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Tamoxifen has been used as an effective treatment against breast cancer for over 30 years. However, tamoxifen resistance has become a major hurdle to effective treatment. Previous studies have implicated up-regulation of the PI3K and MAPK pathways as being involved in the growth exhibited by tamoxifen resistant cells. Data from our lab and others have shown naringenin to have the ability to impair both of these signaling pathways. For this study, I investigated whether naringenin, a flavanone, could reverse the proliferation observed in tamoxifen-resistant cells (TAM-R).

MCF-7 cells were exposed to low levels of 4-OH-tamoxifen (1 μM) for 10 months in order to generate a tamoxifen-resistant cell line, TAM-R. These cells were then cultured under various combinations of treatment with tamoxifen and naringenin. TAM-R cells demonstrated a clear ability to proliferate in the presence of tamoxifen as measured using cell counting and an MTT assay. However, the addition of naringenin reversed that proliferation. The TAM-R cells also exhibited an up-regulation of the MAPK pathway which was also reversed by treatment with naringenin as measured by western blot analysis. In addition, treatment with tamoxifen and naringenin together led to a greater reduction in cell growth than either treatment alone.

Confocal microscopy was also employed to look for any changes in the localization patterns of the estrogen receptor alpha (ERα). ERα was found predominantly in the nucleus in MCF-7 cells. Upon treatment with tamoxifen, this pattern changed to a peri-nuclear localization. Once the cells attained tamoxifen resistance, the ERα displayed

an even pattern across the cell. However, upon treatment of the TAM-R cells with naringenin, the ERα localization returned to a peri-nuclear pattern similar to that seen in the tamoxifen-sensitive cells.

The results from this study clearly demonstrate the ability of naringenin to reverse the proliferation normally seen in TAM-R cells. Naringenin also appears to have a synergistic effect with tamoxifen on growth impairment. There is also evidence these changes are taking place through the MAPK pathway. Taken together, these results open up the possibility of treatment with naringenin as a means to combat tamoxifen resistance.

A POTENTIAL ROLE FOR NARINGENIN IN REVERSING TAMOXIFEN RESISTANCE IN MCF-7 BREAST CANCER CELLS

by

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Approved by

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Dedicated to my wife, Tiffani, and my amazing children who inspire me every day.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Breast Cancer and Estrogen

Cancer is the general term for a group of over 100 diseases which are all characterized as having uncontrolled cellular growth (21). In the US today, half of all men and one third of all women can expect to develop some form of cancer in their lifetime (21). In 2011, over 230,000 new cases of invasive breast cancer were diagnosed resulting in over 39,000 deaths in the United States alone (1). Of those cases, seventy percent were positive for the estrogen receptor (ER+) (4). Estrogens influence breast cancer in ER+ cells because estrogens promote cell proliferation and growth (1). Estradiol is the predominant estrogen found in humans. Estradiol binds well to both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (8). The different estrogen receptors are found at varying levels in different tissues with breast tissue containing mostly ERα. However, the receptors can form homo- or hetero-dimers in a given cell (10). Upon ligand binding, accessory proteins are released from the receptor to facilitate dimerization and DNA binding. This frees the hormone-receptor complex to move into the nucleus and bind to DNA to affect gene transcription (9). As many as 126 genes have been shown to be affected by this hormone receptor complex (19). Many of those genes, such as Human Epidermal Growth Factor Receptor 2 (HER-2), Cyclin-D, and

c-Myc, have been shown to affect cell division, proliferation, and survival (5, 9). HER-2 activates signaling pathways leading to cell growth (5). Cyclin-D acts as a gatekeeper to the G_1/S transition in the cell cycle (9). Cells passing through this restriction point have entered the cell cycle and can now divide and proliferate. c-Myc is a transcription factor and a proto-oncogene. If mutated, it can affect the transcription of multiple genes involved in cellular proliferation (9).

Tamoxifen

In order to combat proliferation in ER+ breast cancer, tamoxifen treatment is routinely employed. Tamoxifen is a selective estrogen receptor modulator (SERM), meaning it acts as an agonist or antagonist to the estrogen receptor depending on the tissue. Tamoxifen is a drug which was originally investigated for its potential use as a contraceptive (11). When it failed to produce the same effects in humans as it did in rats, interest in its contraceptive properties faded. However, once a link began to surface between estrogen and breast cancer, interest in the drug renewed (11). It has since proven to be a powerful, safe, and effective treatment for most breast cancers (11). Tamoxifen has been used as a treatment for breast cancer for more than 30 years. A large study in 1998 also demonstrated its effectiveness as a preventative treatment for high risk subjects. In breast tissue, tamoxifen functions mainly as an antagonist to the estrogen receptor. It does so by binding to the estrogen receptor and preventing it from transcribing estrogen-responsive genes (2). Estradiol binding to its receptor

normally leads to recruitment of co-activators leading to transcription of target genes. Tamoxifen binding leads to the recruitment of co-repressors to prevent the complex from transcribing genes (19). Inhibiting transcription of these genes keeps the cells in the G_0/G_1 phase of the cell cycle making tamoxifen a cytostatic drug. However, treatment with tamoxifen is ineffective for as many as a third of breast cancer patients. Of those subjects who are initially sensitive to tamoxifen, most eventually develop resistance to the drug (3). What is not known is the mechanism behind this resistance. Thus, the identification of the underlying mechanism could lead to more effective treatment options.

Mitogenic Pathways

A recent study found that cells which had become resistant to tamoxifen displayed constitutive activation of the phosphatidylinositol 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways (4). MAPK pathways regulate cellular growth, motility, and survival through phosphorylation of a wide range of substrates including phospholipases, transcription factors, and cytoskeletal proteins (7). There are several MAPK pathways in eukaryotic organisms. Each pathway consists of a set of three sequentially acting kinases. A MAPKKK phosphorylates a MAPKK which in turn phosphorylates a MAPK (7). The classical example of this pathway is the Erk 1/2 MAPK pathway. In this pathway, Ras/Raf activates MEK which activates Erk 1/2. Once active, Erk 1/2 has been shown to phosphorylate several nuclear substrates including c-

Myc, c-fos, and STAT3 all of which are transcription factors as well as proto-oncogenes (7). The PI3K pathway also promotes cellular proliferation. PI3K has been shown to induce activation of Akt via phosphorylation of PIP_2 and subsequent activation of PDK1 (12). Upon activation, Akt can contribute to tumor growth by inhibiting apoptosis and promoting cellular proliferation (13). Razandi et al found that estradiol was able to activate the MAPK pathway (6). In this study, the levels of expression of various proteins in the MAPK pathway were elevated upon exposure to estradiol. This study demonstrated that estradiol was capable of producing nongenomic effects. Migliaccio et al showed that it was not estradiol itself, but rather estradiol complexed with the estrogen receptor which was interacting with the MAPK pathway (14). ERα has also been found to bind to PI3K and activate this pathway in an estrogen-independent manner (9). Taken together, these findings suggest that tamoxifen resistance may be the result of complex interactions between the estrogen receptor and components of kinase signaling pathways. Therefore, finding a way to inhibit the activity of the PI3K or MAPK pathways may restore growth arrest to tamoxifen resistant cells. Indeed, using chemical inhibitors of MEK and PI3K is a promising new strategy for breast cancer patients (4).

Naringenin

One potential candidate to inhibit the MAPK and PI3K pathways is naringenin, a flavanone. Our lab has been investigating the role of naringenin on cell proliferation, motility, and metabolism. Flavanones are secondary metabolites derived from plants. They have low toxicity compared to other plant compounds such as alkaloids and they often function in the body as antioxidants (20). Besides their ability to scavenge free radicals, flavanones have also been shown to affect cell proliferation, angiogenesis, and signaling cascades (16, 18). Naringenin is the predominant flavanone found in grapefruit and gives it its characteristic bitter taste (15). Several studies have shown that naringenin hinders cell proliferation and motility by interfering with the PI3K and MAPK pathways (16, 17). Bulzomi et al have even shown that naringenin can bind directly to the estrogen receptor as an antagonist (22). The ability of naringenin to both impair the MAPK and PI3K pathways and to function as an antagonist to the estrogen receptor may allow it to restore growth arrest to tamoxifen resistant cells.

I hypothesize that by interfering with the PI3K and/or MAPK pathways and by acting as an antagonist to the estrogen receptor, naringenin will be able to reverse the resistance normally exhibited by cells following prolonged treatment with tamoxifen. In addition, application of both treatments simultaneously may allow for a synergistic effect leading to greater proliferative impairment.

CHAPTER II

MATERIALS AND METHODS

Materials

MCF-7 breast cancer cells (HTB-22) and the MTT cell proliferation assay (30-1010K) were purchased from ATCC. Dulbecco's Modified Eagle Medium (DMEM) (11885) was obtained from Gibco. Phenol red-free media (DMEM D5921), L-glutamine (G7513), charcoal-stripped fetal bovine serum (F6765), bovine Insulin (15500), anti-rabbit secondary antibody (A6154) and 4-OH-tamoxifen (H7904) were purchased from Sigma Aldrich. HyClone fetal bovine serum (SH30071) was obtained from Fisher Scientific. Antibodies for p-Erk 1/2 (9101), Erk 1/2 (9102), p-Akt (9271), and Akt (9272) were purchased from Cell Signaling Technology. Anti-actin antibody (ab3280) was obtained from Abcam. Anti-ERα antibody (HC-20) was purchased from Santa Cruz biotechnology.

Cell Culture

A variety of media formulations were employed during this study. Dulbecco's Modified Eagle Medium (DMEM) or phenol red-free Dulbecco's Modified Eagle Medium (PRF-DMEM) were supplemented with 10 % of either Fetal Bovine Serum (FBS) or charcoal-stripped Fetal Bovine Serum (CS-FBS). Viability of the cells in each of the

formulations was determined by culturing MCF-7 breast cancer cells in each (DMEM + FBS, DMEM + CS-FBS, PRF-DMEM + FBS, or PRF-DMEM + CS-FBS). Cells were viable across all treatments after 12 days. Cells grown in CS-FBS exhibited a pronounced decrease in growth as compared to the other treatments.

MCF-7 cells were maintained in DMEM + FBS, 0.01 mg/mL bovine insulin, and 100 U/mL penicillin/streptomycin. Cells were maintained at 37° C with 5% CO_2. Media was changed every two to three days and cells were passed once they attained 80% confluence.

Generation of TAM-R Cells

MCF-7 breast cancer cells were cultured in phenol-red free media (PRF-DMEM) supplemented with 4 mM L-Glutamine, 100 U/mL penicillin/streptomycin, 0.01 mg/mL bovine insulin, 10% charcoal-stripped fetal bovine serum (CS-FBS), and 10^{-7} M concentration of 4-OH-tamoxifen (the main active metabolite of tamoxifen).

Once this formulation failed to provide the desired results, a new formulation was employed and used for the duration of the experiment. A 10^{-6} M concentration of 4-OH-tamoxifen was added to Dulbecco's Modified Eagle media (DMEM) supplemented with 100 U/mL penicillin/streptomycin, 0.01 mg/mL bovine insulin, and 10% fetal bovine serum (FBS).

Cell Counting

Cell counting was carried out using a hemacytometer to determine the number of cells per mL in each sample. Cells (10 µL) were collected and mixed with trypan blue exclusion dye (10 µL). Cells were counted in four large grids. That total was divided by 4 and multiplied by 10^4 to determine cell density for the sample.

MTT Proliferation Assay

Cells were seeded in a 96-well plate (1×10^4 cells/well) in triplicate in media containing their respective treatment. Three plates were seeded for each trial to allow for counting after 24, 48 and 72 hours. At each time point, 10 µL of MTT solution (5 mg/mL) was added to each well and each sample was incubated for 3 hr. at 37 °C. The MTT-formazan crystals formed by metabolically viable cells were dissolved in 200 µL of Dimethyl Sulfoxide (DMSO). Following a 2 h incubation, the absorbance at 570 nm was detected and recorded by using a BioTek Synergy 2 microplate reader.

Western Blot

Cell lysates were collected and subjected to 10% SDS-PAGE to separate the proteins before transfer to an immobilon filter. Antibodies for Akt, p-Akt, Erk 1/2, p-Erk 1/2, and ERα were used for western blot analysis. Membranes were incubated with the antibody of interest and visualized using a Bio-Rad ChemiDoc XRS system using an

enhanced chemiluminescent (ECL) kit. The resulting bands were quantified and compared via densitometric analysis using Quantity One analysis software.

Immunofluorescence

Cells from various treatments were grown on coverslips. Samples were washed with phosphate-buffered saline (1xPBS), fixed with 3.7% paraformaldehyde, and permeabilized in 0.25% Triton. Primary antibodies for the estrogen receptor (ERα) were added and allowed to incubate for 1 h. Following 3 washes with 1xPBS, secondary antibody was added and allowed to incubate for 45 min. The coverslips were washed 3 more times with 1xPBS. Following washing, the coverslips were mounted using permount. After staining and mounting, the slides were visualized using an Olympus iX81 Motorized Inverted Confocal Microscope equipped with Fluoview FV500 software.

Preparation of Nuclear and Cytoplasmic Extracts

Cells were washed with 1xPBS, collected, and then centrifuged at 8000 rpm for 2 min. The supernatant was removed and cells were resuspended in 1 mL of Hank's balanced salt solution. Next, cells were centrifuged at 4000 rpm for 2 min. The supernatant was removed and the cells were resuspended in 100 µL of CE buffer (10 mM Hepes pH7.6, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.7% NP-40). They were placed on ice for 5 min, and then centrifuged at 4000 rpm for 4 min. The supernatant was collected as the cytoplasmic extract. The remaining pellet was resuspended in 500 µL of

CE buffer without NP-40 and then centrifuged at 10,000 rpm for 4 min. The supernatant was removed and the remaining pellet was the nuclear extract.

CHAPTER III

RESULTS

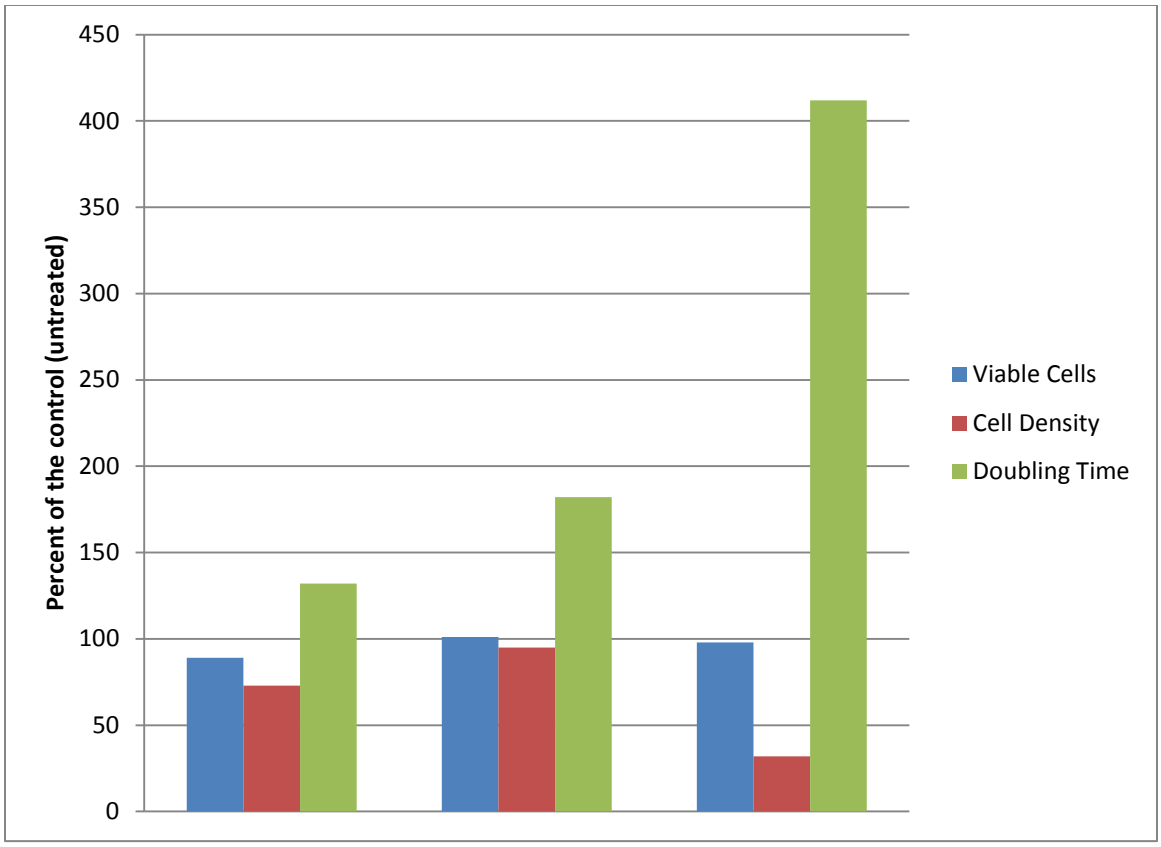
Optimizing Naringenin Concentration

Naringenin has been previously reported to impair both the PI3K and MAPK pathways in MCF-7 breast cancer cells (27). This impairment leads to a decrease in cellular proliferation. In order to explore the anti-proliferative effects of naringenin, we had to determine the optimal concentration at which impairment of proliferation was greatest without causing cell death. To determine the optimal concentration of naringenin, cells were treated with increasing concentrations of naringenin. After five days, cell density was quantified using a hemacytometer and trypan blue exclusion dye, as described in Materials and Methods, and compared to an untreated control. Naringenin treatment at 250 μM showed the greatest impairment of growth while demonstrating comparable cell viability to that of the untreated control (Figure 1).

It is possible that the decrease in cell density was due to cells losing adhesion to the plate upon death and not being counted as they were aspirated with the media. In addition, work in our lab and others demonstrated the ability of naringenin to affect cell adhesion during cell migration (23). It is possible that this interference causes some cells to detach from the plate. To investigate this possibility, cell density and viability

was quantified by a trypan blue exclusion assay on the detached cells to determine if there was a difference in the viability of the cells with increasing concentrations of naringenin. No increase in cell death was found at any of the naringenin concentration levels tested. In addition, there was no observed difference in the percentage of cells detached with increasing concentrations of naringenin (data not shown).

There was a significant increase in the doubling time of cells treated with 250 µM naringenin (Figure 1 green bar). However, there was no decrease in the percentage of viable cells (Figure 1 blue bar) or increase in the percentage of detached cells when compared to the other naringenin concentrations used (data not shown). Thus the data suggest that cells treated with 250 µM naringenin experienced a decrease in cell density by slowing the growth rate, not by an increase in detachment or cell death.



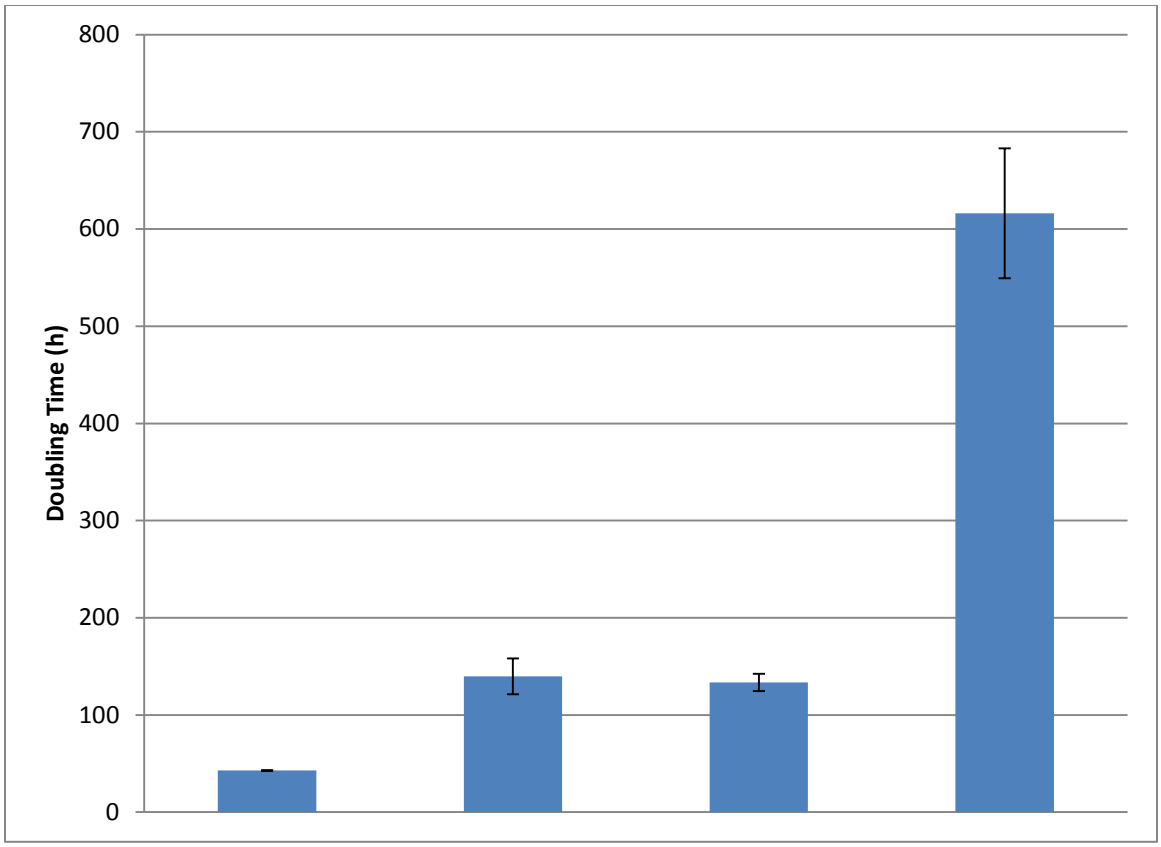
NG (µM)	50	100	250

Figure 1. Increase in doubling time with increasing concentration of naringenin. MCF-7 cells were grown in full media (DMEM + FBS) in the presence of increasing concentrations of naringenin (NG). Media was changed and treatments were reapplied after 3 days. Following five days total of treatment, cell density and viability was quantified using a hemacytometer and trypan blue exclusion dye. The doubling time was calculated using the formula, $T_d = (t_2-t_1) * \log2/\log (q2/q1)$ where "t" is the time in hours and "q" is the number of cells counted at each time point. The values from each treatment group are presented as a percentage of an untreated control.

Naringenin and Tamoxifen Demonstrate a Synergistic Effect

Naringenin has a demonstrated ability to inhibit pathways involved in cellular proliferation (16). Tamoxifen is currently being used as a treatment for breast cancer due to its action as an antagonist to the estrogen receptor which leads to down-regulation of the expression of estrogen responsive genes involved in cell proliferation. Since naringenin and tamoxifen affect separate pathways both involved in cell proliferation, they could have an additive effect if taken concomitantly.

MCF-7 cells were treated with either 4-OH-tamoxifen (the main active metabolite of tamoxifen) (100 nM), naringenin (250 μM), or both. Control cells were treated with the vehicles for 4-OH-tamoxifen (ethanol) and naringenin (DMSO). Cells were plated and cultured for 7 days in full media (DMEM + FBS) in the presence of each treatment. Next, cells were replated on separate plates to be analyzed. Cell proliferation was quantified after 2, 4, and 7 using a hemacytometer. Treatment with naringenin alone resulted in a 2-fold increase in doubling time when compared to the control. Treatment with 4-OH-tamoxifen also resulted in a 2-fold increase. However, the combination treatment demonstrated a synergistic effect with a 13-fold increase in doubling time (Figure 2). In addition, cells were undetectable after 3 weeks in the combination treatment only (data not shown).



Tam	**-**	**+**	**-**	**+**
NG	**-**	**-**	**+**	**+**

Figure 2. Synergistic effect of tamoxifen and naringenin on growth rate of MCF-7 cells. MCF-7 cells were grown for 7 days in full media (DMEM + FBS) in the presence or absence of 4-OH-tamoxifen (Tam) (100 nM) and naringenin (NG) (250 µM). Following 7 days, the cells were replated for an additional 7 days. The media was changed and the treatments were reapplied every 2-3 days. Following treatment cell density was quantified and doubling times were calculated using the formula, $T_d = (t_2-t_1) * \log 2/\log (q2/q1)$. The results are the average of 3 separate trials.

Generation of a Tamoxifen Resistant Cell Line (TAM-R)

Tamoxifen has been in use as a safe and effective treatment for breast cancer for over 30 years (11). However, many patients demonstrate acquired resistance to the drug following long-term treatment (3). Previous studies have shown that MCF-7 cells can be made to become tamoxifen-resistant through prolonged exposure to 4-OH-tamoxifen (24, 26). Results from other studies have shown that tamoxifen resistant cells demonstrated constitutive activation of the PI3K and MAPK pathways (4). The ability of naringenin to impair these pathways may help restore the resistant cells to a more tamoxifen sensitive state.

Before this could be investigated, I first needed to generate a tamoxifen-resistant cell line (TAM-R) to use as a model. I chose MCF-7 breast cancer cells because they are a well characterized ER-positive breast cancer model which has been shown to become tamoxifen-resistant following prolonged exposure to 4-OH-tamoxifen. I attempted to generate TAM-R cells using the estrogen-depleted media formulation in the methods section (PRF-DMEM + CS-FBS) along with 4-OH-tamoxifen treatment (100 nM) (24). The removal of phenol red and lipophilic hormones via the charcoal stripping process eliminates most of the estrogen and estrogen mimetics from the media preventing them from binding to the estrogen receptor. This allows a greater number of estrogen receptors to bind with 4-OH-tamoxifen hastening the onset of resistance.

This particular formulation proved to be problematic as it completely growth arrested the control plates and made culturing the cells very difficult. If MCF-7 cells remained on the same plate for more than a week, they became nearly impossible to detach from the plate. I ultimately opted to switch to a full media (DMEM +FBS) solution and increased the tamoxifen dosage from 100 nM to 1 μM in an attempt to compensate for the additional estrogen that would now be competing with the tamoxifen for binding with the estrogen receptor (28). This system may more closely mimic physiological conditions as estrogen is present during treatment with tamoxifen.

Cells were continually cultured in this new media and the media was changed every 2 to 3 days. The cells were replated at a 1:4 dilution once they attained 80% confluence. Cells were periodically assayed for proliferation by quantification of cell density. Cells treated with 4-OH-tamoxifen consistently demonstrated a reduced proliferation rate when compared to the vehicle over the first 6 month treatment period (Figure 3).

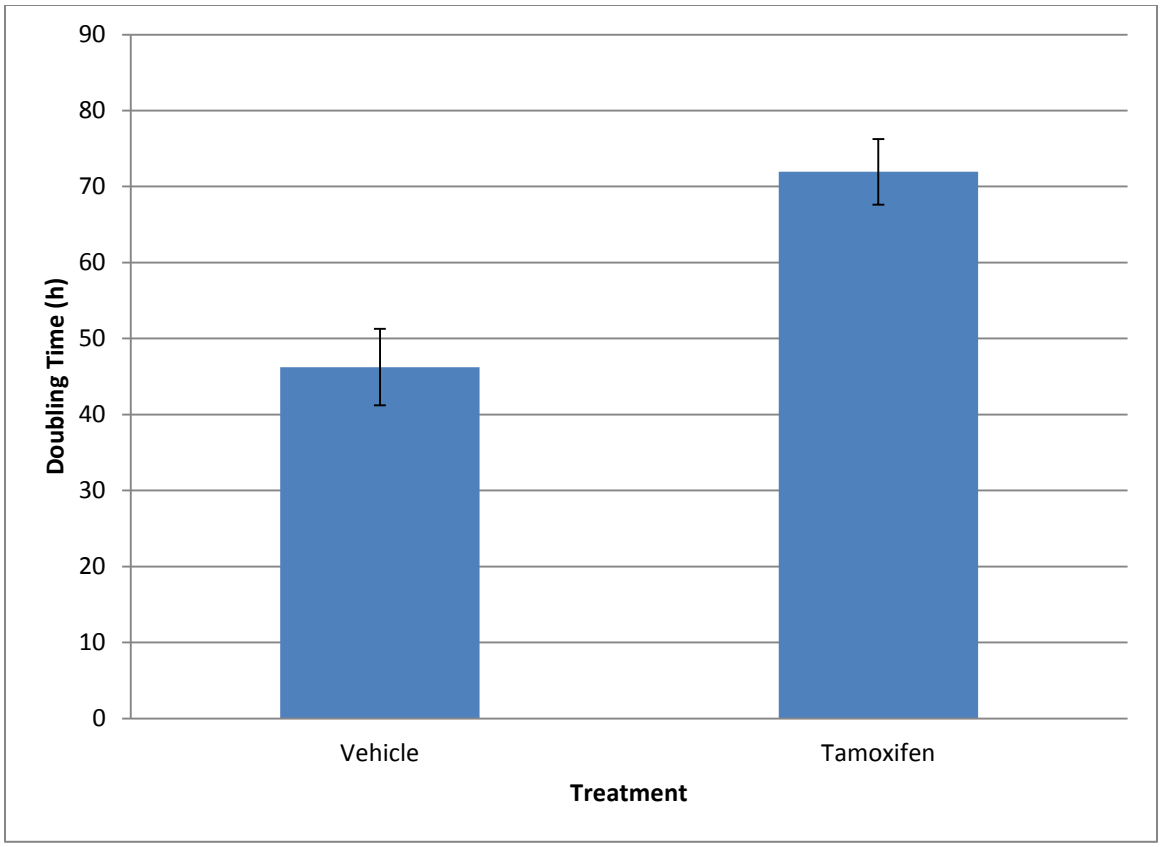


Figure 3. Tamoxifen increases doubling time in MCF-7 cells. MCF-7 cells were cultured in full media (DMEM + FBS) and treated with either 4-OH-tamoxifen (1 µM) or vehicle (ethanol) for 6 months. Cell density was quantified periodically and doubling times calculated using the formula, $T_d = (t_2-t_1) * \log 2/\log (q2/q1)$. The results are an average of 10 separate counts over the 6 month treatment period.

Following 6 months of treatment, cells samples were analyzed for any changes which might indicate the onset of resistance to tamoxifen. Protein lysates were collected to analyze the phosphorylation state of Akt and Erk 1/2. The lysates were subjected to SDS-PAGE and transferred to an immobilon filter. The samples were probed for p-Akt, Akt, p-Erk 1/2, Erk 1/2, and actin. Previous studies have found constitutive activation of both the PI3K and MAPK pathways in tamoxifen resistant cells.

An increase in p-Akt or p-Erk 1/2 in the cells being treated with 4-OH-tamoxifen may indicate the onset of tamoxifen resistance. Total Akt, Erk 1/2, and actin were probed to serve as controls. There was a decrease in the amount of p-Erk 1/2 in the tamoxifen treated cells when compared to the vehicle. p-Erk 1 decreased 48% and p-Erk 2 decreased 42% when compared to the vehicle. The amount of p-Akt, however, showed no difference between the two groups (Figure 4). These data suggest that the cells were tamoxifen-sensitive at the 6-month time point.

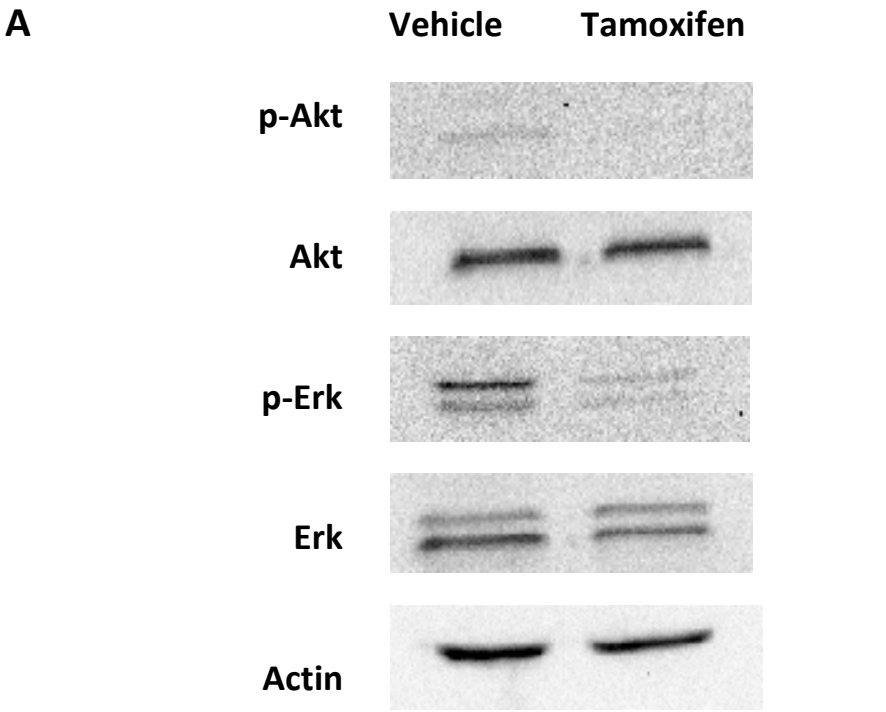
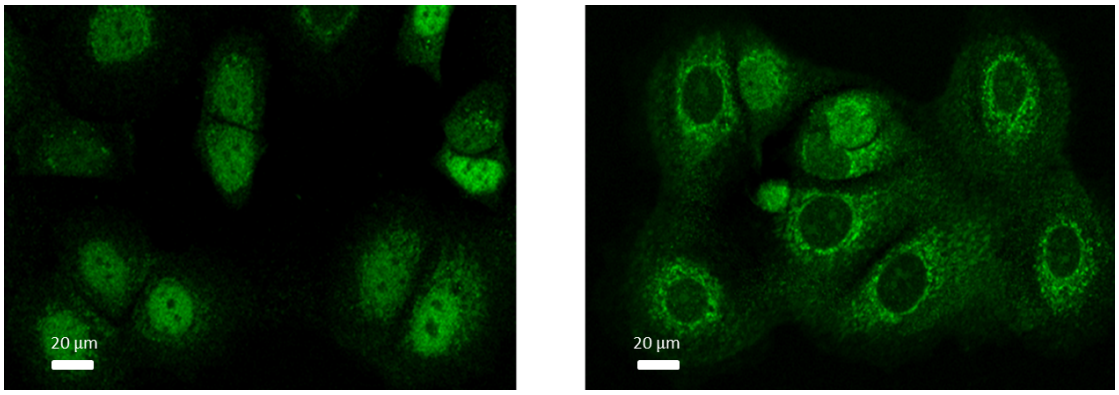


Figure 4. Phosphorylation status of Akt and Erk 1/2 following tamoxifen treatment. MCF-7 cells were exposed to either 4-OH-tamoxifen (1 uM) or vehicle (ethanol) for 6 months. Media was changed every 2-3 days and cells were passed upon reaching 80% confluence. (A) Protein lysates were prepared, subjected to SDS-PAGE and then immunoblotted using antibodies against p-Akt, Akt, p-Erk, Erk, or actin. (B) The resulting bands were quantified using densitometric analysis using Quantity One software and are expressed as a percent of the control (vehicle). The results are representative of 5 separate experiments.

Estrogen Receptor Localization Changes

The estrogen receptor is normally found in the cytoplasm, but binding with estrogen causes dimerization and movement into the nucleus to affect transcription of estrogen-responsive genes. Binding of the estrogen receptor with 4-OH-tamoxifen instead of estrogen may be keeping this complex in the cytoplasm for longer periods of time. This would allow for the possibility of interaction with other signaling pathways such as PI3K and MAPK pathways (9, 14). In order to determine if treatment with 4-OH-tamoxifen was causing changes to the estrogen receptor localization patterns, cells were prepared and visualized using confocal microscopy. The cells were stained with antibodies against the estrogen receptor (ERα). The untreated cells showed localization of the ERα in the nucleus of the cells. Cells treated with 4-OH-tamoxifen exhibited peri-nuclear localization of the ERα (Figure 5).



Tam	-	+

Figure 5. ERα localization pattern in the presence or absence of tamoxifen. MCF-7 cells were treated with either 4-OH-tamoxifen (1 µM) or a vehicle for 6 months. Cells were fixed and then probed with anti-ERα antibody and visualized using confocal microscopy (40x).

In complementary assays, we fractionated cells and collected cytoplasmic and nuclear extracts. Following the protocol in Material and Methods, samples were separated into cytoplasmic extracts (CE) and nuclear extracts (NE) from both treatment groups. The samples were then subjected to SDS-PAGE and transferred to an immobilon filter and probed using an antibody against the estrogen receptor α (ERα). The results showed a decrease in the percentage of the ERα signal in the nuclear extract of the tamoxifen treated cells when compared to the vehicle. In the cells treated with vehicle only, 51% of the ERα signal was found in the nuclear extract and 49% was found in the cytoplasmic extract. In the cells treated with 4-OH-tamoxifen, only 33% of the ERα signal was found in the nuclear extract with 67% found in the cytoplasmic extract (Figure 6A and B). Samples for this assay were collected following treatment with 4-OH-

tamoxifen for 6 months. It is possible this effect happens immediately upon treatment with 4-OH-tamoxifen and would not require 6 months of treatment. Together these studies suggest that tamoxifen treatment is affecting the localization pattern of ERα.

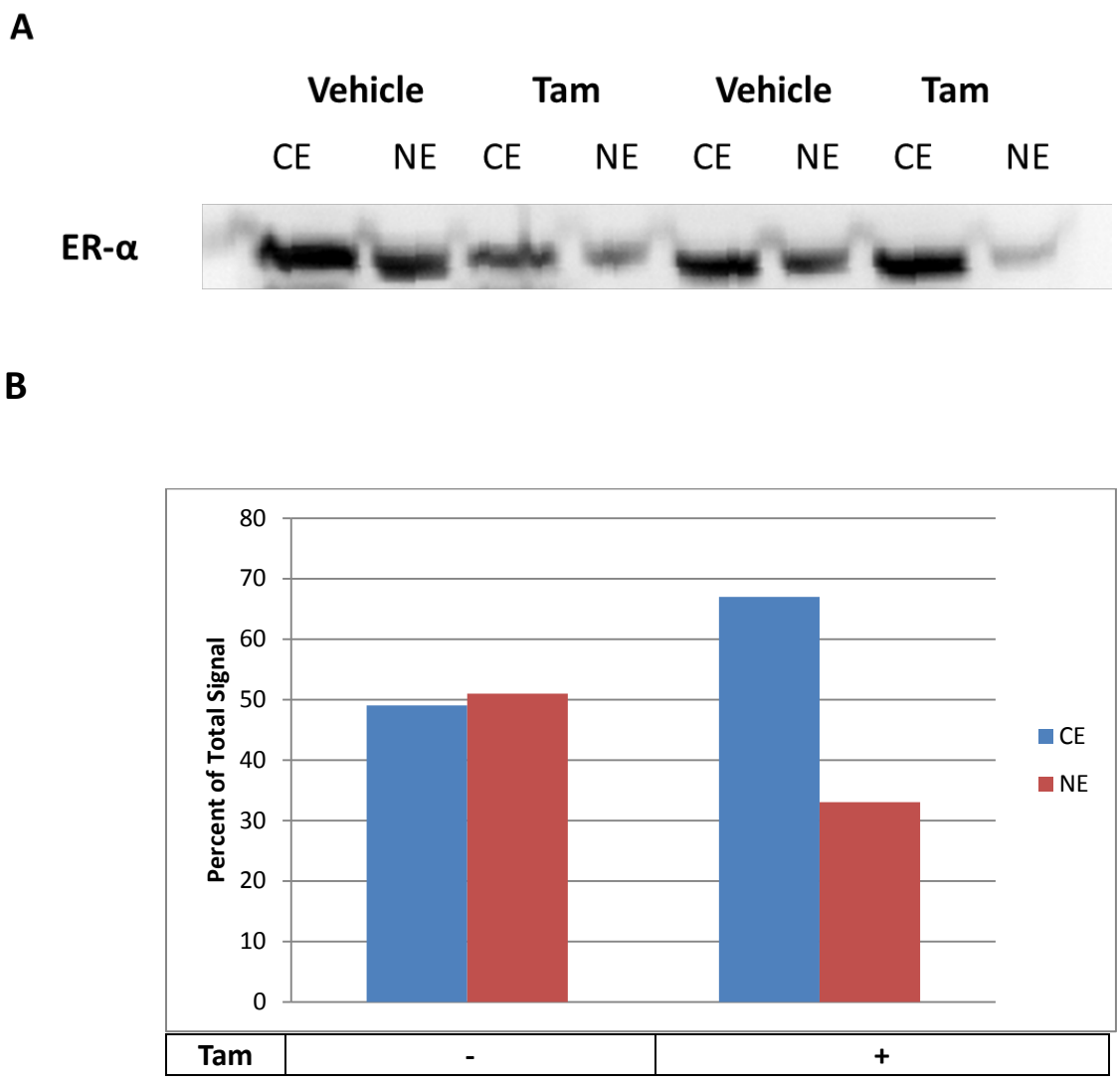


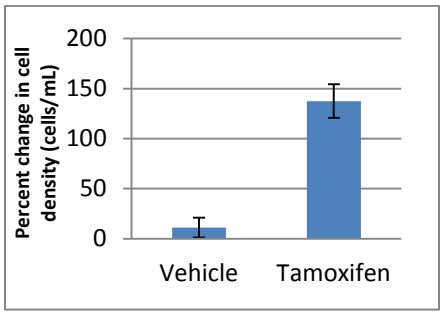
Figure 6. Decreased presence of ERα in nuclei of tamoxifen treated cells. MCF-7 cells were grown to confluence following 6 month treatment with 4-OH-tamoxifen (Tam) (1 µM) or a vehicle (ethanol). Protein lysates were prepared. The lysates were fractionated as described in Materials and Methods in order to separate the nuclear proteins (NE) from the proteins in the cytoplasm (CE). (A) The extracts were subjected to SDS-PAGE and then immunoblotted using an ERα antibody. (B) The signal was quantified by densitometric analysis using Quantity One software.

TAM-R Cell Line Established

After 10 months of treatment with 4-OH-tamoxifen, the proliferation rate of the cells began to increase. This could be an indication that the cells were becoming resistant to tamoxifen. Most of the growth and proliferation of MCF-7 cells is estrogen-dependent and results from the transcription of estrogen-responsive genes (HER-2, Cyclin-D, c-Myc). Estrogen binding to the estrogen receptor allows for transcription of these genes and drives proliferation. Since estrogen was still present in the media, it was possible that this was masking the tamoxifen resistance. To determine whether the proliferation was no longer estrogen-dependent, cells were cultured in estrogen-depleted media (PRF-DMEM + CS-FBS) treated with 4-OH-tamoxifen (1 µM) for 7 days. Samples were assayed for cell proliferation and quantified after 2, 4, and 7 days of treatment. The density of the cells which had been treated with tamoxifen for 10 months increased by 137% in 7 days. The cells which had been treated with vehicle only over that 10 month period had an increase in cell density of 11% over the same time period (Figure 7A). In order to determine if the change in growth rates was associated with a change in phosphorylation of Akt or Erk 1/2, protein lysates were collected to analyze for p-Akt and p-Erk 1/2. The lysates were subjected to SDS-PAGE and transferred to an immobilon filter. The samples were probed for p-Akt, p-Erk 1/2, and actin. p-Akt was not detected in any of the samples (data not shown). However, p-Erk 1/2 showed an increase in the tamoxifen-treated cells as compared to those treated with vehicle only (Figure 7B). Phosphorylation of components of the MAPK pathway

has a demonstrated ability to activate ERα by direct phosphorylation (30). The observed increase in p-Erk 1/2 could lead to an increase in p-ERα. This increased activity could lead to an increase in ERα localization in the nucleus as compared to the tamoxifen-sensitive cells. To assess whether the increase in p-Erk 1/2 was associated with any changes in the localization pattern of ERα, additional samples were grown on cover slips for 4 days. Cells were fixed and probed with an ERα antibody and visualized using confocal microscopy to detect any changes in localization patterns of ERα. The cells showed a relatively even distribution of ERα across the cell. These cells were not exhibiting growth impairment from the tamoxifen and thus were classified as tamoxifen resistant cells (TAM-R) (Figure 7C).

A



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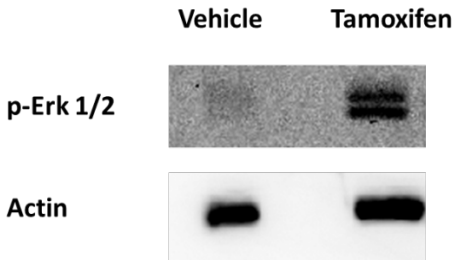

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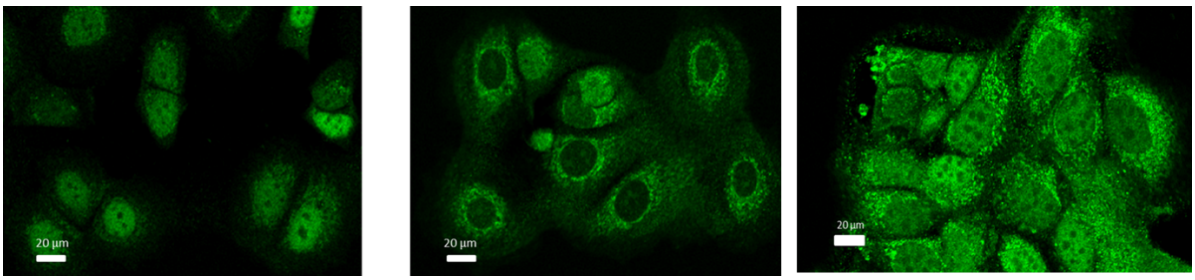


Tam	-	6 months	10 months

Figure 7. Generation of TAM-R cells. Cells were cultured for 10 months in full media (DMEM + FBS) containing either 4-0H-tamoxifen (Tam) (1 µM) or a vehicle (ethanol). Following that treatment cells from each treatment group were cultured into estrogen depleted media (PRF-DMEM + CS-FBS) containing 4-0H-tamoxifen (1 µM). (A) After 7 days, cells were counted and the percent change in cell density was calculated. (B) Protein lysates were prepared, subjected to SDS-PAGE and then immunoblotted using antibodies against p-Erk, or actin. (C) Additional samples were collected after 4 days and were fixed and probed for ERα and visualized using confocal microscopy (40x). These results are representative of 3 separate experiments.

Optimizing Tamoxifen Concentration

To this point I had been using a higher concentration of 4-OH-tamoxifen (1µM) than may now be necessary. This was to compensate for the change to full media containing estrogen (DMEM + FBS). An increase in 4-OH-tamoxifen concentration allowed for greater binding with the estrogen receptor and expedited the onset of resistance. With the onset of resistance, the concentration of tamoxifen may be able to be reduced to 100 nM. A decrease in concentration will reduce the chances of any deleterious side effects. To make certain there was no change in growth rate from the reduction in 4-OH-tamoxifen concentration, TAM-R cells were cultured in media containing 4-OH-tamoxifen at either 100 nM or 1µM. After 7 days, cell density was assayed. There was no difference in the growth rates of the cells with different concentrations of tamoxifen (Figure 8).

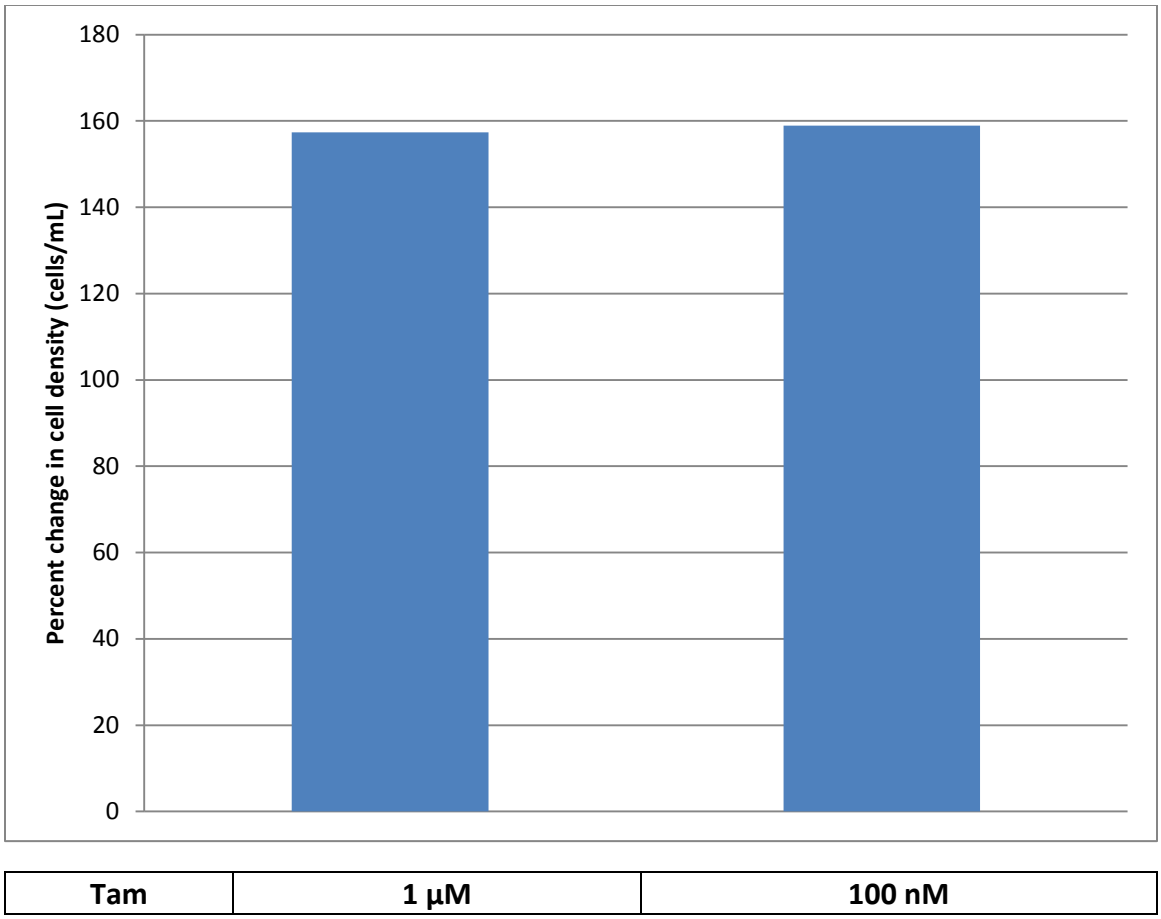


Figure 8. Growth rate unchanged by reduced concentration of tamoxifen. TAM-R cells were cultured in estrogen-depleted media (PRF-DMEM + CS-FBS) with tamoxifen (Tam) at a concentration of either 100 nM or 1 µM. Cells were treated for 7 days and media was changed every 2-3 days. Cell density was quantified and the percent change in cell density over 7 days was calculated.

Determination of the Stability of Resistance in TAM-R Cells

Next, I wanted to determine if we had established a permanently altered line of tamoxifen-resistant cells (TAM-R). Samples of the TAM-R cells were cultured to confluence. Several plates of cells were collected and placed in media designed for long-term storage. The samples were divided into two groups. The groups were

separated according to the presence or absence of 4-OH-tamoxifen (100 nM) in the media. The cells were frozen in liquid nitrogen for 2 months. It was not known whether the cells could maintain tamoxifen resistance after being frozen for 2 months. Further, it was not known whether adding tamoxifen to the freeze media would have an effect on that outcome. After 2 months, one sample from each group was thawed and cultured. Following 6 days of treatment with 4-OH-tamoxifen (100 nM), the cell densities were quantified and growth rates determined. Both the TAM-R cell groups (frozen in the presence or absence of 4-OH-tamoxifen in the media) exhibited much higher growth rates than the control group (MCF-7 cells treated with 4-OH-tamoxifen (100 nM)). These findings indicate the TAM-R cells have retained their resistance to tamoxifen (Figure 9).

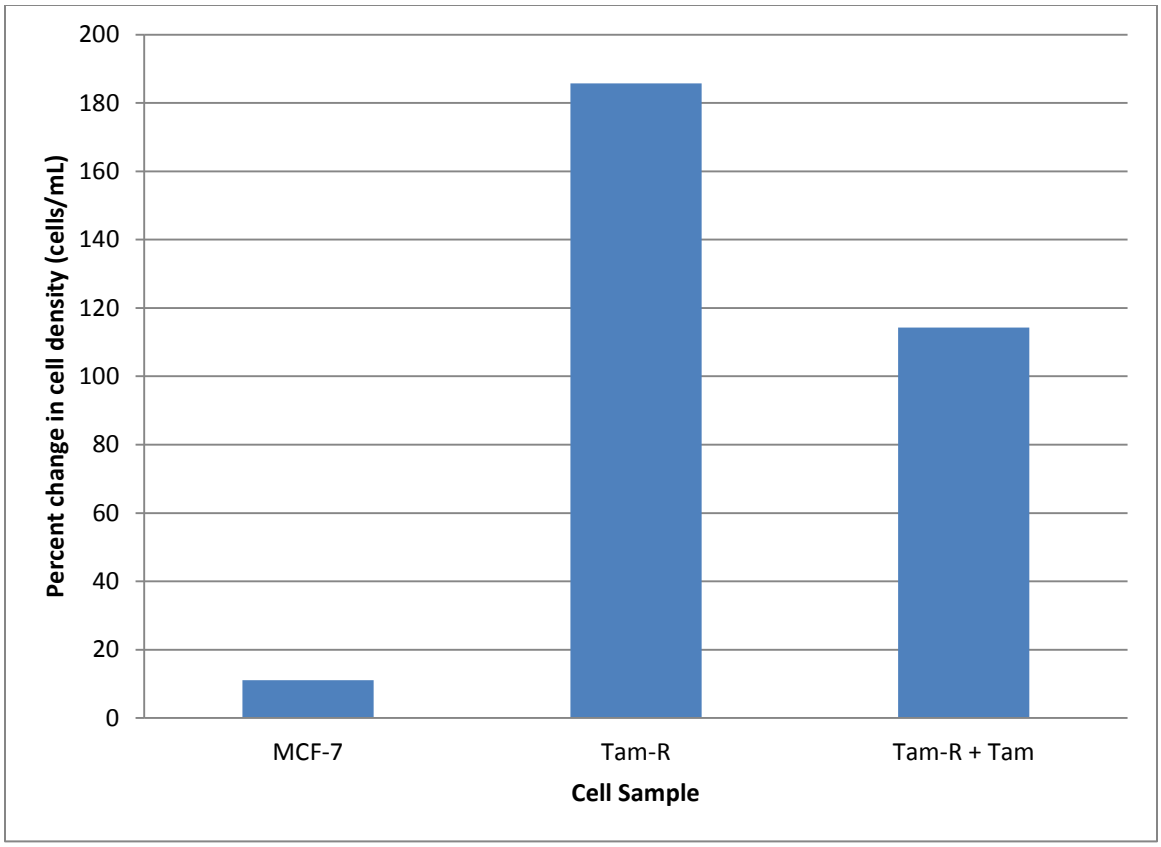


Figure 9. Establishment of stable TAM-R cell line. TAM-R cells were frozen in liquid nitrogen for 2 months in the presence or absence of 4-OH-tamoxifen (100 nM) in the media. These cells were thawed and allowed to proliferate for 6 days in the presence of 4-OH-tamoxifen (100 nM) and then assayed for cell density to measure the growth rate. Growth rate was compared to wild-type MCF-7 cells.

Growth of TAM-R cells is Estrogen-Independent

To this point, the TAM-R cells have been cultured in both the presence of tamoxifen and the absence of most of the estrogen from the media. In order to separate out these effects, cells were grown in estrogen-depleted media (PRF-DMEM + CS-FBS) but without the addition of tamoxifen to the media. MCF-7 cells and TAM-R cells were cultured in this media for seven days and their cell densities were compared

by quantification. The MCF-7 cells exhibited impaired growth when compared to the TAM-R cells. The MCF-7 cells had a 64% change in cell density over the 7 day period, while the TAM-R cells grew by 305% (Figure 10). The MCF-7 cells have impaired growth in the absence of estrogen, but the TAM-R cells proliferate despite the absence of estrogen in the media.

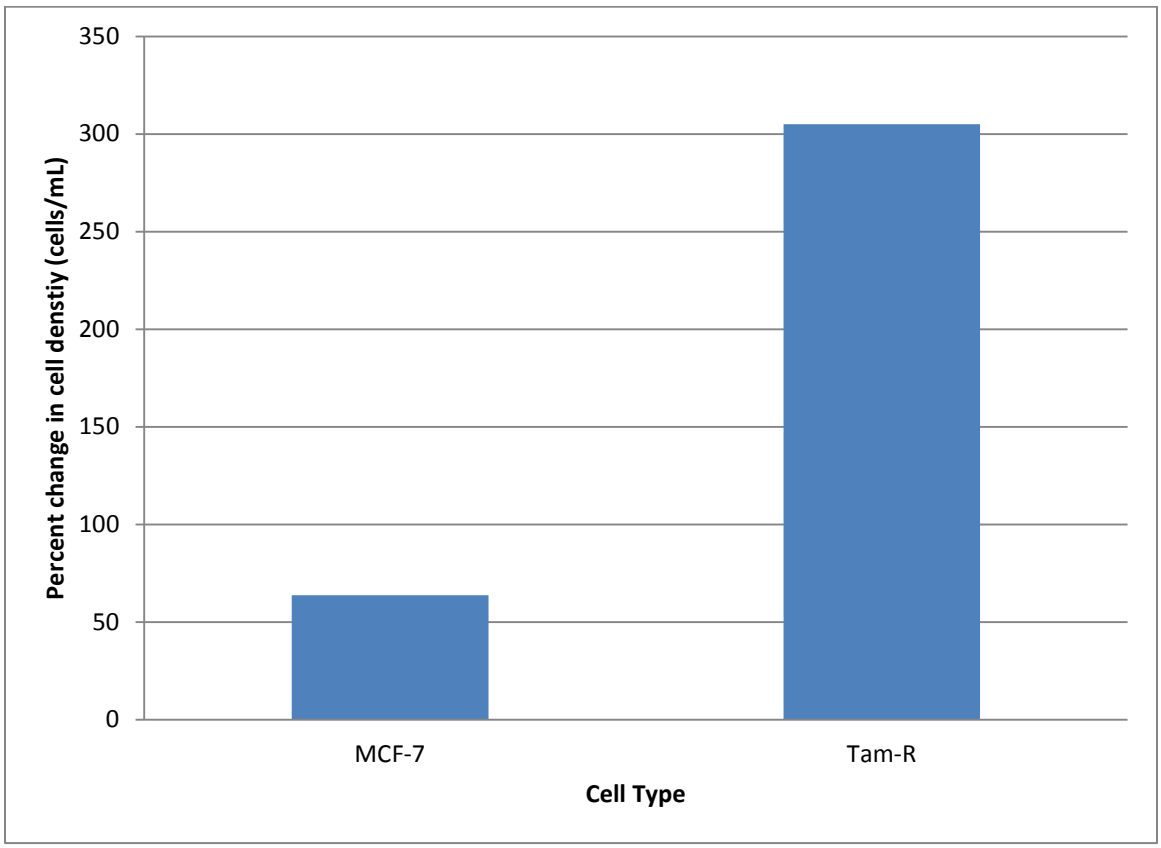


Figure 10. Estrogen-independent cell proliferation. MCF-7 and TAM-R cells were cultured in estrogen-depleted media (PRF-DMEM + CS-FBS) and in the absence of 4-OH-tamoxifen for 7 days. Their cell densities were quantified.

Cells Respond to Addition of Estrogen in the Media

MCF-7 and TAM-R cells were grown in the presence of tamoxifen in full media containing estrogens (DMEM + FBS). These cells were grown for 7 days and their cell densities were quantified. Their growth rate was calculated and compared against samples grown in the absence of estrogen (PRF-DMEM + CS-FBS). While TAM-R cells exhibited some additional growth with the addition of estrogen, that gain was a fraction of what is seen in the MCF-7 cells (Figure 11). The MCF-7 cells still rely on estrogen for the majority of their growth signaling, while TAM-R cells may be using other pathways.

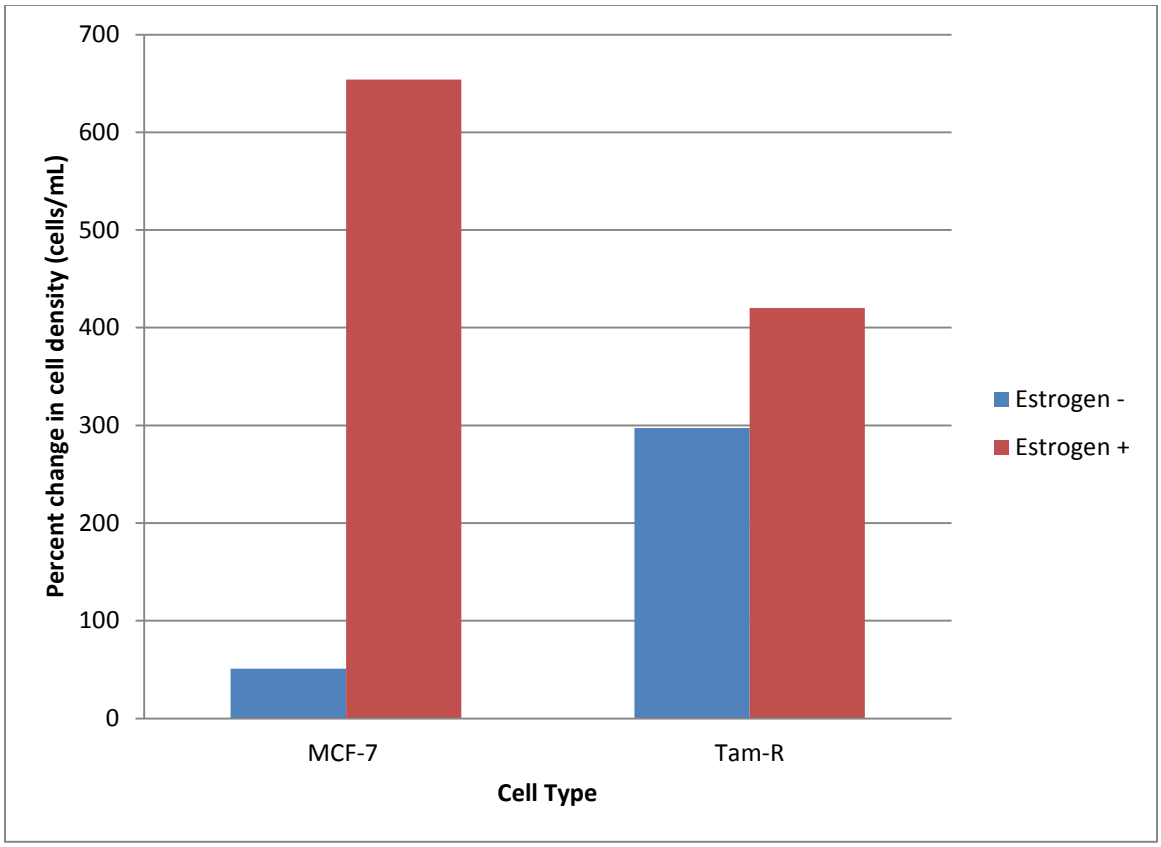


Figure 11. Growth of MCF-7 and TAM-R cells in the presence or absence of estrogen. MCF-7 and TAM-R cells were grown in the presence of 4-OH-tamoxifen (100 nM) and with full media containing estrogen (DMEM + FBS). The cells were cultured for 7 days, their cell densities quantified, and their growth rates calculated. These growth rates were then compared to the growth rate of cells grown in estrogen-depleted media (PRF-DMEM + CS-FBS).

Naringenin Impairs Proliferation of TAM-R Cells

By impairing the PI3K and/or MAPK pathways, naringenin may be able to reverse the resistance exhibited by cells following a 10 month treatment with 4-OH-tamoxifen (Figure 7A). If the TAM-R cells are utilizing the PI3K or the MAPK pathways for growth, naringenin's impairment of those pathways should result in growth arrest. To

determine the potential effect of naringenin, MCF-7 and TAM-R cells were grown in estrogen-depleted media (PRF-DMEM + CS-FBS) containing 4-OH-tamoxifen (100 nM) in the presence or absence of naringenin (250 μM). The cells were cultured in the respective treatment. After 7 days, cells were collected, cell densities were determined, and the growth rates were calculated. The TAM-R cells exhibited an accelerated proliferation rate in the absence of naringenin compared to the MCF-7 cells as shown previously (Figure 7A). However, this accelerated proliferation was completely reversed by the addition of naringenin (Figure 12).

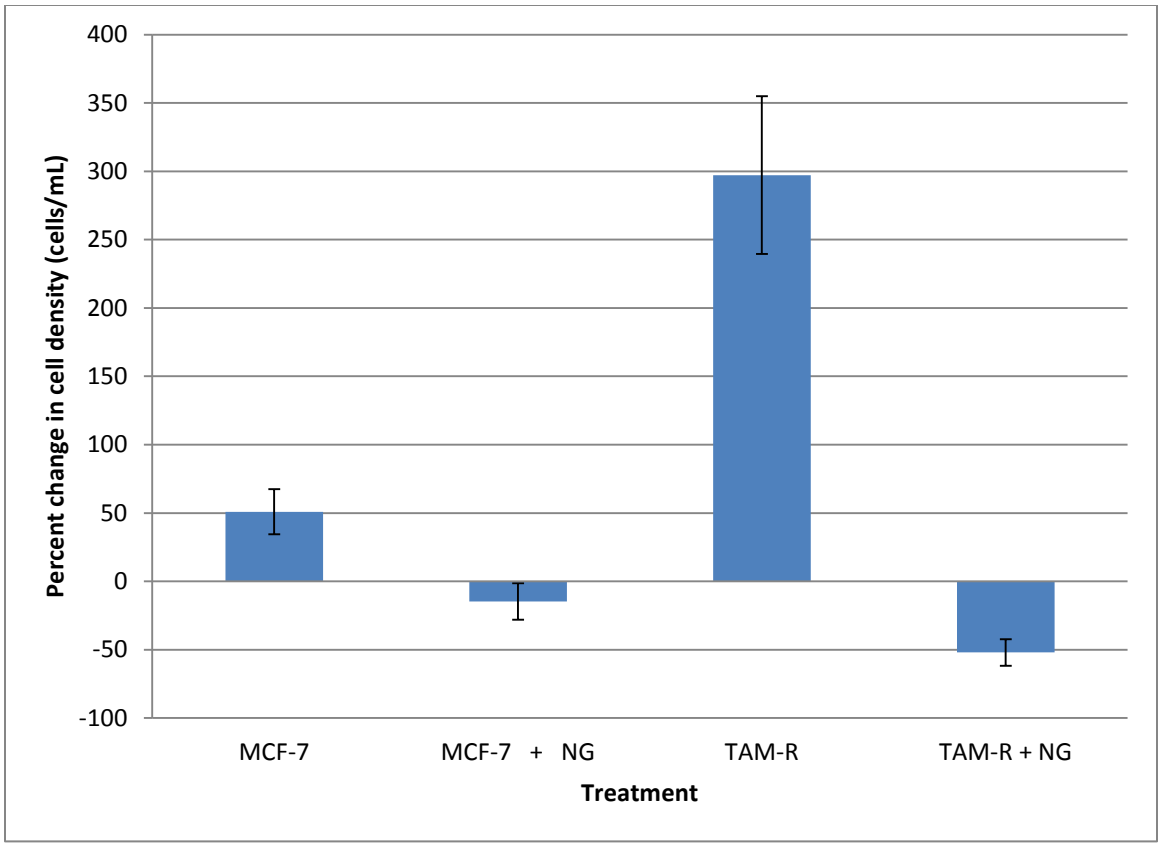


Figure 12. Naringenin reverses tamoxifen resistance effects on cell proliferation. MCF-7 and TAM-R cells were cultured in estrogen-depleted media (PRF-DMEM + CS-FBS) containing 4-OH-tamoxifen (100 nM) in the presence or absence of naringenin (NG) (250 µM). Following 7 days in the indicated treatment, cells were collected, cell densities quantified, and their growth rate calculated. This experiment was performed 5 separate times.

<u>Up-regulation of p-Erk 1/2 in TAM-R Cells is Reversed by Naringenin</u>

An increased presence of p-Erk 1/2 was found in the TAM-R cells when compared to MCF-7 cells (Figure 7B). The increased activation of this pathway may stimulate cell proliferation even in the presence of 4-OH-tamoxifen. Additionally, naringenin's ability to impair these pathways may be the cause of the growth arrest

observed in TAM-R cells treated with naringenin (Figure 12). To test this, MCF-7 and TAM-R cells were cultured in estrogen-depleted media (PRF-DMEM + CS-FBS) containing 4-OH-tamoxifen (100 nM) in the presence or absence of naringenin (250 µM). Cells were cultured for 4 days then protein lysates were collected, subjected to SDS-PAGE, and immunoblotted using antibodies against p-Akt, p-Erk 1/2 and actin. p-Akt was not detected in any of the samples (data not shown). However, p-Erk 1/2 showed an increase in expression in the TAM-R cells which was dramatically reduced by the addition of naringenin (Figure 13).

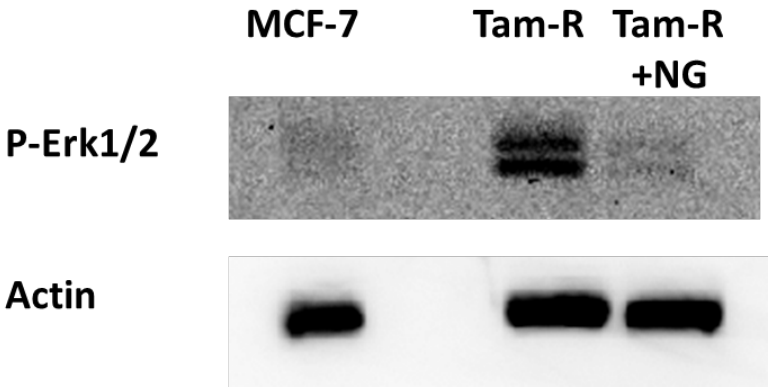
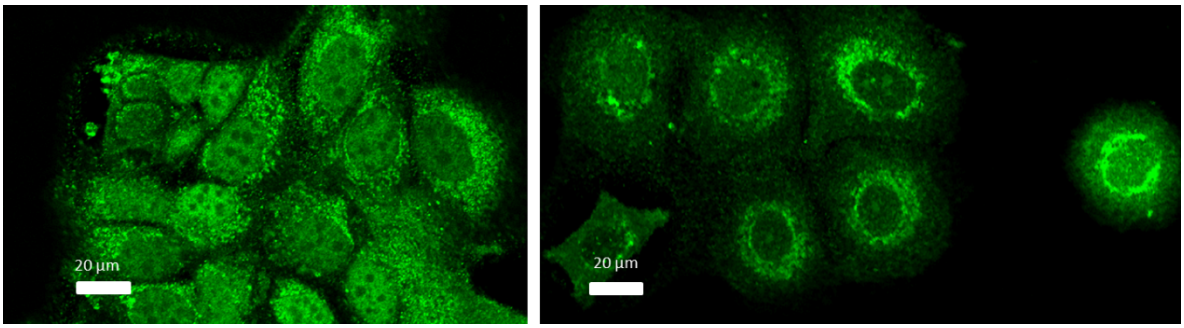


Figure 13. Naringenin impairs Erk 1/2 phosphorylation in TAM-R cells. TAM-R cells were treated with either naringenin (250 µM) or vehicle (DMSO) for 4 days and compared to a control (MCF-7 cells). All cells were cultured in estrogen-depleted media (PRF-DMEM + CS-FBS) and 4-OH-tamoxifen (100 nM). Protein lysates were prepared, subjected to SDS-PAGE and then immunoblotted using antibodies against p-Erk, or actin. These results are representative of 3 separate experiments.

Localization Changes of ERα Following Treatment with Naringenin

Naringenin has a demonstrated ability to impair the phosphorylation of Erk 1/2 (14). A decrease in the amount of p-Erk could lead to a reduction in p-ERα (30). This may result in ERα remaining in the cytoplasm for longer periods of time facilitating interaction between ERα and p-Erk 1/2. To explore this possibility, TAM-R cells were grown on cover slips in estrogen-depleted media (PRF-DMEM + CS-FBS) containing 4-OH-tamoxifen (100 nM) in the presence or absence of naringenin (250 μM) for 4 days. Cells were fixed and probed with an ERα antibody and visualized using confocal microscopy to detect any changes in the localization patterns of ERα. TAM-R cells showed a relatively even distribution of ERα across the cell. However, cells treated with naringenin and 4-OH-tamoxifen showed a perinuclear localization pattern for ERα similar to the pattern seen with MCF-7 cells treated with 4-OH-tamoxifen for 6 months (tamoxifen-sensitive cells) (Figure 14).



NG	-	+

Figure 14. Treatment with naringenin alters ERα localization pattern in TAM-R cells. TAM-R cells were grown for 4 days in the presence or absence of naringenin (NG) (250 µM) in estrogen-depleted media (PRF-DMEM + CS-FBS) containing 4-OH-tamoxifen (100 nM). Cells were fixed and probed for ERα and visualized using confocal microscopy (40x).

CHAPTER IV

DISCUSSION

Breast cancer epithelial cells such as the MCF-7 cell line used in this study are estrogen-dependent; meaning most of their growth signaling is controlled through the estrogen receptor and its responsive genes. Tamoxifen binds to the estrogen receptor and acts in an antagonistic manner to prevent the estrogen receptor from binding with co-activators and the promoters of estrogen responsive genes in the nucleus (19). Estrogen-dependent cells treated with tamoxifen are not completely growth arrested however and some growth is still possible through other pathways such as PI3K and MAPK. Naringenin has been shown to impair both the PI3K and MAPK pathways leading to arrested growth (16, 17). Therefore, treatment with naringenin and tamoxifen simultaneously could lead to a greater effect on growth arrest than could be seen using either treatment individually. The results from this study demonstrated that while treatment with tamoxifen or naringenin each increased the doubling time of the cells 2-fold, using the two treatments in combination resulted in a 13-fold increase in doubling time (Figure 2). This demonstrated a synergistic effect between tamoxifen and naringenin. An MTT assay performed in this same manner also demonstrated the greatest level of growth impairment was with the combination treatment. However, the results from the MTT assay did not show the same level of growth impairment. The

96-well format only allowed for growth for a 3-day timeframe. This reduced timespan did not allow the same effects on proliferation. Still, after only three days of treatment, the cells in the combination treatment demonstrated a 47% increase in doubling time when compared to the control as determined by MTT assay (data not shown). Regardless of the assay, adding naringenin to tamoxifen allowed for more effective control of the growth of these breast cancer cells. It may also allow for a reduction in the dosage of tamoxifen used during treatment which could lead to a reduction in undesirable side effects. Future studies could investigate the effect of the double treatment on non-cancerous cells. Once dosage and toxicity studies are concluded, animal studies could be explored to understand how these treatments might operate at the organismal level.

Tamoxifen resistance is a serious challenge facing the medical community. Recent studies have implicated overexpression of the PI3K and MAPK pathways as being involved in acquired tamoxifen resistance (4). The cells could be compensating for the reduction in estrogen receptor genomic activity by activating these other growth pathways. In addition, the estrogen receptor has a demonstrated ability to activate both the PI3K and MAPK pathways (9, 14). Taken together, these data suggest the possibility that estrogen receptors, once bound to tamoxifen, are remaining in the cytosol for longer periods of time and activating these alternative growth pathways leading to the observed tamoxifen resistance and resumption of proliferation.

To investigate this phenomenon, I first needed to generate tamoxifen resistant cells (TAM-R) to use as a model. MCF-7 cells were grown in the presence of 4-OH-tamoxifen and monitored for any changes in growth rate, phosphorylation status of Akt and Erk 1/2, or estrogen receptor localization. While the cells were still sensitive to tamoxifen, they showed a marked decrease in growth rate as the doubling time increased from 46 h to 72 h (Figure 3). They also exhibited a slight decrease in the expression levels of p-Erk 1/2, but not in p-Akt (Figure 4). In addition, the cells demonstrated a peri-nuclear localization pattern of the estrogen receptor as compared to a nuclear localization pattern for untreated MCF-7 cells (Figure 5). Fan et al also noted a localization change of ERα from the nucleus to the cytoplasm following tamoxifen treatment (29). We are the first to specifically note a peri-nuclear localization pattern. These data show that treatment with tamoxifen is resulting in a decrease in proliferation not only through its effects on estrogen-responsive genes, but also through some reduction in the activity of the MAPK pathway. In addition, the ERα localization pattern demonstrates a clear change from the normal nuclear localization of ERα to a peri-nuclear location. Normally, estrogen binding to ERα leads to dimerization, translocation into the nucleus, and recruitment of co-activators to allow for transcription of estrogen-responsive genes (9). However, tamoxifen binds to the ERα causing a conformation change in the ERα leading to a reduction in binding of co-activators or promoters of estrogen-responsive genes. The tamoxifen/ERα complex may

now spend less time in the nucleus allowing for increased time in the cytosol to interact with other kinase signaling pathways such as the PI3K and MAPK pathways.

Following 10 months of treatment with tamoxifen, the proliferation rate of the cells began to increase. The media used on the cells was changed to an estrogen-depleted media. The cells were cultured in this media and subjected to treatment with 4-OH-tamoxifen to look for differences in growth rates indicating the cells had become resistant to the drug. Results showed greater than an 11-fold increase in the growth rate of the cells which had been treated with 4-OH-tamoxifen for 10 months as compared to the cells which had been treated with vehicle only during the 10-month period (Figure 7A). These cells could now be classified as tamoxifen-resistant cells (TAM-R). The combination of tamoxifen in the media and the absence of estrogen led the MCF-7 cells to an almost total growth arrest. The population of these cells increased only 11% over the seven day trial compared to 137% for the TAM-R cells. The TAM-R cells appear to be mediating their proliferation through pathway(s) other than the estrogen pathway.

In order to separate out the effects of estrogen-deprivation from those of tamoxifen treatment on growth impairment, MCF-7 and TAM-R cells were cultured in estrogen-depleted media which did not contain tamoxifen. This resulted in a 6-fold increase in the growth rate for the MCF-7 cells and only a 3-fold increase for the TAM-R cells when compared to the same cells treated with 4-OH-tamoxifen (Figure 10).

However, the TAM-R cells were still growing at a much faster rate than the MCF-7 cells. The MCF-7 cells appear to be affected more intensely by treatment with tamoxifen and able to mount a greater growth recovery in its absence than the TAM-R cells. On the other hand, the TAM-R cells are demonstrating an ability to proliferate independently of estrogen to a much greater extent than the MCF-7 cells. Likewise, adding estrogen back into the media while treating with tamoxifen allowed for a 13-fold increase in the MCF-7 proliferation rate as compared to less than a 1-fold increase for the TAM-R cells (Figure 11). These findings indicate that the proliferation of TAM-R cells is being mediated by pathway(s) other than the estrogen pathway.

Both the MAPK and PI3K pathways can facilitate proliferation in MCF-7 cells following estrogen-deprivation (4, 31). Ghayad et al found that the PI3K and MAPK pathways were upregulated in tamoxifen resistant cells (4). To investigate this possibility, MCF-7 and TAM-R cells were cultured for 4 days and cell lysates collected for western blot analysis. Antibodies were used to probe for p-Akt and p-Erk 1/2 from both samples. p-Akt was not consistently found in any of the samples. Akt and p-Akt have been found consistently in MCF-7 cells by others (4, 27). Since p-Akt was not detected in any of our samples, these data were discounted as technical error rather than an interpretation of findings (data not shown). p-Erk 1/2, however, was found to be upregulated in the TAM-R cells (Figure 7B) as has been shown previously (4). This finding provides evidence for hormone-independent growth and resistance to tamoxifen of the TAM-R cells. The MAPK pathway in these cells may be activated by ERα bound

with tamoxifen in the cytosol. In this case, the addition of tamoxifen should have less of an impact on proliferation and even removal of estrogen from the media should not impair growth significantly. The results support this explanation. In addition, confocal microscopy studies of ERα localization indicated the TAM-R cells had an even distribution of ERα throughout the entire cell (Figure 7C). This is in contrast to both the nuclear localization of MCF-7 cells and the peri-nuclear localization in tamoxifen-sensitive cells. This could support the idea that the ERα is more active in the cytosol in the TAM-R cells exhibiting non-genomic effects by interacting with the kinase signaling pathways. In addition, phosphorylated Erk 1/2 has been shown to activate ERα by direct phosphorylation allowing ERα to resume transcription of estrogen-responsive genes (30). In this way, ERα would be active in both the cytoplasm and the nuclear region of the cell. The observed ERα localization patterns from this study support this idea. Future studies could investigate whether the ERα is active in the TAM-R cells. An assay could be conducted to detect transcription of estrogen-responsive genes.

Since naringenin has been shown to impair both PI3K and MAPK pathways, an investigation was conducted to learn whether naringenin could restore a state of growth arrest to cells which had become tamoxifen resistant. The addition of naringenin caused a complete reversal of proliferation in MCF-7 and TAM-R cell lines (Figure 12). Not only did naringenin abolish cell proliferation, but it also reduced the total number of cells. This result implies cell death in both MCF-7 and TAM-R cell lines. The TAM-R cells which have demonstrated both hormone-independent growth as well

as resistance to treatment with tamoxifen were now growth arrested. This finding opens up the possibility that naringenin could be used to impair cell proliferation in cancer cells which have become resistant to tamoxifen.

In addition, western blot analysis demonstrated that the increased phosphorylation of Erk 1/2 in TAM-R cells was reduced by naringenin treatment. The addition of naringenin reduced the p-Erk 1/2 expression to a similar level as that observed under basal conditions (Figure 13). This finding suggests that naringenin is hindering proliferation in the TAM-R cells by blocking the activity of the MAPK pathway. Future studies could investigate the specific point in the MAPK pathway in which naringenin is interacting.

Finally, the results of an immunofluorescence assay revealed a localization change in the ERα with the addition of naringenin. The TAM-R cells exhibited an even distribution of ERα across the cell. However, with the addition of naringenin, the ERα was localized to the peri-nuclear region similar to the pattern in tamoxifen-sensitive cells during treatment with tamoxifen alone (Figure 14). One interpretation of this finding is that the ERα-tamoxifen complex which had been bound to components of the MAPK signaling pathway in the cytosol was now being disrupted by naringenin. Naringenin is blocking the MAPK signaling pathway, preventing phosphorylation of Erk 1/2, and the ERα-tamoxifen complex is spending less time bound to that pathway in the cytosol. In addition, the reduction in phosphorylation of Erk 1/2 is reducing the

activation of ERα. Phosphorylated Erk 1/2 may have been activating ERα leading to transcription of estrogen-responsive genes. Naringenin is now hindering phosphorylation of Erk 1/2 and may be returning the cells to a tamoxifen-sensitive state. Future studies could explore whether naringenin treatment alone could induce a change in the ERα localization pattern.

Finding new treatment options for a disease which claimed the lives of over 39,000 people in the US alone in 2011 is a high priority. Further testing must be conducted before naringenin can secure its place as a treatment option. However, naringenin's potential as a treatment to combat tamoxifen resistant is clearly demonstrated from this study.

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