Published in final edited form as:

J Steroid Biochem Mol Biol. 2010 February 28; 118(4-5): 277–282. doi:10.1016/j.jsbmb.2009.10.011.

Molecular Characterization of Aromatase Inhibitor-Resistant, Tamoxifen-Resistant and LTEDaro cell lines

Selma Masri, Sheryl Phung, Xin Wang, and Shiuan Chen

Division of Tumor Cell Biology, Beckman Research Institute of the City of Hope, Duarte, CA

Abstract

To determine potential genes involved in mediating resistance to aromatase inhibitors (AI), a microarray study was performed using MCF-7aro (aromatase overexpressing) cells that are resistant to letrozole (T+LET R), anastrozole (T+ANA R) and exemestane (T+EXE R), as well as LTEDaro and tamoxifen-resistant (T+TAM R) lines for comparison. Based on hierarchical clustering, estrogen-responsive genes were found to be differentially expressed in AI-resistant lines versus LTEDaro and T+TAM R. Additional genome-wide analysis showed that gene expression profiles of the non-steroidal AI-resistant lines were most closely correlated and that T+EXE R lines exhibit differing profiles. Also, LTEDaro and T+TAM R lines are inherently different from expression profiles of AI-resistant lines. Further characterization of these resistant lines revealed that T+LET R, T+ANA R and LTEDaro cells contain a constitutively active estrogen receptor α (ER α) that does not require the ligand estrogen for activation. Ligand-independent activation of ER α does not activate identical estrogen-responsive gene profiles in AI-resistant lines as in LTEDaro lines, thereby establishing differing mechanisms of resistance. This ligand-independent activation of ER was not observed in the parental cell lines MCF-7aro, T+EXE R or T+TAM R cells. Based on the steroidal structure of EXE, our laboratory has shown that this AI has weak estrogen-like properties, and that EXE resistance involves an ER-dependent crosstalk with EGFR growth factor signaling. Recent studies in our laboratory pertaining to pre-clinical models of AI treatment revealed that intermittent use of EXE delays the onset of acquired resistance in comparison to continuous treatment. Specific molecular mechanisms involved in intermittent use of EXE are currently being explored, based on microarray gene expression profiling. Lastly, our laboratory has initiated a study of microRNAs and their potential role in regulating target genes involved in AI-resistance. Overall, we propose a model of acquired resistance that progresses from hormone-dependence (T+TAM R and T+EXE R) to hormone-independence (T+LET R and T+ANA R), eventually resulting in hormone-independence that does not rely on conventional ER signaling (LTEDaro).

Introduction

The mitogenic effects of estrogen play a crucial role in mediating survival and proliferation of hormone-dependent breast cancer. Targeting estrogen-dependent growth pathways using endocrine therapy agents such as tamoxifen and aromatase inhibitors (AI) have shown good efficacy in treating hormone-responsive breast cancer. Tamoxifen functions as an antagonist of the estrogen receptor (ER), whereby binding of tamoxifen competes with the estrogen ligand to prevent hormone-dependent activation of ER [1]. In contrast, AIs diminish estrogen production by inhibition of the aromatization reaction that converts androgens into estrogens.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Currently, there are three FDA approved AIs available for the clinical treatment of post-menopausal and hormone-responsive breast cancer patients. The steroidal AI exemestane has structural similarity to the androgen substrate of aromatase and is a mechanism-based inhibitor which requires the catalytic ability of aromatase to yield an active intermediate [2,3]. The non-steroidal AIs letrozole and anastrozole are structurally unique and contain a triazole functional group that interacts with the heme prosthetic group of aromatase for inhibition [2]. The AIs approved for clinical use are effective at inhibiting aromatase at very low doses and depending on the inhibitor, can achieve significant whole-body estrogen depletion.

The increased efficacy of aromatase inhibitors (AI) over tamoxifen therapy has been demonstrated by clinical trials, whereby a significant increase in disease-free survival has been shown using three third-generation aromatase inhibitors [4–7]. In addition, these major clinical trials, including ATAC, BIG 1–98, MA 17 and IES, demonstrated a significant decrease in the cases of contralateral breast cancer in the AI treated arm versus tamoxifen arm. Though these endocrine therapy agents are clinically effective, the main hindrance is acquired resistance to both tamoxifen and AIs after long-term exposure. Studies pertaining to tamoxifen-resistance, long-term estrogen deprivation (LTED) and letrozole-resistant model systems have provided some mechanistic information that can be applied towards understanding resistance to AIs, yet the specific molecular characteristics of AI-resistance are not fully elucidated.

To further understand mechanisms of resistance to endocrine therapy agents, our laboratory is studying the following aspects of acquired resistance. 1) Genome-wide expression profiling was performed using microarray analysis to determine genes that were differentially expressed in AI or tamoxifen-resistant cells lines, which would potentially confer resistance to these agents [8]. 2) Exemestane was further studied and in addition to its action as an aromatase inhibitor, this agent also has weak ER α agonist properties [9]. 3) Looking further at the mechanisms of acquired resistance to exemestane, our laboratory has identified the role of the growth factor amphiregulin in driving an ER and EGFR dependent crosstalk responsible for survival of these cells [10]. 4) Using the MCF-7aro cell line, which is suitable for studying acquired resistance in an ER+ and aromatase expressing model, we have demonstrated that the intermittent use of exemestane is effective in delaying the onset of acquired resistance. 5) Lastly, we have initiated a microRNA study to determine the role of small RNAs in post-transcriptional mechanisms of regulation involved in acquired resistance to AIs.

1. Genome-wide expression profiling of AI-resistance

Using the hormone-responsive breast cancer cell line, MCF-7, our laboratory overexpressed aromatase (MCF-7aro) to obtain a suitable system to study acquired resistance to endocrine therapy agents [11]. Using the parental MCF-7aro cells, derivative resistant lines were generated with long-term treatment of letrozole (T+LET R), anastrozole (T+ANA R) and exemestane (T+EXE R), as well as LTEDaro and tamoxifen-resistant (T+TAM R) lines for comparison. As an additional control, the testosterone-only lines (T cells) were grown in the presence of hormone only. Multiple biological replicates were generated for each type of resistant line for statistical analysis of microarray data. To determine that these cells had acquired resistance to their inhibitor, cell proliferation studies were carried out to show that resistant lines proliferated similar to the MCF-7aro parental lines [12]. Real-time PCR as well as western analysis showed that no major changes had occurred in aromatase or ER α mRNA and protein levels in the LTEDaro or resistant cell lines, as compared with MCF-7aro. The aromatase enzyme was still functional, as determined by an in-cell aromatase activity assay, with the exception of T+EXE R lines that lose aromatase activity as well as protein expression, due to mechanism-based inhibition and destabilization of the aromatase protein, respectively [3,13]. Also, the resistant cell lines were responsive to ICI treatment in cell proliferation assays [8].

Using an unbiased approach to determine pathways involved in mediating resistance to AIs, affymetrix microarray chips were utilized to determine which genes were differentially expressed between parental MCF-7aro and derivative resistant lines. Significantly expressed genes were determined using RMA transformation followed by 1-way ANOVA analysis, with a false discovery rate (FDR) <1% (for detailed methodology, [8]). Hierarchical clustering was used to visualize these 264 significant genes, with the AI-resistant lines clustering separately from the LTEDaro and T+TAM R lines. Also, of the AI-resistant lines, non-steroidal T+LET R and T+ANA R cells clustered more closely than the steroidal T+EXE R lines. Similar results were observed using correlation coefficients visualized with a similarity matrix, showing higher correlation with the AI-resistant lines than with LTEDaro or T+TAM R (Figure 1). Using two known estrogen-responsive gene databases [8], we determined that a large number of the significantly regulated genes in our clustering analysis were hormone-responsive but that the expression of these genes was not identical in all cell lines. Three major types of estrogen-responsive genes were observed in our hierarchical clustering, up-regulated in all cell lines (e.g., CTSD, TFF1, CCND1, BIRC5), up-regulated in all lines except LTEDaro (e.g., PGR, GJA1, GREB1, PDZK1), and up-regulated in all lines except LTEDaro and T+TAM R (e.g., MGP, EGR3, AREG, CA2). This data suggests inherent differences between AI-resistant lines versus expression profiles of LTEDaro and T+TAM R, while also emphasizing the pivotal role ER plays in resistance.

Based on the function of AIs in suppressing estrogen production, it would be expected that treatment with these inhibitors would suppress or down-regulate expression of estrogen-responsive genes. Our microarray data demonstrates that hormone-responsive genes are not affected by AI treatment, confirming that these cells have acquired resistance. Looking at the top 20 up-regulated genes in our cell lines, the majority are estrogen-responsive genes and show high levels of expression in the AI-resistant lines, but show minimal up-regulation or even down-regulation in the LTEDaro lines (Supplementary Table S3, ref [8]). In addition, a group of 70 significant genes were selected based on up-regulation only in the LTEDaro lines, and of which, 40% were estrogen-responsive (Supplementary Table S6, [8]). This select number of hormone-responsive genes was not found in the other AI-resistant lines, and suggests an altered mechanism of ER-dependent transcriptional activity or even ER-independent action present only in the LTEDaro system.

The action of ER was further investigated in our resistant lines, due to the microarray data that suggested altered functionality of the nuclear receptor. Using a luciferase-based activity assay to assess endogenous ER functionality, it was found that T+LET R, T+ANA R and LTEDaro contained a constitutively active ER α that did not require the addition of the ligand 17- β estradiol for activation [8]. This was not found in the hormone-dependent parental MCF-7aro, T+EXE R or T+TAM R lines. To validate this result, chromatin immunoprecipitation (ChIP) assays were performed to functionally confirm ER promoter occupancy of an estrogen-responsive gene (TFF1 or pS2). Similar results were observed to the ER activity assays, where the T+LET R, T+ANA R and LTEDaro lines showed a hormone-independent recruitment of ER to the estrogen-response element (ERE) while the control, T+EXE R and T+TAM R lines retain hormone-dependent ER functionality. These results were further confirmed with western blot analysis that demonstrated serine 118 phosphorylation of ER α , a mark of hormone-independent activation, in the T+LET R, T+ANA R and LTEDaro lines.

The microarray analysis of our AI and tamoxifen-resistant cell lines was extremely useful in determining gene profile signatures that have been altered, especially those genes that are estrogen-responsive and potentially driving survival and proliferation of resistant breast cancer cells. In cells that have acquired resistance to endocrine therapy agents, ER is still a crucial regulator of cell viability, whether in a hormone-dependent system (T+TAM R and T+EXE R) or in a hormone-independent system (T+LET R, T+ANA R and LTEDaro). This work not

only establishes inherent differences between AI-resistance versus LTEDaro and tamoxifen-resistance, but also suggests the mechanisms of resistance between steroidal and non-steroidal AIs are not identical.

2. Exemestane as a weak ER agonist

Microarray analysis that was performed to determine differentially expressed genes involved in AI-resistance revealed new information about the steroidal AI exemestane (EXE). Using a similarity matrix that compares genome-wide gene expression correlation coefficients of resistant lines, we observed that the EXE O resistant lines, that do not contain hormone, did correlate with the hormone-containing T lines (Figure 2). In contrast, both ANA O and LTEDaro did not correlate with the T cell lines, suggesting the correlation between EXE O and T lines is specific to exemestane. Analysis of differentially regulated genes revealed a large number of estrogen-regulated genes, especially in the EXE O lines versus the ANA O cells [9]. In addition, the fold-changes of estrogen-responsive genes were significantly greater than expression values of these same genes in ANA O lines, suggesting the ER-dependent transcription in the absence of ligand in the EXE O lines. It is important to point out that androgen-responsive genes were not found to be differentially expressed in the EXE O, with the exception of KLK11.

Using the hormone-responsive breast cancer cell lines, MCF-7 and T47D, we further validated the effect of exemestane on ER function. Both cell lines contain very low levels of aromatase activity, but endogenously express ER α , while ER β is expressed moderately in T47D with low expression levels in MCF-7. Exemestane was able to active ER activity in a luciferase-based reporter assay, at a 1 μ M concentration. This exemestane-mediate ER activity was blocked using the pure ER antagonist ICI as well as the ER α -specific antagonist MPP, but not with the ER β -selective antagonist, PHTPP [9]. Using our MCF-7aro/ERE stable cell line that is a dual reporter system of both aromatase activity as well as ER α functionality, dose-response evaluation of exemestane, in comparison to T and E2 was performed. Based on these dose-response studies, the effective agonist concentration of exemestane is 1000 times less potent than both T (once converted to E2) and E2 supplemented directly into the system. Additionally, exemestane was able to induce proliferation of hormone-responsive MCF-7 and MCF-7aro cells as well as transactivation of ER target genes, PGR and pS2 at the 1 μ M concentration. Similar to the microarray data, these experiments suggest that exemestane does act as a weak agonist of ER α .

This information does establish a unique property of exemestane which may explain key differences that have been observed related to side-effects with steroidal versus non-steroidal AIs in the clinic. Loss in bone mineral density (BMD) and increases in bone fracture rates have often been reported with AI treatment, which are a result of estrogen depletion [14–16]. Yet, recent reports reveal that the effects of exemestane on bone are less severe than non-steroidal AI therapy (using letrozole or anastrozole), and that rates of bone fractures were lower in exemestane-treated patients [5,17]. In addition, Goss *et al.* [18] compared short-term (24 weeks) exemestane versus letrozole and anastrozole treatment on bone turnover markers. Results showed that high levels of procollagen type I N-terminal propeptide (PINP), a serum marker of bone formation, were present in exemestane-treated patients, but not in non-steroidal AI-treated patients. Our data potentially supports the idea that the hormone-like property of exemestane may have a protective effect on BMD profiles, which are adversely effected by AI treatment and estrogen deprivation.

3. Amphiregulin-driven exemestane-resistance

Gene expression profiling by microarray was also useful in the identification of crucial genes/pathways involved in acquired resistance to exemestane. Up-regulation of amphiregulin

(AREG) was most elevated in exemestane-resistant lines, though other hormone-containing AI-resistant cells or T-only lines do express AREG. AREG is a known estrogen-responsive gene [19,20], and expression of AREG was not seen in LTEDaro lines (hormone-free), though AREG was highly expressed in hormone-free EXE O resistant lines. Based on the hormone-like properties of exemestane, this result is expected, though we speculated whether AREG is responsible for driving the proliferation of exemestane-resistant lines specifically.

AREG is a growth factor ligand of the epidermal growth factor (EGF) family that binds and activates EGFR, which has a known function in breast cancer survival [21,22]. Our laboratory also demonstrated by western blot analysis that in the MCF-7aro cells, expression of AREG is induced by both testosterone (T) alone as well as exemestane alone [10]. This expression was inhibited with the addition of the ER antagonist ICI. In addition, using ELISA to detect AREG protein levels secreted in the media of resistant lines, significantly higher levels of AREG were seen in EXE O lines versus LTEDaro. To determine the importance of AREG in driving cell growth of EXE O resistant cells, a siRNA was used to block AREG expression followed by cell proliferation assays. In the EXE O cells where the siRNA targeting AREG was used, cell proliferation was significantly inhibited compared to a siRNA scrambled control that did not affect proliferation [10]. In contrast, the proliferation of LTEDaro cells when transfected with the same siRNA targeting human AREG was unaffected. Similarly, use of human recombinant AREG (hrAREG) was able to significantly drive the proliferation of parental MCF-7aro cells [10].

Using various small molecule inhibitors in cell proliferation assays, the role of different signaling pathways was delineated in exemestane-resistant lines. As expected, the ER antagonist ICI significantly decreased cell growth of the EXE O lines, in a dose-dependent manner [10]. The EXE O cells are believed to rely on ER-mediated signaling, especially because of the hormone-like nature of exemestane which can activate ER directly. In addition, the EGFR-specific inhibitor AG1478 as well as the MAP kinase MEK inhibitor UO126, both significantly and dose-dependently decreased proliferation of the EXE O resistant lines [10]. Based on this data, a model of exemestane-resistance has been developed where exemestane itself can bind to ER and activate expression of the estrogen-responsive gene AREG. AREG can then bind and activate EGFR-dependent signaling, which is mediated by the survival properties of the MAP kinase pathway. This data provides novel information about the mechanism of exemestane-resistance, which is different from pathways involved in non-steroidal AI-resistance. In addition, signal transduction inhibitors targeting EGFR can potentially be used as a treatment option for exemestane-resistant breast cancers.

4. Intermittent treatment of exemestane to delay acquired resistance

Based on the hormone-like property of exemestane and its inherent ability to activate AREG and the EGFR pathway, we examined whether intermittent treatment with exemestane could delay the onset of acquired resistance. Using the MCF-7aro hormone-responsive cell line that overexpresses aromatase, we treated these cells with testosterone (T) and exemestane (EXE). As a control for the development of acquired resistance, EXE was added throughout the entire selection process, and these cells were called T+EXE continuous. In contrast, cells were treated continuously with T and intermittently with EXE, 1 week ON/1 week OFF EXE, 2 weeks ON/1 week OFF and 3 weeks ON/1 week OFF. Cell viability was monitored by number of total cells under each condition, with three biological replicates generated simultaneously for each treatment type.

In the T+EXE continuous condition, cells acquired resistance to EXE treatment at 12–14 weeks. In contrast, the T+EXE intermittent cells under all three conditions showed a delay in acquired resistance to EXE, which was consistent up to 34 weeks. Identical experiments were carried out with letrozole and anastrozole, with no added benefit observed between intermittent

treatment versus continuous treatment. Total RNA was harvested from replicates of MCF-7aro control cells, the T+EXE continuous and the T+EXE intermittent cells (2 weeks ON/1 week OFF) for microarray analysis. Genome-wide profiling revealed that numerous significant genes were differentially expressed between the continuous and intermittent T+EXE treated cells. As with previous microarray analysis, estrogen-responsive genes were found among the top regulated genes, which are crucial in survival pathways of breast cancer cells. Of particular interest, AREG expression was much higher in the T+EXE continuous cells versus the intermittent cells both with microarray as well as real-time PCR confirmation. As previously discussed, we have implicated the estrogen-responsive gene AREG in the EGFR-dependent growth of exemestane-resistant cells. Based on the decreased expression of AREG as well as other estrogen-responsive genes in intermittent cells versus continuous cells, the ON/OFF scheduling of exemestane may hinder the initiation of acquired resistance. We are currently further exploring results obtained from this microarray study to elucidate genes implicated in the delay of acquired resistance.

The idea of intermittent treatment has been suggested to delay drug-resistance, though results from clinical trials will be more definitive. Sabnis *et al.* have reported a model where stopping letrozole treatment re-sensitizes letrozole-resistant tumors to letrozole [23]. Though this study is not identical to intermittent treatment at the onset of exposure to letrozole, there is still a lesson to be learned regarding scheduling of endocrine therapy agents. In terms of a prostate model, intermittent androgen ablation therapy has been used to delay the onset of a more aggressive androgen-independent phenotype. This was done both in the Shionogi tumor model where time to progression was prolonged 3-fold [24], as well as LNCaP prostate cancer cells where androgen-independence was delayed with intermittent treatment, using prostate-specific antigen (PSA) as a marker of androgen-response [25]. Based on these ideas as well as preliminary data from our laboratory, intermittent treatment may provide a valuable therapy option to prolong responsiveness to aromatase inhibitors.

5. microRNA expression profiling in AI-resistant lines

Our laboratory has examined transcriptional profiles of genes involved in AI-resistance by microarray. We next decided to analyze pathways involved in resistance that may be regulated by microRNAs, an additional level of control that may be post-transcriptional. Recent reports have cited the role of microRNAs in resistance to tamoxifen as well as chemotherapeutic agents, though microRNA involvement in AI-resistance has not been reported. Zhao *et al.* identified miR-221/222 in the negative regulation of ER which subsequently confers breast cancer cells resistant to tamoxifen [26]. Miller *et al.* also found that miR-221/222 is up-regulated in tamoxifen-resistant versus tamoxifen-sensitive breast cancer cells and that miR-221/222 is significantly overexpressed in HER2+ breast cancers that are *de novo* resistant to endocrine therapy [27]. In addition to tamoxifen-resistance, numerous microRNAs have been reported to be involved in multi-drug resistance or resistance to chemotherapy agents in breast, neuroblastoma, gastric, lung and ovarian cancers [28–32].

Microarray work was performed in our laboratory to elucidate microRNAs that are differentially expressed between the parental MCF-7aro cells in comparison to the AI-resistant lines. In terms of global profiles, significantly expressed microRNAs differed among the AI-resistant, LTEDaro and tamoxifen-resistant cells, as compared to MCF-7aro. This observation is opposite to results obtained with gene expression profiling, where AI-resistant lines were highly correlated in terms of significantly regulated genes. At a preliminary level, this data suggests that microRNA expression is unique to each type of resistant line. We are currently working to identify gene targets of significantly regulated microRNAs, to better understand potential novel pathways involved in AI-resistance. This microarray data has also been valuable in the identification of novel hormone-responsive microRNAs. Comparing

microRNA profiles of the T-only cells with the parental MCF-7aro as well as hormone-free LTEDaro lines, we identified 48 microRNAs that are estrogen-responsive and therefore up-regulated in the T lines. Though this work is preliminary, we have generated new data about microRNAs and their role in AI-resistance.

Concluding Remarks

Using a cell culture system, we established a pre-clinical model that replicates AI-resistance in the laboratory. This system has provided information regarding genome-wide gene expression profiles that are unique to AI-resistant cells, LTEDaro and tamoxifen-resistant lines, and has confirmed the pivotal role of ER α in determining these gene signatures. Based on estrogen-responsive gene profiles using microarray, the functionality of ER α was suggested to be altered, establishing inherent differences in hormone-dependence of these resistant lines. The non-steroidal AI resistant cells contain a constitutively active ER α that renders these cells hormone-independent, yet phosphorylation of the nuclear receptor is responsible for driving ER-dependent survival and proliferation pathways. In contrast, the LTEDaro lines do contain a constitutively active ER α , but based on microarray analysis, few estrogen-responsive genes are significantly differentially expressed compared to parental MCF-7aro. Alternatively, a select number of estrogen-responsive genes, not found in other resistant lines, were identified in LTEDaro cells. This suggests that LTEDaro lines are not only hormone-independent with a constitutively active ER α , but that the transcriptional program of ER is altered and these cells have adapted non-hormone dependent pathways for survival. This work does reveal inherent differences in mechanisms of signaling for survival and growth adaptation between LTEDaro versus T+LET R and T+ANA R lines.

The steroidal AI exemestane is not only functionally and structurally unique from its non-steroidal counterparts, but also was identified by our laboratory to have weak estrogenic properties. In addition, our group elucidated a mechanism of exemestane-resistance that relies on the hormone-like property of this AI to activate the estrogen-responsive gene AREG, which drives EGFR dependent signaling and subsequent cell growth. Based on this data, as well as ER functionality studies, our laboratory established that exemestane-resistant lines are hormone-dependent, unlike the non-steroidal AI-resistant cells. Our working model for endocrine therapy resistance is based on a spectrum of estrogen-dependence. The hormone-dependent tamoxifen as well as exemestane-resistant lines differ from the hormone-independence letrozole and anastrozole-resistant lines, while the late-stage LTEDaro cells bypass the need for ER-dependent signaling (Figure 3).

The next critical direction for this pre-clinical work is to shift from model systems into examination of clinical material with AI-resistant breast tumors. Nevertheless, the use of cell culture systems has provided a subset of data that can be further investigated with patient material from the clinic. Laboratory studies have proven to be useful in confirming clinical results, and we have utilized this idea to perform cross-resistance studies to determine response of endocrine therapy resistant lines to a second-line inhibitor (Figure 4). The hormone-responsive tamoxifen-resistant cells respond extremely well to second-line treatment with an AI. Additionally, the AI-resistant lines significantly respond to second-line treatment with another AI, but not with tamoxifen. This suggests that AI-resistant breast cancers may respond well to a change in AI treatment, a potential therapy option in the clinic. The LTEDaro lines represent a very late stage breast cancer that no longer responds to endocrine therapy, as shown by second-line treatment with an AI or tamoxifen, potentially due to altered ER signaling pathways as previously discussed. Overall, this data provides insight into differences in mechanisms of AI-resistance versus that of LTEDaro and tamoxifen-resistance, as well as valuable information for sequencing of endocrine therapy agents in the clinic.

References

1. Fabian CJ, Kimler BF. Selective estrogen-receptor modulators for primary prevention of breast cancer. *J Clin Oncol* 2005;23(8):1644–1655. [PubMed: 15755972]
2. Brueggemeier RW, Hackett JC, Diaz-Cruz ES. Aromatase inhibitors in the treatment of breast cancer. *Endocr Rev* 2005;26(3):331–345. [PubMed: 15814851]
3. Hong Y, Yu B, Sherman M, Yuan YC, Zhou D, Chen S. Molecular basis for the aromatization reaction and exemestane-mediated irreversible inhibition of human aromatase. *Mol Endocrinol* 2007;21(2):401–414. [PubMed: 17095574]
4. Coates AS, Keshaviah A, Thurlimann B, Mouridsen H, Mauriac L, Forbes JF, Paridaens R, Castiglione-Gertsch M, Gelber RD, Colleoni M, et al. Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer: update of study BIG 1–98. *J Clin Oncol* 2007;25(5):486–492. [PubMed: 17200148]
5. Coombes RC, Hall E, Gibson LJ, Paridaens R, Jassem J, Delozier T, Jones SE, Alvarez I, Bertelli G, Ortmann O, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *N Engl J Med* 2004;350(11):1081–1092. [PubMed: 15014181]
6. Goss PE, Ingle JN, Martino S, Robert NJ, Muss HB, Piccart MJ, Castiglione M, Tu D, Shepherd LE, Pritchard KI, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. *N Engl J Med* 2003;349(19):1793–1802. [PubMed: 14551341]
7. Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, Hocht-Boes G, Houghton J, Locker GY, Tobias JS. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 2005;365(9453):60–62. [PubMed: 15639680]
8. Masri S, Phung S, Wang X, Wu X, Yuan YC, Wagman L, Chen S. Genome-wide analysis of aromatase inhibitor-resistant, tamoxifen-resistant, and long-term estrogen-deprived cells reveals a role for estrogen receptor. *Cancer Res* 2008;68 (12):4910–4918. [PubMed: 18559539]
9. Masri S, Lui K, Phung S, Ye J, Zhou D, Wang X, Chen S. Characterization of the weak estrogen receptor alpha agonistic activity of exemestane. *Breast Cancer Res Treat.* 2008
10. Wang X, Masri S, Phung S, Chen S. The role of amphiregulin in exemestane-resistant breast cancer cells: evidence of an autocrine loop. *Cancer Res* 2008;68 (7):2259–2265. [PubMed: 18381432]
11. Sun XZ, Zhou D, Chen S. Autocrine and paracrine actions of breast tumor aromatase. A three-dimensional cell culture study involving aromatase transfected MCF-7 and T-47D cells. *J Steroid Biochem Mol Biol* 1997;63(1–3):29–36. [PubMed: 9449203]
12. Chen S, Masri S, Wang X, Phung S, Yuan YC, Wu X. What do we know about the mechanisms of aromatase inhibitor resistance? *J Steroid Biochem Mol Biol* 2006;102(1–5):232–240. [PubMed: 17055257]
13. Wang X, Chen S. Aromatase destabilizer: novel action of exemestane, a food and drug administration-approved aromatase inhibitor. *Cancer Res* 2006;66 (21):10281–10286. [PubMed: 17079446]
14. Chien AJ, Goss PE. Aromatase inhibitors and bone health in women with breast cancer. *J Clin Oncol* 2006;24(33):5305–5312. [PubMed: 17114665]
15. Lonnig PE. Bone safety of aromatase inhibitors versus tamoxifen. *Int J Gynecol Cancer* 2006;16 (Suppl 2):518–520. [PubMed: 17010062]
16. Perez EA. Safety profiles of tamoxifen and the aromatase inhibitors in adjuvant therapy of hormone-responsive early breast cancer. *Ann Oncol* 2007;18(Suppl 8):viii26–35. [PubMed: 17890211]
17. Coleman RE, Banks LM, Girgis SI, Kilburn LS, Vrdoljak E, Fox J, Cawthorn SJ, Patel A, Snowdon CF, Hall E, et al. Skeletal effects of exemestane on bone-mineral density, bone biomarkers, and fracture incidence in postmenopausal women with early breast cancer participating in the Intergroup Exemestane Study (IES): a randomised controlled study. *The lancet oncology* 2007;8 (2):119–127. [PubMed: 17267326]
18. Goss PE, Hadji P, Subar M, Abreu P, Thomsen T, Banke-Bochita J. Effects of steroidal and nonsteroidal aromatase inhibitors on markers of bone turnover in healthy postmenopausal women. *Breast Cancer Res* 2007;9(4):R52. [PubMed: 17692126]

19. Frasor J, Stossi F, Danes JM, Komm B, Lyttle CR, Katzenellenbogen BS. Selective estrogen receptor modulators: discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells. *Cancer Res* 2004;64(4):1522–1533. [PubMed: 14973112]
20. Martinez-Lacaci I, Saceda M, Plowman GD, Johnson GR, Normanno N, Salomon DS, Dickson RB. Estrogen and phorbol esters regulate amphiregulin expression by two separate mechanisms in human breast cancer cell lines. *Endocrinology* 1995;136(9):3983–3992. [PubMed: 7649107]
21. Berasain C, Castillo J, Perugorria MJ, Prieto J, Avila MA. Amphiregulin: a new growth factor in hepatocarcinogenesis. *Cancer letters* 2007;254(1):30–41. [PubMed: 17321672]
22. Shoyab M, Plowman GD, McDonald VL, Bradley JG, Todaro GJ. Structure and function of human amphiregulin: a member of the epidermal growth factor family. *Science* 1989;243(4894 Pt 1):1074–1076. [PubMed: 2466334]
23. Sabnis GJ, Macedo LF, Goloubeva O, Schayowitz A, Brodie AM. Stopping treatment can reverse acquired resistance to letrozole. *Cancer Res* 2008;68 (12):4518–4524. [PubMed: 18559495]
24. Akakura K, Bruchovsky N, Goldenberg SL, Rennie PS, Buckley AR, Sullivan LD. Effects of intermittent androgen suppression on androgen-dependent tumors. Apoptosis and serum prostate-specific antigen. *Cancer* 1993;71 (9):2782–2790. [PubMed: 7682149]
25. Hsieh JT, Wu HC, Gleave ME, von Eschenbach AC, Chung LW. Autocrine regulation of prostate-specific antigen gene expression in a human prostatic cancer (LNCaP) subline. *Cancer Res* 1993;53 (12):2852–2857. [PubMed: 7684949]
26. Zhao JJ, Lin J, Yang H, Kong W, He L, Ma X, Coppola D, Cheng JQ. MicroRNA-221/222 Negatively Regulates Estrogen Receptor{alpha} and Is Associated with Tamoxifen Resistance in Breast Cancer. *J Biol Chem* 2008;283(45):31079–31086. [PubMed: 18790736]
27. Miller TE, Ghoshal K, Ramaswamy B, Roy S, Datta J, Shapiro CL, Jacob S, Majumder S. MicroRNA-221/222 Confers Tamoxifen Resistance in Breast Cancer by Targeting p27Kip1. *J Biol Chem* 2008;283(44):29897–29903. [PubMed: 18708351]
28. Kovalchuk O, Filkowski J, Meservy J, Ilnytsky Y, Tryndyak VP, Chekhun VF, Pogribny IP. Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin. *Mol Cancer Ther* 2008;7 (7):2152–2159. [PubMed: 18645025]
29. Fontana L, Fiori ME, Albini S, Cifaldi L, Giovannazzi S, Forloni M, Boldrini R, Donfrancesco A, Federici V, Giacomini P, et al. Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. *PLoS ONE* 2008;3(5):e2236. [PubMed: 18493594]
30. Xia L, Zhang D, Du R, Pan Y, Zhao L, Sun S, Hong L, Liu J, Fan D. miR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. *International journal of cancer* 2008;123(2):372–379.
31. Garofalo M, Quintavalle C, Di Leva G, Zanca C, Romano G, Taccioli C, Liu CG, Croce CM, Condorelli G. MicroRNA signatures of TRAIL resistance in human non-small cell lung cancer. *Oncogene* 2008;27(27):3845–3855. [PubMed: 18246122]
32. Yang H, Kong W, He L, Zhao JJ, O'Donnell JD, Wang J, Wenham RM, Coppola D, Kruk PA, Nicosia SV, et al. MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. *Cancer Res* 2008;68(2):425–433. [PubMed: 18199536]

Figure 1. Similarity matrix for resistant cell lines

Similarity matrix visually displaying correlation coefficients for the resistant cell lines using Partek Genomics Suite. Correlation coefficients range from 0.36 to 0.95, using MCF-7aro parental cells for baseline transformation of gene expression values for all biological replicates.

Figure 2. Similarity matrix of hormone and non-hormone containing resistant lines

EXE and ANA-resistant lines, as well as T-Only (T) and LTEDaro controls, were normalized with parental MCF-7aro and the correlation coefficients of 5007 significant genes were displayed as a similarity matrix, using Pearson's correlation. Correlation coefficients ranged between 0.5 to 0.98, with red indicating good correlation and green representing less correlated lines.

Figure 3.
Working model for endocrine therapy resistance.

Figure 4. Cross-resistance studies of resistant lines

Resistant cell lines were treated second-line with either DMSO, LET, ANA, EXE or TAM to assess response after acquired resistance to first-line agent. Cross-resistance was determined using cell proliferation assays, with protein concentration as a reference. Cell growth was shown as a percent of control (resistant line treated with same inhibitor), with 3 independent experiments shown. Student's t-test was used for statistical analysis in comparison to control. ** indicates p-value < 0.001 and * indicates p-value < 0.01.