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What do we know about the mechanisms of aromatase inhibitor resistance?

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Abstract

Clinical trials have demonstrated the importance of aromatase inhibitor (AI) therapy in the effective treatment of hormone-dependent breast cancers. Yet, as with all prolonged drug therapy, resistance to aromatase inhibitors does develop. To date, the precise mechanism responsible for resistance to aromatase inhibitors is not completely understood. In this paper, several mechanisms of de novo/ intrinsic resistance and acquired resistance to AIs are discussed. These mechanisms are hypothesized based on important findings from a number of laboratories.

To better understand this question, our lab has generated, *in vitro*, breast cancer cell lines that are resistant to aromatase inhibitors. Resistant cell lines were generated over a prolonged period of time using the MCF-7aro (aromatase overexpressed) breast cancer line. These cell lines are resistant to the aromatase inhibitors letrozole, anastrozole and exemestane and the anti-estrogen tamoxifen, for comparison. Two types of resistant cell lines have been generated, those that grow in the presence of Testosterone (T) which is needed for cell growth, and resistant lines that are cultured in the presence of inhibitor only (no T). In addition to functional characterization of aromatase and ER α in these resistant cell lines, microarray analysis has been employed in order to determine differential gene expression within the aromatase inhibitor resistant cell lines versus tamoxifen, in order to better understand the mechanism responsible for AI resistance on a genome-wide scale. We anticipate that our studies will generate important information on the mechanisms of AI resistance. Such information can be valuable for the development of treatment strategies against AI resistant breast cancers.

1. Introduction

The strategy for treatment of hormone-dependent breast cancers has typically depended on estrogen-deprivation, either via ovarian ablation or targeting estrogen receptor (ER) action, using Tamoxifen. Another type of estrogen-deprivation therapy for breast cancer treatment is inhibition of aromatase, the enzyme that catalyzes the conversion of androgens into estrogens. The increased efficacy of aromatase inhibitors (AI) over tamoxifen therapy has recently been demonstrated by clinical trials, whereby a significant increase in disease-free survival has been shown using three third-generation aromatase inhibitors (AIs) (1-3).

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The three FDA-approved third-generation AIs, i.e., two non-steroidal derivatives [anastrozole (Arimidex) and letrozole (Femara)] and one steroidal derivative [exemestane (Aromasin)], are now widely used as first-line drugs in the endocrine treatment of estrogen-dependent breast cancer in postmenopausal patients. The structures of these AIs are shown in Figure 1. Anastrozole and letrozole have the triazole functional group that interacts with the heme prosthetic group of aromatase, and they act as competitive inhibitors with respect to the androgen substrates. Exemestane is a mechanism-based inhibitor that is catalytically converted into a chemically reactive species, leading to irreversible inactivation of aromatase.

AIs are thought to be of value in treating estrogen-dependent breast cancer, especially in postmenopausal patients. Estrogens in postmenopausal patients are mostly produced in peripheral adipose tissues and in cancer cells, and the peripheral aromatase is not under gonadotropin regulation (4). Therefore, in postmenopausal patients, complications due to a feedback regulatory mechanism which increases luteinizing hormone (LH) and follicle-stimulating hormone (FSH) after AI treatment does not occur. In premenopausal women, LH and FSH stimulate the synthesis of aromatase in ovaries and may counteract the effects of AIs.

Although AI treatment of hormone-dependent breast cancers in postmenopausal women has shown to be effective in the clinic, resistance to these endocrine therapies still occurs. A number of laboratories have carried out research to examine the mechanisms of endocrine resistance. Most of these studies concerning resistance mechanisms focus on ER antagonists such as tamoxifen. Several laboratories have initiated research to examine the resistance mechanisms of AIs. Recently, several excellent reviews on this topic have been published, e.g., Normanno et al. (5), Dowsett et al. (6), Moy and Goss (7) and Ali and Coombes (8).

There are two types of endocrine resistance. **De novo/intrinsic resistance** refers to lack of response at initial exposure to endocrine therapy of aromatase-positive and estrogen receptor (ER)-positive breast cancers. **Acquired resistance** is developed during endocrine therapy of patients who respond to the treatment initially. We and other investigators believe that elucidating the mechanisms of resistance to AIs/antiestrogens, on the molecular level, will be extremely valuable for the effective treatment of hormone-dependent breast cancers and for the development of novel approaches to treat patients who fail endocrine therapy.

2. De novo/intrinsic resistance

It is understood that only aromatase-positive and ER-positive breast cancer would respond to the treatment of aromatase inhibitors. Abnormally higher expression of aromatase in breast cancer cells and/or surrounding adipose stromal cells than normal breast tissue, have been demonstrated by a number of laboratories by aromatase activity measurement (9-11), immunohistochemical analysis (12-15) and RT-PCR analysis (16,17). The *in situ* estrogen biosynthesis is thought to have a significant influence on tumor maintenance and growth in breast cancer patients who can be treated with aromatase inhibitors. In a recent study, Ma et al. identified and characterized genetic polymorphisms in the human aromatase gene (18). These investigators have identified nucleotide polymorphisms [including 85 single nucleotide polymorphisms (SNPs), 2 insertion-deletion events and 1 polymorphic TTTA repeat] with most of them present in the non-coding regions. Although the physiological consequence of most genetic polymorphisms have not been determined, it is possible that some genetic variations influence the expression of aromatase/levels of estrogen. The TTTA microsatellite polymorphism is present in intron 4 of the aromatase gene and has been studied by a number of laboratories. While the results generated from these laboratories are not exactly consistent with each other, the TTTA polymorphism has been suggested to associate with circulating estrogen, bone structure and breast cancer risk. As an example, two such papers discussing disparities related to this polymorphism are Dick et al. (19) and Okobia et al. (20). There are

four cSNP in the coding region. These cSNPs alter the following amino acids: Trp39Arg, Thr201Met, Arg264Cys and Met364Thr (18). The Met364Thr variant was found to be less stable and to have significantly lower affinities for the androgen substrate and for the inhibitor exemestane. Met364Thr was only observed in the Han Chinese-American samples, with allele frequency of 0.8%. The physiological significance of this cSNP has not yet been determined. However, as we believe that aromatase is expressed in all breast cancer tissues at higher levels than normal breast tissue, it is possible that women with the Met364Thr would have lower levels of estrogen through their life time or breast cancer patients with this cSNP would not respond well to exemestane. Thus far, no known genetic polymorphisms of the aromatase gene have been reported that affect binding affinities of anastrozole or letrozole.

Approximately 75% of breast cancer in postmenopausal patients is estrogen-dependent, as indicated by ER positivity through immunohistochemical analysis. It is well known that not all ER positive breast cancers respond to endocrine therapy with anti-estrogens or AIs. Typical AI response rates vary from 20 to 50% [as discussed by Dowsett et al. (6)]. Several mechanisms of resistance have been suggested. Most of them are associated with alterations of ER α function. Fuqua et al. have reported that Lys303Arg mutation leads to a hypersensitive ER α (21). Increased expression of coactivators and decreased expression of corepressors associated with nuclear receptors have also been suggested to lead to hypersensitive response of ER α to estrogen, as shown by Osborne et al. (22).

Progesterone receptor (PgR) expression has recently been shown to be associated with increased benefit from tamoxifen (23). PgR is an estrogen-responsive gene. It is logical to think that ER+/PgR+ tumors are truly estrogen dependent. Therefore, such tumors respond well to the treatment of tamoxifen or AIs. ER+/PgR- tumors could be less responsive to estrogen or through non-genomic action of estrogen. These mechanisms would support the findings that anastrozole was more effective than tamoxifen in patients with tumors that are ER+/PgR- (23b). Furthermore, it has been hypothesized that activation of the PI3K/AKT pathway down regulates the transcription of the PgR gene (24). On the other hand, results from trials using letrozole or exemestane have not yet confirmed that these two AIs are more effective than tamoxifen in the treatment of ER+/PgR-patients (5).

Cross talk between ER and growth factor pathways (e.g., EGFR/ErbB2 and IGFR mediated pathways) is certainly attractive mechanism of endocrine resistance. Ellis et al. found that patients with EGFR and/or ErbB2 over expression and ER+ tumors responded significantly better to letrozole than tamoxifen (25). It was hypothesized that tamoxifen could act as an agonist upon the phosphorylation of ER by EGFR or ErbB2-associated kinase. However, most of cell culture model studies have found that over-expression of EGFR/ErbB2 or down stream members of these pathways leads to the insensitivity to tamoxifen as well as AIs. Furthermore, trials involving kinase inhibitors or in combination with anti-hormonal agents have not yet generated encouraging outcomes (e.g., reference (26)). Therefore, exact mechanism of endocrine resistance associated with growth factor pathways is not yet clearly defined.

Cytochrome P450 2D6 has been shown to be an important tamoxifen-metabolizing enzyme (27). Genetic polymorphism of the p450 2D6 gene has been identified, and women with a variant allele are suggested to respond to tamoxifen differently from women with the wild-type allele. We do not yet have any information as to how AIs are metabolized in men.

At the molecular level, ER has been shown to be capable of binding to a number of non-conventional estrogen responsive elements, e.g., AP1 and SP1. However, the physiological significance of such findings and relevance of these interactions to endocrine resistance are not yet demonstrated in a definitive manner.

3. Acquired resistance

The most obvious mechanism of acquired resistance involves a selection process. All tumors are heterogeneous. Each breast tumor contains ER+/estrogen responsive as well as ER- or estrogen independent cells. During endocrine therapy, the population of ER+/estrogen responsive cells reduces, and with time, ER- or estrogen independent cells become the dominant group of cells in tumors. At this stage, the tumors will stop responding to endocrine therapy using anti-estrogens or AIs, which is referred to as acquired resistance.

However, clinically, many of the endocrine resistant tumors are still ER positive. The mechanisms of such acquired resistance are probably similar to those discussed for de novo/ intrinsic resistance, except that resistance develops during treatment. It is very unlikely that acquired resistance results from aromatase or ER mutation developed during endocrine treatment. Most likely, such resistance results from cross talk between ER and growth factor pathways, or other currently unidentified pathways.

Aminoglutethimide (AG) is the first generation aromatase inhibitor. It was found that in some patients, the aromatase activity in breast tumors is significantly increased after AG treatment (9). Using an aromatase-positive breast cancer cell line, SK-BR-3, as a model, our primer-specific RT-PCR analysis revealed that AG enhanced the action of a promoter which is different from promoter I.1, I.3, or II (28). Furthermore, since the AG induction was found to be suppressed by SQ 22536, an adenylate cyclase inhibitor, a cAMP-dependent mechanism might be involved. However, such acquired resistance mechanism has not been demonstrated for the three third-generation AIs.

Almost all the data on acquired resistance are at present derived from laboratory studies. A major hypothesis is that the adaptation to estrogen withdrawal is involved in the resistance to both tamoxifen and AIs. Due to the ability of breast cancer cells to be adaptive, these endocrine therapies that function to block hormone-dependent signaling cascades required for breast cancer proliferation, may cause novel signaling mechanisms which circumvent the effects of an AI or anti-estrogen. An attractive hypothesis is that the resistance results from estrogen hypersensitivity or estrogen-independent activation of ER. To address this question, studies have been undertaken to investigate long-term estrogen deprivation (LTED), since AIs function to effectively block the synthesis of estrogens. It has been reported by Dr. R. Santen's laboratory that an up-regulation in the crosstalk between the insulin-like growth factor receptor (IGF-1R) and ER α induce rapid non-genomic effects in LTED cells, which are responsible for activation of MAP kinases and PI-3K/AKT that drive breast cancer proliferation by estrogen in a hypersensitive manner, i.e., the proliferation of LTED cells can be stimulated at concentrations four-log lower as compared with wild-type MCF-7 cells (29,30). In addition, the ER α levels in LTED cells have been found to be 4- to 10-fold of those in MCF-7 cells. The ER transcriptional activity is similar to the wild-type MCF-7 cells. Estrogen deprivation cells have been generated and characterized in four laboratories. Important findings from these studies are summarized in Table 1.

LTED cells were also generated in Dr. M. Dowsett's laboratory, but different mechanisms of resistance were found. After prolonged culture in the absence of estrogen (eight weeks), ER α was found to function without the need of estrogen (31,32). Elevated levels of Ser118 phosphorylation of ER α and PgR expression were detected. The ER transcriptional activity was found to be 7-10 times that in the wild-type MCF-7 cells. ER crosstalk with ErbB2 signaling, instead of IGF-1R signaling, was shown to be important in the LTED cells generated by Dowsett and his colleagues.

Nicholson et al. have also prepared an estrogen withdrawal cell line, i.e., MCF-7X (33,34). These cells have an estrogen-dependent ER α pathway, and their growth is mainly supported

by the PI3K/AKT pathway. In addition, Dr. A. Brodie's laboratory (35) has produced an estrogen withdrawal cell line using MCF-7ca, an aromatase over-expressing cell line originally generated in our laboratory (36). The cells from Brodie's laboratory have 4-5 fold higher levels of ER α and ErbB2 than the wild-type cells. Elevated levels of S167 phosphorylation of ER α were found, but the ER α transcriptional activity was not determined.

Studies from these laboratories have revealed that growth factor pathways are activated in these estrogen withdrawal cell lines. This results in increases in ER expression and phosphorylation, which further activates the receptor in a ligand-independent manner or estrogen hypersensitivity, leading to breast cancer proliferation.

Although the actions of tamoxifen (ER antagonist) and aromatase inhibitors (prevention of estrogen synthesis) are inherently different, understanding acquired resistance to tamoxifen can be useful in elucidating mechanisms responsible for AI-resistance. Several groups have studied tamoxifen-resistance quite extensively. It is believed that primarily ErbB1 and ErbB2 crosstalk with ER. This crosstalk leads to enhanced cell survival pathways via PI3K/AKT activation in addition to activation of various MAP kinases that mediate transcriptional effects resulting in cell proliferation (37-42). In addition to tamoxifen-resistance, the Brodie laboratory has initiated the first direct study of aromatase inhibitor resistance, using the non-steroidal AI letrozole (43). It was observed that letrozole-resistance involves HER2 crosstalk with ER α , leading to activation of MAPK and phosphorylation of ER α , resulting in breast cancer cell proliferation (43,44). Interestingly, levels of ER α in the letrozole-resistant cells were found to be 50% of those in the wild-type cells.

Overall, previous studies that have described the mechanisms responsible for acquired resistance in breast cancer cells (LTED, MCF-7X, tamoxifen-resistance and letrozole-resistance) have focused on the crosstalk between ER and growth factor signaling pathways that mediate cell survival and proliferation.

4. Generation of AI-resistant and tamoxifen-resistant cell lines in our laboratory

By reviewing what has been accomplished in other laboratories, we have learned several important lessons.

- A. The estrogen withdrawal cell lines generated from different laboratories are not exactly identical as indicated by the stimulation of different molecular pathways in these lines.
- B. The estrogen withdrawal cell lines are not exactly equivalent to AI resistant cell lines, as demonstrated through the comparison of molecular features between the estrogen withdrawal cell lines and letrozole resistant cell line generated in Brodie's laboratory (35,43). In addition, it is logical to believe that different AIs may have unique resistance mechanisms, as supported by the fact that after treatment failure of one AI, patients can still respond to the treatment with another AI. Bertelli et al. (45) have carried out sequential treatment with exemestane and non-steroidal AIs in women with advanced breast cancer. In their study, 40 patients received exemestane 25 mg daily as first AI, with a clinical benefit (CB) rate of 67.5% (95% CI 52.9-82.0%) and a median time to progression (TTP) of 9.6 months. Eighteen patients were then treated with a second AI [letrozole (n=17) and anastrozole (n=1)] after failure of exemestane, with a CB rate of 55.6% (95% CI 32.6-78.5%) with a median TTP of 9.3 months. The CB rate was defined as: complete response + partial response + stabilization of disease for ≥ 24 weeks. In addition, 23 patients received exemestane as the second AI, with a CB rate of 43.5% (95% CI 23.2-63.7%) with a median TTP of 5.1 months. Bertelli

et al. have concluded that exemestane is active after prior failure of letrozole or anastrozole, and the partial non-cross resistance between steroidal and non-steroidal AIs is independent of the sequence employed.

- C. AIs will only be effective in the aromatase-positive cells when the enzyme is actively converting androgen to estrogen. Therefore, androgen should be present in the resistant breast cancer cells. Recently, Macedo et al. (46) have indicated that AI treatment may suppress estrogen-dependent proliferation as well as unmask the inhibitory effect of androgen.
- D. The information generated thus far on acquired resistance has been very informative, but the possibility exists of other currently unidentified pathways that may further augment resistance. Thus, it is important to apply a non-biased method, e.g., cDNA microarray analysis, to identify additional novel genes or pathways that play a role in AI resistance.

To investigate AI resistance, our laboratory has generated AI resistant lines (using letrozole, anastrozole and exemestane) and anti-estrogen resistant lines (using tamoxifen) for comparison. In terms of a suitable cell line that can be used for resistance studies, an ER-positive breast cancer line that does express high levels of aromatase is needed, but does not exist. MCF-7aro cells [stably transfected with the aromatase gene (47)] were generated in our lab and are used as a model system to study AI response and were therefore used to produce the drug resistant lines.

In order to generate drug-resistant cell lines, MCF-7aro cells were cultured in the presence of testosterone plus the appropriate inhibitor. Initially, inhibitor treatment of these breast cancer cells induced massive cell death, but after prolonged culture (2-6 months) in the presence of the inhibitor, resistance to these drugs was acquired (i.e., T+LetR, T+AnaR, T+ExeR, and T+TamR). As proper controls, MCF-7aro cells were also cultured in the presence of testosterone only (i.e., AroT), in addition to cells that were grown in medium alone [long term estrogen deprived (LTEDaro) cell lines]. Six independent sets of each resistant line were generated. We feel that study of a single resistant line for each inhibitor is not a nonbiased approach. Furthermore, our lab has also generated three independent sets of each resistant line that are grown without testosterone (i.e., LetR, AnaR, ExeR and TamR). This was done to determine if differential resistance pathways exist between cells cultured with or without testosterone. In addition, we prepared three independent sets of MCF-7aro cells cultured for 5 days without testosterone or inhibitor. These cells (Aro) served as reference cells that did not reach resistance status, and are used as reference cells for our AI response studies (discussed below).

We feel that it is important to examine the effects of AIs on gene expression in MCF-7aro cells before the analysis of resistant cell lines. In most of the recently published studies about the effects of hormones or other chemicals on gene expression, cells were cultured in the presence of chemicals for only a few hours to a couple of days prior to expression analysis (48,49). These studies have identified early responsive genes whose expression may or may not remain up-regulated after a longer period of treatment. The cells in our study were exposed to chemicals for 10 days because we wanted to identify genes whose expression are modulated under conditions that model breast cancer patients who are treated with medication long-term (50). In addition, we would like to compare, in the future, the microarray results produced from hormone responsive cells and those from AI-resistant cells that were generated following a period of drug treatment (as described above). We treated MCF-7aro cells with 2 steroid hormones: 1nM testosterone (androgen) or 1nM 17 β -estradiol (estrogen). Androgen is converted to estrogen by aromatase in MCF-7aro cells, so we expected to see similar effects for the androgen and estrogen treatments (Figure 2). We also cultured the cells with four different inhibitors, three aromatase inhibitors (letrozole, anastrozole and exemestane) and one

ER antagonist (tamoxifen), in the presence of testosterone. The results generated from treatment of letrozole, anastrozole or tamoxifen have been published in 2005 (50).

We found that testosterone or 17 β -estradiol induced the proliferation of MCF-7aro cells at a rate six times faster than the untreated cells. In addition, the testosterone-induced proliferation of MCF-7aro cells was effectively suppressed by letrozole, anastrozole or tamoxifen (Figure 2). Microarray analyses on Affymetrix Human Genome U133A GeneChips were carried out using total RNA isolated from the control and treated cells. At the false discovery rate of 0.05 and a minimum fold change criteria of 1.5, 104 genes were identified that were up-regulated, and 109 genes were identified that were down-regulated by both androgen and estrogen. More than 50% of these hormone-regulated genes were counter-regulated by all 3 inhibitors, and more than 90% were counter-regulated by at least one of the inhibitors. When comparing the effect of each inhibitor on gene expression we observe that letrozole and anastrozole are more similar in terms of the genes they affect, compared to treatment with tamoxifen. To validate the gene expression profiles identified from microarray analyses, the expression patterns of 13 representative genes were examined by Northern analysis. The results of this study provide us with a better understanding of the actions of both aromatase inhibitors and anti-estrogens at the molecular level. We believe that the results of this study serve as the first step in identifying unique expression patterns following drug treatment, and that this will ultimately be useful in customizing patient treatment strategies for hormone-dependent breast cancer.

In contrast to AI/tamoxifen-responsive cells (as shown in Figure 2), cell proliferation assays demonstrate that the AI-resistant cell lines all proliferated similar to the testosterone control, implying that these cells had adapted a mechanism to grow despite the presence of the inhibitor (Figure 3). This large panel of resistant cell lines generated in our study will be useful in determining any heterogeneity that may exist in signaling mechanisms specific to each inhibitor. Also, for microarray and other experiments, multiple biological replicates will allow for a more thorough experimental and statistical analysis. As an example, six T+LetR resistant cell lines have different growth rates (Figure 4), suggesting that each of them has its unique features that would be expected in real clinical situation.

In addition to cell proliferation assays, aromatase and ER expression and activity levels in the resistant cell lines were examined in order to determine if these proteins play a role in resistance. The aromatase activity of the resistant cell lines is commonly measured using an 'in cell' aromatase assay (51). The aromatase and ER α mRNA levels in the resistant cell lines remained at similar levels as the original MCF-7aro cell line, except for the LTEDaro and AnaR cells in which ER α levels were elevated. Our results indicate that our LTEDaro cell lines are similar to LTED/estrogen withdrawal cell lines generated in other laboratories, where ER α expression is elevated. Interestingly, ER α expression only increases in AnaR, but not other type of resistant cell lines. The aromatase protein level and activity in the T+LetR, T+AnaR, and T+TamR-resistant cell lines are similar to the control AroT cell lines. In addition, aromatase is still functional in these resistant lines and is responsive to the treatment of AIs. These results indicate that AI resistance is not a result of change in aromatase expression or in its response to AI. As expected, we detect a low level of aromatase activity and aromatase protein in the T+ExeR cell lines because exemestane is a mechanism-based inhibitor that inactivates the aromatase enzyme.

Beside these basic characterizations of the resistant lines, microarray analysis of these resistant cell lines has been performed in order to observe changes in gene expression profiles that could be unique to aromatase inhibitor resistance. Preliminary data suggests that the mechanism of resistance to aromatase inhibitors differs between steroidal and non-steroidal AIs. Yet, the literature describing resistance to aromatase inhibitors is limited to one study which uses a letrozole-resistant line. To date, no study has addressed differences between AI-resistance

mechanisms and variations that may exist between these signaling pathways. To address this question, microarray analysis has been employed to elucidate AI-resistance on a genome-wide basis, making this analysis an unbiased approach to study resistance. Previously, microarray analysis has been employed with tamoxifen-resistant cell lines, in an attempt to better understand the anti-estrogen resistance mechanism. It has been reported that genes involved in the apoptotic response, the growth factor signaling pathway and many estrogen-responsive genes were found to be differentially regulated in tamoxifen-resistant cells (52-54). Therefore, microarray analysis will be a useful tool to understand differences in drug resistance in steroidal versus non-steroidal aromatase inhibitors, in contrast to the anti-estrogen tamoxifen.

To check the quality of our microarray analysis, a hierarchical clustering analysis of the data has been carried out (Figure 5). As a crucial quality control assessment, we are very pleased with our analysis in which replicates of each type of resistant lines are clustered together. The results demonstrate the high quality of our data where similar genes are modulated in each type of resistant lines although they have different growth rates. The only exception is AroT.3 that is very different from AroT.1 and AroT.2, therefore, data of AroT.3 have been removed from our analysis. As expected, our results indicate that data of T+LetR, T+AnaR and T+ExeR lines are more similar than those of T+TamR and AroT lines. The data of AroT lines are very different from those T+TamR lines. Furthermore, the data of Aro lines are in a group different from the resistant lines. In addition, clustering analysis has revealed that the testosterone-containing resistant lines (T+LetR and T+AnaR) cluster separately from the inhibitor-only lines (no T) (i.e., LetR and AnaR). This does suggest that for non-steroidal inhibitors, inherent differences do exist between the hormone containing lines versus the inhibitor-only lines. Interestingly, the gene profiles of T+ExeR and ExeR are very similar.

While we are working very hard to functionally characterize our AI resistant cell lines, our results generated so far indicate that not only do inherent differences exist between AI and tamoxifen-resistant lines, but that AI-resistance does vary between the anastrozole, letrozole and exemestane-resistant cell lines.

In summary, resistance to AIs is emerging as a complex phenomenon, based on new experimental information discussed in this paper. Thus far, analysis of acquired resistance pathways has focused primarily on growth factor and nuclear receptor crosstalk. This information has been quite valuable, but may not be the complete story. In addition, previous studies using LTED of breast cancer cells has been viewed as a model system to understand AI-resistance. Yet, LTED will not address differences between resistance mechanisms to steroidal and non-steroidal AIs, as variations are suspected in these pathways. Therefore, analysis of a large panel of resistant cell lines by microarray, is an unbiased genome-wide examination of signaling pathways responsible for steroidal and non-steroidal AI-resistance. Acquired resistance to AIs is a hindrance in the clinic and better understanding the molecular mechanisms responsible for such occurrences will be beneficial for effectively treating hormone-dependent breast cancers. Furthermore, information generated from such studies will also help us better understand the mechanisms of De novo/intrinsic resistance, which may be similar to those for acquired resistance.

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Figure 1.
Structures of exemestane, anastrozole and letrozole.

Figure 2.

The effect of vehicle, testosterone (androgen, A), 17 β -estradiol, A + Letrozole, A + Anastrozole and A + Tamoxifen on the growth of MCF7aro cells. Cells were plated on 6-well plates and cultured in phenol red-free culture medium including 10% of charcoal/dextran-treated FBS for 3 days. Triplicate wells were then treated with the drugs and/or hormones. The cells were harvested with 0.5N NaOH on days 4, 7 and 10, and the values were expressed as mean of protein concentration $\pm$ Standard Deviation. [Itoh et al. (50)]

Figure 3.

The effect of inhibitors on the growth of resistant cells (T+LetR, T+AnaR, T+ExeR and T+TamR). The growth rates of resistant cells in the presence of testosterone and inhibitor (used for the selection) were compared to MCF-7aro cells that treated with 1nM testosterone. The treatments were performed in triplicate. The cells were harvested with 0.5N NaOH on days 4, 7 and 10, and the values were expressed as mean of protein concentration $\pm$ Standard Deviation.

Figure 4.
Cell proliferation rates of six lines of T+LetR. The cells were cultured in the presence of 1 nM testosterone + 200 nM letrozole. Cell proliferation was examined using the Real Time Cell Electronic Sensor (RT-CES) system (ACES Biosciences Inc, San Diego, CA).

Figure 5.

Hierarchical clustering of microarray data generated from our resistant cell lines. All the analysis was performed in R statistical environment v2.3.0 (<http://www.r-project.org>). The data were processed and normalized using GCRMA method implemented in Bioconductor package *gcrma* v2.4.1 (55). The expression values of the transcripts were median centered across arrays and Pearson correlation was used to calculate the distance. Average linkage method was used to generate the clusters and visualized in R. Removing transcripts with low intensities does not affect the hierarchical clustering results.

Table 1

Summary of Long Term Estrogen Deprived (LTED) lines and Letrozole Resistance Study

Reference	Aromatase Expression	Aromatase Activity	ER Expression	ER activity	ER phosphorylation	Growth Factors	Kinases Involved
Santen et al. (28)	Unavailable	Unavailable	Increased	Increased	Unavailable	IGF-1R	MAPK, PI3K/AKT
Martin et al (31)	Unavailable	Unavailable	Increased	Increased	S118 Increased	HER2 & IGF-1R	MAPKs
Sabnis et al. (34)	Unavailable	Unavailable	Increased	Unavailable	S167 Increased	HER2	PI3K/AKT
Staka et al. (33)	Unavailable	Unavailable	Increased	Increased	S118 Increased	Slight decrease IGF-1R	PI3K/AKT
Jelovac et al. * (42)	Unavailable	Unavailable	Decreased	Unavailable	S167 Increased	HER2	MAPK, PI3K/AKT

* Letrozole-Resistance Study