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## The development, application and limitations of breast cancer cell lines to study tamoxifen and aromatase inhibitor resistance

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### Abstract

Estrogen plays important roles in hormone receptor-positive breast cancer. Endocrine therapies, such as the antiestrogen tamoxifen, antagonize the binding of estrogen to estrogen receptor (ER), whereas aromatase inhibitors (AIs) directly inhibit the production of estrogen. Understanding the mechanisms of endocrine resistance and the ways in which we may better treat these types of resistance has been aided by the development of cellular models for resistant breast cancers. In this review, we will discuss what is known thus far regarding both *de novo* and acquired resistance to tamoxifen or AIs. Our laboratory has generated a collection of AI- and tamoxifen-resistant cell lines in order to comprehensively study the individual types of resistance mechanisms. Through the use of microarray analysis, we have determined that our cell lines resistant to a particular AI (anastrozole, letrozole, or exemestane) or tamoxifen are distinct from each other, indicating that these mechanisms can be quite complex. Furthermore, we will describe two novel *de novo* AI-resistant cell lines that were generated from our laboratory. Initial characterization of these cells reveals that they are distinct from our acquired AI-resistant cell models. In addition, we will review potential therapies which may be useful for overcoming resistant breast cancers through studies using endocrine resistant cell lines. Finally, we will discuss the benefits and shortcomings of cell models. Together, the information presented in this review will provide us a better understanding of acquired and *de novo* resistance to tamoxifen and AI therapies, the use of appropriate cell models to better study these types of breast cancer, which are valuable for identifying novel treatments and strategies for overcoming both tamoxifen and AI-resistant breast cancers.

### Keywords

Models; endocrine resistance; tamoxifen; aromatase inhibitors

## 1. Introduction

Breast cancers are characterized based on whether they express several protein receptors: estrogen receptor  $\alpha$  (ER $\alpha$ ), progesterone receptor (PR), and ErbB2/HER2 receptor. There are two estrogen receptors:  $\alpha$  and  $\beta$  [1]. While ER $\alpha$  has been implicated to play a role in breast cancer, the role of ER $\beta$  in breast cancer is not fully defined [2–3]. Further discussion

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of ER $\alpha$  in this review will be referred to as ER. Approximately 70% of breast cancers are ER positive and/or PR positive (ER+/PR+) [4], about 20% overexpress HER2 [5], and about 10% do not express ER, PR, or HER2 (triple negative) [6]. Breast cancer is also characterized by an overexpression and increased activity of the cytochrome P450 aromatase enzyme [7–10]. Aromatase is predominantly expressed in the ovaries in premenopausal women under the regulation by the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both FSH and LH are players in a feedback regulatory mechanism in which they stimulate the synthesis of aromatase and subsequent secretion of estrogen in the ovaries. However, in postmenopausal women, aromatase is primarily expressed in the adipose tissue, which is stimulated by glucocorticoids, and in cancer cells, which is regulated through a cAMP-mediated mechanism [11]. Thus, aromatase expression in postmenopausal women is not regulated in a gonadotropic manner and makes aromatase inhibitors (AIs) useful for the treatment of estrogen-dependent breast cancers in postmenopausal patients. Unlike in postmenopausal women, aromatase expression is regulated by FSH and LH in premenopausal women and use of AIs would be rendered ineffective.

ER+ breast cancers require the hormone estrogen for their growth. The standard mode of treatment of ER+ breast cancers in postmenopausal women is estrogen deprivation or blockade of the estrogen-regulated pathways. This is through the use of ovarian ablation, or more commonly through the use of endocrine therapies such as tamoxifen or fulvestrant (ICI 182,780) to block the ER action, or by directly inhibiting the production of estrogen by AIs. The efficiency of AIs over tamoxifen, demonstrated by a number of clinical trials such as ATAC [12–13], IMPACT [14], and BIG 1–98 [15], has resulted in AIs becoming the first line of therapy for ER+ breast cancer.

While the use of antiestrogens and AIs is effective in the clinic, resistance towards these therapies has been observed. There are two types of resistance: *de novo*/intrinsic and acquired. *De novo* resistance occurs when the patient commences on a treatment but does not respond to the therapy. Acquired resistance is when the patient responds to the prescribed treatment, but after some time, no longer responds and breast cancer recurs. The occurrence of resistance to endocrine therapy presents a difficult challenge for the treatment of breast cancer, particularly determining the next line of treatment when all options have been exhausted. A number of laboratories have taken up the aim of elucidating and learning more about the mechanisms of resistance in order to identify new avenues of treating these types of breast cancers.

## 2. Cell Models

A collection of breast cancer cell lines derived from patients to serve as simplified *in vitro* models to study the inner workings of breast cancers are readily available and used by a number of laboratories. The growth of ER+ breast cancer is promoted via the ER-mediated pathway. Endocrine therapies are those which target and disrupt the ER pathway. Therefore, endocrine therapies are administered to breast cancer patients whose cancer cells express ER $\alpha$ . Since ER $\alpha$  is absolutely required for estrogen-dependent breast cancer, studies on hormone-responsive and endocrine resistant breast cancers have utilized a number of ER $\alpha$ + breast cancer cell lines. ER-negative (ER–) cell lines do not express ER $\alpha$ . Therefore, ER– cells do not respond to endocrine therapies and should **not** be considered models of endocrine resistance. Furthermore, the roles of ER $\beta$  in endocrine resistance are not totally understood. Therefore, studies using ER $\beta$ + cell lines require clear physiologically relevant justifications. Many endocrine therapies target the hormone activation of the ER signaling pathway, including AIs and anti-estrogens. One ER $\alpha$ + line predominantly used is the MCF-7 cell line that responds well to estrogen stimulation and to anti-estrogen-mediated

suppression. MCF-7 cells express aromatase at very low levels [16]. To engineer an aromatase-positive breast cancer cell model for the evaluation of AI responses, our laboratory stably overexpressed aromatase in MCF-7 cells, which were referred to as MCF-7aro [16–17]. The MCF-7aro cell line has been shown in several laboratories to be a suitable cell model to study AI-responsive breast cancer. Two additional ER $\alpha$ + cell lines, T47D and ZR75-1, are commonly used to study ER-mediated response. Aromatase-expressing versions of these lines, T47Daro and ZR75-1aro, have also been generated to study AI response. Through the use of these cell lines we can study resistance mechanisms and test new therapeutic agents for their ability to overcome endocrine therapy resistance.

### 3. Acquired Endocrine Resistance

#### 3.1 Acquired tamoxifen resistance models

Tamoxifen was the first approved line of endocrine therapy to treat postmenopausal women before AIs were proven to be superior and superseded tamoxifen for this spot. Tamoxifen is a SERM, which demonstrates selective antagonist activity in the breast and agonist activity in the endometrium and bone [18–19]. While tamoxifen has increased survivorship from breast cancer, recurrence still occurs. Due to its dual agonist/antagonist activity, tamoxifen can demonstrate weak estrogenic effect, which can prevent a total blockade of estrogen-stimulated breast cancer cell growth [20]. To study other mechanisms involving acquired tamoxifen-resistance, cells were generated by culturing the MCF-7 ER+ breast cancer cell line long-term in the presence of tamoxifen until growth was achieved in the presence of this anti-estrogen [21–22], such as LCC2 [23] and MCF-7/TAMR-1[24].

Increased expression of EGFR [25] or HER2 [26–27] in cancer patients have been observed, potentially leading to tamoxifen-stimulated cancer cell growth. Furthermore, several groups have proposed that resistance to endocrine therapies such as tamoxifen may be due to the cross-talk between ER and growth factor signaling proteins, especially EGFR/HER2. This has been determined through the use of several tamoxifen-resistant cell lines which have been generated by overexpressing a specific protein, including growth factor signaling proteins and kinases [28–31]. In these events, growth factor signaling proteins such as EGFR and mitogen-activated protein kinase (MAPK) display increased activity. These kinases can phosphorylate and induce ER activation in the absence of ligand or in the presence of tamoxifen [32–34].

Considering the ratio of coactivators and corepressors in the cells may explain another possible mechanism for acquired tamoxifen resistance. It has been shown that the differential expression or activity of coactivators and corepressors in the cell can modulate the agonist versus antagonist activity of drugs [35]. Coactivators and corepressors may play a part in acquired tamoxifen resistance. Tamoxifen displays both agonist and antagonist activities, which may be due to the activation and recruitment of coactivators or corepressors to the ER. For example, the ER coactivator AIB-1 is overexpressed in more than 50% of breast tumors and its gene is amplified in 5–10% of breast tumors [36]. In cell culture, AIB-1 enhances tamoxifen agonist activity [37]. Moreover, AIB-1 is phosphorylated and activated by a number of different kinases, such as p42/44 MAPK, which can be activated by HER2 [38]. Therefore, in cells overexpressing HER2 the role of tamoxifen may depend on the activation of AIB-1, which confers tamoxifen agonist activity.

As previously mentioned, ER $\alpha$  cross-talks with a number of growth factor receptors and kinases, which activate ER $\alpha$  in a ligand independent manner. This suggests that ER $\alpha$  is an important factor for tamoxifen resistance. Studies have demonstrated that expression of ER $\alpha$  in ER– breast cancer cells resulted in inhibition of growth [39–41]. To determine the response of increased ER $\alpha$  protein levels in an ER+ breast cells, ER $\alpha$  was overexpressed in

MCF-7 cells, which are referred to as MLET5 [42]. Microarray analysis comparing the parental MCF-7 cells to MLET5 revealed that genes involved in the apoptosis mechanism were differentially expressed between the two cell lines, such that MLET5 cells could evade apoptosis and survive. These findings support the possibility that breast cancer tumors which display elevated levels of ER $\alpha$  may develop estrogen-independent mechanisms of growth, likely through activation by growth factor receptors and kinases, and devise ways to circumvent apoptosis.

Models for acquired resistance to ICI 182,780 have also been developed [43–44]. MCF-7 cells were cultured for several months in the presence of ICI 182,780 until the cells gained the ability to proliferate in the presence of the antiestrogen. Work from the Lykkesfeldt group has demonstrated that ICI 182,780-resistant cells retain ER protein expression [43] and growth factor proteins ErbB3, EGFR, and kinase ERK are necessary for growth [45]. Both mRNA and protein levels as well as their downstream kinases pAKT and pERK are elevated. The Nephew lab has shown that ICI 182,780-resistant cells are also cross-resistant to tamoxifen [46]. In addition, these cells contain an increased number of growth-stimulatory pathways, including EGFR, HER2, and Wnt/ $\beta$ -catenin which may drive resistant cell growth in an estrogen-independent manner [44]. Clark and colleagues also reported that their ICI 182,780-resistant cells, MCF-7/LCC9, retain their ER expression and that upregulation of PR mRNA and protein was observed independent of estrogen [47]. Interestingly, in ICI 182,780 resistant cells, NF- $\kappa$ B p65 was upregulated while interferon regulatory factor-1 (IRF-1) was downregulated [48]. Interferon gamma induction of interferon regulatory factor-1 resulted in suppression of NF- $\kappa$ B p65 activity and antiestrogen response in ICI 182,780 resistant cells via inhibiting prosurvival gene expression and inducing proapoptotic genes [49]. These results suggest new treatment options which may include those that induce IRF-1.

### 3.2 Acquired AI resistance models - LTED

AIs, another type of treatment for breast cancer have shown efficacy in the clinic, however, acquired resistance to these inhibitors have also been observed. In order to investigate the mechanisms governing acquired AI resistance, cell models were generated. Several groups used the MCF-7 cell line and cultured cells long-term in the absence of hormone until the cells acquired the ability to proliferate without estrogen. In these culture conditions, a majority of the cells died, but a few remained and proliferated. This fraction of proliferating cells was considered resistant and termed long-term estrogen deprived (LTED). Characterization and mechanistic studies using these cells were performed in order to elucidate the mechanisms of acquired resistance [17, 50–53]. LTED cells appear to undergo three phases: a quiescent phase; a hypersensitive phase in which growth is induced at lower concentrations of estradiol compared to the MCF-7 control; and the final estrogen-independent phase [53]. This last phase occurs when the cells no longer require estrogen for growth. The process from responsiveness to resistance has been the subject of numerous studies. It has been observed that hypersensitivity of ER to estrogen may result from the action of different kinases, especially p42/44 MAPK [52, 54]. Kinases such as MEK1/2, c-Raf, p42/44 MAPK, p90RSK, c-MYC, display increased expression levels of both their unphosphorylated and phosphorylated forms in resistant cells [51]. Moreover, these activated growth factors and kinases influence the action of transcription factors to augment estrogen action through ER. For example, the AIB1 coactivator, a ligand-dependent ER coactivator, is enhanced by p42/44 MAPK phosphorylation leading to the recruitment of CBP and an associated increase in acetyl transferase activity [38]. In contrast to the thought that estrogen may still induce growth in AI-resistant cells, it has been reported that after 24 months, LTED cells evolved into estrogen-inhibitory cells [55]. Estrogen treatment of these cells resulted in a marked increase in Fas ligand and activation of Bcl-2 family protein [56].

It is regarded that when a cell is responsive to endocrine therapy, the ER signaling pathway is the most important mode of growth. During periods of estrogen deprivation, the estrogen-induced ER pathway is no longer viable. As a result, the cell develops an alternative method of ER activation. This is through increased expression and activation of growth factors such as HER2, Akt [57–58], and MAPK [51, 59], which can phosphorylate ER. Phosphorylation of ER at serine 118 by MAPK [51] or serine 167 by Akt [59] has been shown to induce transcription of growth-promoting genes. To confirm the importance of the ER pathway in AI resistance, LTED cells were treated with the pure antiestrogen fulvestrant, which prevents the activation of AF-1 and AF-2 and reduces the ER $\alpha$  half-life [54]. Fulvestrant markedly inhibits LTED cell growth, while excess estradiol restores cell growth [54]. Based on these results, it could be concluded that the ER-mediated pathway is solely responsible for proliferation.

Work from the Brodie laboratory has focused on acquired AI resistance and potential treatment options for AI resistance. Two AI-resistant cell models have been described. UMB-1Ca cells, an LTED model for acquired AI resistance, was generated by culturing MCF-7aro cells established by the Chen laboratory [16], in estrogen-free medium for several months until cells acquired the ability to grow in the absence of hormone [57]. Long-term letrozole-treated (LTLT-Ca) cells, a model for acquired letrozole resistance [59] was also generated from the parental MCF-7aro cells. A tumor xenograft was grown using MCF-7aro cells injected into ovariectomized mice, which were treated with letrozole for up to 56 weeks. Afterwards, cells from this tumor were extracted and maintained as a cell line. Studies using these cell lines have revealed changes in the protein levels of ER $\alpha$  [57, 59], increased protein expression and enhanced activation of growth factor receptor pathways, in particular HER2 and MAPK [59]. UMB-1Ca cells were characterized by increased protein levels of epidermal growth factor receptor 2, ER $\alpha$ , and phosphorylated levels of Akt [57]. In addition, these cells were less sensitive to AIs, tamoxifen, and ICI 182,780 compared to the parental cells.

UMB-1Ca and LTLT-Ca treated with inhibitors against the activated growth factor receptors and their downstream kinase targets showed abrogated activation of these proteins [57, 59]. Furthermore, ER $\alpha$  protein levels in the resistant cells were restored to levels seen in the AI responsive cells, such that they regained responsiveness to tamoxifen [59] and letrozole [60]. Combination treatment using signal transduction inhibitors for the activated kinases and an AI resulted in regained sensitivity to AIs. Interestingly, the use of an ER $\beta$  agonist DPN has been shown to overcome acquired AI resistance *in vivo* [61]. Combination usage of an ER $\beta$  agonist and letrozole resensitized acquired letrozole resistance in a LTLT-Ca xenograft model. Inhibition of tumor growth was associated with decreased ER $\alpha$ /ER $\beta$  ratio and blocked G1/S phase progression. Additionally, interesting results have been also generated through the use of histone deacetylase inhibitors.

The Gee group has also developed an acquired AI resistant cell line referred to as MCF-7X [58]. Studies using these cells suggest an important role for the PI3K/Akt pathway in promoting growth via phosphorylation and activation of ER. Single treatment of ICI 182,780 or an inhibitor for MAPK or PI3K inhibited growth to a degree. Cotreatment resulted in an enhanced inhibitory effect, indicating that a combination therapy to block both ER and kinases which modulate it should be used for greater suppression of AI resistant cell growth.

Findings from the Dowett laboratory have shown that the HER2/MAPK pathways were activated and IGF-1R was upregulated in LTED cells [51, 53]. Furthermore, these pathways crosstalk with ER to phosphorylate and subsequently activate it. Santen and colleagues have reported that long-term hormone deprivation causes cells to develop hypersensitivity to



estrogen [62–63]. These cells display a 4–10 fold increase in ER expression. Furthermore, insulin growth factor receptor (IGF-1R) activates nongenomic ER signaling and downstream MAPK and PI3K/Akt/mTOR pathways, which induce proliferation in an estrogen hypersensitive state.

### 3.3. Alternative methods of estrogen biosynthesis

Resistance to AIs may also result from incomplete inhibition of estrogen production. In addition to aromatase, estrone sulfatase, and 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) are enzymes involved in estrogen biosynthesis in human breast cancer tissues. Estrone sulfatase converts estrone sulfate to estrone. Estrone can then be converted into estradiol by 17 $\beta$ -HSD. This route of estradiol synthesis is independent of aromatase and may be a source for AI resistance. It is known that estrogen sulfate concentrations are several times higher in breast cancer tissue compared to normal breast tissue [64–66]. Therefore, the high concentration of estrogen sulfate in the tissue has potential for producing estradiol, which can then activate ER and promote cancer cell growth regardless of whether AIs are present.

### 3.4 ER-independent mechanism

Few investigators have suggested that the ER may not be important for the proliferation of resistant cells. Several clinical studies have observed cases in which patients who are initially ER+ lose the expression of ER, and develop resistance to endocrine therapy [67–68]. In addition, breast tumors can be categorized based on their expression of HER2, ER and PR protein receptors. Luminal A tumors are highly ER+ and PR+ and have a better response to antiestrogen and AI therapy. Luminal B tumors are those which are ER+ and display high cellular proliferation. These tumors typically display poor response to antiestrogen and AI therapy. It is possible during antiestrogen or AI therapy, tumors lack the estrogen-induced pathway and can evolve from luminal A to luminal B tumors which become entirely ER-independent. In these instances, resistance to antiestrogen or AI therapies may be the result of selection for cells which have lost the expression of ER.

### 3.5 Suppression of apoptotic pathways in endocrine resistant cells

Endocrine resistant cell lines generated by overexpression of certain growth factor signaling proteins and kinases have shown that increased levels and activity of these proteins may lead to pathways which block apoptosis and promote cell growth via estrogen-independent mechanisms [28–29, 69]. Growth factor signaling proteins overexpressed in cells cross-talk and activate ER and can circumvent tamoxifen-induced apoptosis through the upregulation of antiapoptotic proteins [70] and induce growth independently of estrogen-dependent pathways. In addition, serum- and glucocorticoid-inducible kinase 3 (SGK3) kinase, an ER regulated protein, was stably overexpressed in MCF-7 cells and conferred resistance to ICI 182,780-induced apoptosis [71]. Moreover, the anti-apoptotic proteins BCL2 and BCL-W have also been implicated in contributing to resistance [72]. Inhibitors to these proteins indicate that sensitive and resistant cells survive by different mechanisms. The mechanism of resistance may have developed by altering the programmed cell death pathway [72]. Moreover, these results further support the thought that sensitive and resistant cells grow and survive by different mechanisms.

### 3.6 Differential gene expression between tamoxifen and AI resistant cells

It is well recognized that there is a lack of complete cross-resistance among three FDA approved AIs [73]. Therefore, we believed that resistance mechanisms for each AI and tamoxifen may differ. Therefore, in order to study the mechanisms of tamoxifen and AI resistance, our laboratory also developed a set of acquired endocrine resistant cell lines. We generated several replicates of the MCF-7aro LTED cell line, in addition to several

replicates of MCF-7aro derived cell lines specifically resistant to a particular FDA approved AI (exemestane, anastrozole, or letrozole): T+EXE R, T+ANA R and T+LET R, or tamoxifen: T+TAM R. Inhibitor-only resistant cell lines were also generated [17]. This was performed by culturing MCF-7aro cells with the appropriate inhibitor with or without testosterone for several months. Initially, many cells died, while few remained. These cells proliferated and took several months for complete resistance to be achieved. Using these cell lines, we sought to identify genes and pathways involved in the mechanisms of AI-resistance through the use of microarray analysis [17]. The significance of our microarray study compared to others is two-fold: first, we are using true resistant cell lines which were generated after several months of AI-treatment or hormone deprivation. Second, we utilized a nonbiased genome-wide approach in order to identify genes and pathways involved in the mechanisms of AI-resistance from these individual cell lines. To check the quality of our microarray results, we carried out hierarchical clustering analysis. We were pleased to see that our nonbiased genome-wide approach resulted in the replicates for each AI-resistance clustering together. Based on the gene expression profiles T+LET R, T+ANA R, and T+EXE R cells are similar to each other, and distinct from T+TAM R and LTEDaro cells, which display similarities [74]. Moreover, results from our microarray study revealed that cells resistant to a specific AI possess unique gene expression signatures and are different from the LTEDaro cell model, suggesting that the LTED model alone is not an accurate representation of AI resistance. Rather, we must take into consideration the uniqueness of each AI and study these cells which have acquired resistance to the particular AI in order to obtain a better understanding of AI resistance.

Through the use of these cell lines as models of acquired AI or tamoxifen resistance, we can determine that the ER remains important for cell growth. This is demonstrated by their growth inhibition to the pure antiestrogen inhibitor ICI 182,780 [17]. These results are similar to those reported previously [54] and support our cell lines as true models of endocrine resistance. Results from our studies using these cells also revealed that ER in T+ANA R, T+LET R, and LTEDaro cells is constitutively activated through its phosphorylation on serine 118 in the absence of ligand, confirmed by luciferase reporter assays and western blot analysis [17]. However, the ER activity in T+EXE R and T+TAM R cells remained estrogen-dependent. The steroidal AI exemestane has been reported to display mild estrogenic activity [74–75]. Our microarray results also indicated that most of the upregulated genes from EXE-only resistant cells were estrogen-dependent. In addition, we discovered that there are notable differences between the expression profiles of the upregulated genes, such as PgR, in the AI- and T+TAM R cell lines. While PgR is upregulated in testosterone-only, T+ANA R, T+LET R, T+EXE R, EXE R, and T+TAM R, it is not in LTEDaro and ANA R. Taking the data together, it delineates four types of AI-resistant cell lines. The first type is LTEDaro and ANA R, in which ER is overexpressed and is constitutively active. The second type consists of T+ANA R and T+LET R, which have constitutively active ER. The third type consists of EXE R and T+EXE R, which contain ER that depends on estrogen for its activation and exemestane which acts as a weak estrogen [74]. The fourth type includes T+TAM R, which has a unique gene expression profile compared to LTEDaro and the individual AI-resistant cell lines [76].

### 3.7 Cross-resistance studies

Determining which secondary therapy to use once a tumor develops resistance is extremely important. Using our cell lines as models of tamoxifen-resistance or a specific AI resistance, we conducted cross-resistance studies to determine whether cells resistant to tamoxifen or a particular AI, are responsive to a different AI. These studies are important as it has the potential to identify possible alternative treatment options once a breast cancer patient develops resistance to a particular AI therapy. We found that tamoxifen-resistant cells are

growth inhibited by all three AIs. Cells resistant to a particular AI were still responsive to treatment with a different AI. However, LTEDaro cells were unaffected by either tamoxifen or AI. These results, together with our previous findings that exemestane displays estrogenic activity [74], suggest that our cell lines may be models for what occurs in cells as they progress towards acquired resistance. Cells begin as estrogen-dependent cells (MCF-7aro) which are responsive to both tamoxifen and AIs. During prolonged treatment with AIs, cells become less dependent on estrogen and develop alternative pathways to activate ER. These involve activation of ER by growth factor signaling proteins in a ligand-independent manner. Cells which begin to develop this phenotype are represented by the tamoxifen-resistant and exemestane-resistant cells. In these resistant cells, estrogen-mediated ER $\alpha$ -signaling is blocked; however, tamoxifen and exemestane may function as weak estrogens which drive cell growth. This is the first stage of resistance. Over time, the growth pathways become more estrogen-independent and rely on an increased number of growth factor signaling pathways. It is believed that cells at this stage are demonstrated by the anastrozole-resistant and letrozole-resistant cells. Finally, once the cells have achieved total estrogen-independence, they no longer respond to any AI or tamoxifen. These cells are exemplified by the LTEDaro cells [17].

#### 4. *De novo* resistance

Most ER+ patients respond well to ER-targeted therapy; however, there is a number of patients who do not respond to endocrine therapy (*de novo*/intrinsic resistance) [77]. For example, approximately 30% of ER+ breast cancers do not respond to tamoxifen treatment [78]. Several proteins are being considered as markers for *de novo* resistance. Increased expression of type 1 growth factor receptors (e.g. EGFR and HER2) and kinases such as Akt have been reported to be associated with indices of reduced likelihood of responding to or benefiting from tamoxifen.

##### 4.1 HER2

HER2 overexpression in clinical tumor samples has been shown to positively correlate with resistance to tamoxifen. Studies using clinical samples have revealed that tumors with high circulating levels of HER2 or overexpression of HER2 as observed in luminal B cancers are less responsive to endocrine therapy with lower response rate and shorter duration of response to antiestrogen therapy [79–81] than luminal A subtype which overexpress ER but not HER2.

To date, there is a paucity of *in vitro* models for *de novo* endocrine therapy resistance. Those which exist typically overexpress the HER2 protein to mimic the cancer cells observed in the clinic [30–31, 69]. *In vitro* studies correlate with findings from the clinic. In cell-based studies, HER2 has been implicated in playing a role in resistance. In these studies, cell lines used have generally been an ER+ MCF-7 line which has been transfected to overexpress HER2. Evidence suggests that overexpressed HER2 confers tamoxifen resistance in ER+ breast cancer [29, 69]. Whether HER2 overexpression results in estrogen-dependent or -independent growth is unclear [30–31, 82]. Moreover, increased EGFR/HER2 signaling is associated with tamoxifen resistance, in which case tamoxifen may function as an agonist and stimulate cell growth [69, 83].

It has been proposed that HER2-mediated tamoxifen resistance is through the MAPK pathway. *In vitro* studies using ER+ MCF-7 cells overexpressing HER2 showed that treatment with the EGFR inhibitor AG1478 + 4OH tamoxifen or MAPK inhibitor U0126 + 4OH tamoxifen enhances the inhibitory effect of 4OH tamoxifen on ER transcriptional activity [83–84]. This was the result of restoration of the interaction between ER and nuclear corepressor (N-CoR) with tamoxifen treatment. This in turn, resulted in cell growth



inhibition [84] and decreased colony formation [83]. HER2-overexpressing MCF-7 xenografts in athymic mice also confirmed the findings that the combination of both AG1478 and tamoxifen arrested tumor growth relative to untreated controls. This arrest in growth was due to the anti-proliferative effect of AG1478 and apoptosis [83]. Resistance to tamoxifen may be a result of anti-apoptotic mechanisms employed by the cell. Tamoxifen resistance mediated by HER2 was correlated with overexpression of the anti-apoptotic protein, Bcl-2, in MCF-7 cells [70].

#### 4.2 Akt

Akt has also been demonstrated to play a role in *de novo* endocrine resistance. Akt overexpression is associated with HER2 overexpression and aberrantly activated Akt has been reported in many tumors and associated with resistance to endocrine therapy. MCF-7 cells overexpressing constitutively active Akt display activation of ER in a ligand-independent manner through phosphorylation of ER $\alpha$  at serine 167 by Akt. These cells demonstrate reduced sensitivity to tamoxifen, yet are growth inhibited by the pure-antiestrogen ICI 182,780 [28]. This suggests that AKT is responsible for 4OH tamoxifen resistance by modulating the ER $\alpha$  activity. Further studies showed that AKT expression prevents apoptosis by increasing the expression of the anti-apoptotic Bcl-2 gene, which is regulated by ER $\alpha$  [28]. Akt regulation of ER $\alpha$  on a post-translational level, may lead to increased Bcl-2 expression. This may result in the cells ability to resist endocrine therapy via its capability of evading apoptosis.

#### 4.3 De novo AI resistance model

To our knowledge, currently there are no cell models for *de novo* AI resistance. Moreover, little is known about the mechanisms governing *de novo* AI resistance and suitable therapeutic options available for this type of resistant breast cancer. Therefore, as proof-of-concept studies, our laboratory has generated *de novo* AI therapy resistant cell lines. In an effort to model breast cancer patients who are thought to be suitable candidates for endocrine therapy, however, later discover that their breast cancer cells are intrinsically resistant to endocrine therapies, we generated cells which stably overexpress both aromatase and HER2 or Akt at high levels. These cells are denoted MCF-7HER2aro and MCF-7Aktaro. Our *de novo* AI-resistant cells are the first of its kind. To address the lack of information regarding *de novo* AI resistant breast cancer cell models, we treated our MCF-7HER2aro and MCF-7Aktaro cells with AIs and ICI. Both cell lines did not respond to the AI treatment and were mildly growth inhibited by the ICI treatment. These results indicated that these cell lines are models for *de novo* endocrine therapy resistance. We further characterized these cells and learned that the overexpression of aromatase had no role in contributing to the *de novo* AI resistant phenotype. The ER activity remained intact; however, the cells were not heavily dependent on the ER activation for proliferation (unpublished data). We are currently investigating with these cell lines to elucidate their mechanisms of resistance, how they differ from acquired AI-resistance, and to determine possible treatment options for *de novo* AI resistance. Currently, there are no good targeted therapeutic options for *de novo* AI resistant breast cancer. It is imperative that we invest our time and effort into studying this type of resistance and identifying novel treatment options for this type of cancer.

### 5. Importance of cell models

*In vitro* cell models are important tools for studying both tamoxifen and AI responsive and resistant breast cancer. Through the use of these simplified disease models, we can elucidate and better understand the mechanisms governing breast cancer. Clearly, these cells are valuable in that we may rapidly test a number of novel drugs for their anti-cancer abilities

and their modes of action. One such novel drug to treat AI resistant breast cancers is an inhibitor for the heat shock protein 90 (HSP90) [85]. HSP90 is a chaperone protein that assists a number of “client” proteins in assuming a conformationally active form. Many of these client proteins are factors and key players in the promotion of oncogenesis. Inhibition of this critical chaperone protein results in proteasome-mediated degradation of these client proteins. The finding from our laboratory is the first report which determines the efficacy of HSP90 inhibitors on acquired AI-resistant breast cancer cells [85]. In our studies we used the HSP90 inhibitor 17-DMAG to treat our AI-responsive MCF-7aro cells and acquired AI-resistant LTEDaro cells. We found that 17-DMAG inhibits both AI-responsive and AI-resistant cell growth at low nanomolar concentrations and was specific at inhibiting cancer cells rather than normal breast cells. HSP90 inhibitor treatment resulted in induced apoptosis and cell cycle arrest. Importantly, a number of client proteins thought to be important for cancer cell growth promotion, including ER $\alpha$ , HER2, and Akt were degraded upon HSP90 inhibitor treatment. Notably, HER2 and Akt are important proteins believed to crosstalk with ER $\alpha$  and activate it in a hormone-independent manner in AI-resistant breast cancers. Collectively, our results provide evidence for further examination of HSP90 inhibitors as a second line therapy for acquired AI resistance. The use of cell models has also enabled novel treatment regimens to be considered. Continuous treatment with an anticancer therapy forces the cell to adapt and depend on alternative pathways for promoting growth. Studies have demonstrated that drug treatment in an intermittent fashion may delay resistance from occurring and reduce the experience of side-effects [50].

Moreover, cross-talk between steroid hormone receptors such as ER and a number of kinases has been demonstrated in the presence of tamoxifen and AI therapy which results in the activation of ER in the absence of hormone. The discovery of cross-talk between these key proteins involved in cancer promotion has resulted in both single therapy to block kinase action as well as combination therapy treatment to simultaneously inhibit ER activation as well as the signaling proteins which cross-talks with ER. The HER2 antibody inhibitor, trastuzumab (Herceptin), is currently used for the treatment of HER2-positive metastatic breast cancer. Combination therapy involving endocrine therapy (i.e. tamoxifen) and the EGFR tyrosine kinase inhibitor (gefitinib) is being evaluated as a therapeutic option to overcome *de novo* resistance and delay acquired resistance. Results from a phase II study trial combining gefitinib with and without anastrozole did not show significant biological or clinical differences between outcomes for gefitinib with anastrozole or anastrozole alone [86]. Lapatinib, a dual EGFR and HER2 inhibitor, blocks the autophosphorylation of both receptors. A phase III trial comparing lapatinib with or without letrozole in ER+, HER2-positive metastatic cancer concluded that lapatinib with letrozole was superior to letrozole alone in terms of the progression-free survival as well as clinical benefit rate [87]. These findings resulted in the accelerated approval of lapatinib with letrozole as a therapy for hormone receptor-positive, HER2-overexpressing breast cancer in postmenopausal women. Others have proposed that inhibiting the function and activity of growth factor signaling pathways through the use of inhibitors may force the cell to revert to the initial hormone-dependent ER regulated pathway to promote growth. Tamoxifen-resistant and LTED cells were treated with lapatinib and were growth suppressed. Interestingly, LTED cells demonstrated resensitization to tamoxifen and estrogen deprivation, while tamoxifen-resistant cells were resensitized to tamoxifen [88]. These exciting findings suggest new treatment options, but require further testing in animal models as well as in the clinic. Combination therapy appears to be effective in overcoming resistance. Moreover, if signal transduction inhibitor treatment can reverse cells to their former endocrine therapy responsive state, this type of therapy is promising and may be a method to combat resistance.

Drug testing can also be conducted in a more complex model than cell lines through the use of xenograft models developed using these cell models. Testing *in vivo* is necessary to determine whether a drug's efficacy can be translatable to the clinic. Determining the proper dosage, the pharmacodynamics of the drug, clearance from the body, and retention in the tumor are important factors to consider when deciding whether to proceed with the investigational drug.

## 6. Limitations of cell culture studies for endocrine resistance

*In vitro* cell models for various types of breast cancers are valuable tools which can be used to study the different mechanisms of oncogenesis and resistance, as well as to test novel therapeutic agents for their ability to block cancer cell growth and the development of resistance. As important as these cell models are, they are not without limitations.

While various cell lines allow us to study differing types of breast cancers, the population of breast cancer patients is quite diverse and the number of cell lines available to study the variety of breast cancers is limited. To study AI resistance, many laboratories have used the MCF-7 cell line in the past to generate models for acquired resistance. Only recently are multiple models of acquired resistance being developed using other ER+ breast cancer cell lines [89]. Currently, our laboratory is generating other ER+ AI-resistant cell lines which overexpress aromatase in order to obtain a more comprehensive collection of AI-resistant breast cancer cell lines that reflect the diverse ER+ breast cancer patient population. These cell lines will be characterized and examined for their mechanisms of resistance in order for us to gain a better understanding of different types of acquired resistance in breast cancer patients. It will be interesting and informative to detect differences in resistance mechanisms among the cell lines, which may provide clues as to how ER+ breast cancer patients respond differently to various endocrine therapies. Moreover, these valuable cell lines will enable us to test a variety of novel inhibitors with the hope of identifying new alternative therapies which can overcome resistance.

To study a specific protein or type of resistant cancer, cells have been generated to stably overexpress or silence a particular protein or gene. While these are useful and important models to gain better insight into the roles of these proteins of interest and disease models, we must keep in mind that these cell lines are simply models and not true representations of the disease itself. Overexpression of a protein or silencing a gene may be a simplified version of the disease model and does not account for other alterations that may be present in the disease state which we are unaware of and may lead us to incorrect conclusions and not allow us to obtain a comprehensive understanding of the disease.

Most AI-resistant cell models are epithelial breast cancer cells which express high levels of aromatase. However, in breast tumor tissue, the stromal cells surrounding the epithelial cells also express aromatase mRNA at high levels [9–10]. By focusing our *in vitro* studies solely with epithelial cells, the paracrine effects contributed by stromal cells in the breast could be overlooked. The current models would be improved once stable aromatase-expressing human stromal cell lines become available.

Testing various therapeutic agents in cell models may not translate in the whole body system. On occasion, a therapy may show strong potency in the cell, but may not have an effect on the tumor in the animal model. In other instances, the drug may demonstrate potency in *in vivo* models, which is due in part to the high toxicity of the therapy in the animal system, thus rendering the drug unusable in the clinic. Animal models provide a platform which allows us to take into consideration the tumor microenvironment and the entire system of the organism that cell models cannot. For example, a small population of women carry the inactive allele for cytochrome P450 2D6 (*CYP2D6*) which converts

tamoxifen to its active metabolite, endoxifene [90]. As a result, these women are unable to metabolize tamoxifen properly and are less responsive to tamoxifen. In this era of science, simply designing a study using solely cell models is not enough. To fully determine the significance of the science, our findings from cell models must be compared to those conducted in animal models, and then finally correlated with the data from the clinic. However, we must also realize that cell models are important and provide useful information in a timely manner for the design of animal experiments and clinical trials.

Ideally, in order to obtain useful data from the clinic, procedures in the clinic must be modified to allow the collection of breast tumor samples at different stages of the treatment; such as before and after therapy, pre- and post-resistance to therapy and during therapy which would allow us to compare and confirm molecular targets from both *in vitro* and clinical studies. However, it is not easy to justify obtaining tumor biopsies at different stages of disease or to monitor patients over extended periods of time until relapse occurs. Moreover, it is not feasible to collect tissue samples pre- and post-resistance to therapy from the same patient.

Finally, it is currently difficult to obtain clinical samples relevant to laboratory studies. Laboratory and clinical groups should collaborate and carefully design studies to incorporate clinical samples in the laboratory data analysis in order to provide more convincing and substantial conclusions. Likewise, it is important for clinical and laboratory groups to work together in order to determine the mechanism of action of an effective drug and to identify the cancer growth mechanisms.

## 7. Summary

Despite the obvious limitations associated with cell lines as models of disease, cell models have been extremely important tools in helping us dissect the mechanisms which govern both endocrine responsive as well as resistant breast cancer cell growth. These *in vitro* models have allowed us to test a variety of novel endocrine therapies, small molecule inhibitors to block proteins which cross-talk with ER and inhibitors that can simultaneously block multiple proteins in an effort to shut-down a number of pathways involved in promoting growth and survival. Furthermore, different treatment regimens and combinations of therapies have been tested using these cell lines which have yielded interesting and potentially promising results, including several currently being used in the clinic.

Based on the available literature, it is important to note several points. First, regarding cell models for AI resistance, it is absolutely necessary that the cell line express aromatase. This allows us to test the effect of AIs in ER+ breast cancers as well as inhibitors that may modulate aromatase activity or aromatase expression. Second, new models for antiestrogens and AI resistance are strongly needed in order to gain a better understanding of the disease of resistance. Using these models derived from various ER+ breast cancer cells we must compare the results obtained from them to those from the MCF-7aro LTED model in order to verify whether our current knowledge of endocrine resistance is consistent in other ER+ antiestrogen- or AI-resistant breast cancers. Moreover, it is interesting to note the parallels between the mechanisms proposed for *de novo* and acquired resistance, in particular for tamoxifen. In both of these types of resistance, growth factor signaling proteins are upregulated and activated, which can modulate ER activity and downstream ER-regulated genes. However, there are differences between the two types of resistance. *De novo* resistance is characterized by inherent upregulation of growth factor signaling pathways while acquired resistance is an adaptation process of the cell to activate these pathways in order to bypass the suppression of ER-mediated pathways. Therefore, more studies are required to distinguish the differences between *de novo* and acquired resistance.

### Highlights

- Two types of endocrine resistance: *de novo* and acquired.
- Resistance involves activation of ER $\alpha$  by growth factor receptors and kinases.
- Cross-resistance studies show resistance mechanisms are different.
- Focus on treatment therapies with multiple targets to overcome resistance.

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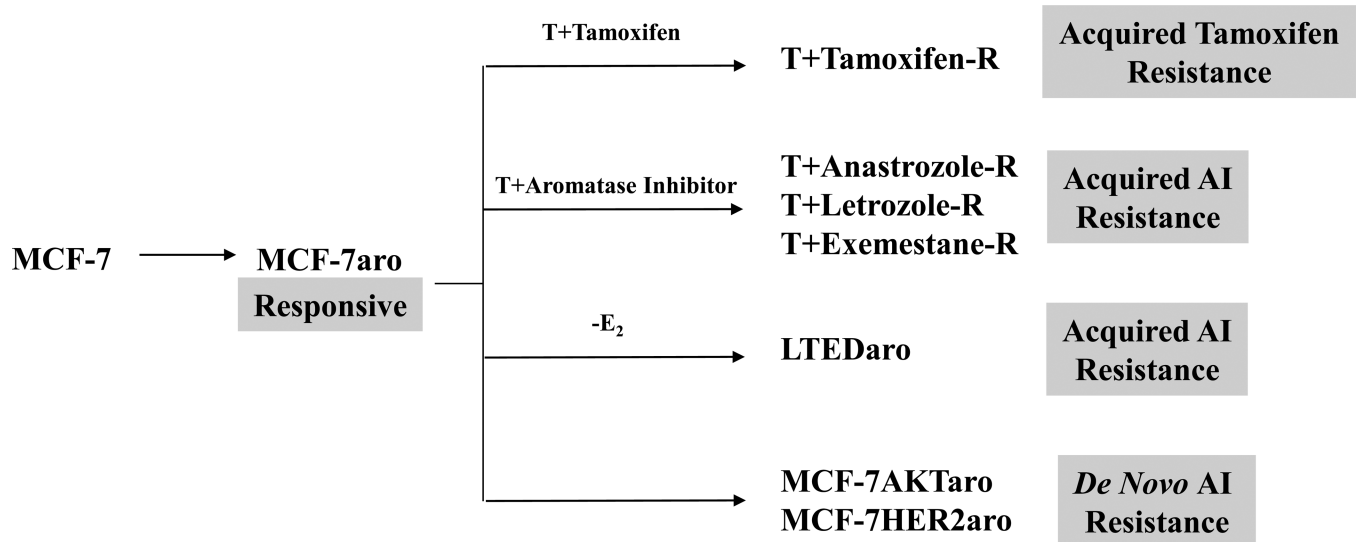
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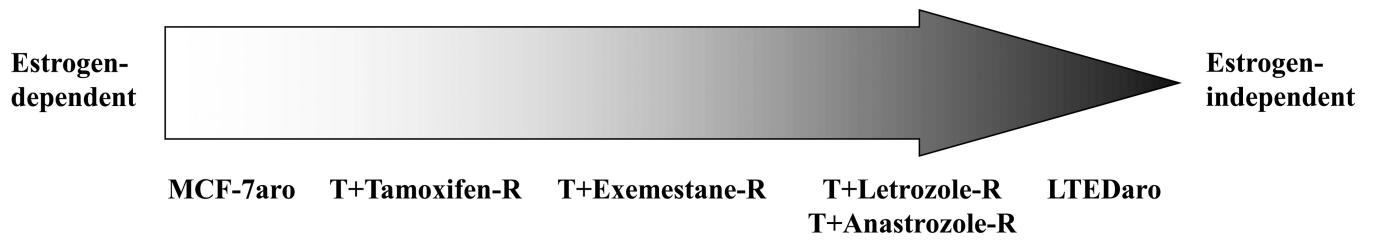
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**Figure 1.**

Generation of Tamoxifen and Aromatase inhibitor resistant cell lines. AI and Tamoxifen responsive cells were generated by engineering MCF-7 cells to overexpress aromatase. MCF-7aro cells were cultured long-term in the presence of tamoxifen or in the absence of estrogen until cells gained the ability to proliferate in these conditions. These cells are considered cells which have acquired either tamoxifen resistance (Tamoxifen-R) or AI resistance (LTEDaro). Anastrozole resistant (Anastrozole-R), Letrozole resistant (Letrozole-R), and Exemestane resistant (Exemestane-R) cells that have acquired resistance to a specific AI were generated by culturing MCF-7aro cells in the presence of testosterone and the appropriate inhibitor until cells gained the ability to proliferate in these conditions. Finally, models for *de novo* AI resistance were generated by overexpressing aromatase and HER2 or Akt.



**Figure 2.**  
Estrogen dependency of growth pathways in endocrine-resistant cell lines.

Table 1

Summary of *de novo* and acquired resistance

		Growth factors, kinases	ER protein levels	ER activity	Response to ICI
<i>De novo</i> resistance	Tamoxifen	HER2, Akt	Unaltered	Unaltered	Responsive
	AI	HER2, Akt	Unaltered	Unaltered	Nonresponsive
Acquired resistance	Tamoxifen	EGFR, HER2	Changed <sup>a</sup>	Ligand-independent	Responsive
	AI	HER2, PI3K/Akt, MAPK	Changed <sup>b</sup>	Ligand independent	Responsive

<sup>a</sup>unaltered, decreased;<sup>b</sup>increased, decreased according to different reports