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Phytoestrogens in Breast Cancer

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Abstract

Lignans, natural phytoestrogens found in flaxseed, have garnered increased attention due to their documented capabilities of inhibiting breast cancer development. However, to what extent and the specific mechanisms by which lignans inhibit cancer growth is still subject to scrutiny. Secoisolariciresinol diglucoside (SDG), a type of lignan that has not been thoroughly studied, was combined with tamoxifen (TAM) and administered to T47D breast cancer cells to investigate its anti-proliferative effects and its interactions with the well-known cancer therapy drug. Results showed that SDG alone, TAM alone, and SDG combined with TAM significantly reduced T47D proliferation in comparison to untreated cells, and luminescence data showed that SDG was acting through the estrogenic pathway. Significant decrease in T47D cell count also hinted at the possibility of SDG and TAM acting through the apoptosis pathway, but further experimentation is required to support this theory.

1. Introduction

Phytoestrogens found in the vegetarian/soy diet have sparked an interest in the general public due to their promising inhibitory effects on breast cancer. Recent epidemiological studies have demonstrated that the soy-rich diet in Asian populations may be a critical factor in their respective low breast cancer incidence rates (Lamartiniere 2000). Further clinical studies suggest that phytoestrogens, especially when combined with other breast cancer therapies such as tamoxifen, may have inhibitory effects on cell growth (Nurrochmad et. al, 2013, van Duursen et. al, 2013).

Phytoestrogens share similar structural properties with mammalian estrogen 17-oestradiol, enabling them to bind to estrogen receptors and mimic their effects. Their mechanism of action, although not entirely understood, relies on phytoestrogens binding to either ER-α or ER-β, and thus preventing the binding of estrogen, the natural driver of breast cancer (Velentzis et. al, 2008). This would label phytoestrogens generally as estrogen receptor antagonists. Researchers have tried to combine plant-derived phytoestrogens and tamoxifen therapies to combat cancer proliferation, but results have varied.

Nurrochmad et. al, (2013) showed that low doses of phytoestrogen (8,9)-furanyl-pterocarpan-3-ol (FPC), co-administered with a low dose of tamoxifen, showed proliferative effects on T47D breast cancer cells, compared to inhibitory effects at higher doses and simply tamoxifen alone. A similar study testing against MCF-7 cells also found that four different phytoestrogens, along with genistein, had proliferative effects when combined with Letrozole (aromatase inhibitor) and tamoxifen (estrogen receptor antagonist) (van Duursen et. al, 2013). Interestingly, other studies suggest that lignans (a plant derived phytoestrogen) in flaxseed combined with tamoxifen have an inhibitory effect on breast cancer cells (Chen and Thompson

2003, Lindahl et. al, 2011, Thompson et. al, 2004). Another study suggests that at higher doses, combination therapy of genistein (a phytoestrogen) and tamoxifen has inhibitory effects, but has proliferative effects at low dosages (Liu et. al, 2005). It is clear that there is some relevance to the use of phytoestrogens in combination with preexisting synthetic cancer therapy drugs, such as tamoxifen.

Tamoxifen (TAM) is a non-steroidal drug that has both estrogenic and antiestrogenic effects, depending on the type of tissue that is being targeted. Therefore, it is appropriate to refer to TAM as an "selective estrogen receptor modulator" (SERM). In the case of breast tissue, it exhibits an antiestrogenic effect, so it is frequently used as a treatment for breast cancer tumors which express estrogen receptors in their cytoplasm. The mechanism of action of TAM works in several ways. For example, it acts as a competitive estrogen receptor antagonist (on both ER-α and ER-β receptors) with very similar affinities, meaning it inhibits the binding of estrogen to these receptors and therefore suppresses cellular proliferation. The only difference between ER-α and ER-β is that they may induce different effects depending which agonist it binds to; however, it has been shown that TAM induces an inhibitory effect when bound to either receptor (Helguero, 2005). Once the drug binds to the receptor, it recruits other molecules such as corepressor proteins to the site, which modulate gene expression in the cell's DNA. It also halts metastatic cell growth in the G_0 and G_1 phases of the cell cycle while preventing precancerous cells from dividing, without killing them (Sporn, 1970).

Few studies about lignans and their interactions with tamoxifen exist, so it is difficult to make significant conclusions. This study attempts to shed light on the previous conflicting results, while also detailing if and how lignans may be used in combination therapies for breast

cancer. It will also be useful to understand the exact mechanism of action of inhibition by lignans on breast cancer cells.

Secoisolariciresinol diglucoside (SDG), a type of lignan found in flaxseed, will be tested against T47D breast cancer cells, both alone and in combination with tamoxifen, to observe potential effects through regulation of estrogen receptors. We hypothesize that a combination therapy of TAM and SDG will enhance the inhibitory effects of TAM, by downregulation of ER-α or ER-β.

2. Materials and Methods

2.1 Cell Lines

T47D cells, a breast cancer epithelial cell line expressing both estrogen receptors α and β were used as a model system to analyze the effects of tamoxifen and SDG on cell proliferation. T47D K-Bluc cells are T47D cells stably transfected with a reporter plasmid containing three estrogen response elements upstream of a luciferase reporter (Wilson et. al, 2004). These cells were used to examine the binding of Tamoxifen and SDG to estrogen receptors, due to their fluorescent behavior when activated at their receptor.

2.2 Cell Maintenance

T47D and T47D KBluc cells were grown in 89% Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS—composed of ≤1 mg/mL bovine immunoglobulin and ≤20 mg/mL hemoglobin), and 1% penicillin-streptomycin (PS, 5,000 units/mL penicillin and 5 mg/mL streptomycin). The cells were plated onto a T75 flask and incubated at 37°C, 5% CO_2. They were fed approximately every 2 days and split at a 1:2 or 1:3 ratio approximately every 5 days once confluence was observed. Splitting of the cells required the use of trypsin because

they are adherent to the flask and to each other. To split cells, the medium was aspirated, and the flask was rinsed with phosphate buffered saline (PBS). 1 mL of 0.05% trypsin-EDTA was added and cells were incubated at 37℃ until cells began to lift from the flask. Cells were then washed from the flask surface in culture medium, centrifuged to pellet and resuspended for plating in culture medium. As necessary, cell counting was performed prior to plating.

2.3 Cell Counts:

Cells were counted using the trypan blue dye exclusion test to determine total cell count and viability using a Neubauer hemocytometer. Alternatively, cells were counted in a Cellometer Auto T4 Cell Counter (Nexelcom) following manufacturer's directions.

2.4 Cell Plating

50,000 T47D cells were plated in each well of a 24-well plate in phenol-red DMEM, and were incubated for 2 days. The media was then aspirated and replaced with phenol red-free, charcoal stripped media, in which the FBS component is removed of lipid-related materials, including steroids (Thermo Fisher Scientific Inc., 2020). The cells were incubated again for 24 hours before drug treatments in triplicates were added. All drug treatments were left to incubate for 72 hours before cells were then aspirated, washed with PBS, and trypsinized for cell counting.

For luciferase assays, each well of a black, clear-bottomed 96-well plate contained 30,000 T47D-KBluc cells in phenol-red DMEM, which were left to incubate for 2 days. The media was then aspirated and replaced with phenol red-free, charcoal stripped media and incubated again for 24 hours before drug treatments were added. All drug treatments were left to incubate for 72 hours before they were aspirated off, followed by the addition of 100 uL of ONE-

Step luciferase reagent (mixture of 100 uL luciferase assay substrate and 10 mL buffer) (BPS Bioscience Inc., 2020). Luminescence was recorded using a Victor3TM plate reader.

2.4(a) Estrogen

A 20 ug/mL stock solution was created by combining 1 mg of β-estradiol-17 (E2) with 1 mL of absolute ethanol and 49 mL of sterile medium. The stock solution was held in dark storage at -20°C in 1 mL aliquots. Aliquots were diluted to a final concentration of 20nM and administered in each experiment to appropriate wells.

Serial dilutions were performed to decrease the concentration of β-estradiol (E2) in each well by a factor of 10 with each dilution, for a total of 5 different concentrations, starting at the physiological concentration of 20 nM and reaching the minimum 0.2 pM. The concentration of 20 nM was determined to be optimal based on previous research of physiological β-estradiol concentrations (Celojevic et al., 2011). 5 uL of each E2 concentration was added to their subsequent wells, each containing 50,000 cells/mL of phenol red-free, charcoal stripped DMEM, and pipetted up and down to ensure even distribution.

2.4(b) Tamoxifen

A stock solution of tamoxifen (TAM) and ethanol (1 mg/mL) was held in dark storage at -20°C. Stock solution was then diluted with PBS for a final concentration of 20 nM. 20 nM TAM was administered in each experiment to appropriate wells, each containing 50,000 cells/mL.

2.4(c) Secoisolariciresinol diglucoside (SDG)

A stock solution of SDG dissolved in ethanol and water (1 mg SDG, 400 uL ethanol, 1 mL of water) was held in dark storage at 0°C. Stock solution was then diluted with PBS for a

final concentration of 100 uM, which was administered in each experiment to appropriate wells containing 50,000 cells/mL.

2.5 Statistical Analyses

Data from the experiments were expressed as mean +/- SEM, with statistical analysis performed using one-way ANOVA on Microsoft Excel. A p-value of 0.05 was used to as the criteria for statistical significance, using 3 biological replicates to increase the validity of the data.

3. Results and Discussion

3.1 Estrogen Response

In order to perform experiments that analyze SDG's and TAM's effects on T47D cells, we first needed to ensure the cells were estrogen responsive. The results from the E2 positive control experiment showed that at an estrogen concentration of 20 nM, T47D cells showed maximal increase in proliferation with less responsiveness as concentrations decreased, as shown in *Figure 1* below. These results indicate that the cells are most responsive to E2 at a concentration of 20 nM; therefore, this is the concentration that we proceeded to utilize in further experiments when treating with E2.

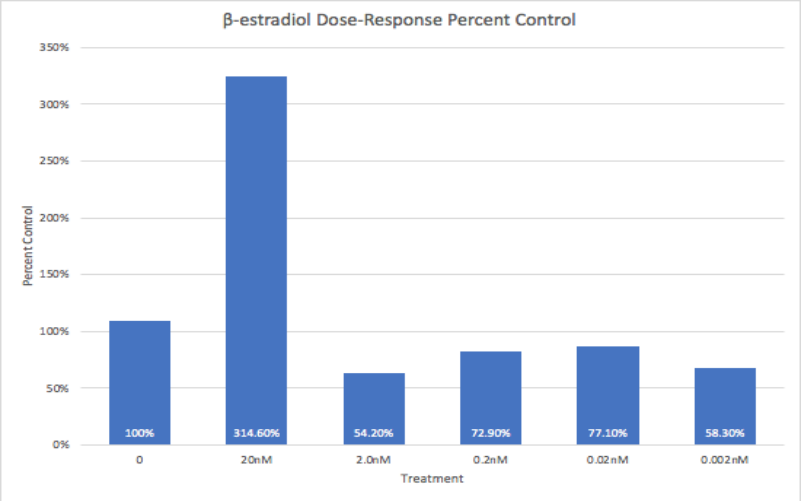


Figure 1 (left): Proliferation of T47D cells when administered β-estradiol. Data represents counts of cells growing in 1mL of media on a 24-well plate following 48hrs incubation.

"Percent control" values were calculated for this experiment and for all following experiments because it allowed us to calculate a standard deviation if multiple tests were run, minimizing variation in absolute counts between individual tests.

3.2 Co-administration of TAM and SDG on T47D Cells

T47D cells were initially treated with increasing SDG concentrations from 1 uM to 100 uM, combined with 20 nM E2, in order to determine the optimal concentration of SDG for treatments. *Figure 2* shows that growth inhibition from SDG peaked at 100 uM, while greater concentrations led to minimal difference in inhibition. This provided the basis of administering 100 uM SDG concentration for further experiments. It is important to note that the optimal concentration was based on the average inhibitory response for the triplicate trials; while the error bars shown in *Figure 2* overlap with the other treatments and the interpretation that 100 uM showed the highest inhibitory ability is not definite, the data here support such an interpretation.

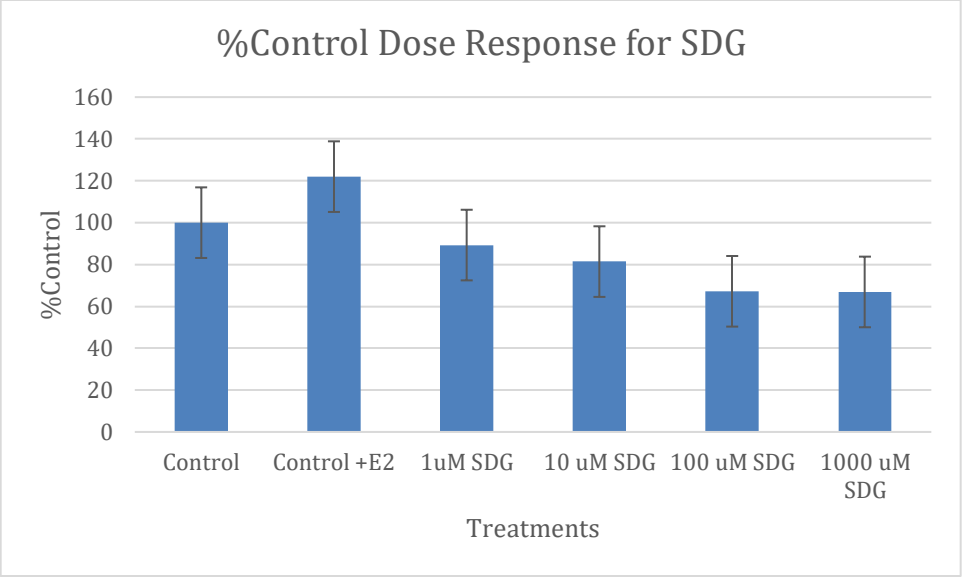


Figure 2: Dosage response for SDG compared to negative control (only T47D cells) ranging from 1 uM to 1000 uM. Peak growth inhibition was found at 100 uM SDG.

In order to determine the effects of SDG and TAM on T47D inhibition, cells were treated with either estrogen alone (positive control), estrogen + tamoxifen, estrogen + SDG, estrogen + SDG + TAM, and cells + EtOH (negative control). The wells were set up in triplicates and the experiment was replicated three times. *Figure 3* compiles this data into one graph. All treatments administered with SDG, TAM, or a combination of both resulted in significant inhibition compared to that of the positive control ($p < 0.01$, $N = 3$, see *Figure 3*). Combination of TAM and SDG treatments showed the greatest cell inhibition compared to the positive control ($p < 0.01$, see *Figure 3*), but was not significantly different to treatments of just TAM or SDG alone. In comparison to the drug treatments, cells treated with only E2 continued to show the highest cell counts, as expected. This data indicates that there is a significant increase in inhibition of E2 induced T47D cell proliferation when treatments of SDG and TAM are administered individually or in combination, but not enough evidence is found to conclude whether there is a synergistic effect of combination treatments vs. individual treatments.

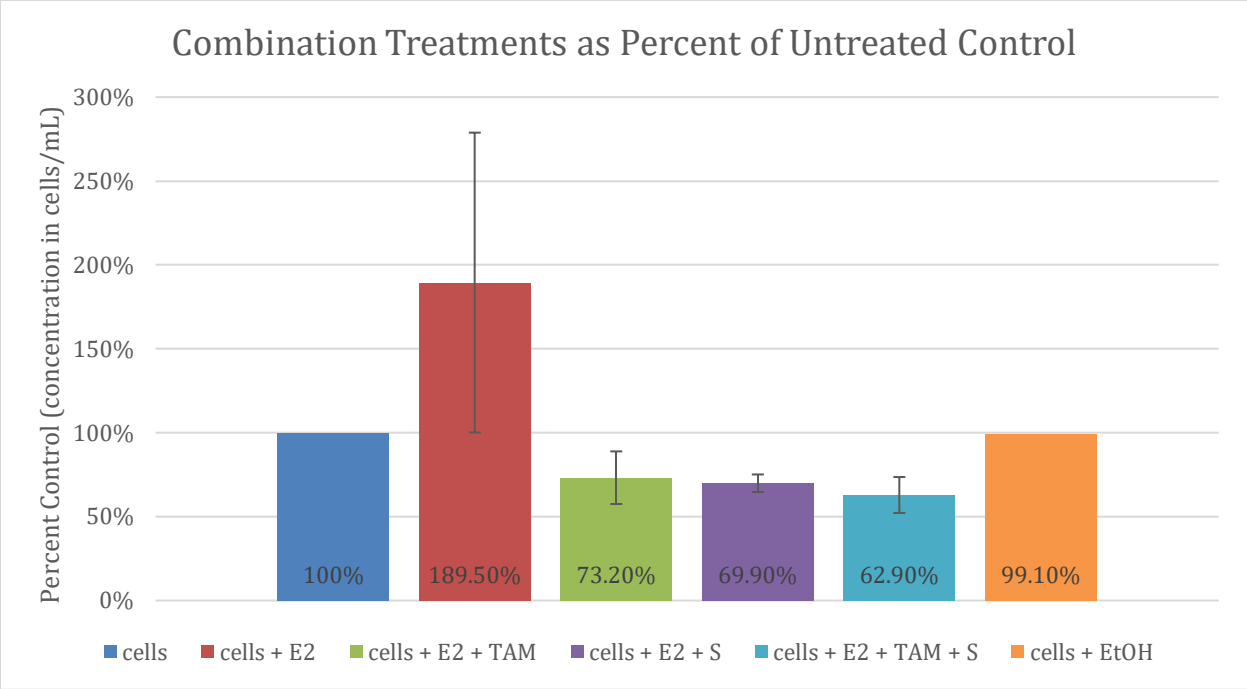


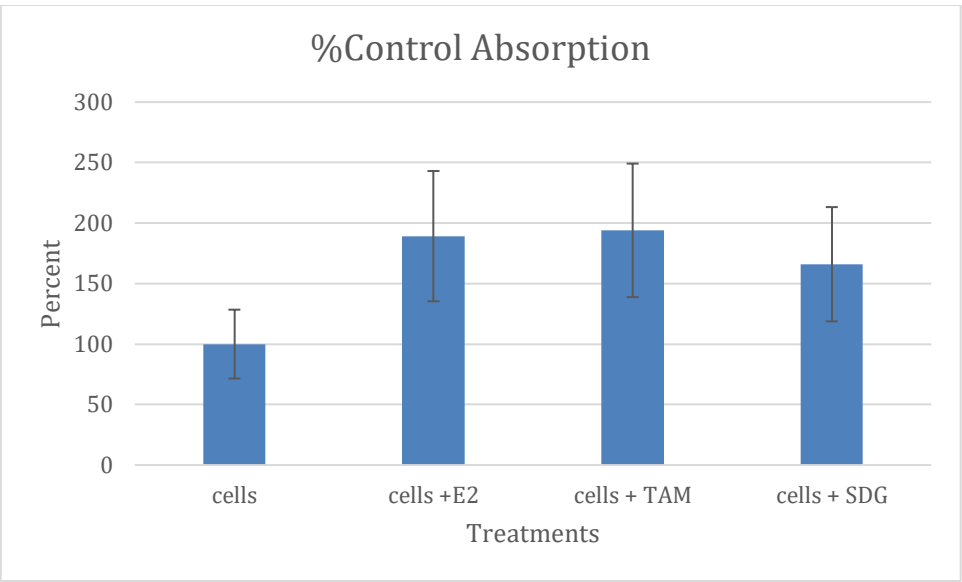
Figure 3: Percent control calculations for each cell treatment based on the control wells containing cells only. Experiment was repeated 3 times and average percent control was calculated. Standard error bars are shown on the data bars which contain treatments. Since the data are being compared to the average control, the control has no standard deviation. Cells + EtOH also has no standard deviation considering the test was run only once.

The vehicle control treatment, consisting of cells treated with just 5 nM EtOH, showed similar counts to the untreated control (cells that were not treated with any drugs) and were not significantly different, indicating that the addition of EtOH had no anti-proliferative effect on the cells. The purpose of administering such a small concentration of EtOH to cells was to ensure that the decrease in proliferation may not have been due to the EtOH which was used to dissolve the three drugs. The maximum concentration of ethanol in cell culture that does not cause toxic damage to the cells is approximately 5% (Tapani et. al, 1996). Since the maximum concentration of ethanol that ended up in the wells after all dilutions were performed was only 5 nM, approximately 5.0 x 10^{-9} percent of the final volume consisted of pure ethanol. This percentage is much less than the limit of 5% of the total volume, it can be concluded that ethanol was not the primary cause of anti-proliferation.

The data in *Figure 3* suggests that the combination of these two drugs on cells that are exposed to estrogen further suppresses growth compared to either treatment alone by a factor of 7% regarding SDG and 10.3% regarding TAM, but this was not significant. However, it is important to note that the standard error bars for both of these suggestions are overlapping. Although averages were used in data analysis, there is not enough statistical evidence to conclude that combination treatments of SDG and TAM have greater inhibitory effects than either drug alone. However, the results obtained are promising; future experiments are required to continue to explore this topic. Furthermore, cell counts shown in *Figure 3* of TAM treatment, SDG treatment, and combination treatment were much lower compared to that of the untreated control, indicating a possibility of cell apoptosis. This claim would be the basis of future experiments involving SDG, TAM, and proteins that are released from cells when cell apoptosis is triggered.

3.3 Luciferase Assay

In order to determine the mechanism of TAM and SDG inhibition on T47D growth, a luciferase assay was performed. The luciferase reporter assay was used to determine whether SDG is acting through an estrogen receptor or via a different metabolic pathway when inhibiting cellular proliferation. The T47DKBluc cells are tagged at their estrogen receptors with a construct of estrogen response elements, promoters, and luciferase, allowing them to luminesce in response to 17-beta-estradiol. It is expected that the addition of E2 and TAM will induce fluorescence, because it has been shown through prior research that estrogen and Tamoxifen act through both the alpha and beta estrogen receptor (ER). The addition of SDG explored whether it also acted through the ER, by determining whether the cells luminesce at a similar emission as those treated with E2 and TAM. The results from this experiment are shown below.



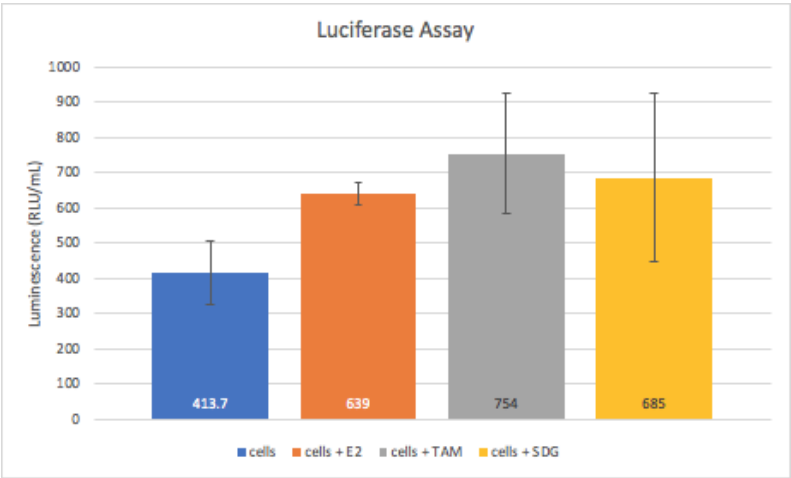


Figure 4a (above) and 4b (left): Luciferase reporter assay data. Standard error bars are shown. Percent control is shown in panel A. The numerical values represent luminescence units of RLU/mL in panel B.

Cells were administered either E2, TAM, or SDG, along with a vehicle control. The experiment was repeated in triplicates in order to calculate statistical significance. Emission of the "cells + SDG" treatment and the "cells + TAM" treatment were significantly greater than that of just cells alone (p-value < 0.05, see *Figure 4a* and *4b*), and were not significantly different than the positive control of just "cells + E2". Since the positive control would have maximum luminescence due to the binding of the ER, there is evidence to suggest that both SDG and TAM are acting through an estrogen receptor when inhibiting cellular proliferation. The luminescence of cells that were treated with E2 was 639 units, and that of the cells treated with TAM was 754

units, meaning that both E2 and TAM act through the receptor. The luminescence of cells that were treated with SDG fell close by that of the control treatment with a value of 685 units. This suggests that SDG does act through the ER. However, which receptor (alpha or beta) it is acting through is still unclear and requires further experimentation. It is possible that this ER binding is not the mechanism responsible for the decrease in cell proliferation. Experimentation with antagonizing both the alpha and beta receptors would have provided further insight on this information.

4. Future Experiments

4.1 Immunoblots

In order to determine if SDG and TAM are acting through another metabolic pathway that is triggering cell apoptosis, an immunoblot would be performed using antibodies to probe for apoptosis related proteins that would be upregulated, such as caspase 3. (Papaliagkas et. al, 2007). If these proteins appeared on the blot and were upregulated compared to controls along with either SDG or TAM, then there would be evidence to conclude that SDG or TAM (or both) are acting through another metabolic pathway to trigger cell apoptosis, combined with antagonizing the estrogen receptor. This experiment only provides evidence that SDG and TAM are acting through the apoptosis pathway, but does not necessarily identify if the decreased cell numbers are from the estrogenic pathway or the apoptosis pathway. It is hypothesized that both SDG and TAM are acting through both the apoptosis and estrogen pathway, and the results would show that apoptosis-indicating proteins such as caspase-3 would appear upregulated in the gel, along with other cell cycle regulatory proteins such as p21 (Porter & Jänicke, 1999).

Immunoblotting will be used to search for two proteins of interest: caspase-3 and p21. Concentration of proteins from lysed cells would be calculated, and samples would be vortexed to denature proteins in a 95°C temp block for 5 minutes. The samples would then be loaded onto 12% acrylamide 10-well Mini Protean Precast Gels (Bio-Rad) based on the amount of total protein for all samples. Gel electrophoresis would be conducted at 200 V until the dye runs off, and the gel would be cut with a razor to remove well lanes and dye runoff. The gel would then be put in a transfer buffer. Polyvinylidene difluoride (PVDF) membrane would be wetted with methanol, then soaked in the transfer buffer and placed on top of the gel along with filter paper. Following transfer, PVDF membranes would be cut in half using a razor and put in a blocking solution of 1% low fat instant dried milk in 1X Tris-Buffered Saline with 0.1% Tween-20 (TBS-T). Primary antibody dilutions of 1:1000 of caspase-3 p17 (sc-271028-Santa-Cruz Biotechnology) and p21 would be made in 1X TBS-T and kept cold. The antibodies would then be incubated and washed on a rocking platform. Secondary antibody, IgGk BP-HRP (sc-516102-Santa-Cruz Biotechnology), would be diluted by 1:5000 in 1X TBS-T. Immunoblots would be incubated in secondary antibody for 1 hour at room temperature on a rocking platform, and then stained with colorimetric 1-Step Ultra TMB Blotting-Solution (ThermoFisher Scientific) for 5-30 minutes or until bands develop. Blots would finally be imaged using the ChemiDoc XRS Gel Photo Documentation System (BioRad).

Using image J, we would assess the density of bands in the treated and control lanes. We would be looking for increased density for bands of caspase-3 and p21 that would indicate to us that these proteins were upregulated by either SDG or TAM in T47D cells.

4.2 Receptor Identification

Although the data suggest that the anti-proliferation effects of SDG and TAM are due to the binding of an estrogen receptor, it does not conclude that the anti-proliferation observed is strictly due to this binding. Therefore, an experiment that antagonizes the α- or β-receptors of T47D-KBluc cells is appropriate to conduct. According to the literature, TAM is known to work through ER-α in order to competitively inhibit binding of estrogen to the receptor. However, there is not enough evidence in the literature to indicate to which receptor SDG binds. Therefore, T47D-KBluc cells where either the α- or β-receptor individually is inhibited would be treated with SDG and TAM to determine through which receptor each drug may be functioning. Following a similar experimental setup to that seen in *Figure 4*, T47D-KBluc cells would be cultured in a charcoal 96-well plate. A charcoal-plate would be used to remove steroid-related materials from the medium. However, before the addition of SDG, TAM, and E2, α- and β-receptor antagonists would be added in separate experiments. SDG, TAM, and E2 would be administered to both cell lines in triplicates, and a luciferase assay would be performed. It is hypothesized that since TAM acts primarily through ER-α, SDG would bind to ER-β in order to achieve the synergistic effects displayed in *Figure 3*. Essentially, luminescence levels of cells when treated with SDG that are α-receptor-inhibited would be high compared to that of the vehicle control.

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