen levels and diets of Caucasian American and Oriental immigrant women". *The American journal of clinical nutrition*, 44 (6), p. 945.

Hilakivi-Clarke, L (2000). "Estrogens, BRCA1, and breast cancer". *Cancer research (Baltimore)*, 60 (18), p. 4993.

Holmberg, L., Iverson, O., Rudenstam, C., Hammar, M., Kumpulainen, E., Jaskiewicz, J., et al. (2008). Increased risk of recurrence after hormone replacement therapy in breast cancer survivors. *Journal of National Cancer Institute, 100*(7), 475-482.

Kennemans, P., and A. Bosman. (2003). Breast cancer and postmenopausal hormone therapy. *Best Practice & Research Clinical Endocrinology & Metabolism*, 17(1)

Kuiper, George, Lemmen, Josephine, Carlsson, Bo, Corton, Christopher, & Safe, Stephen, et al. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor b. *Endocrinology*, *139*(10), Retrieved from http://endo.endojournals.org/cgi/reprint/139/10/4252

McGuire, W.L., K.B. Horwitz, O.H. Pearson, and A. Segaloff. (2006). Current status of estrogen and progesterone receptors in breast cancer. *Cancer,* 39(6), Retrieved from <http://www3.interscience.wiley.com/cgi-bin/fulltext/112679751/PDFSTART>.

Ososki, Andreana, and Edward Kennelly. (2003). Phytoestrogens: A Review of the Present State of Research. *PhytoTherapy Research,* 17, Retrieved from <http://www.humboldt.edu/~ao32/OsoskiKennelly2003.pdf>.

Pratt, SE, and MN Pollak. (1993). Estrogen and antiestrogen modulation of MCF7 human breast cancer cell proliferation is associated with specific alterations in accumulation of insulin-like growth factor-binding proteins in conditioned media. *Cancer Research,* 53(21), Retrieved from < http://cancerres.aacrjournals.org/cgi/reprint/53/21/5193 >.

Raasumaa, C. (2008). *Phytoestrogen Effects on Breast Epithelium.* Worcester Polytechnic Institute, Worcester, MA.

"Ranges of Normal Estrogen Levels." *The Anti Aging Guide*. N.p., 2010. Web. 23 Apr 2010. <http://antiagingguide.com/estrogen_levels.htm>.

Rice, Suman, & Whitehead, Saffron. (2006). Phytoestrogens and breast cancer - promoters or protectors?. *Endocrine-Related Cancer*, *13*(4), Retrieved from http://erc.endocrinology-journals.org/cgi/reprint/13/4/995

Ross, Ronald, Annlia Paganini-Hill, Peggy Wan, and Malcolm Pike. (2000). Effect of hormone replacement therapy on breast cancer risk: Estrogen versus estrogen plus progestin. *Journal of the National Cancer Institute,* 92(4), Retrieved from <http://jnci.oxfordjournals.org/cgi/reprint/92/4/328>.

Santa Cruz Biotechnology. (2010). *PCNA (F-2) Antibody sc-25280.* Retrieved April 24, 2010, from Santa Cruz Biotechnology Inc. < http://datasheets.scbt.com/sc-25280.pdf>.

Strom, Anders, Hartman, Johan, Foster, James, Kietz, Silke, Wimalaasena, Jay, & Gustafsson, Jan-Ake. (2003). Estrogen receptor β inhibits 17β-estradiol-simulated proliferation of the breast cancer cell line T47D. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(6), Retrieved from http://www.jstor.org/stable/pdfplus/3371229.pdf

Thermo Scientific. (2010). *Coomassie Bradford Protein Assay Kit.* Retrieved April 24, 2010, from Thermo Scientific: http://www.piercenet.com/files/0129kc9.pdf

Trock, B., Hilakivi-Clarke, L., & Clarke, R. (2006). Meta-analysis of soy intake and breast cancer risk. *Journal of the National Cancer Institute, 98*

Warren, B., & Devine, C. (2002). *Phytoestrogens and breast cancer.* Retrieved from http://envirocancer.cornell.edu/FactSheet/Diet/fs1.phyto.cfm

Wood, Charles, Appt, Susan, Clarkson, Thomas, Franke, Adrian, & Lees, Cynthia, et al. (2006). *Chemical structures of the isoflavonoids genistein, daidzein, and*. [Web]. Retrieved from http://www.biolreprod.org/content/75/3/477.full.pdf+html

Wu, A., Ziegler, R., Horn-Ross, P., Nomura, A., West, D., Kolonel, L., et al. (1996). Tofu and risk of breast cancer in Asian-Americans. *Pub Med, 5*(11), 901-906.

Yuan, J., Wang, Q., Ross, R., Henderson, B., & Yu, M. (1995). Diet and breast cancer in shanghai and Tianjin, china. *British Journal of Cancer, 71*