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Pathways to Tamoxifen Resistance

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Abstract

Therapies that target the synthesis of estrogen or the function of estrogen receptor(s) have been developed to treat breast cancer. While these approaches have proven to be beneficial to a large number of patients, both *de novo* and acquired resistance to these drugs is a significant problem. Recent advances in our understanding of the molecular mechanisms that contribute to resistance have provided a means to begin to predict patient responses to these drugs and develop rational approaches for combining therapeutic agents to circumvent or desensitize the resistant phenotype. Here, we review common mechanisms of antiestrogen resistance and discuss the implications for prediction of response and design of effective combinatorial treatments.

Keywords

Tamoxifen; antiestrogen; aromatase inhibitor; fulvestrant; estrogen receptor

1. Endocrine therapies for breast cancer

Estrogen and the steroid estrogen receptors (ERs) are critical regulators of breast epithelial cell proliferation, differentiation, and apoptosis. Mammals express two ERs, ER α and ER β , which show distinct tissue distributions and functions (for review, see [1]). Mice with targeted deletions of one or both ER genes have established that ER α is the key regulator of mammary gland development. ER α is expressed in 15-30% of the luminal epithelial cells present in normal breast tissue; estrogen stimulation of these cells results in transcription of various genes, including many that are involved in cell cycle regulation (for review, see [2]). However, estrogen-dependent proliferation of breast epithelial cells is thought to occur in a paracrine fashion, such that ER α -containing cells produce growth factors that induce proliferation in adjacent ER-negative cells. In malignant breast tissue, the action of estrogen is deregulated, resulting in a shift to proliferation without differentiation or apoptosis. The percentage of epithelial cells expressing ER α is significantly increased under these conditions. Moreover, these cells now proliferate, marking a shift from paracrine to autocrine growth.

ER β is more ubiquitously expressed throughout mammary tissue than is ER α [3]. ER β is often found co-expressed with ER α in the luminal epithelial cells but has also been detected in myoepithelial cells and surrounding stromal cell nuclei. While its specific function(s) in normal

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and neoplastic tissues remain unknown, ER β is essential for the fully differentiated phenotype of the normal mammary gland in mice [4]. In ER β $-/-$ mice, the mammary gland develops normally until puberty. However, several abnormalities are apparent during pregnancy and lactation, including incomplete penetration of the fat pad by glandular tissue, an abnormally large increase in the size of the lumen of ducts and alveoli, and a decrease in the total number of alveoli. Also, in luminal epithelial cells of these mice, the loss of ER β disrupts the formation of tight junctions and alters the expression of β -catenin, features often associated with a malignant phenotype. It has been suggested that the function of ER β in these processes may contribute to the protection conferred by pregnancy and lactation against breast cancer.

Several reports describe a loss of ER β during carcinogenesis (for review see [5,6]), providing support for its possible role as a tumor suppressor. Indeed, ER β has been reported to suppress the function of ER α as a transcriptional activator when both receptors are co-expressed [5,7] and to promote anti-proliferative and pro-apoptotic effects [8]. Several genes have been identified that are regulated by ER β but not ER α ; studies are underway to determine whether these genes may have anti-proliferative or pro-apoptotic properties that could account for the putative tumor suppressor function of ER β [5,9,10].

A majority of invasive breast tumors express ER α , and as discussed above, ER β is often down-regulated. These findings provide a strong rationale for therapies that disrupt the actions of estrogen and ER α . Current approaches include inhibiting the function of ERs with the use of antiestrogens such as tamoxifen, raloxifene, and ICI 182,780 (faslodex, fulvestrant), or blocking the conversion of androgens into biologically active estrogens through the action of aromatase inhibitors such as letrozole, anastrozole, and exemestane (see Section 1.1). Recent studies on ER β have also highlighted its growing value as a diagnostic and prognostic marker in breast cancer and its potential for use as a new strategy for delaying and retarding breast tumorigenesis (see Section 1.1.1).

1.1. Classes of therapeutic agents

1.1.1. Selective Estrogen Receptor Modulators (SERMs)—SERMs have been developed as agents that function as ER antagonists in some tissues, including breast, and agonists in other tissues such as the heart and bone (for review, see [11]). The antagonist effects of tamoxifen in breast tissue are thought to result from its ability to bind to the ligand-binding domain (LBD) of the ER, effectively blocking the potential for estrogen stimulation. Tamoxifen binding further prevents critical ER conformational changes that are required for the association of coactivators. However, tamoxifen treatment is associated with an increased risk of endometrial cancer [12]. Other SERMs, such as raloxifene, have been developed with the goal of reducing some of the deleterious effects of tamoxifen.

New classes of SERMs are being developed that exhibit mixed agonist/antagonist activities that are subtype specific (“dual SERMs”) or preferentially affect one ER over the other (“subtype-selective SERMs”) (reviewed in [13]). However, it is important to note that selectivity is often assessed in the context of binding affinity relative to 17 β -estradiol. Therefore, a “selective” compound may actually interact with both receptors but have a higher affinity for one versus the other. The relative binding affinities of several agents have been determined in HeLa cells engineered to express ER α or ER β [14]. Propyl pyrazole triol (PPT) was shown to be a selective agonist for ER α in this system, while the synthetic ligands diarylpropionitrile (DPN) and 8 β -VE2, and the natural products genistein and biochanin A, were preferential agonists of ER β . In contrast, the antiprogesterin RU486 was shown to be a strong ER β antagonist with only modest agonist activity toward ER α . DPN has been shown to inhibit proliferation and induce apoptosis in the HC11 mouse mammary cell line but this compound has not been widely studied in human breast cancer cell lines [8]. It has also been

reported that a modified androstenediol is a potent activator of ER β by ligand binding assay, but its biological activity in breast cancer cells requires further investigation [15].

Dual SERM compounds that inhibit ER α while activating ER β are particularly attractive, as ER β often performs growth-inhibitory functions in breast tissues and cell lines (see above, Section 1). An example of this type of SERM is TAS-108, a novel, steroidal antiestrogen that inhibits ER α while acting as a partial agonist of ER β ([16] and references therein). *In vitro*, TAS-108 effectively inhibits the growth of tamoxifen-resistant breast cancer cells, and Phase I studies have established that this agent is not only well-tolerated but appears to induce stable disease in women with advanced breast cancer. Where and how this particular SERM will be used in the clinic, particularly in sequence with the other classes of therapeutic agents described below, has yet to be determined. However, this and future compounds that have similar profiles will be important additions to the current repertoire of endocrine therapies.

1.1.2. Selective estrogen receptor downregulators (SERDs)—The pure ER antagonist ICI 182,780 (hereafter called fulvestrant) binds to ER α with 100-fold greater affinity than does tamoxifen and in so doing, inhibits receptor dimerization and abrogates estrogen signaling [17]. Both laboratory and clinical studies have shown a decrease in overall ER protein levels in response to fulvestrant treatment (discussed in [18]). Importantly, a majority of ER α -expressing tamoxifen-resistant breast cancers remain sensitive to fulvestrant treatment. This has led to its approval in the United States for use in treating ER-positive patients following failure to respond to first-line antiestrogen therapy.

1.1.3. Aromatase Inhibitors (AIs)—Aromatase inhibitors (AIs) represent a new class of endocrine therapy drugs that inhibit the action of aromatase, an enzyme necessary for the conversion of androgens to estrogens (for review, see [19]). Clinically, AIs have had success as a second line of therapy for post-menopausal patients who have progressed after tamoxifen treatment. They also show promise as first-line adjuvant treatment for ER α -positive breast cancers.

1.2 Resistance to endocrine therapies

While considerable progress has been made in developing strategies to target estrogen and ERs in breast cancer, approximately 30% of ER α -positive breast cancers do not respond to tamoxifen treatment (*de novo* resistance). In addition, the majority of tumors that initially respond to treatment develop resistance over time (acquired resistance), despite continued expression of ER α (reviewed in [11,20]). Interestingly, many of these resistant tumors still respond to fulvestrant and AIs, indicating that estrogen remains an important regulator of tumor growth under these circumstances [11]. These data provide support for the idea that endocrine-targeted therapies can lead to the activation of novel signaling pathways that circumvent the effects of antiestrogens [21]. *De novo* and acquired resistance to AIs have also been reported. The appearance and development of resistant tumors are therefore major obstacles in the struggle to control the growth and metastasis of breast carcinomas.

1.3. Biological effects of endocrine therapies

Homeostasis of non-cancerous cells requires a balance between proliferation, cell cycle arrest, and apoptosis; this equilibrium is disrupted during cancer progression. Endocrine therapies induce cell cycle arrest and apoptosis through a reduction in expression of cell cycle regulators such as c-Myc and cyclin D1, accumulation of hypophosphorylated Rb, induction of the cell cycle inhibitors p21^{WAF1/CIP1} and p27^{Kip1}, induction of the putative tumor suppressor interferon regulatory factor-1 (IRF-1), and inhibition of pro-survival pathways through decreased Bcl-2 expression and increased Bax expression ([22-25]; for review, see [20,26]). Breast tumor cells that are resistant to the growth-inhibitory and apoptotic effects of

antiestrogens such as tamoxifen overcome these influences through a variety of mechanisms that are the focus of this review.

2. Molecular mechanisms of antiestrogen resistance

Emerging data from breast tumor biopsies indicate that altered expression and/or modification of several growth factor receptors and downstream signaling molecules correlate with tamoxifen resistance (Figure 1). Epidermal growth factor receptor (EGFR), human epidermal growth factor receptor type 2 (HER2), and insulin-like growth factor-1 receptor (IGF-1R) signaling pathways are often elevated in non-responsive tumors that exhibit either *de novo* or acquired resistance [27-30], as is the activity of kinases that function downstream of these receptors such as extracellular-regulated kinase (ERK) 1/2, p38, AKT, and p21-activated kinase-1 (Pak1) [30-32]. Expression and/or phosphorylation of substrates of these receptor and non-receptor kinases are also elevated in tumors resistant to tamoxifen; such molecules include the cytoplasmic adapter molecule “breast cancer antiestrogen resistance 1” (BCAR1; also known as p130^{Cas}; hereafter called Cas), the coactivator “amplified in breast cancer 1” (AIB1), and ER α itself [28,33-35]. Finally, dysregulated expression of molecules involved in cell survival, such as c-Myc, Bad, and Bcl-2, can also be predictive of a poor response to endocrine therapies such as tamoxifen [36-39]. Clinical data addressing the correlation between some of these markers and antiestrogen resistance are presented in greater detail in Section 4.1. While these studies are limited in both scope and number, however, the association between expression/activation of these molecules and resistance to endocrine therapies has been corroborated (and in fact extended) in tissue culture and animal models. These data are reviewed below.

2.1. ER α , ER β , and receptor co-regulatory proteins

Expression of ER α has long been considered the primary determinant of a clinical response to endocrine or antiestrogen therapy. However in 1996, the concept of estrogen signaling and ER function in breast cancer was significantly altered by the discovery of ER β [40]. The DNA binding domain of full-length ER β 1 shares 96% identity with that of ER α , and although the affinity of this receptor for estrogen is similar to that of ER α , the response to antagonists like tamoxifen and fulvestrant is often different. Both receptors can modulate transcription, either directly from estrogen response elements (EREs), or indirectly through tethering to AP-1 and Sp1 binding sites (reviewed in [41]). However, although ER α perceives tamoxifen and fulvestrant as antagonists, it has been shown that ER β can utilize these compounds as agonists when associated with AP-1 and Sp1 sites [42,43]. Additionally, two naturally occurring variants, ER β 2/ER β cx and ER β 5, contain carboxy-terminal deletions that prevent their binding to ligand, and it has been suggested that these receptors may function as dominant-inhibitory mutants of full-length ER β 1. Murphy *et al.* have recently reviewed the evidence for a role for ER β 1 and its variants in breast cancer diagnosis and prediction of endocrine therapy response [6].

The transcriptional activities of ER α , ER β , and other steroid hormone receptors are modulated by coregulatory proteins in either a positive or negative fashion. The association of coactivators and corepressors with estrogen receptors is regulated by conformational changes induced by ligand binding, with estrogen inducing coactivator recruitment and antiestrogens leading to association with corepressors. Several excellent reviews have recently detailed the expression, function, and clinical relevance of these coregulators in breast cancer and endocrine resistance [44-47].

2.1.1. ER α expression and downregulation—In human breast tumors, the vast majority of ER α -negative tumors exhibit *de novo* or intrinsic resistance to tamoxifen and other antiestrogens; however, small numbers of ER α -negative but progesterone receptor-positive

(PR-positive) tumors respond to antiestrogen treatment (reviewed in [48]). One possible explanation for this finding is that PR-positive tumors retain a functional ER α signaling pathway, particularly since expression of PR is an estrogen-regulated event, and that ER α is present in these tumors at levels that are below the limit of detection for ligand-binding or immunohistochemical assays. The importance of PR as a marker of antiestrogen response is substantiated by studies showing that 70% of ER α -positive/PR-positive tumors effectively respond to tamoxifen, while only 34% of ER α -positive/PR-negative patients respond to tamoxifen therapy [49].

In the context of *de novo* antiestrogen resistance displayed by ER α -negative breast cancer cells, several studies suggest that it may be possible to restore tamoxifen sensitivity through re-expression of ER α . Epigenetic silencing of the ER due to hypermethylation of the promoter region has been reported both *in vitro* and *in vivo* [50,51]. For example, ER α expression can be restored in the ER α -negative MDA-MB-231 breast cancer cell line by either treatment with the demethylating agent 5-aza-2'-deoxycytidine or the histone deacetylase inhibitor trichostatin A [52]. Importantly, when endogenous ER α expression was restored in this way, MDA-MB-231 cells became sensitive to growth inhibition by tamoxifen. Based on data such as these, it has been suggested that restoration of ER α expression may be a viable option for the treatment of some ER-negative breast cancers (discussed in [53]). However, a separate study showed that ectopic expression of ER α in MDA-MB-231 cells was not sufficient to render the cells sensitive to tamoxifen [54]. This suggests that the level and cellular context of ER α expression will be important factors to consider when attempting to modulate ER α levels in "ER-negative" tumors.

Although downregulation of ER α expression can contribute to *de novo* antiestrogen resistance, loss of the receptor during the acquisition of tamoxifen resistance is not commonly observed *in vivo* ([55,56]; reviewed in [57]). However, loss of ER α has been reported in a few models of acquired antiestrogen resistance. For example, the ZR-75-derived 9a1 cell line becomes ER α -negative in the presence of tamoxifen but reverts to being ER α -positive when tamoxifen is removed and estrogen is reintroduced [58].

Like tamoxifen, fulvestrant inhibits ER α transcriptional activity. In addition, fulvestrant has also been shown to accelerate ER α protein degradation via the ubiquitin-proteasome pathway, leading to its reclassification as a SERD (see above). Loss or downregulation of ER α has the potential to be a viable mechanism for antiestrogen resistance in the context of acquired resistance to fulvestrant or other SERDs, but current evidence to this effect is contradictory. ER α protein is completely lacking and only minimal mRNA is detectable in several fulvestrant-resistant cell lines, including MCF-7-r, T47D-r, and ZR-75-r cells [59]. MCF-7-F resistant cells show a 90% decrease in both ER α mRNA and protein, accompanied by significant changes in estrogen-induced expression of ER target genes [60]. In contrast, the MCF-7/182^R-1, -6, and -7 models of fulvestrant resistance maintain ER α expression and function, although its expression at the protein level is reduced by one-third as compared to the parental cell line [61,62]. MCF-7/LCC9 breast cancer cells, which display acquired resistance to fulvestrant and cross-resistance to tamoxifen, show no downregulation of ER α [63]. This cell line was originally derived from the estrogen-independent MCF-7/LCC1 cell line [64]; interestingly both MCF-7/LCC1 and MCF-7/LCC9 cells express higher levels of ER α mRNA and protein than the parental MCF-7 cell line [65]. MCF-7/LCC9 cells have also been shown to contain increased basal ER α transcriptional activity that is not inhibited by fulvestrant [66]. Factors that drive the differential expression and activity of ER α among these cell culture models of fulvestrant resistance are not known, but one likely explanation for these disparities may be the distinct schemes of drug selection that have been used to generate resistant cells *in vitro*. Clinical studies confirm that fulvestrant can reduce ER α levels following either short- (21 days) or long- (6 months) term treatments, but that expression is not completely eliminated (reviewed

in [67]). Interestingly, patients who do not show a clinical response to fulvestrant treatment also do not show a decrease in ER α expression.

2.1.2. ER α mutation—Mutation of the ER may also impact endocrine therapy responsiveness. For example, one tamoxifen-resistant variant of the ER α -positive T47D cell line, T47D_{CO}, has acquired three ER α deletion mutations that affect the hormone- and DNA-binding domains of the receptor [68]. This cell line also expresses different levels of PR [69], suggesting that these variants are functionally distinct from the wild type receptor. These and many other deletions, exon duplications or insertions, point mutations, and alternative splicing events have the potential to regulate ER α function in breast and other tissues. Herynk and Fuqua have recently published a review of known ER mutations and their connection to human disease, several of which are linked to tamoxifen response and resistance [70]. For example, duplication of exons 6 and 7 in an estrogen-independent variant of the MCF-7 cell line leads to the production of an ER that can no longer bind to estrogen or tamoxifen [71]. Short insertions in exon 6 have also been identified in tamoxifen-resistant tumors [72]. A tyrosine-to-asparagine substitution at residue 537 (Y537N), originally identified in metastatic breast cancer, confers constitutive transcriptional activity *in vitro* and renders the ER insensitive to both estrogen and tamoxifen, while mutation of this tyrosine to either serine or alanine results in a constitutively active receptor whose activity is still inhibited by tamoxifen and fulvestrant [73]. In contrast, a lysine-to-arginine substitution at residue 303 (K303R) that was first identified in breast hyperplasia and a number of breast tumors results in a receptor that is hypersensitive to substantially lower concentrations of estrogen than normal (10^{-12} vs. 10^{-9} M) [74]. The prevalence of this mutation in patient populations is unclear, as more recent studies have not found ER α K303R in other patient groups [75]. The difficulty in identifying functional ER α mutations *in vivo* that affect tamoxifen therapy response may stem from the fact that such mutations are relatively rare, estimated to be present in only 1% of breast tumors [51].

2.2. Growth factor receptors

As discussed above, tumors resistant to tamoxifen often show high expression and/or activation of EGFR, its close relative HER2, IGF-R1, and proteins that function downstream of these receptors such as ERK1/2, and AKT (for review, see [28,76-78]). *In vitro* data corroborate a role for these growth factor receptors in mediating antiestrogen resistance, both through crosstalk with the ER and through independent pathways.

2.2.1. EGFR and HER2—Several studies have established that overexpression of EGFR or HER2 in ER-positive, antiestrogen-sensitive, breast cancer cells can confer resistance to these drugs [79-81]. Both ERK1/2 and AKT activity appear to contribute to the resistant phenotype under these conditions. Other groups have isolated antiestrogen-resistant cell lines following long-term treatment of ER-positive breast cancer cells with either tamoxifen or fulvestrant. In these studies, acquisition of resistance often coincides with overexpression of the EGFR and activation of downstream molecules such as ERK1/2, PI3K, and AKT [59,63,82,83]. Expression of one of the ligands for EGFR, transforming growth factor α (TGF α), is elevated in one of these cell models, resulting in an autocrine loop that is required for maintenance of the resistant phenotype [84]. Unlike the parental cells, which are relatively impervious to EGFR signaling pathways, the resistant clones are highly dependent on these pathways for growth and survival.

While the mechanism(s) by which these receptors promote antiestrogen resistance are not clear, several lines of evidence suggest that they may function to provide survival signals that override apoptotic programs in the cell. First, when EGFR functions are blocked by either the tyrosine kinase inhibitor Iressa or blocking antibodies, apoptosis is induced [85-88]. Second, the expression and/or activity of several anti-apoptotic molecules such as AKT, Bcl-2 and Bcl-xL

are upregulated under conditions of HER2 overexpression in MCF-7 cells [89,90]. However, events considered in other contexts to be generally pro-apoptotic are also associated with overexpression of EGFR or HER2 in human tumors. For example, elevated levels of phospho-p38 have been reported in HER2-expressing tumors as well as in tamoxifen-resistant xenografts [29,30]. While its role in regulating apoptosis under these conditions is not clear, activated p38 may promote resistance to tamoxifen through its ability to enhance the nuclear functions of ER [91]. Finally, recent data from our group indicate that the EGFR may work together with the non-receptor protein tyrosine kinase c-Src and the adapter molecule Cas to promote survival [92].

In addition to the direct effect of activated growth factor receptors on cell proliferation and survival, crosstalk between these receptors and ER plays a significant role in regulating the cellular response to antiestrogens. Signaling through EGFR and HER2 can result in downregulation of ER α expression in cultured cells [93]. This inverse relationship has been corroborated in a recent clinical study by the Southwest Oncology Group [27]. Not only does growth factor signaling affect ER levels, but it can also strongly impact ER nuclear functions, at least in part through promoting phosphorylation of the ER, its coactivators, and/or its corepressors (for review, see [94]).

Conversely, ER α appears to regulate growth factor receptor activities through rapid, non-genomic functions. This may be augmented by an accumulation of cytoplasmic ER α at the expense of nuclear ER α under conditions of high HER2 expression [95]. Interestingly, it appears that tamoxifen may be able to function as an agonist for these non-genomic ER activities under some circumstances, perhaps explaining how cells expressing high levels of HER2 and the coactivator AIB1 show increased proliferation in the presence of both estrogen and tamoxifen [90]. Additional support for this crosstalk comes from preclinical studies showing enhanced efficacy and/or delays in the appearance of resistance in the presence of combined therapies that target both tyrosine kinase receptors and estrogen signaling ([87,96]; and see below, Section 4.3).

2.2.2. IGF-1R signaling—The involvement of IGF signaling in antiestrogen resistance is less clear than is the role for EGFR and HER2. While data are mixed about whether IGF-1R levels are elevated or decreased in resistant tumors and cell lines [28,97,98], evidence for a role of IGF-1R signaling in acquired resistance is emerging. This may be mediated at least in part through crosstalk with EGFR, HER2, and/or ER α and likely involves the scaffolding molecule insulin receptor substrate 1 (IRS-1) [77,99,100].

2.3. Cas/c-Src/BCAR3

Numerous cytoplasmic proteins whose functions are coupled to growth factor receptors have been linked to antiestrogen resistance, further supporting a role for these pathways. Two of these molecules, Cas and “Breast Cancer Anti-Estrogen Resistance 3” (BCAR3, also known as AND-34 and Nsp2), were identified in a screen designed to isolate proteins capable of inducing antiestrogen resistance in sensitive MCF-7 and ZR-75-1 cells [101,102]. Interestingly, both of these molecules are expressed and interact with one another in breast cancer cells (Schreengost and Bouton, unpublished data), supporting a role for this molecular pathway in the promotion of resistance.

2.3.1. Cas and c-Src—Cas functions in a wide variety of cellular processes, including proliferation, survival, cell adhesion, and migration [103,104]. Cas overexpression promotes increased proliferation and inhibition of apoptosis in MCF-7 breast cancer cells treated with tamoxifen [92,105]. This requires the association of Cas with c-Src, as documented by the finding that, unlike wildtype Cas, overexpression of a Cas mutant that does not interact with

c-Src does not promote growth in the presence of tamoxifen [92]. Interactions between c-Src and Cas result in activation of c-Src catalytic activity, promotion of serum- and anchorage-independent growth, and enhancement of cellular migration [106,107]. Interestingly, two substrates of c-Src, Tyrosine 845 (Tyr-845) on the EGFR and signal transducer and activator of transcription (STAT) 5b, have been implicated in Cas-dependent tamoxifen resistance [92]. These data suggest a mechanism whereby c-Src activation by Cas results in the phosphorylation of a number of c-Src substrates and subsequent activation of various proliferative and survival pathways that allow the cells to grow in the presence of tamoxifen. Interestingly, ER nuclear functions remain unaffected by this pathway [92], suggesting that therapies that target the Src-Cas-EGFR-STAT5b signaling axis may be able to synergize with tamoxifen for the treatment of “resistant” tumors.

Several other studies provide additional insight into how c-Src and Cas may contribute to tamoxifen resistance. First, ER α and phosphatidylinositol 3-kinase (PI3K) have been found in the c-Src/Cas complex in T47D breast cancer cells following rapid estrogen stimulation [108]. While it is clear that these complexes help regulate estrogen-dependent cell proliferation and survival, however, it is not yet known whether they are important for promoting resistance to antiestrogens. Second, pharmacological inhibition of c-Src in MCF-7 cells enhances the growth-inhibitory and apoptotic-inducing effects of tamoxifen, further strengthening a role for c-Src in this process [109]. Finally, cells treated over a period of two weeks with tamoxifen or fulvestrant show a c-Src-dependent increase in Cas phosphorylation that correlates with activation of a survival pathway involving AKT [110].

Together, these studies point to a key role for Cas and c-Src in tamoxifen resistance in cell culture. Whether these pathways also play a role in clinical resistance remains to be determined. c-Src/Cas interactions are favored under conditions in which both proteins are expressed at high levels [106]. Thus it is intriguing that a large percentage of human breast tumors contain high levels of both c-Src and Cas, and elevated c-Src kinase activity [33,111,112]. Moreover, c-Src/Cas complexes can be isolated from a majority of breast cancer cell lines (Schreengost and Bouton, personal communication). This suggests that one of the mechanisms by which c-Src activity may be elevated in breast cancer cells is through its association with Cas. Cas overexpression in human tumors correlates with poor overall and relapse-free survival and a poor intrinsic response to tamoxifen treatment [33]. The majority of the Cas-overexpressing tumors were shown to be ER-positive, suggesting that Cas promotes *de novo* resistance to tamoxifen through mechanisms that do not involve downregulation of the ER. In contrast, Cas expression levels do not appear to correlate with acquired tamoxifen resistance [113]. c-Src and the EGFR are also frequently (~30%) co-overexpressed in breast tumors, while 20-30% co-overexpress HER2 and c-Src [114,115]. Based on these data, it is reasonable to postulate that, in at least a subset of tumors, tamoxifen resistance may be mediated through a coordinated mechanism involving these receptor tyrosine kinases, c-Src, and Cas.

2.3.2. BCAR3/AND-34—BCAR3/AND-34 (hereafter called BCAR3) is a Cas binding partner that was identified in a genetic screen along with Cas as a molecule whose overexpression conferred antiestrogen resistance [102]. Since its discovery, BCAR3 has been implicated in altering the activity, expression, and intracellular location of many proteins, including Cas, Ras and Rho family GTPases, and Pak1 ([107,116-118]; for review, see [119]). BCAR3 overexpression induces antiestrogen resistance through a process that involves PI3K and Rac1 [118]. Estrogen-independent growth can also be induced by BCAR3 expression, a process that requires R-Ras activation of AKT [120]. While these data suggest that BCAR3 overexpression may promote antiestrogen resistance in cultured cells, however, it is yet to be determined whether this molecule contributes to resistance in the clinical setting.

2.4. PI3K/AKT

Downstream of growth factor receptor and adapter protein activation, signaling intermediates such as AKT play an important role in cell proliferation, survival, and endocrine resistance. AKT, also known as protein kinase B, is activated by PI3K, leading to substrate phosphorylation and cell survival. AKT activity is subsequently counter-balanced by the tumor suppressor PTEN. Historically, all three isoforms of AKT have been considered oncogenic, although recent studies have reported that different isoforms may play distinct roles in invasion and cell migration (discussed in [121]). AKT is most often considered to be a survival factor, preventing apoptosis under conditions of cell stress. However, it has become increasingly clear that AKT also participates in cell cycle regulation (reviewed in [122]). Cell cycle proteins with established connections to antiestrogen resistance, including those that are AKT targets, will be detailed in Section 2.6.

In ER-positive breast cancer cell lines, PI3K-mediated AKT activation is linked to rapid phosphorylation of ER α and ligand-independent receptor activation [123]. AKT can also stimulate ER β transcriptional activity and enhance coactivator recruitment to this receptor [124]; whether this has an impact on antiestrogen resistance has yet to be determined.

Overexpression of constitutively active AKT in breast cancer cell lines can induce estrogen independence and resistance to tamoxifen and fulvestrant [125], while inhibition of PI3K or AKT restores tamoxifen sensitivity [126]. In addition to endocrine agents, activated AKT can also confer resistance to conventional chemotherapeutics such as doxorubicin [127]. The link between AKT and tamoxifen resistance is not solely observed in cell lines engineered to overexpress this kinase; MCF-7 cells selected for resistance to tamoxifen exhibit upregulated endogenous AKT activity that is required for antiestrogen resistance [128,129]. However, a separate study showed that an aromatase-expressing MCF-7 cell line (MCF-7/Ca), when grown as xenografts in nude mice, did not upregulate the PI3K/AKT pathway in the process of acquiring resistance to the aromatase inhibitor letrozole [130].

The mechanisms by which AKT may confer antiestrogen resistance are varied. Phosphorylation of the ER on serine 167 is discussed below (Section 2.5). AKT can also activate the mammalian target of rapamycin (mTOR), which controls cap-dependent translation, cell growth, and survival (recently reviewed in [131]). This can occur in response to activation of AKT by growth factor receptors such as EGFR and IGF-1R; AKT then phosphorylates and inactivates the tuberous sclerosis complex, which normally keeps mTOR kinase activity in check. Rapamycin and its analog CCI-779 are able to restore tamoxifen sensitivity in MCF-7 cells overexpressing constitutively active AKT [132]. Another mTOR inhibitor (RAD-001) has also been shown to inhibit the growth of wild type MCF-7 cells, as well as MCF-7/Aro cells that stably over-express aromatase [133]. Moreover, a combination of the aromatase inhibitor letrozole with RAD-001 was shown to more effectively inhibit MCF-7/Aro proliferation than either drug alone. However, given that MCF-7/Ca cells with acquired resistance to letrozole do not contain elevated levels of activated AKT [130], it would seem that a definitive role for the PI3K/AKT/mTOR pathway in aromatase inhibitor resistance remains to be determined. Nonetheless, mTOR and rapamycin-like drugs that target mTOR are considered to be attractive molecular targets and therapeutic agents, respectively, for treating breast cancer (reviewed by [134,135]; see Section 4.3).

2.5. ER phosphorylation

The estrogen receptor is a target of serine/threonine as well as tyrosine phosphorylation (reviewed in [136]). While ER phosphorylation is a key element of non-genomic or rapid estrogen actions, several studies have shown that it can also affect conventional, transcription-

mediated events. Reported ER phosphorylation sites include tyrosine 537, serines 104, 106, 118, 167, 236, and 305, and threonine 311 (Figure 2, and discussed in [137]).

Tyr-537, which is located in the activation function-2 (AF-2) domain, is phosphorylated by Src family kinases [138]. Mutation of Tyr-537 to phenylalanine increases basal ER transcriptional activity without changing the DNA binding capacity of the receptor [139]. Mutation of Tyr-537 to either serine or alanine also results in estrogen-independent transcription, but both 4-hydroxytamoxifen and fulvestrant inhibit this activity [140,141]. Although the full function(s) of Tyr-537 phosphorylation are still under investigation, the evidence that “constitutively active” Tyr-537 mutants are still inhibited by antiestrogens argues against a critical role for this residue in endocrine therapy resistance. It has also yet to be determined whether Tyr-537 phosphorylation is observed in breast tumors.

The effect of serine phosphorylation on ER functions is better established than is tyrosine phosphorylation and it is more likely to contribute to the resistant phenotype. Ser-104 and Ser-106 are located in the AF-1 domain and are targets of the cyclin A/CDK2 complex; mutation of these sites to alanine prevents cyclin A-mediated enhancement of ER transcriptional activity [142]. Conversely, cyclin A enhances ER transcriptional activity in the presence of tamoxifen. Together, these data suggest that inappropriate cyclin A/CDK2-mediated phosphorylation of Ser-104 and Ser-106 may play a role in endocrine resistance in breast tumors. Cyclin A has been implicated as an independent prognostic factor for poor outcome in tamoxifen-treated breast cancer patients [143].

Ser-167, also located within the AF-1 domain, was initially proposed to be phosphorylated in response to estrogen binding [144]. However, subsequent studies have revealed that its phosphorylation is ligand-independent, and that this site is a major target of ERK, RSK, and AKT kinases (reviewed in [136]). Overexpression of either EGFR or AKT increases Ser-167 phosphorylation and reduces tamoxifen sensitivity, whereas RNAi-mediated inhibition of AKT abrogates Ser-167 phosphorylation and restores tamoxifen sensitivity [145]. *In vitro*, AKT-mediated phosphorylation of Ser-167 can increase ER binding to DNA and enhances the interaction of ER with the coactivator SRC3 in the presence of estrogen [146]. Despite the evidence that Ser-167 phosphorylation appears to play an important role in promoting antiestrogen resistance in cell culture, however, clinical evidence of a definitive role for phosphorylated Ser-167 in antiestrogen resistance is less convincing (see Section 4.1).

Ser-236, located within the DNA binding domain of the receptor, has been shown to be phosphorylated by protein kinase A (PKA) [147]. While phosphorylation at other sites seems to positively regulate ER transcriptional activity, PKA phosphorylation at Ser-236 appears to inhibit receptor dimerization in the absence of ligand. This suggests that Ser-236 represents a negative regulatory site that has yet to be implicated in the process of tamoxifen resistance.

Ser-305 is located in the hinge region of the receptor, and its phosphorylation by Pak1 has been shown to increase ER transcriptional activity [148]. Substitution of glutamic acid for serine at this site recapitulates increased receptor activation and induces expression of the ER target gene cyclin D1 [149]. Pak1 is a well-known mediator of cell survival and migration, and has itself recently been shown to contribute to tamoxifen resistance. Pak1 overexpression in MCF-7 cells enhances cyclin D1 expression and prevents tamoxifen-mediated inhibition of ER activity [150,151]. Conversely, lower expression of Pak1 in primary breast tumors is associated with improved disease-free survival in patients treated with tamoxifen [32]. Pak1 co-immunoprecipitation with ER is enhanced by tamoxifen treatment of resistant cell lines [150, 151]. Pak1-mediated stimulation of ER transcriptional activity through Ser-305 is reported to require processive phosphorylation of Ser-118, suggesting that these two sites work together to regulate ER activity. Interestingly, Ser-305 can also be phosphorylated by PKA. While PKA-

mediated phosphorylation of Ser-236 appears to negatively regulate receptor activities (see above), phosphorylation of Ser-305 by PKA is thought to prevent tamoxifen from inducing the inactive conformation of the ER [152].

Ser-118 is perhaps the best studied site of ER phosphorylation and is widely considered to be a target of ERK1/2, although other kinases such as the TFIID cyclin-dependent kinase, cyclin-dependent kinase 7 (CDK7), and glycogen synthase kinase-3 (GSK-3) may also phosphorylate this site [153-156]. Interestingly, estrogen-induced phosphorylation of Ser-118 occurs within 10 minutes of stimulation, and this can be inhibited by 4-hydroxytamoxifen but not fulvestrant [155]. Recent studies by Likhite *et al.* have helped to clarify the function of Ser-118 phosphorylation in tamoxifen-sensitive and resistant cells with respect to hormone, DNA, and coregulator binding [146]. Stable overexpression of HER2 in MCF-7 cells, which induces tamoxifen resistance, also results in increased basal phosphorylation of Ser-118 that is no longer enhanced by estrogen stimulation. Using purified wild type and serine-mutated ER in *in vitro* kinase assays, this group subsequently demonstrated that phosphorylation of Ser-118 by ERK1/2 specifically enhances recruitment of the coactivator SRC3 to estrogen-bound receptor, but has no effect on the affinity of ER for estrogen or DNA. In contrast, phosphorylation at Ser-118 reduced the association of ER with trans-hydroxytamoxifen, which has the potential to attenuate the growth inhibitory effects of this antiestrogen.

The role of Ser-118 phosphorylation in antiestrogen resistant cell culture models is not fully understood. Kuske *et al.* have recently reported that estrogen-induced Ser-118 phosphorylation levels are comparable in parental MCF-7 cells and the estrogen-independent MCF-7/LCC1 cell line, but that basal and estrogen-induced levels of phospho-Ser-118 are significantly reduced in the antiestrogen-resistant MCF-7/LCC9 cell line [65]. In studies by other investigators, baseline phosphorylation of Ser-118 is increased in tamoxifen-resistant MCF-7 cells as compared to the parental cell line and is reduced by MEK and EGFR tyrosine kinase inhibitors, which also inhibit cell growth [157]. Finally, Weitsman *et al.* suggest that, although both HER2 overexpression and activation of the MAPK pathway can induce antiestrogen resistance, neither has any effect on Ser-118 phosphorylation of ER in MCF-7 cells [158]. Instead, they suggest that the activity of I κ B kinase- α (IKK α) is involved. A potential role for IKK α in ER phosphorylation has only recently been suggested [159], and further studies are needed to clarify its role in antiestrogen resistance.

2.6. Cell cycle regulators

Cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors are the major regulators of cell cycle progression. Antiestrogens like tamoxifen induce cell cycle arrest during the G1 phase; therefore, genes that control cell cycle progression have the potential to significantly impact drug sensitivity and resistance. In breast cancer, normal expression and function of cyclins D1 and E, and the CDK inhibitors p21 and p27, are often specifically disrupted. Caldon *et al.* have recently published a comprehensive review of cell cycle control in breast cancer that summarizes how cyclins and CDK inhibitors influence cancer progression and may serve as markers of treatment outcome [160]. Butt *et al.* have also published a summary of the literature concerning the role of cyclins D1 and E in endocrine resistance [161]. Given the timeliness of these reviews, we will only summarize key information about these molecules and focus on the most recent literature in this area.

2.6.1. Cyclin D1—Cyclin D1 is a well-studied contributor to antiestrogen resistance in breast cancer. Essential for early progression through the G1 phase, cyclin D1 both modulates CDK4/6-dependent phosphorylation of the tumor suppressor Rb and sequesters the CDK inhibitors p21 and p27 [160,162]. Cyclin D1 is a direct transcriptional target of estrogen signaling, where its induction is mediated by ER/AP-1 and ER/Sp1 transcription complexes;

conversely, antiestrogens like tamoxifen inhibit cyclin D1 expression (discussed in [161]). AKT can indirectly affect cyclin D1 expression by phosphorylating and inhibiting the activity of GSK3 β , which normally induces cyclin D1 degradation [163]. In cell culture, stable overexpression of cyclin D1 can confer resistance to tamoxifen as well as fulvestrant [164]. Moreover, some cells that have acquired resistance to tamoxifen show increased expression of cyclin D1 [165]. However, these cells are still growth-inhibited by fulvestrant.

Recent studies have shed some light on potential mechanisms of cyclin D1 upregulation in the context of antiestrogen resistance, some of which involve crosstalk between growth factor receptors and other signaling molecules. Tamoxifen-resistant MCF-7 cells overexpressing HER2 upregulate cyclin D1 [90]. Similarly, the tamoxifen-resistant phenotype of MCF-7 cells overexpressing Pak1 is partly due to the increased expression of cyclin D1 [32]. BCAR3 overexpression in MCF-7 also induces cyclin D1 promoter activity, coincident with the adoption of antiestrogen resistance [117]. In some instances, tamoxifen has been reported to stimulate breast cancer, although this has been observed more widely in cell culture and xenograft studies than in the clinic. Under conditions in which tamoxifen or raloxifene functions as an ER agonist, cyclin D1 regulates cell cycle progression much as it does in parental MCF-7 cells stimulated by estrogen. For example, cyclin D1 expression was found to be significantly elevated in raloxifene-stimulated MCF-7 xenografts [166]. Treatment with the HER2 inhibitor trastuzumab decreased cyclin D1 expression in these xenografts and concomitantly inhibited tumor growth. Similarly, cyclin D1 was shown to be essential for cell cycle progression induced by tamoxifen in resistant MCF-7 cells grown in tissue culture [167].

Evidence for a role of cyclin D1 in clinical endocrine resistance is still emerging (reviewed in [160]). Cyclin D1 is overexpressed in ~50% of breast cancers. Some studies have reported that cyclin D1 overexpression is more often observed in highly differentiated, slower growing tumors than in highly proliferative tumors. Other studies report that cyclin D1 amplification is more frequent in ER-positive tumors, whereas overexpression in the absence of amplification is more common in ER-negative tumors [168]. Not surprisingly, it is in the ER-positive cases where cyclin D1 amplification is associated with a worse outcome. How these findings will affect the clinical management of ER-positive breast tumors and future use of tamoxifen therapy remains to be determined.

2.6.2. Cyclin E—In response to inhibition of Rb by cyclin D1 early in G1 phase, the transcription factor E2F strongly induces the expression of cyclin E, which associates with CDK2 to form an active complex that promotes entry into S phase [160]. Cyclin E/CDK2 activity is subsequently counterbalanced by the CDK inhibitors p21 and p27. While not a direct target of the ER, estrogen-dependent induction of c-Myc can activate cyclin E complexes by stimulating release of the inhibitor p21. CDC25A can also participate in estrogen-mediated cyclin E activation [169].

The contribution of cyclin E to tamoxifen resistance is less clear than for cyclin D1. This may be due to the presence of multiple low molecular weight forms of cyclin E that are generated by post-translational proteolytic cleavage events (reviewed in [170]). Low molecular weight cyclin E variants, which exhibit greater activity than full-length cyclin E, can induce antiestrogen resistance in MCF-7 cells [171]. Further studies are required to clarify the mechanism(s) by which low molecular weight cyclin E is induced in breast cancer, and its function in antiestrogen resistance. Other groups have reported that full-length cyclin E can also confer resistance to tamoxifen and fulvestrant [164,172].

2.6.3. p21 and p27—The CDK inhibitors p21 and p27 are important negative regulators of cell cycle progression that counteract the activities of cyclin D1 and cyclin E. p27 is largely

regulated in a post-transcriptional manner, while p21 is primarily controlled at the level of transcription (reviewed in [160]). In MCF-7 cells, both tamoxifen and fulvestrant increase the expression of p21 and p27 during cell cycle arrest [173]. p27 induction by tamoxifen induces quiescence in breast cancer cells and prevents a positive proliferative response following re-stimulation by growth factors such as IGF-I and EGF [174]. Combined treatment with tamoxifen and the farnesyl transferase inhibitor FTI-277, which causes a potent G1 arrest, maintains the expression and association of p21 and p27 with cyclin E/CDK2 complexes [23]. These two studies suggest that preservation of p27 and p21 is critical for the inhibitory effects of antiestrogens like tamoxifen in controlling breast cancer cell growth. In contrast, downregulation of these CDK inhibitors prevents the growth inhibitory effects of these drugs [173].

p21 and p27 functions are regulated in part by AKT through phosphorylation of residues within their nuclear localization sequences, leading to sequestration in the cytoplasm where they are unable to interact with cyclin E and CDK2 (discussed in [175]). c-Myc also plays a role in regulating p21, although the mechanism is less clear. Most significantly, c-Myc appears to block tamoxifen- and fulvestrant-induced expression of p21 [176]. In MCF-7/LCC9 breast cancer cells that have acquired resistance to fulvestrant and cross-resistance to tamoxifen, p21 expression is no longer regulated by estrogens and antiestrogens [176]. Studies of other models of antiestrogen resistance also support the idea that p21 and/or p27 play important roles in antiestrogen sensitivity and resistance. Unlike parental MCF-7 cells in which tamoxifen or fulvestrant treatment increases p27 binding to cyclin E, antiestrogens do not alter p27 binding in the tamoxifen-resistant LY-2 variant [177]. These LY-2 cells display constitutive activation of MEK and ERK1/2, and inhibition of these kinases partially restores antiestrogen sensitivity and p27 function. Other cell culture models of resistance, such as MCF-7/LCC2 and MCF-7/HER2-18 cells, exhibit increased MEK/ERK activity, although whether this affects antiestrogen resistance through modulating p27 remains to be determined [177]. These data supporting a role for p21 and p27 in antiestrogen responses and resistance are corroborated by clinical evidence (see Section 4.1).

3. Endocrine therapies; effect on motility/invasion/metastasis

Metastatic breast cancer is ultimately the greatest cause of disease mortality. Tamoxifen is beneficial for decreasing secondary disease occurrence, both at local and distant sites [178]. However, the major phenotype of endocrine resistance is cancer recurrence. While clinical data have not definitively linked antiestrogen resistance with metastasis, many of the proteins/pathways discussed above in Section 2 that are involved in circumventing antiestrogen-induced blocks in cell cycle progression and survival are also implicated in increased migration and metastasis of breast cancer cells.

Hiscox *et al.* have begun to tackle the molecular relationship between antiestrogen resistance and invasion/metastasis [179]. They found that MCF-7 cells selected for tamoxifen resistance showed enhanced basal motility and an invasive phenotype. Treatment of these cells with the pharmacological inhibitors gefitinib and AZD0530 inhibited migration and invasion, indicating that the invasive phenotype of these resistant cells was dependent on EGFR and c-Src, respectively. The ability of tumor cells to metastasize requires multiple genotypic and phenotypic changes. Signaling perturbations associated with the epithelial to mesenchymal transition (EMT) contribute significantly to invasive and metastatic disease [180]. These include increased expression of extracellular matrix proteins, downregulation of E-cadherin, disruption of adherens junctions complexes, accumulation of β -catenin and NF- κ B in the nucleus, and transcription of target genes such as c-Myc and cyclin D1 [181,182]. Interestingly, the tamoxifen-resistant MCF-7 cells investigated by Hiscox *et al.* displayed an altered morphology reminiscent of an EMT, marked by an elongated, fibroblast-like phenotype that

lacked characteristic cell-cell contacts [179]. Tyrosine phosphorylation of β -catenin was elevated in the tamoxifen-resistant cells, coincident with accumulation of the protein, loss of its association with E-cadherin, increased interactions with EGFR, and increased transcription of c-Myc and cyclin D1. These effects were reversed in the presence of gefitinib, supporting a mechanism whereby the EGFR drives phosphorylation of β -catenin and activation of c-Src to initiate EMT-mediated metastatic progression.

Many of the other molecules that have been mentioned above in the context of promoting antiestrogen resistance also play a role in promoting invasiveness. For example, Pak1 is functionally linked to regulation of the actin cytoskeleton through Rho family GTPases [150]. The *Pak1* gene is closely linked to *CCND1* (cyclin D1) at 11q13, a region that is often amplified in breast cancers [183]. Similarly, amplification of the gene encoding c-Myc in breast tumors appears to correlate with the transition from *in situ* to invasive carcinoma [38,184]. Finally, in a study of matched primary and metastatic breast tumors, Bcl-2 expression was found to be elevated in metastatic tumors coincident with a retention of ER α expression [38]. These results conflicted with those from a similar study performed on paired primary and recurrent tamoxifen-resistant tumors, which found that the recurrent tumors did not show a correlation between ER status and Bcl-2 expression [30]. However, there was a correlation between ER α and phosphorylated p38 and ERK in these relapsed specimens. These discrepancies may be due to differences in inclusion criteria and the source of recurrent tissue between studies. The former study evaluated mostly tumors at distant sites after discontinuation of hormonal therapy, while the latter study evaluated locoregional recurrences while the patients continued therapy.

4. Antiestrogen resistance: implications for treatment

4.1. Prediction of response

It is clear from the discussion above that resistance to endocrine therapy likely occurs through diverse mechanisms that vary from patient to patient. Consequently, it is imperative to develop molecular signatures that can predict the likelihood of response and potential for relapse of individual tumors. Initially, predictive markers for tamoxifen resistance included expression of ER and receptor tyrosine kinases such as EGFR and HER2. As our understanding of antiestrogen resistance has evolved, these markers have broadened. Currently, the overall expression of proteins, protein modifications, and intracellular localization are all being analyzed for predictive value of response to endocrine therapy.

As mentioned above, survival pathways regulated by PI3K, AKT, PTEN, and Bcl-2 family members play an important role in antiestrogen resistance. Studies are currently underway to determine whether increased activation and/or expression of AKT, decreased activity of PTEN, or altered expression of Bad could be predictive markers for response to anti-hormonal treatments. For example, a retrospective analysis of 402 ER α -positive breast carcinoma biopsies treated with tamoxifen for a median of 5 years revealed that high expression of AKT2, as measured by immunohistochemistry, was predictive of improved overall survival [31]. Patients who relapsed following tamoxifen treatment were more likely to have low AKT2 expression and high cytoplasmic phospho-AKT (Ser-473). Interestingly, expression of AKT1 and AKT3 did not have a significant predictive correlation. Bad expression could also predict tamoxifen-treatment outcome. Patients in this same clinical study whose tumors had high levels of Bad expression had improved disease-free survival rates compared to patients whose tumors had low levels of Bad [36]. There was no association between phospho-Bad (Ser-112), Bax, Bcl-2 or Bcl-xL expression and response to tamoxifen treatment in this group of patients. A related study examined 252 primary ER α human breast carcinomas to determine whether activated AKT or loss of heterozygosity (LOH) of PTEN was predictive of response to tamoxifen [185]. Phospho-AKT correlated with HER2 status in this study, as did PTEN LOH.

From this patient cohort, 36 patient samples were used to examine the relationship between activated AKT and the efficacy of endocrine therapy for metastatic breast cancer. Patients with phospho-AKT-positive tumors received less benefit from endocrine therapy than did patients with phospho-AKT-negative tumors. A third study evaluated PTEN expression in 100 patients with ER α breast carcinomas who had been treated with tamoxifen [186]. High levels of PTEN were recorded for 81% of patients who had non-recurrent disease and only 41% of patients with recurrent disease. Conversely, low PTEN expression was associated with a shorter relapse free survival and reduced disease-specific survival. PTEN expression was an even stronger predictor of relapse and survival when the analysis was restrained to only stage I breast cancer patients.

Like the PI3K/AKT survival pathway, cyclin D1 expression is associated with multiple mechanisms of antiestrogen resistance. Patients with ER α -positive tumors and high levels of cyclin D1 mRNA had a worse prognosis for overall survival [187]. In a small subset of these patients who were treated with endocrine therapy, an apparent association between high cyclin D1 mRNA and decreased duration of response was made. Similar results were obtained by evaluating protein levels of cyclin D1 in 248 post-menopausal patients who had been randomized into 2-year tamoxifen treatment or no-treatment groups [188]. Interestingly, the 55 strongly ER α -positive patients with tumors showing moderate or low cyclin D1 levels responded to tamoxifen treatment, whereas the 46 patients with both high ER α and cyclin D1 expression in their tumors showed no difference in survival between tamoxifen and no treatment.

The association between cyclin E levels and antiestrogen response is even less clear. Some of the clinical studies focused on cyclin E have shown a correlation between the presence of low molecular weight and/or full-length cyclin E and poor survival, while others have not found such an association ([189] and discussed in [161]). A recent study examining the expression of cyclin E and two proteases implicated in the generation of low molecular weight cyclin E (proteinase 3 and neutrophil elastase) in 205 breast cancer patients revealed that cyclin E was significantly associated with poor disease-free survival in the entire cohort, and with tamoxifen resistance in the 110 treated patients [190]. However, expression of neutrophil elastase was not associated with poor outcome or treatment resistance. Further studies are required to clarify the role of these variants in clinical endocrine resistance.

Coincident with cyclin D1 and cyclin E expression, p21 and p27 levels may also prove to be predictive for clinical response to antiestrogen resistance. High levels of p27 expression in a group of 512 patients with premenopausal breast cancer patients treated with tamoxifen and the gonadotropin-releasing hormone agonist goserelin were strongly associated with improved overall and disease-free survival [191]. Data for p21 are less consistent, perhaps because they may be confounded by the complex regulation of p21 expression and contributions from other proteins such as p53 and IRF-1 that may independently affect breast cancer outcome (discussed in [20,161]). It may also be that subcellular localization of p21, rather than overall expression levels, will prove to be a better marker of therapeutic response and treatment outcome. In line with this suggestion, Perez-Tenorio *et al.* have recently reported that cytoplasmic p21 is associated with increased AKT activity and poor tamoxifen response in a cohort of 280 women [192].

As mentioned above, serine phosphorylation of ER α may also prove to be useful for predicting therapeutic response (see Section 2.5). Recent studies have centered on the role of Ser-118 phosphorylation in predicting clinical outcome in breast cancer patients. In a series of 117 lymph node-negative primary breast tumors from patients treated with tamoxifen following surgery with or without radiation, Murphy *et al.* observed Ser-118 phosphorylation only in tumors with positive estrogen binding activity [193]. Phosphorylated Ser-118 was also

observed more frequently in PR-positive tumors than PR-negative tumors, and patients with tumors positive for Ser-118 phosphorylation showed increased disease-free survival. Bergqvist *et al.* reported that increased MAPK activation and expression of phosphorylated Ser-118 correlated with one another and also predicted better survival [194]. However, overexpression of HER2 was inversely correlated with activated ERK1/2 and phosphorylated Ser-118, suggesting that at least in this cohort, HER2 activation does not drive phosphorylation and hyperactivation of ER. Moreover, this apparent improved outcome observed in patients with Ser-118 phosphorylation appears to contradict some of the cell culture experiments that showed a link between Ser-118 phosphorylation and antiestrogen resistance (see Section 2.5). This discrepancy may be explained by the possibility that Ser-118 phosphorylation of the ER may have distinct functions in different phases of breast cancer; there is some evidence to suggest that it may be associated with a better outcome prior to endocrine therapy but a worse outcome following acquisition of tamoxifen resistance [35].

The correlation between Ser-167 phosphorylation and clinical response to antiestrogens is equally complex. Kirkegaard *et al.* found that high levels of active AKT correlated with reduced survival and increased Ser-167 phosphorylation in tamoxifen-treated patients [31]. However, Yamashita *et al.* reported that phosphorylated Ser-167, along with expression of ER and PR, were associated with an improved response to endocrine therapy and survival [195]. The fact that Ser-167 can be phosphorylated by kinases other than AKT may suggest that it is AKT rather than phosphorylated Ser-167 that is responsible for the poor outcome in the Kirkegaard study [31].

In addition to the ER α modulation, PR status has also been suggested to be predictive of response to antiestrogens. As discussed above (Section 2.1), the presence of PR is reflective of a functional ER pathway because PR synthesis is initiated by ER-induced transcription. Bardou *et al.* analyzed two databases containing information on ER and PR status of a large number of breast cancer patients [196]. They found that PR expression was associated with disease-free survival independently of ER α status. However, ER α -positive/PR-positive tumors received the greatest benefit from adjuvant hormonal therapy when both ER α and PR were taken into consideration, while loss of expression of either receptor reduced responsiveness by nearly half. Additional studies have also found that ER/PR-positive breast tumors have significant improvement in prognosis after either adjuvant tamoxifen or letrozole treatments [197,198]. This supports the hypothesis that ER α positive tumors that lack PR expression are less likely to respond to anti-hormonal therapy because they are no longer dependent on estrogen for survival. Conversely, ER β expression appears to be predictive of a positive response to endocrine therapy and better overall survival [6]. This is potentially due to the putative tumor suppressor functions of ER β discussed above (see Section 1), and provides the rationale for developing SERMs that exhibit selective activities to ER subtypes (see Section 1.1.1).

4.2. Molecular profiling studies of antiestrogen resistance

Gene expression analysis and molecular profiling have added to our understanding of the mechanisms of antiestrogen response and resistance. Several groups have used these approaches on tumor samples to develop gene signatures that can predict clinical responses to antiestrogen treatment [199,200]. However, in some instances, these profiles have not been validated by independent studies [201]. Expression profiling of cell culture and xenograft models has also been extensively utilized to help clarify the molecular actions of antiestrogens and aromatase inhibitors. Several of these studies have confirmed biological evidence indicating that SERMs like tamoxifen and raloxifene can behave as both antagonists and agonists of the ER, whereas fulvestrant is a pure antagonist. For example, expression of cell cycle-associated genes such as cyclin A1 and CDC25B was unaffected by estrogen, tamoxifen,

or raloxifene but strongly regulated by fulvestrant [202]. The effects of tamoxifen and raloxifene were also similar to estrogen with respect to expression of Bcl-2-interacting killer (BIK), an estrogen-inhibited, pro-apoptotic gene that has been demonstrated to play a critical role in fulvestrant-induced breast cancer cell death [203].

Other studies have examined regulation of gene expression by letrozole and anastrozole as compared to tamoxifen [204]. As might be expected, the effects of these aromatase inhibitors were more similar to each other than they were to tamoxifen. However, the major functional classes of genes regulated by all three drugs were comparable, and included apoptosis, Wnt, and MAPK signaling pathways. This suggests that, rather than looking at individual genes, these approaches may be most useful for identifying functional groupings of genes that work together in specific signaling pathways to regulate responses to antiestrogens. A recent study in MCF-7 cells comparing acquired resistance to tamoxifen and fulvestrant found that tamoxifen resistance was strongly associated with changes in the PKA pathway, MAPK phosphatases, and calcium binding proteins. In contrast, downregulation of ER and major alterations in growth factor receptor and interferon signaling were observed in cells resistant to fulvestrant [60].

Interferon-inducible genes have also been found to be differentially regulated in the MaCa 3366/TAM xenograft model of acquired tamoxifen resistance when compared to the tamoxifen-sensitive MaCa 3366 counterpart [205]. Interestingly, loss of the putative tumor suppressor interferon regulatory factor 1 (IRF-1) has been specifically implicated in fulvestrant resistance [25] and is required for tamoxifen-induced cell death of normal mammary epithelial cells [206]. Another interferon-induced gene, calgranulin A (S100A8) [207], is upregulated in MCF-7, T47D, and ZR-75-1-derived models of fulvestrant resistance [59], providing additional support for a role of interferon-inducible genes in antiestrogen resistance.

While expression profiling has provided insight into molecular pathways that may contribute to antiestrogen resistance, there are some challenges associated with these approaches that must be carefully considered. Multiple expression array platforms and technologies can make comparing the findings of different investigators difficult, especially at the level of individual genes. Statistical analysis of these data is also very complex, and the risk of excluding biologically relevant genes while including others that don't contribute to the phenotype is a significant one. Unfortunately, these challenges are greatly magnified in the context of analysis of tumor tissues [208-210]. Nonetheless, these approaches can be quite powerful, particularly when one considers functional grouping of genes rather than individual molecules.

4.3. Combinatorial therapies

Strategies for treating breast tumors have significantly broadened in recent years because of a growing appreciation for the fact that resistance to endocrine therapies can and will invariably arise through a variety of distinct mechanisms. Our increasing understanding of the molecular basis for these failures has brought to the forefront the possibility of using combination therapies for treating breast cancer. There are two separate rationales for using combination therapies. First, targeting multiple pathways at once can potentially delay or halt the acquisition of a resistant phenotype. Alternatively, treatments can be administered to effectively resensitize tumors to endocrine therapies for which they are resistant. In many cases, the practical strategies for addressing these distinct phenomena are the same. For example, as discussed above, growth factor receptor signaling is often increased in antiestrogen-resistant breast tumors, leading to upregulation of proliferation and survival pathways that may be distinct from estrogen signaling per se. Thus, treatment with gefitinib or trastuzumab to inhibit the EGFR or HER2, respectively, along with hormonal therapies would potentially block both the pro-proliferative activity of the ER and the proliferation/survival pathways activated by the growth factor receptors. Some of the current trials evaluating the efficacy of these combinations include a

Phase II randomized trial (Translational Oncology Research International trial) that is investigating fulvestrant and trastuzumab in combination as a first-line treatment for patients with ER α /HER2-positive breast tumors, a comparative study evaluating anastrozole versus fulvestrant with the addition of gefitinib in women with ER α -positive tumors who have metastatic disease and have failed chemotherapy and/or endocrine therapy, and a combination of fulvestrant with the dual inhibitor lapatinib in postmenopausal women with ER α -positive and EGFR-and/or HER2-positive tumors who have had previous exposure to an aromatase inhibitor (reviewed in [211,212]).

In addition to targeting growth factor receptors, several clinical trials have been initiated to investigate the effect of farnesyl transferase inhibitors such as tipifarnib in combination with endocrine therapies. However, these trials have been somewhat disappointing in that they have not thus far corroborated pre-clinical models showing that tipifarnib in combination with tamoxifen resulted in greater tumor regression than either treatment alone [213]. Additional studies are underway that examine the efficacy of combining tipifarnib with AIs (reviewed in [214]).

Clinical studies with mTOR antagonists in combination with hormonal therapies are also ongoing. As a single agent, CCI-779 has been somewhat effective in Phase II studies of metastatic breast cancer [215]. Currently, Phase II and Phase III trials are testing either CCI-779 or RAD-001 in combination with letrozole, as compared to letrozole alone. Interim results show that the combination of CCI-779 and letrozole translates into a clinical benefit rate of 77% versus 66% for letrozole alone, although these values have not yet reached statistical significance [135].

Finally, extensive efforts are being made to determine whether pre-clinical data showing that tumors resistant to one class of hormonal therapy may still retain sensitivity to alternate endocrine therapies hold true in the clinic. Results of the Phase II Swiss Group for Clinical Research Trial (SAKK 21/00), which compared the efficacy of fulvestrant treatment in AI-sensitive versus AI-resistant patients, were recently published (reviewed in [216]). Overall fulvestrant yielded a clinical benefit (measured as an objective response or stable disease >24 weeks) in 30% of patients, irrespective of whether they had previously been treated with AIs. There are currently multiple planned or ongoing clinical trials combining AIs with antiestrogens. These include Phase II and Phase III trials evaluating use as first line and neoadjuvant treatment (reviewed in [214]).

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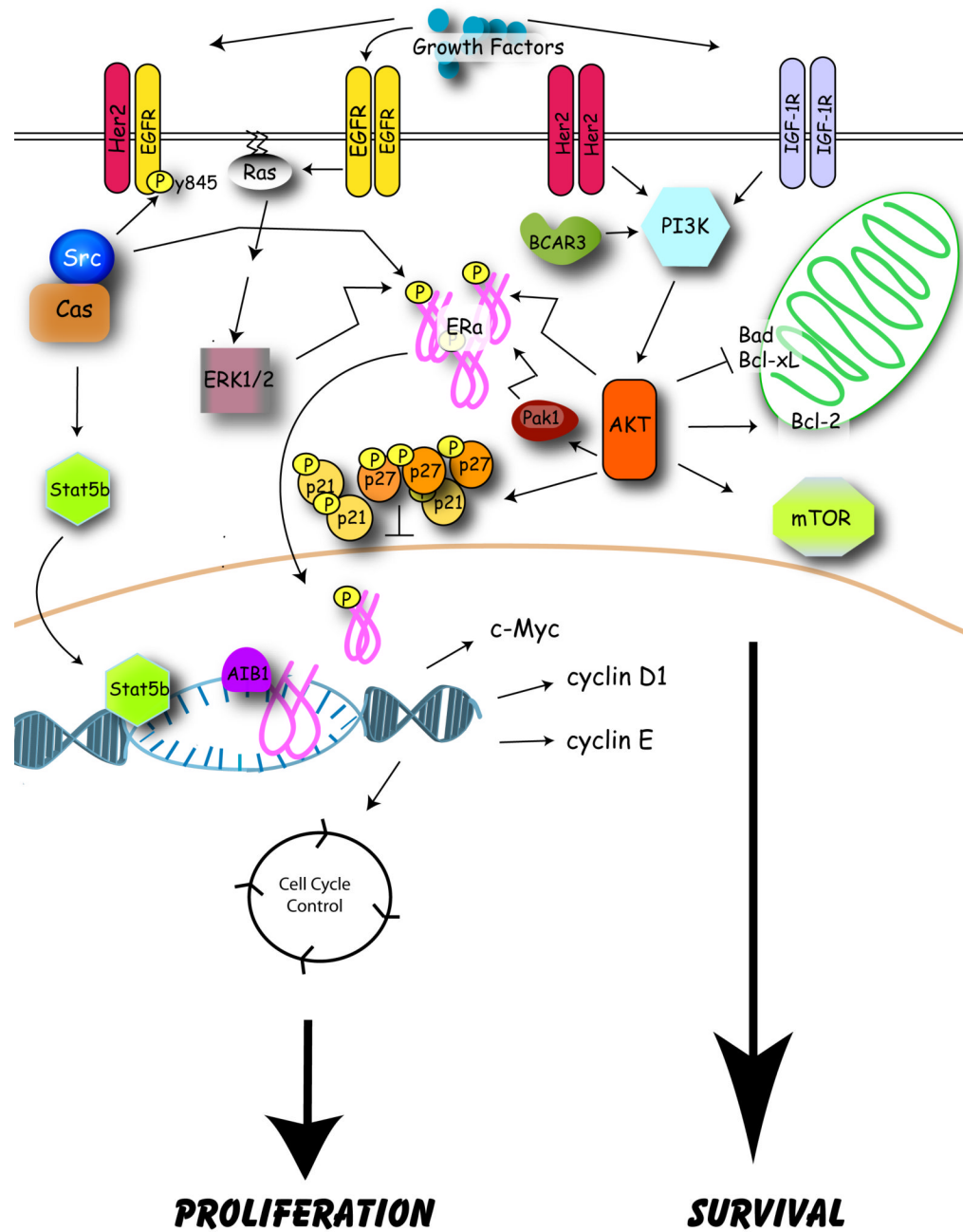


Figure 1. Molecular mechanisms of tamoxifen resistance. Model depicting molecules implicated in antiestrogen resistance and discussed in this review.

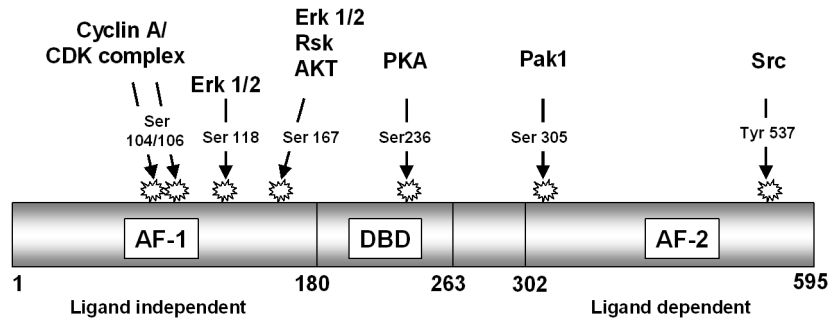


Figure 2. ER α phosphorylation. Domain structure of ER α showing phosphorylation sites and the kinases that mediate these phosphorylations.