Breast Cancer Resistance to Hormone Therapy

The Role of the ErbB2/PI 3-K/Akt1 Pathway in the Development of Hormone Resistance in Breast Cancer

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Abbreviations: ER-α: estrogen receptor α; Tam: 4-hydroxy tamoxifen; ICI 182,780: fulvestrant; ErbB2: epidermal growth factor receptor II (Her 2); PI 3-K: Phosphoinositide 3-kinase; AKT1: Serine/threonine protein kinase (human isoform 1); E2: estradiol; PCR: polymerase chain reaction; PGR: progesterone receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SERMS: selective estrogen receptor modulators; ER: estrogen receptor; ER-β: estrogen receptor β; EGFR: epidermal growth factor receptor; ERE: estrogen response element; AF-1: activating function 1; IMEM: improved minimal essential medium; FCS: fetal calf serum; CCS: charcoal-treated calf serum; AF-2: activating function 2
Abstract

The presence of estrogen receptor α (ER-α) in breast tumors predicts the patient response to hormone therapy. The most common treatments are the antiestrogens 4-hydroxy tamoxifen (Tam) and fulvestrant (ICI 182,780). While Tam exhibits partial agonistic activities, ICI is a total antagonist. Most patients eventually develop resistance to antiestrogens. Identifying cellular pathways which enable hormone sensitive and hormone resistant breast cancer cells to continue growing will help determine the direction of future therapy. To study the acquired hormone resistance of MCF7 breast cancer cells during endocrine treatment, specifically the role of antiestrogens, epidermal growth factor receptor II (ErbB2), and the phosphoinositide 3-kinase/serine/threonine protein kinase 1 (PI 3-K/Akt1) pathway, I used three variants of MCF7 cells. Parental MCF7 cells are estrogen-dependent and responsive to antiestrogens, Tam and ICI 182,780. LCC1 cells grow independently of estrogen, but are sensitive to Tam and ICI 182,780. LCC2 and LCC9 cells were generated from LCC1 cells that underwent long-term treatment with antiestrogens, Tam and ICI 182,780, respectively. LCC2 and LCC9 grow estrogen-independently and are antiestrogen resistant. RNA was extracted from MCF7/LCC1, 2, and 9 cells under estradiol (E2) treatment in the presence or absence of antiestrogens, ErbB2 and/or PI 3-K inhibitors to determine the effect of these treatments on ER-α activity by real time RT-polymerase chain reaction (PCR). The mRNA induction was measured for the progesterone receptor (PGR), an estrogen-dependent gene, and compared to the internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA induction. Results are compared in MCF7 cells versus LCC cells. In MCF7/LCC cells, PGR mRNA is induced upon E2 treatment. This effect is inhibited by both Tam and ICI 182,780 only in MCF7 and LCC1 cells. Tam and ICI 182,780 also increase PGR mRNA in LCC2 and LCC9 cells, respectively. The E2 effect and antiestrogen resistance was stably inhibited by both inhibitors of ErbB2, AG825, and PI 3-K, LY 294,002. The data support that activation of ErbB2 and PI 3-K/Akt1 signaling pathway can overcome the antiestrogenic effects of Tam and ICI 182,780. Results demonstrate the ability of the selective ErbB2 inhibitor, AG825, and the PI 3-K inhibitor, LY 294,002 to inhibit cell growth after breast cancer cells have developed resistance to antiestrogens, Tam and ICI 182,780. Further studies of the effects of the AG825 and LY 294,002 are needed to determine their reliability and the practical clinical use of these findings for those patients who have developed resistance to antiestrogens.
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Introduction

Today in the United States, breast cancer is the most prominent cause of cancer in white and African American women (1). In 1960, breast cancer incidence in women was one in twenty, but today breast cancer incidence in women is one in eight (2). For many, this diagnosis is a death sentence. In 2006, 214,640 new cases of invasive breast cancer were diagnosed and, due to this disease, 41,430 people died in the same year (1). In more tangible terms, a woman is diagnosed with breast cancer every two minutes in the US and approximately every 12 minutes, the disease takes a life.

Among the many risk factors for breast cancer, including age, family history, and alcohol use, the most important etiological agent of breast cancer is the hormone estrogen, especially estradiol (3). Exogenous and endogenous exposures to estrogens are the most prominent risk factors associated with the initiation of metastasis and abnormal cellular proliferation (3). Therefore, prevention and treatment are based upon the hormonally dependent process of tumor development and metastasis in the breast. The prevention and treatment methods most commonly used in the clinic include selective estrogen receptor modulators (SERMS) and aromatase inhibitors (3). SERMS, or antiestrogens, competitively bind to the estrogen receptor (ER), inhibiting estrogen from binding and initiating oncogenic gene expression and cellular proliferation (3). Aromatase inhibitors block estrogen-synthesizing enzymes to prevent the synthesis of estrogen and thereby decrease the amount of E2 that could potentially bind to ER (4). This suppresses breast cancer growth. Tamoxifen is the most commonly used anti-estrogen (4).

Insert Figure 1 Here (6)
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Most breast cancers are initially responsive to this drug but then develop resistance which leads to further disease progression (5). Several mechanisms of acquired resistance have been suggested, most of which propose that signaling pathways utilized for typical cellular functioning are used for tumor genesis in breast cancer. Some of these mechanisms involve the ER, especially activation of ER-α. This receptor binds the estrogen ligand and triggers appropriate cell growth (4). Yet ER-α is commonly over-expressed in the early stages of breast cancer and serves as a predictor of patient response to hormone therapy (7). Another isoform of ER in the steroid receptor family is estrogen receptor β (ER-β), which is also present in breast cancer cells (4). A hypothesis which is becoming a more predominant topic of investigation is that ER-β may modulate signaling pathways in breast cancer (8). Members of the epidermal growth factor receptor (EGFR) family are also thought to contribute to breast cancer resistance to hormonal treatment (9). They are typically involved with standard activation of the PI 3-K/Akt1 pathway (9). Specifically, the EGFR II isoform ErbB2 makes cells less sensitive to the antiestrogen tamoxifen and other cytotoxic drugs (10). This EGFR is thought to contribute to antiestrogen resistance in three ways, the first being that ErbB2 enhances ligand-independent ER transcription enabling cellular proliferation even when estrogen is not bound to ER. ErbB2 is also thought to contribute to antiestrogen resistance by increasing ER association with coactivators of transcription, and/or by decreasing ER association with corepressors (10). ErbB2 activation triggers the PI 3-K pathway, resulting in cellular proliferation of breast cancer cells (11). Akt1, a downstream target of PI 3-K, is over-expressed in some breast cancer cells, specifically the hormone dependent MCF7 cell line (12) The cellular target, Akt1, contributes to growth factor signaling cascades.
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Several mechanisms involving these receptors and pathways can potentially explain the development of hormone resistance in breast cancer. Most research suggests that many of these mechanisms do in fact contribute to acquired resistance and the modes in which they do so vary within each tumor (4).

The mechanisms of acquired resistance must be identified so that clinical therapies can be tailored to preventing the development of resistance. The basis of one suggested route of resistance lies in the most commonly used antiestrogen, tamoxifen (5). Though intended to competitively block estrogens from binding to the ER, Tam is a possible agonist for estrogen-responsive gene expression (13). Tam can activate estrogen response elements (EREs) mediated by the activating function 1 (AF-1) domain of ER-α (10). Another proposed mechanism involves ER-β, the counterpart to ER-α in the ER. Antiestrogen resistance may be achieved through a switch from ER-α to ER-β expression (8). A switch from ER-α to ER-β possibly desensitizes breast tumor cells to antiestrogens, Tam and ICI 182,780, that specifically target ER-α. However, this article focuses on the proposed mechanism that antiestrogen resistant breast cancer cells continue to proliferate by activating various signaling pathways that promote cell growth. This proposal is supported by the enhanced expression of the members of the EGFR family in MCF7 cells upon E2 activation and in antiestrogen resistant variants of MCF7 cells (LCC2 and LCC9) (14). Previously, inhibiting ErbB2 has blocked phosphorylation of the PI 3-K pathway and consequently restores the inhibitory effects of Tam, decreasing cancer growth in MCF7 cells (15). Blocking phosphorylation of the PI 3-K pathway also inhibits the downstream target Akt1 and prevents Akt1 from inducing transcription of gene promoters containing EREs.
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The aim of this article is to report findings from three years of biotechnological research efforts to further support a possible explanation for acquired antiestrogen resistance during hormone treatment of breast cancer. The investigated hypothesis is that resistance to antiestrogen treatment in hormone-dependent breast cancer is achieved through activation of the ErbB2/PI 3-K/Akt1/ER-α signaling pathway. Data supporting this hypothesis will be reported and discussed in the context of previous findings and investigations of the relationship between breast cancer resistance and the ErbB2/PI 3-K/Akt1/ER-α signaling pathway.

Methods & Materials

MCF7 Cell Culture

MCF7 cells were cultured in a monolayer in improved minimal essential medium (IMEM), and were maintained in this medium with a supplement of 5% fetal calf serum (FCS). The medium was replaced with phenol-red-free IMEM containing 5% charcoal-treated calf serum (CCS) (16). The calf serum was pretreated with sulfatase and dextran-coated charcoal. This enabled removal of exogenous steroids (17). The medium was again changed after 2 days and replaced with serum-free, phenol-red-free IMEM supplemented with fibronectin, glutamine, HEPES, trace elements, and transferrin. 10^-9 M estradiol was then added in the presence or absence of antiestrogens, Tam and ICI 182, 780, (5x10^-7M), LY 294,002 (10^-5M), and/or AG825 (10^-7M). Colonies were quantified after 7-10 days. Estradiol was purchased from Sigma (St. Louis, MO, USA). The selective ErbB2 inhibitor AG825 and PI 3-K inhibitor LY 294,002 were purchased from Calbiochem (San Diego, CA, USA).
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**LCC Cell Culture**

Experiments were performed using the model system MCF7/LCC to simulate the development of hormone resistance in human breast cancer.

Insert Figure 2 Here (Clarke, R.)

Estrogen-dependent, parental MCF7 cells were inoculated into ovariectomized NC+ *nu/nu* athymic nude mice as shown in Figure 2. Prolonged exposure of MCF7 cells to estrogen led to the isolation of LCC1 cells that can grow independently from estrogen. LCC1 cells were then selected by treatment with 4-hydroxy tamoxifen (Tam) and ICI 182,780. Consequently, LCC2 and LCC9 cells, respectively, were isolated. This process, as shown in Figure 2, resulted in isolation of the four cell lines used in this experiment, MCF7, LCC1, LCC2, and LCC9. LCC1, 2, and 9 were maintained in IMEM supplemented with 5% CCS as previously described (17).

**RNA Extraction**

RNA was extracted using the TRIzol method (Life Technologies, Rockville, MD.) following the protocol as described by the manufacturer’s instructions. RNA was separated from MCF7/LCC1, 2, and 9 cells under estradiol treatment in the presence or absence of an antiestrogen, an ErbB2-inhibitor and/or a PI 3-K inhibitor. As the breast tumor cells were disrupted and cellular components dissolved, the integrity of the total RNA isolated from MCF7/LCC breast cancer cells was maintained by the TRIzol Reagent. RNA was separated into the aqueous phase and recovered by alcohol precipitation. The RNA was maintained at -80°C in the presence or absence of estrogen, an antiestrogen, an ErbB2-inhibitor and/or a PI 3-K inhibitor until processing.
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**Measurement of PGR mRNA**

The real time RT-PCR technique was chosen to measure PGR mRNA because it is the most sensitive technique for mRNA detection currently available, possessing the ability to quantify RNA from a single cell (18). PGR and GAPDH mRNA induction levels were determined by real time RT-PCR using the 2-△△CT Method and the SDS 2.1 software on the Applied Biosystems 7900HT Fast Real Time PCR System. GAPDH served as the endogenous control so PGR mRNA was normalized to this internal standard. RNA of MCF7/LCC cells which had been treated with one or combinations of E2, Tam, ICI 182,780, LY 294,002, and/or AG825 was amplified by RT-PCR. Various amounts of cDNA were generated through reverse transcription using random hexamer primers. These cDNA samples underwent real time PCR so that PGR mRNA could be amplified and measured. Real time PCR quantifies reaction products for each sample after every cycle, so amplification can be tracked over time as oppose to end-point RT-PCR. The Taqman gene expression probes used in this experiment, PGR and GAPDH, hybridize to an internal region of a PCR product so that the fluorescent signal of the probe can then be detected. The amplification lengths and nucleotide sequences for PGR and GAPDH are presented in Figure 3.

Insert Figure 3 Here (Applied Biosystems, 19, 20)

**Results**

**Previous Results**

All four cell lines, MCF7, LCC1, 2, and 3 express ER-α. Previous data from our lab has shown that ER-α is involved in breast cancer cell growth, as oppose to ER-β
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which most likely promotes suppression of tumor growth. When estrogen binds to ER-α, signaling pathways trigger estrogen responsive genes to undergo transcription and translation. The subsequent protein expression can be measured to track estrogenic activity at the cellular level. Previous results from our laboratory have shown that E2 rapidly activated Akt1 in MCF7, LCC1 and LCC9 cells and basal Akt1 activity was greater in LCC cells compared to their activity in parental MCF7 cells. Both the selective ErbB2 inhibitor, AG825, and the PI 3-K inhibitor, LY 294,002, blocked the growth of MCF7/LCC1, 2, and 9 cells in vitro and in vivo, supporting that activation of ErbB2 and/or PI 3-K leads to downstream activation of Akt1, which promotes cellular proliferation in MCF7/LCC cells. Results were compared from western blot analysis of the LCC cells (with the ligand E2 in the presence or absence of the antiestrogens, the PI 3-K inhibitor, LY 294,002, and/or the selective ErbB2 inhibitor, AG825) versus MCF7 cells. There was an elevated expression in all three LCC cell lines of ErbB2 and ErbB3 expression and activity. The effect of ErbB2 and PI 3-K on anchorage-dependent cell growth was found by quantifying cell cultures 7-10 days after the cells underwent treatment in the presence or absence of E2, Tam, ICI 182,780, AG825, and/or LY 294,002 (16).

New Results

Background

In this experiment, the estrogen regulated gene PGR was measured on the mRNA level by real time RT-PCR. My results will be compared to previous data showing the effects that E2, Tam, and ICI 182,780 have on ER-α and estrogen-regulated genes. ER-α
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has two activating function domains, AF-1 and AF-2, which mediate transcriptional activity. Antiestrogens inhibit these specific domains of ER-α to interrupt estrogen-regulated gene transcription. Tam competes with estrogens and other steroids to bind to ER-α and then, once bound, acts as a partial agonist, inhibiting transcriptional activity mediated by activating function 2 (AF-2) domain (16). The antiestrogen ICI 182,780 has recently become more popular for its complete antagonistic effects, since it not only blocks the transcriptional activation of the estrogen-dependent AF-2 domain but also inhibits transcriptional activation of the estrogen-independent AF-1 domain (16). The effective inhibiting actions of Tam and ICI 182,780 have been previously supported by data.

In addition to confirming the inhibiting actions of Tam and ICI 182,780 on MCF7/LCC cells in this experiment, the inhibiting actions of LY 294,002 and AG825 on MCF7/LCC cells were tested. Previous data support that cellular proliferation in breast tumor cells is triggered by the Akt1 pathway and the PI 3-K inhibitor, LY 294,002, can block this action (16). AG825 is a selective ErbB2 inhibitor, meaning it does not block all EGFRs but only ErbB2. ErbB2 is part of a heterodimer (usually with ErbB3) that has been shown to interact with ER-α to lead to tyrosine phosphorylation resulting in the activation of PI 3-K and Akt1 (16). Akt1 can then interact with ER-α and promote cellular proliferation by encouraging ligand-independent gene transcription, decreasing ER-α association with co-repressors, and/or by increasing ER-α association with co-activators (16).

\[ PGR \text{ mRNA Induction} \]
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The PGR mRNA induction in MCF7 and LCC1, 2, and 9 variants was measured at different intervals after cells were treated with E2 in the absence or presence of Tam, ICI 182, 780, AG825, and/or LY 294, 002. The resulting PGR mRNA induction for parental MCF7, LCC1, 2, and 9 cells is represented in Figure 4. The relative quantifications or the amount of gene expression of the estrogen regulated gene PGR was compared in the E2-treated breast tumor cells in the presence or absence of the ErbB2/PI 3-K inhibitors to the untreated control. The results displayed in Figure 4 correlate with previous results, supporting that the activation of ErbB2 and PI 3-K/Akt1 signaling pathway enables certain breast cancer variants to grow in the presence of Tam and ICI 182,780.

**MCF7 PGR mRNA Induction**

Figure 4 shows MCF7 cells respond to treatment with E2, Tam, and ICI 182,780 in a manner that correlates with previous findings. Parental MCF7 cells are positive for ER-α, estrogen-dependent, and antiestrogen sensitive, which parallels data shown in Figure 4. MCF7 PGR mRNA induction represents the standard control, simulating a patient’s reaction to breast cancer treatment when they do not yet possess estrogen-independence or antiestrogen resistance. Since MCF7 control generated expected results the data shown in Figure 4 for MCF7 cells can be considered reliable. MCF7 cells treated with E2 show a marked increase in PGR mRNA induction, as is expected based upon MCF7 growth-dependence on E2 and estrogen’s ability to induce transcription of estrogen-regulated genes. However, MCF7 cells treated with either Tam, ICI 182,780, LY 294,002, or AG825 did not show any estrogenic activity or significant PGR mRNA
induction. This is to be expected, since MCF7 cells do not normally induce transcription of promoter regions containing EREs unless exposed to estrogen. Furthermore, none of these drugs are known to have estrogenic effects in antiestrogen sensitive cell lines. MCF7 results do confirm that the antiestrogens, the ErbB2 inhibitor, and the PI 3-K inhibitor have no estrogenic effects on a cell line which maintains sensitivity to all these drugs and is estrogen-dependent.

Figure 4 also displays the effectiveness of Tam, ICI 182,780, LY 294,002, and AG825 when administered in conjunction with E2 in the MCF7 cell-line. Since combining each of these drugs with E2 simulates what occurs in the human body, the findings are vitally important for future direction in breast cancer treatments. Comparing the PGR mRNA induction of MCF7 cells treated solely with E2 to those cells treated with E2 & Tam, E2 & ICI 182,780, E2 & LY 294,002, or E2 & AG825, the effectiveness of each drug against estrogenic activity can be evaluated. MCF7 results confirm that the cells maintain sensitivity to all four drugs, each of which exhibits the ability to overcome estrogen-induced transcription of PGR. MCF7 data shows a low PGR mRNA induction in response to E2 & LY 294,002 and E2 & AG825. LY 294,002 and AG825 do not inhibit PGR transcription by occupying ER-α, but instead indirectly block the Akt1 signaling pathway. Therefore, the low PGR mRNA induction in response to E2 & LY 294,002 and E2 & AG825 implies that the ErbB2/PI 3-K/Akt1 pathway is being blocked by these treatments. An active Akt1 pathway in MCF7 cells enables transcription of estrogen-regulated genes, but, according to this data, using the PI 3-K inhibitor, LY 294,002, or the ErbB2 blocker, AG825, in the presence of E2 impedes the transcription induced by the Akt1 pathway.
Treatments were also used to determine whether the PI 3-K inhibitor, LY 294,002, and the ErbB2 inhibitor, AG825, administered in combination with ICI 182,780 and Tam, respectively, would promote transcription of PGR. Results show a low PGR mRNA induction corresponding with these treatments supporting that the PI 3-K inhibitor, LY 294,002, and the ErbB2 inhibitor, AG825, in combination with ICI 182,780 and Tam, respectively, inhibit PGR transcription in estrogen-dependent, antiestrogen sensitive MCF7 cells.

**LCC1 PGR mRNA Induction**

LCC1 cells derived from parental MCF7 cells are positive for ER-α and were selected to maintain sensitivity to antiestrogens and grow independently of estrogen. Since LCC1 variants are sensitive to both Tam and ICI 182,780, the PGR mRNA induction values for LCC1 cells shown in Figure 4 simulate an antiestrogen-sensitive patient response to breast cancer treatment. Figure 4 displays results which confirm that E2 promotes transcription of PGR while Tam and ICI 182,780 inhibit transcription of PGR in LCC1 cells. Results in Figure 4 correlate with the fact that LCC1 cells are estrogen-independent and antiestrogen sensitive. Since LCC1 control generated expected results, the data shown in Figure 4 for LCC1 cells can be considered reliable. LCC1 cells treated with E2 show a marked increase in PGR mRNA induction, as is expected based upon estrogen’s ability to induce transcription of PGR. LCC1 cells treated with one of Tam, ICI 182,780, LY 294,002, or AG825 did not show any estrogenic activity or significant PGR mRNA induction. This is to be expected, since none of these drugs are known to have estrogenic effects in antiestrogen sensitive breast cancer cells. LCC1
results do confirm that the antiestrogens, the ErbB2 inhibitor, and the PI 3-K inhibitor have no estrogenic effects on a cell line which maintains sensitivity to all these drugs and is estrogen-independent. Figure 4 also displays the effectiveness of Tam, ICI 182,780, LY 294,002, and AG825 as they each overcame the estrogenic effects of E2 in the LCC1 cell line. Comparing the PGR mRNA induction of E2-treated cells to those cells treated with E2 & Tam, E2 & ICI 182,780, E2 & LY 294,002, or E2 & AG825, the effectiveness of each drug against estrogenic activity can be evaluated. LCC1 results confirm that the cells maintain sensitivity to all four drugs, each of which exhibits the ability to successfully block the effects of E2. Results show a low PR mRNA induction in LCC1 cells in response to E2 & LY 294,002 and E2 & AG825, implying that the PI 3-K pathway is activated in LCC1 cells to enable transcription of estrogen-regulated genes. Blocking the ErbB2/PI 3-K/Akt1 pathway in LCC1 cells is, according to this data, vital to inhibiting transcription of PGR.

Treatments were also used to determine whether the PI 3-K inhibitor, LY 294,002, and the ErbB2 inhibitor, AG825, in combination with ICI 182,780 and Tam, respectively, would promote transcription of PGR. Results show a low PGR mRNA induction corresponding with these treatments supporting that the PI 3-K inhibitor, LY 294,002, and the ErbB2 inhibitor, AG825, in combination with ICI 182,780 and Tam, respectively, inhibit PGR transcription in estrogen-independent, antiestrogen sensitive LCC1 cells.
**LCC2 PGR mRNA Induction**

LCC2 cells are positive for ER-α, Tam-resistant, and estrogen-independent. Since LCC2 variants are not sensitive to Tam but maintain sensitivity to ICI 182,780, the PGR mRNA induction values for LCC2 cells shown in Figure 4 simulate a Tam-resistant patient response to breast cancer treatment. Figure 4 displays that LCC2 cells responded to treatment with E2, Tam, and ICI 182,780 in the expected manner, exhibiting Tam-resistance and ICI-sensitivity. Since LCC2 control generated expected results, the data shown in Figure 4 for LCC2 cells can be considered reliable. LCC2 cells treated with E2 show a marked increase in PGR mRNA induction, as is expected based upon estrogen’s ability to induce transcription of PGR. When treated with either ICI 182,780, LY 294,002, or AG825, LCC2 cells show no PGR mRNA induction so no estrogenic activity. None of these drugs are known to have estrogenic effects in breast cancer cells. However, LCC2 cells are resistant to Tam and show a slightly greater PGR mRNA induction upon treatment with Tam than they show in response to treatment with E2. This is expected since Tam has been shown to have estrogenic effects on Tam-resistant breast tumor cells (5).

Figure 4 displays the effectiveness of Tam, ICI 182,780, LY 294,002, and AG825 as they each block the effects of E2 in LCC2 cells. The conditions in this experiment simulate the treatment response of a patient with Tam-resistant breast cancer. These results are therefore vitally important for future direction in breast cancer treatments. Comparing the PGR mRNA induction of cells treated solely with E2 to those cells treated with E2 & Tam, E2 & ICI 182,780, E2 & LY 294,002, or E2 & AG825, the effectiveness of each drug against estrogenic activity can be evaluated. The lack of PGR mRNA
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induction for LCC2 cells treated with E2 & ICI 182,780 shows that ICI 182,780 can overcome the estrogenic effects of E2 by binding to ER-α and inhibiting estrogen-regulated gene transcription. However, LCC2 treatment with E2 & Tam results in a greater PGR mRNA induction than the induction that resulted from LCC2 treatment with E2 alone. This is expected, considering that Tam has been shown to trigger estrogenic activity in Tam-resistant breast cancer cells (5).

Treating LCC2 cells with E2 & LY 294,002 and then with E2 & AG825 tests whether or not the PI 3-K pathway is responsible for inducing PGR transcription in a Tam-resistant cell line. The PGR mRNA induction levels for LCC2 treated with E2 & LY 294,002 and E2 & AG825 are approximately the same as the untreated control and lower compared to LCC2 treated with E2 alone. This data confirms that antiestrogen resistant LCC2 cells employ an active ErbB2/PI 3-K/Akt1 pathway to continue promoting transcription of PGR. LY 294,002 and AG825 block transcription of the estrogen-regulated PGR gene. In addition, because LCC2 cells treated with E2 and LY 294,002 showed decreased PGR mRNA field induction relative to LCC2 cells treated with E2 alone, cross-resistance between Tam and LY 294,002 most likely does not exist. AG825 also decreased PGR mRNA expression, which not only shows its inhibitive effects at the cellular level but implies a lack in cross-resistance between Tam and AG825. If this cross-resistance did exist, the results would show high PGR mRNA induction in response to AG825. By blocking ErbB2, AG825 blocks the cell from inducing transcription of estrogen-regulated genes through activation of the ErbB2/PI 3-K/Akt1 pathway. Therefore, if a patient develops resistance to Tam and their tumor has
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ErbB2 expression and Akt1 activity, data support that the patient should be administered AG825 and/or LY 294,002.

LCC2 cells were also treated with ICI & LY and Tam & AG. These treatments were used to determine whether the PI 3-K inhibitor, LY 294,002, and the ErbB2 inhibitor, AG825, can inhibit transcription of PGR in a Tam-resistant cell line when administered in conjunction with an antiestrogen. The low PGR mRNA induction corresponding with these treatments shows that PI 3-K inhibitor, LY 294,002, and the ErbB2 inhibitor, AG825, are able to inhibit PGR transcription in estrogen-independent, Tam-resistant LCC2 cells.

**LCC9 PR mRNA Induction**

LCC9 cells were selected for resistance against ICI 182,780, which resulted in cross-resistance to Tam (20). Since LCC9 cells are positive for ER-α, antiestrogen resistant, and estrogen-independent, the PGR mRNA induction values for LCC9 cells shown in Figure 4 simulate an antiestrogen resistant patient response to breast cancer treatment. LCC9 control generated expected results, so the rest of the data shown in Figure 4 for LCC9 cells can be considered reliable. LCC9 cells treated with E2 show a marked increase in PGR mRNA induction, as is expected based upon estrogen’s ability to induce transcription of PGR. When treated with one of ICI 182,780, LY 294,002, or AG825, LCC9 cells did not show any estrogenic activity or significant PGR mRNA induction. This is expected since none of these drugs are known to have estrogenic effects in breast cancer cells. However, Tam is known to exhibit estrogenic effects in Tam-resistant cell lines such as LCC9. This explains why LCC9 cells treated only with
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Tam show an increased PGR mRNA induction compared to the untreated control. Because LCC9 cells have developed a cross-resistance to Tam, it is not surprising that Tam exhibits estrogenic activity in this cell line (5).

Figure 4 displays the effectiveness of Tam, ICI 182,780, LY 294,002, and AG825 as they each block the effects of E2 in LCC9 cells. The conditions in this portion of the experiment simulate an antiestrogen resistant breast cancer patient response to hormone treatment, so the findings are vitally important for future direction in breast cancer treatments. Comparing the PGR mRNA induction of cells treated solely with E2 to those cells treated with E2 & Tam, E2 & ICI 182,780, E2 & LY 294,002, or E2 & AG825, the effectiveness of each drug against estrogenic activity can be evaluated. LCC9 cells treated with E2 & Tam show a PGR mRNA induction greater than the PGR induction resulting from LCC9 treatment with E2 alone. This supports Tam’s ability to act in a similar fashion to estrogen at ER-α in Tam-resistant breast cancer cells (5). In LCC9 cells, LY 294,002 and AG825 exhibit the ability to inhibit estrogenic activity by blocking the PI 3-K/Akt1 pathway, which is an important finding considering that LCC9 cells are resistant to Tam and ICI 182,780. The Tam-resistance of LCC9 cells is displayed by an increase in PGR induction in response to treatment with E2 & Tam compared to treatment with E2 alone. The ICI-resistance of LCC9 cells is displayed by PGR mRNA induction in response to treatment with E2 & ICI. Though LCC9 PGR mRNA induction resulting from treatment with E2 & ICI is less compared to LCC9 treated only with E2, it is markedly greater compared to the untreated control. A decrease in PGR mRNA induction in response to E2 & LY 294,002 and E2 & AG825 confirms that the PI 3-K/Akt1 triggers transcription of estrogen-regulated genes. LY 294,002 and AG825 can
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Block transcription of estrogen regulated genes in LCC9 cells. The data presented in Figure 4 show that LY 294,002 and AG825 can inhibit tyrosine phosphorylation and activation of PI 3-K/Akt1 pathway and, in patients, could potentially block cancerous cellular proliferation.

In addition, because LCC9 cells treated with E2 & LY 294,002 show decreased PGR mRNA induction relative to LCC9 cells treated with E2 alone, cross-resistance between Tam and LY 294,002 or ICI 182,780 and LY 294,002 most probably does not exist. AG825 also decreased PGR mRNA expression, which not only shows its inhibitive effects at the cellular level but implies a lack in cross-resistance between Tam and AG825. If this cross-resistance did exist, the results would show high PGR mRNA induction in response to AG825. By blocking ErbB2, AG825 inhibits estrogenic effects in antiestrogen resistant LCC9 cells. Therefore, if a patient develops resistance to Tam and ICI 182,780 and their tumor has ErbB2 expression and Akt1 activity, the patient should be administered AG825 and/or LY 294,002.

LCC9 cells were also treated with ICI & LY and Tam & AG, treatments used to determine whether the PI 3-K inhibitor, LY 294,002, and the ErbB2 inhibitor, AG825, can inhibit transcription of PGR in an antiestrogen resistant cell line when administered in conjunction with an antiestrogen. The low PGR mRNA induction corresponding with these treatments shows that PI 3-K inhibitor, LY 294,002, and the ErbB2 inhibitor, AG825, are able to inhibit PGR transcription in estrogen-independent, antiestrogen resistant LCC9 cells.
Comparative Analysis

Comparing the respective PGR mRNA induction levels of MCF7/LCC variants shows how the different drugs used in breast cancer treatments affect the various types of tumors that can be found in breast cancer patients. All four cell lines show a marked increase in PGR mRNA induction in response to treatment with E2 compared to the untreated control. This is because MCF7/LCC cells express ER-α, the receptor to which E2 binds and triggers transcription of estrogen-regulated genes in the nucleus. In response to treatment with Tam, MCF7 and LCC1 variants show no PGR mRNA induction. LCC2 and LCC9 variants treated solely with Tam show an increase in PGR mRNA induction compared to the untreated control. This confirms Tam’s ability to adapt estrogenic activities in cell lines which are Tam-resistant (5). None of the cell lines show that ICI 182,780, LY 294,002, or AG825 have estrogenic effects, not even in the ICI-resistant cell line LCC9, confirming the total antagonistic effect of ICI 182,780.

All four cell lines display no PGR mRNA induction in response to treatment with E2 & LY 294,002 and E2 & AG825, supporting that LY 294,002 and AG825 can successfully block estrogenic activity in breast cancer cells. This supports that the ErbB2/PI 3-K/Akt pathway is active in all four cell lines, enabling transcription of PGR. In both of the Tam-resistant cell lines, LCC2 and LCC9, an elevated PGR mRNA induction resulted from treatment with E2 & Tam and in LCC9 an elevated PGR mRNA induction resulted from E2 & ICI 182,780 treatment. Tam not only fails to block the estrogen-induced transcription of PGR as it did in Tam-sensitive MCF7 and LCC1 variants, but also has estrogenic effects on LCC2 and LCC9 cells. Though antiestrogen resistance creates complications clinically, data in Figure 4 show that breast cancer cells
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maintain sensitivity to the PI 3-K inhibitor, LY 294,002, and the ErbB2 inhibitor, AG825. When all four cell lines individually underwent treatment with ICI & LY and Tam & AG, PGR mRNA induction levels were low.

Discussion

Estrogen plays a vital role in the maintenance of reproductive tissue as well as the development and progression of breast cancer. E2’s effect on ER-α in breast cancer cells has been well defined, yet the effect of E2 on specific signaling mechanisms and involvement of MAPK and Akt1 remains an important topic of investigation. Estrogen is thought to passively diffuse through the cell membrane and the nuclear membrane where it binds directly to ER-α (22). This binding triggers transcription, translation, and expression of estrogen regulated genes, such as PGR. My experiments do not provide information on the protein level, so findings are limited to expression of mRNA through real time RT-PCR.

The data generated from this particular study support the hypothesis that an active ErbB2/PI 3-K/Akt1 signaling pathway enables MCF7/LCC cells to circumvent the inhibition caused by tamoxifen and ICI 182,780. An inhibitor of the PI 3-K pathway, LY 294, 002, and a selective ErbB2 inhibitor, AG825, significantly blocked the effect of estradiol, which was measured through PGR induction. Current literature correlates with these findings. In 2003, Stoica, G., et al. published results stating that, in addition to the two mitogenic growth factors epidermal growth factor and IGF-I, estradiol can rapidly activate the PI 3-K pathway (11). The ErbB2 signaling pathway was shown to mediate this effect. In this study and in mine, LY 294,002, an inhibitor of PI 3-K, AG825, the
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selective ErbB2 inhibitor, and the antiestrogens Tam and ICI 182,780 all blocked Akt1 activation in antiestrogen sensitive cell lines. Tam and ICI 182,780 inhibited E2 from rapidly activating the PI 3-K pathway by competing directly with E2 at ER-α. LY 294,002 and AG825 blocked E2 from rapidly activating the PI 3-K pathway by blocking ErbB2 and PI 3-K. Though activation of PI 3-K due to a membrane ER-α –E2 complex normally triggers Akt1 activation, blocking the ErbB2 component of the heterodimer on the cell membrane inhibits this effect. Therefore, using an ErbB2 blocker like AG825 inhibits transcription, translation, and proliferation of breast cancer cells. Additionally, Stoica, G., et al. showed restored Akt1 activation in response to transient transfection of ER-α into the cells that had previously exhibited Akt1 inhibition, which supports that E2-induced activation of Akt1 requires the ER-α (11). My data shows correlation since MCF7/LCC cells express ER-α and each of these cell lines showed no PGR mRNA induction in response to treatment with E2 & LY 294,002 and E2 & AG825, implying that MCF7/LCC cells have an activated Akt pathway.

The role of ErbB2, in this experiment, was specifically studied by treatment of MCF7 and LCC cells with AG825, the selective ErbB2 inhibitor. Results from real time RT-PCR suggest that, by blocking ErbB2, AG825 overcomes the cell’s proliferative response to estrogen. In the Tam-resistant cell lines, LCC2 and LCC9, AG825 also blocked the estrogenic effects of tamoxifen by indirectly inhibiting PI 3-K/Akt1 activation. Stoica, G., et al. also showed that activation of ErbB2 leads to downstream activation of the Akt1/PI 3-K pathway (11). They supported this by stably transfecting MCF7 cells with anti-ErbB2-targeted ribozyme and then noting the failure of estradiol to activate Akt1 (11). Blocking ErbB2 via administration of AG825 could potentially serve as a reliable
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treatment to give to those breast cancer patients who express ER-α and present ErbB2 expression and Akt1 activation. Using Tam and AG825 or LY 294, 002 in conjunction with one another could prove a beneficial treatment since AG825 and LY 294,002 can block Akt1 activation, something Tam cannot do. Blocking Akt1 activation would prevent an unwanted increase in cell number.

**Limitations & Future Directions**

This study used only PGR as a marker for estrogen function, yet to further explore the relationship between breast cancer resistance and the ErbB2/PI 3-K/Akt1/ER-α signaling pathway, expression of other estrogen-related genes such as pS2 and cathepsin D must be studied. The effects observed on PGR mRNA need to be reproduced at the protein level to confirm current findings. Other mechanisms of antiestrogen resistance should be explored; one being the possibility that Akt1 mutants may trigger abnormal cellular growth. Stable transfection of LCC cell lines with constitutively active and dominant negative Akt1 should be performed to determine the effect of Akt1 mutants on cell growth and gene expression in the antiestrogen resistant cell lines.
References


7. **Kuske, B.** Endocrine therapy resistance can be associated with high estrogen receptor alpha (ERα) expression and reduced ERα phosphorylation in breast cancer models. *Endocrine Related Cancer*, 13: 1121-33, 2006.


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**Figure Legends**

**Figure 1: Tamoxifen binds to ER**
In a Tam-sensitive cell, Tam diffuses through the cell membrane and nuclear membrane and competes with E2 to bind directly to the ER. After binding, Tam changes the conformation of the ER so that no cofactors can bind to its surface receptors. Even though ER can still bind DNA, it cannot, without the help of cofactors, activate transcription of promoter regions containing EREs.

**Figure 2: Deviation of MCF7 variant cells**
Estrogen-dependent MCF7 cells were grown in ovariectomized, nude mice and were then differentiated into LCC1 cells by becoming estrogen independent. LCC1 cells underwent extended exposure to 4OH-Tam, which created LCC2 cells possessing selective resistance to Tam. Other LCC1 cells underwent extended exposure to ICI 182,780, creating the LCC9 variant which possesses resistance to ICI 182, 780 and cross-resistance to Tam.

**Figure 3: Taqman Gene Expression Assays**
The Applied Biosystems Taqman Gene Expression Assays PGR and GAPDH were used during real time RT-PCR in this experiment. The information shown was not made available by Applied Biosystems but was found in other publications. The sense and antisense nucleotide sequences are shown, as well as the amplification length of each probe. The fluorescent probe sequence for GAPDH is also shown. PGR served as the target while GAPDH was the endogenous control.

**Figure 4: PGR mRNA Induction**
These graphs display the PGR mRNA induction for MCF7/LCC cells. Each graph shows PGR mRNA induction for the untreated control and for cells treated with E2, Tam, ICI 182,780, LY 294,002, and/or AG825. Results correspond with previously found data and also support the hypothesis that the ErbB2/PI 3-K/Akt1 pathway contributes to breast cancer resistance. Blocking this pathway prevents transcription of the estrogen regulated gene PGR in both antiestrogen sensitive and antiestrogen resistant cell lines.
Figures

Figure 1: Tamoxifen binds to ER

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NC+ nu/nu mice - athymic nude mice

**MCF7**: ER positive and antiestrogen sensitive, estrogen-dependent growth

**LCC1**: ER positive, estrogen-independent growth, antiestrogen sensitive

**LCC2**: ER positive, estrogen-independent growth, Tam resistant, ICI 182, 780 sensitive

**LCC9**: ER positive, estrogen-independent, antiestrogen resistant (Tam and ICI 182,780)

*estrogen: a mitogenic ligand with partial agonistic activity
*4 hydroxy-tamoxifen: an antiestrogen
*ICI 182,780: antiestrogen, total antagonist

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**Figure 2: Deviation of MCF7 variant cells**

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*The PGR and GAPDH primer sets and corresponding probes were designed by and purchased from Applied Biosystems. The sequences above were located in publications.*

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**Figure 3: Taqman Gene Expression Assays**

*View Legend: pg. 26*
Figure 4: PGR mRNA Induction

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