G PROTEIN-COUPLED ESTROGEN RECEPTOR IS ACTIVATED

BY G-1 WHICH INDUCES A SIGNALING CASCADE THAT

INHIBITS BREAST CANCER CELL PROLIFERATION

A Senior Scholars Thesis

by

HEATHER WATTS

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Biomedical Scienc

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Approved by:

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ABSTRACT

G Protein-Coupled Estrogen Receptor is Activated by G-1 Which Induces a Signaling
Cascade that Inhibits Breast Cancer Cell Proliferation. (May 2012)

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Breast cancer is the most common cancer in women and is predominantly estrogen

dependent. About 1 in 8 women in the United States (12%) will develop invasive breast

cancer over the course of her lifetime. Failure in the current treatment of breast cancer

has led to an increased desire to find the specific mechanism of how cancer cell

proliferation is inhibited. G protein-coupled estrogen receptor (GPER) is very

controversial when concerning breast cancer cell proliferation. Some studies report

evidence that GPER is strongly associated with increased cancer proliferation,

migration, invasion, differentiation and metastasis. Other studies have found that GPER

inhibits proliferation of MCF-7 breast cancer cells. The objective of this study was to

clarify whether GPER works to promote or inhibit MCF-7 breast cancer cell

proliferation. I found that G-1 (GPER agonist) acted on GPER to inhibit MCF-7 cell

proliferation. Manual cell counting was used to find the doubling time of the MCF-7

cells. To determine the effect of G-1 on the cells proliferation I used a cell proliferation

assay kit. Through this method it was found that G-1 inhibits proliferation in a concentration dependent manner. Western blot was used to detect the target proteins such as GPER, ERα, ERβ, p53 and p38. I tested antibody on these cells through Western blot and discovered that the correct antibodies were used and p38 and p53 proteins were present in the MCF-7 cells. Immunocytochemistry was also used to test specific antibodies. Proliferating Cell Nuclear Antigen (PCNA) antibody and α-actin antibody were tested using this method. These results demonstrated that GPER activation inhibits MCF-7 cell proliferation.

DEDICATION

I would like to dedicate this thesis to Becky Taylor and Alice Butler

who have survived breast cancer.

And in loving memory of my grandmother, Audrey Veola

May God heal me,
body and soul.
May my pain cease,
May my strength increase,
May my fears be released,
May blessings, love,
and joy surround me.
Amen.
--Naomi Levy

ACKNOWLEDGEMENTS

NOMENCLATURE

MCF-7   Breast Cancer Cells

GPER   G Protein-Coupled Estrogen Receptor

ERα Estrogen Receptor α

ERβ Estrogen Receptor β

PCNA   Proliferating Cell Nuclear Antigen

G-1   GPER Agonist (drug used to treat MCF-7 cells)

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CHAPTER I
INTRODUCTION

Breast cancer is one of the most common human malignancies and the second leading

cause of cancer-related deaths in women, and its incidence in the developing world is on

the rise. About 30-40% of women with breast cancer will develop metastasis and

eventually die from this disease. Unfortunately, breast cancer is particularly challenging,

because it is highly resistant to radiation and conventional chemotherapeutic agents, and

such resistance is associated with a poor prognosis for this metastatic disease,

particularly in hormone receptor-positive breast cancer [1]. There is considerable

emphasis in searching for novel agents that selectively activate cell death machinery of

breast cancer cells without producing cytotoxic effects on normal cells [2]. The newly

reported, membrane-associated G protein-coupled estrogen receptor (GPER) is

structurally unrelated to nuclear ERα or ERβ. It has drawn much attention as a potential

mediator of estrogen action in breast cancer [3]. Estrogen binds to GPER and acts to

increase breast tissue proliferation. G-1 is a chemical substrate that binds to G protein-

coupled estrogen receptor. G-1 has a similar structure to estrogen which allows it to bind

to GPER. However G-1 decreases MCF-7 proliferation unlike estrogen. G-1 binds only

to GPER and not to ERα or ERβ. Therefore ERα and ERβ will not interfere with

experiments. Activation of ERα is known to promote MCF-7 growth [4]. It is not yet

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This thesis follows the style of *American Association for Cancer Research Journal.*

known whether ERβ promotes or inhibits MCF-7 proliferation [4]. When GPER is activated it induces a signaling pathway. I would like to determine whether this GPER activation will further activate p38 and p53. I hypothesize that GPER will then activate p38 to p38 MAPK by phosphorylating it. p38 MAPK will then activate p53 which then inhibits MCF-7 proliferation. Studies have shown that incubation of active p38 kinase with p53 protein causes the phosphorylation of p53 protein [5]. p53 can cause cells to growth arrest at certain stages in the cell cycle, of which the best understood is late G_2 phase arrest [6]. Preliminary data in our lab also shows that GPER slows human and porcine coronary artery smooth muscle cell proliferation at G_2 and M phases of the cell cycle. Therefore I suspect GPER to inhibit MCF-7 cell proliferation at G_2 and M phases as well.

CHAPTER II

METHODS

Cell lines

MCF-7 cell lines were obtained from American Type Culture Collection and were cultured at $37.0°C$ and 5% CO_2.

Propagation if cells:

The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, I added the following components to the base medium: 0.01 mg/ml bovine insulin; fetal bovine serum to a final concentration of 10% .

Subculturing

MCF-7 cells are cultured and should be monitored every day for growth. The cells should be passed at the appropriate growth levels and should be split into appropriate number of dishes to perform Western blot and other experiments.

Protocol: I strictly followed the protocol given by the manufacturer's instructions. Volumes used in this protocol are for 75 sq cm flasks; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove culture medium to a centrifuge tube.

2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.

3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37C to facilitate dispersal.

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

5. Transfer the cell suspension to the centrifuge tube with the medium and cells from step 1, and centrifuge at approximately 125 x g for 5 to 10 minutes. Discard the supernatant.

6. Resuspend the cell pellet in fresh growth medium. Add appropriate aliquots of the cell suspension to new culture vessels.

7. Incubate cultures at 37C.

Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:6 is recommended

Medium Renewal: 2 to 3 times per week

Cell counting

A drop of the prepared treatment groups was placed in the sink of a hemocytometer slide that enabled cell counting. Typhan Blue dye was used to visualize the cells. By looking under a microscope a grid was used to manually count the number of cells. I used three dishes of cells and counted them each three times every twenty-four hours for 2 days. I was then able to determine the doubling time of the MCF-7 cells.

Cell proliferation assay

Cell proliferation assay is a good method for evaluating a cell population's response to external factors, whether it is an increase in cell growth, no effect, or a decrease in growth due to necrosis or apoptosis [3]. I treated the MCF-7 cells with different concentrations of G-1 and inserted them into a 96 well plate. I then measured the DNA content by using CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen) by following the manufactures manual. Data obtained from this will show me how the G-1 affected the proliferation of MCF-7 cells.

Cell protein assay

The harvested cells were obtained after incubation and samples were placed in a 96 well plate using BCA protein assay according to the manufacturer's instructions. AlamarBlue reagent, BCA protein assay reagent A and BCA protein assay reagent B were added to

the wells along with the samples and the well plate was incubated. The fluorescence intensity data was then measured on image J program. The intensity of the fluorescence is directly proportional to the number of cells. This data helped determine the volume of the samples that should be loaded onto the SDS-PAGE gel.

Western blot

 Gel electrophoresis was used to separate the proteins. Equal amount of protein (40 or 60 µg) was subjected to 4-12% SDS–PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. Membranes were blocked, and then incubated with anti-α-Actin (1:100), anti-β-Actin (1:1000), anti-p38 (1:1000), anti-p53 (1:1000), anti-PCNA (1:100), anti-GPER(1:1000), anti-ERα mA1 (1:500), anti-ER21 (1:200), anti-ERβ 311 (1:500) antibodies (from Santa Cruz Biotechnology, Abcam, or Cell Signaling) overnight, and then with the horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology; 1:5000-10000) for 2 h. These primary antibodies bound to their specific target proteins. The secondary antibody bound to the primary antibody which then allowed the protein to be seen as bands in imaging. The blot was detected with the chemiluminescence detection system. Image J software was used for data analysis. Detecting bands in the imaging allowed me to determine if the protein was present. I used this Western Blot procedure to determine that p53, p38, GPER, ERα, ERβ, and α–actin were present in the MCF-7 cells.

Immunocytochemistry

Immunocytochemistry is another technique that uses an antibody to detect a specific protein antigen. When the antibody binds to the antigen of the protein of interest it stains the protein. This protein can then easily be seen using fluorescent light microscopy. MCF-7 cells were cultured on 12 mm glass coverslips were serum-starved for 48 h, and then treated for 1-2 days in media alone. Cells were then fixed in 10% buffered formalin for 10 min, washed twice with PBS, and permeabilized in 0.2% Triton X-100 and PBS for 10 min. MCF-7 cells were washed twice with PBS, and incubated with 4% BSA-PBS for 1 h and then with anti-α-Actin antibody overnight at 4°C. After three washes with PBS, MCF-7 cells were incubated with FITC-conjugated anti-Rabbit IgG antibody for 1 h at room temperature in darkness. After three washes with PBS, coverslips were mounted for imaging.

CHAPTER III

RESULTS

MCF-7 doubling time

I first proceeded by culturing MCF-7 cells. I let the cells grow in the medium while changing the medium every few days to ensure optimum growth. I split the cells evenly into dishes and counted the cells after 24 hours and 48 hours to monitor the growth and determine the doubling time. I observed the cells through an inverted microscope using a hemocytometer slide. I counted 2 dishes 3 times after the first 24 hours and got an average of 17.5 X 10^4 and 20.75 X 10^4 cells in the dishes. I counted 3 dishes after 48 hours and got an average of 28.6 X 10^4, 44.16 X10^4, and 21X10^4 cells in the dishes. From this data indicated in table 1 I determined the doubling time of the MCF-7 cells to be 38 hours. Figure 1 shows the MCF-7 cellular concentration graph. These cells were to be used for cell proliferation assay, protein assay and Western blot. I had to repeat this process several times because I had problems with cell contamination. My averages were not always very consistent and I expected my cellular concentrations to be higher than they were.

Table 1- MCF-7 cellular concentration

	24 HOURS	48 HOURS
DISH 1 AVERAGE	17.75×10^4	28.66×10^4
DISH 2 AVERAGE	20.75×10^4	44.16×10^4
DISH 3 AVERAGE		21.00×10^4
TOTAL AVERAGE	19.25×10^4	31.25×10^4

Figure 1-MCF-7 doubling time
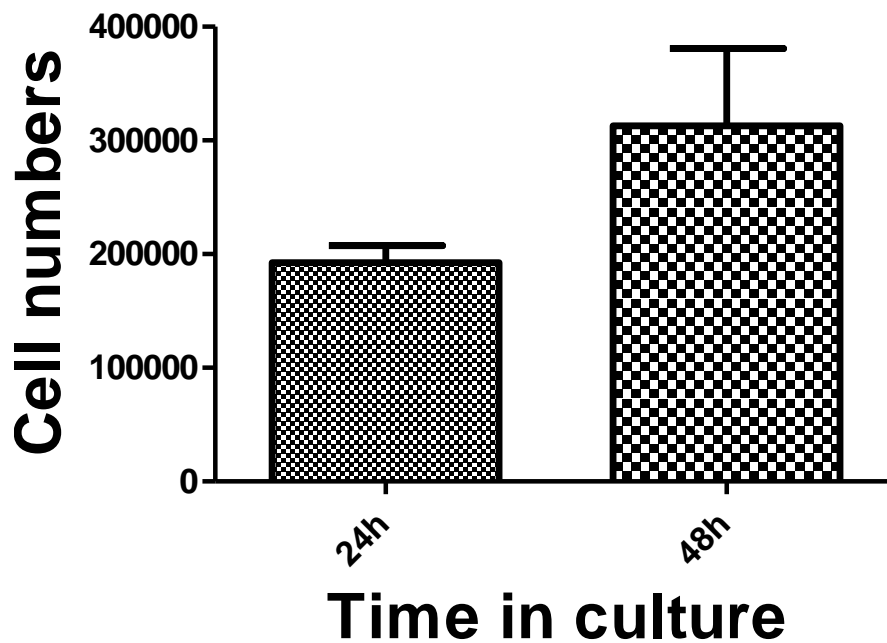
G-1 inhibits MCF-7 proliferation

The results in Figure 2 were obtained by doing a cellular proliferation assay. Cellular concentration assay measured the DNA content of the cells. Certain concentrations of G-1 were used to treat MCF-7 cells and the proliferation of these cells was measured. Dimethyl sulfoxide (DMSO) solvent can be considered a vehicle control. It was tested against a standard to make sure that the DMSO solvent does not have an effect on the cells. The total DMSO concentration is less than 0.001. This makes DMSO a good vehicle control. G-1 concentrations of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M were used to treat the cells. Through this graph we can see that G-1 treatment inhibits MCF-7 cell proliferation by comparing it to the DMSO. G-1 concentration of 10^{-5} M caused the most inhibition of MCF-7 cells. Therefore, when the cells are stimulated with higher concentrations of G-1 then MCF-7 proliferation decreases. This shows that G-1 inhibits MCF-7 cell proliferation in a concentration dependant manner. Table 2 shows the statistical analysis of G-1 effect on MCF-7 cell proliferation. The P value for this data is 0.0022, which is less than 0.05. P values of less than 0.05 indicate that the results are significant. This data shows that G-1 significantly inhibited MCF-7 proliferation.
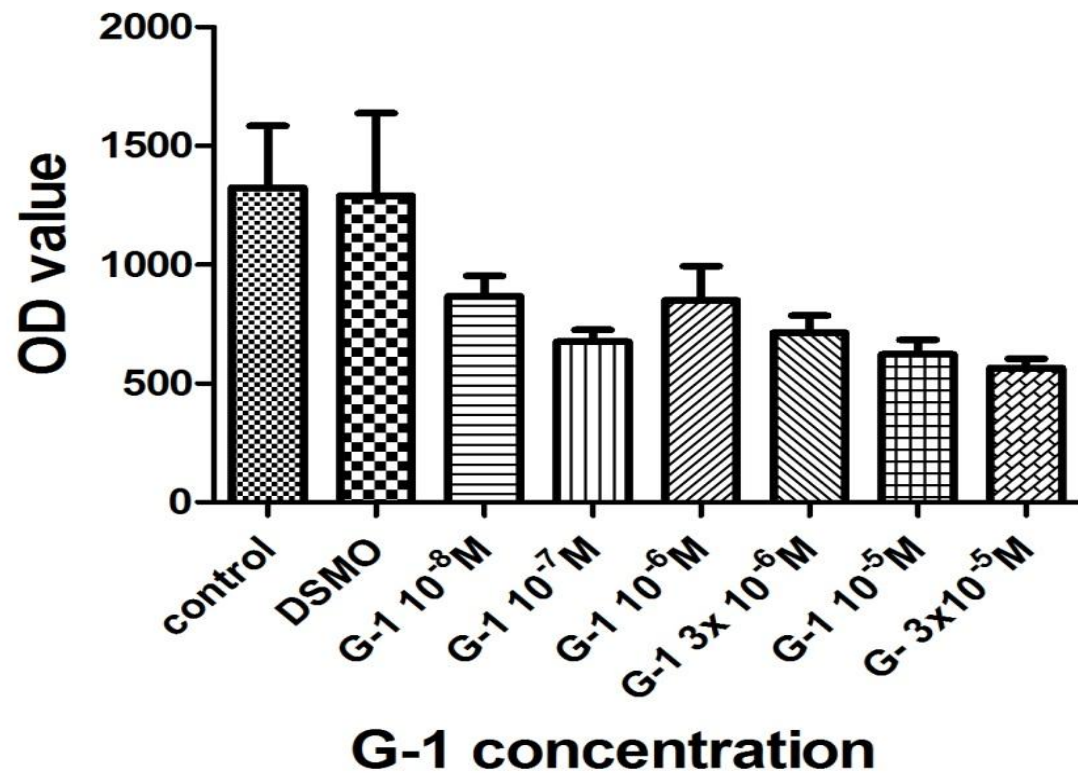
Figure 2- G-1 inhibits MCF-7 cells

Table 2- Statistical analysis of G-1 effect on MCF-7 cell proliferation

Table Analyzed	Data 1		
One-way analysis of variance			
P value	0.0022		
P value summary	**		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	8		
F	4.384		
R square	0.5229		
ANOVA Table	SS	df	MS
Treatment (between columns)	2.118e+006	7	302527
Residual (within columns)	1.932e+006	28	69008
Total	4.050e+006	35	

MCF-7 expression of p38 and p53

Using a protein assay gave me the concentration of each sample. The concentration was used to find the appropriate volume that should be added to the SDS-PAGE gel. The electrophoresis gel separated the proteins according to their molecular weight. The proteins on the gel were then transferred to a membrane through Western blot. The proteins were then probed with primary antibodies. It is important that the antibody used in Western blot be the correct antibody. The antibody has to recognize and bind to specific target proteins. When antibodies bind to a target protein, only that protein will show up as a band when doing imaging. Figure 3 shows that the p38 antibody and p53 antibody were the correct antibodies because they detected the target proteins of p38 and p53 as seen by the bands. The proteins were detected at the concentrations of 35μg and 50μg. β-Actin serves as the internal control. This shows that the conditions were good and the antibodies were working. When I treat the cells at a later time I now know the correct antibodies to use when running the western blot.

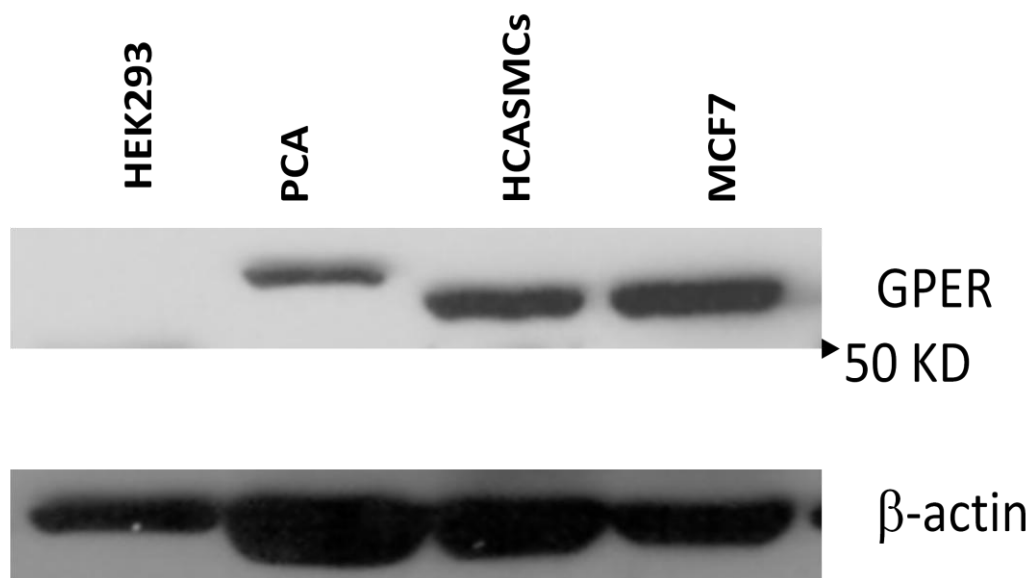Figure 3- The expression of p53 and p38 in MCF-7 cells
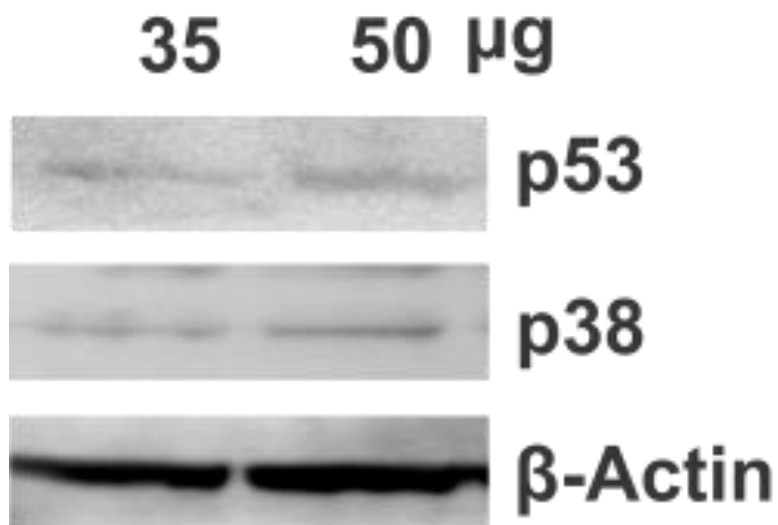
MCF-7 expression of estrogen receptors

Figure 4 was obtained through Western blot procedures. HEK293 is the negative control and β-actin is the internal loading control. GPER antibody was used to determine if the MCF-7 cells contained GPER. Based on the band it can be concluded that these cells contain GPER. From this we know that the expression of the GPER did not go away with the passage of the cells. GPER is also expressed in pig coronary artery smooth muscle cells (PCA) and human coronary artery smooth muscle cells (HCSMCs) as shown in figure 4. In future experiments I know that GPER is present in these cells and I know the correct antibody to use. Therefore, G-1 should be able to bind to GPER when

the cells are treated. Without GPER present the signaling cascade desired cannot be

activated.

Figure 4-GPER expression in MCF-7 cells

Estrogen receptor α (ERα) is known to increase MCF-7 cell proliferation when

stimulated. ERα's presence in the MCF-7 cells is indicated in Figures 5 and 6. In figure

5 ER21 antibody binds to ERα showing 2 bands responsible for the detection of ERα.

This antibody was used in a 1:200 dilution ratio. This was found using Western blot. The

bands are detected at 46kD and 66kD. It produced a truncated form of the full length

ERα when it forms the 46kD band along with the full length 66kD band. The ERα mA1

antibody binds to ERα and only produces one band, as seen in figure 6. The only band

detected is the full length, 66kD band. This antibody is used in a 1:500 dilution ratio.

This shows that the ERα concentration did not fade with passage of the cells. These

antibodies will be used in future experiments.
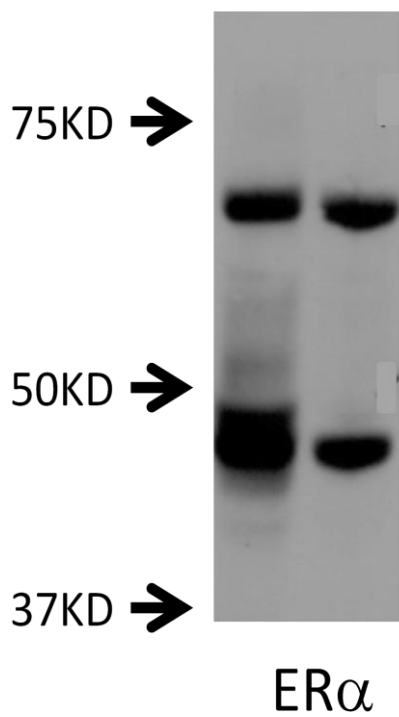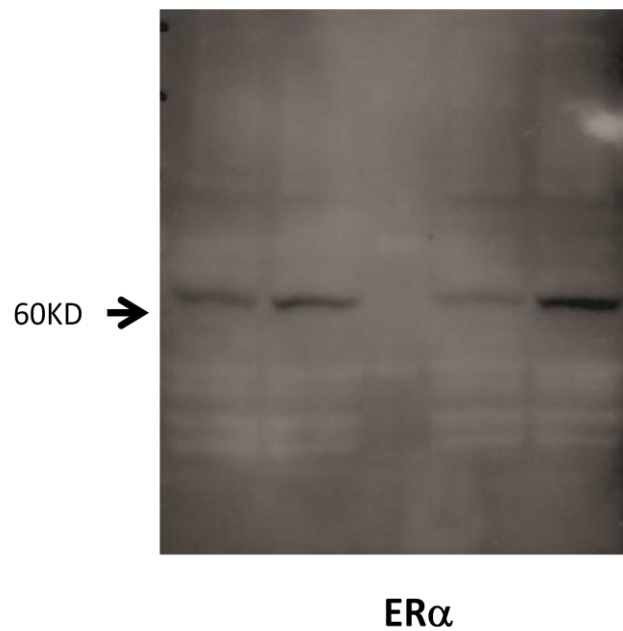
Figure 5- Two isoforms of ERα
present in MCF-7 cells

Figure 6- The 66 kD ERα expression in
MCF-7 cells

75KD ➔

50KD ➔

37KD ➔

ERα

60KD ➔

ERα

Estrogen receptor β (ERβ) is a classic estrogen nuclear receptor. It is not yet known

whether its activation promotes or inhibits MCF-7 cellular growth. Figure 7 shows that

ERβ is present in the MCF-7 cells using the method of Western blot. ERβ 311 antibody

was used at a dilution ratio of 1:500. This antibody bound to ERβ in the cells; therefore

ERβ was not lost with passage of the cells. ERβ is about 50kD which correctly

corresponds to what is seen in the band, further confirming the presence of ERβ. This

information will be useful in future experiments because I now know the correct

antibody to be used and I know that ERβ is found in MCF-7 cells.

Figure 7- ERβ present in MCF-7 cells

50KD →

ERβ
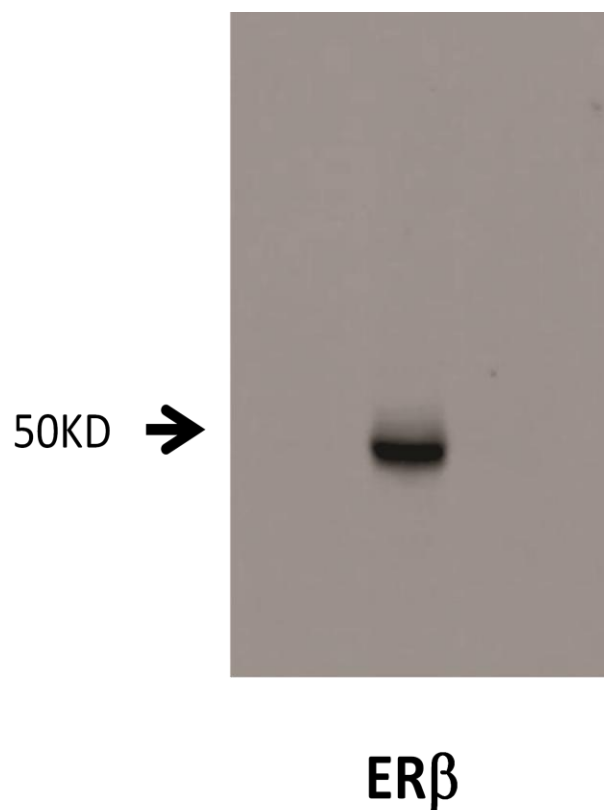
MCF-7 expression of PCNA and α-actin

Immunocytochemistry is another technique that uses an antibody to detect a specific

protein antigen. When the antibody binds to the antigen of the protein of interest it stains

the protein. This protein can then easily be seen using fluorescent light microscopy.

Proliferating Cell Nuclear Antigen, commonly known as PCNA, is a protein that acts as

a processivity factor for DNA polymerase in eukaryotic cells. Multiple proteins involved

in DNA replication, DNA repair, and cell cycle control bind to PCNA rather than

directly associating with DNA, thus facilitating fast processing of DNA. PCNA protein

expression is a well-accepted marker of cellular proliferation [5]. This information was

gathered from Wikipedia. The MCF-7 cells were treated with G-1. PCNA antibody was

then used. This antibody was used at a dilution ratio of 1:100. The PCNA antibody then

bound to the MCF-7 cell nucleus and stained only the nucleus. After this was stained the

proliferation of MCF-7 cells were visualized as seen in figure 8. The next step will be to

use this method and this antibody to do Flow Cytometry. Flow Cytometry will allow me

to visualize what step in the cell cycle is inhibited by the action of G-1.

Figure 8-Immunocytochemistry of PCNA in MCF-7 cells
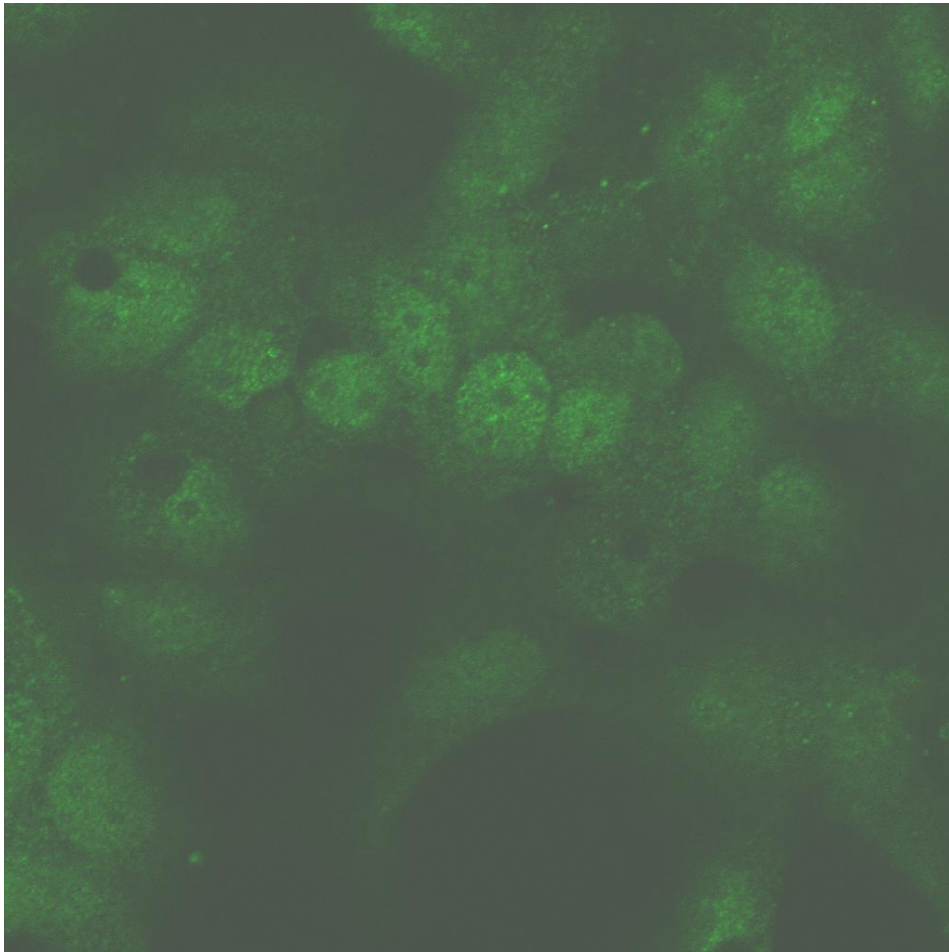
α-actin antibody was used in immunocytochemistry of MCF-7 cells. The purpose of this was to test the α-actin antibody. This is another way to see the effect of G-1 on α-actin other than using Western blot. The MCF-7 cells were treated by G-1. The antibody then bound to these cells allowing the cells to be visualized as seen in figure 9. α-actin antibody was used in a 1:100 dilution ratio. If the proliferation of MCF-7 cells caused by G-1 involves changes in α-actin florescence, then I will study the mechanism of the GPER action.

Figure 9- Immunocytochemistry of α-actin in MCF-7 cells
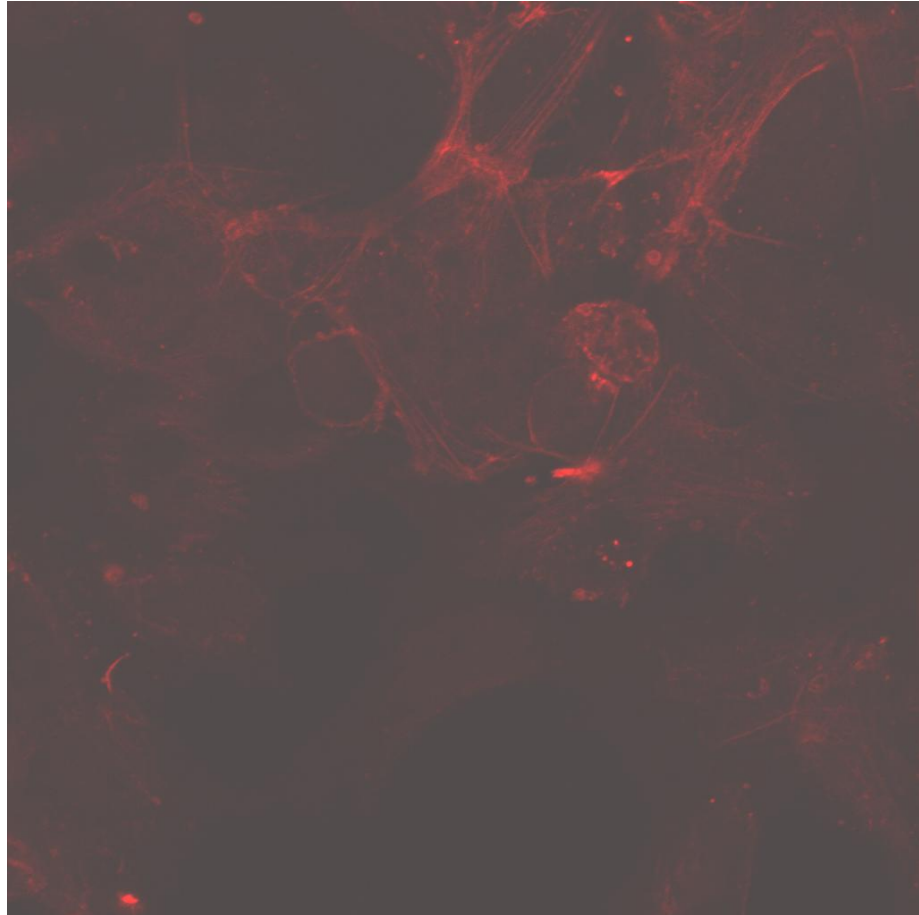
CHAPTER IV

SUMMARY AND CONCLUSTIONS

G protein-coupled estrogen receptor (GPER) is a seven transmembrane domain protein that makes a major contribution to the growth regulation of differing normal and cancer cells [7,8]. GPER likely plays important roles in modulating estrogen responsiveness and in the development and/or progression of hormonally responsive cancers [5]. GPER is a source of much controversy; however my study shows that GPER inhibits breast cancer. This discovery of GPER's inhibition of MCF-7 cells makes it a potential therapeutic target to combat breast cancer and potentially other types of cancer as well [5]. Others believe that GPER increases the proliferation of MCF-7 cells and that blocking (as opposed to activating) GPER may prove to be a better strategy for the treatment of estrogen-related tumors [9]. However GPER has been shown to decrease the proliferative effects of cells of cancers involving not only the breast but also the thyroid, ovaries, and prostate [4].

In my experiments I began by culturing MCF-7 cells and monitoring the growth of the cells. I counted the cell growth over 24 and 48 hours. This allowed me to get the average cellular concentration and doubling time. These cells were then prepared for cell proliferation assay, protein assay and Western blot. I then treated the MCF-7 cells with different concentrations of G-1. The DNA content in this assay decreased as the concentration of G-1 increased, indicating a decreased cellular concentration. This meant that higher concentrations of G-1 inhibited MCF-7 proliferation. By using Western blot I

was able to find the correct primary antibodies that bound to the specific target proteins of, p38, p53, GPER, ERα, and ERβ. Knowing these antibodies is necessary when performing experiments in the future. This also allowed me to see that the proteins were not lost with passage of the cells. At a later time I will test the cross action between GPER, ERα, and ERβ and how they function together to effect MCF-7 proliferation. I was able to determine the primary antibodies of PCNA and α-actin by using the immunocytochemistry staining technique. The nucleus of the cells were stained which allowed the cells to be easily visualized with a fluorescent microscope. PCNA allows you to visualize the proliferation of the cells.

In the near future I will be determining the mechanism of GPER's role in the inhibition of MCF-7 cells by measuring the amounts of p38 and p53 in response to G-1 treatment. G-1 binds to only GPER, but not ERα or ERβ. I will be treating the cells with G-1, which will activate GPER. When GPER is activated it induces a signaling pathway. I anticipate that GPER will phosphorylate p38 to p38 MAPK. Phosphorylated p38 MAPK activates p53 to its active phospho-p53. I anticipate that the phospho p53 stops the MCF-7 proliferation at G2 and M phases. Flow Cytometry will be used to determine the stage of the cell cycle that will be inhibited. I will use protein assay and Western blot to monitor the change in the concentrations of proteins when the MCF-7 cells are treated with G-1. I expect that when the cells are treated with G-1 the concentration of phospho p38 MAPK and phospho p53 will increase leading to a decrease of MCF-7 cell numbers. Increasing evidence indicates a central role for p53 in mediating cell cycle arrest or

apoptosis [10]. Results from Cellular Proliferation Assay have proved that G-1 treatment leads to decreased concentration of MCF-7 cells. G-1 is an agonist of GPER; therefore G-1 must have activated GPER which then led to the inhibition of MCF-7 cell proliferation. This is further evidence that GPER leads to inhibition of breast cancer. If this signaling cascade leading to MCF-7 cellular inhibition proves to be correct then it will contribute to the effort in targeting GPER for chemotherapeutic treatment.

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