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Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling

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Antiestrogens include agents such as tamoxifen, toremifene, raloxifene, and fulvestrant. Currently, tamoxifen is the only drug approved for use in breast cancer chemoprevention, and it remains the treatment of choice for most women with hormone receptor positive, invasive breast carcinoma. While antiestrogens have been available since the early 1970s, we still do not fully understand their mechanisms of action and resistance. Essentially, two forms of antiestrogen resistance occur: *de novo* resistance and acquired resistance. Absence of estrogen receptor (ER) expression is the most common *de novo* resistance mechanism, whereas a complete loss of ER expression is not common in acquired resistance. Antiestrogen unresponsiveness appears to be the major acquired resistance phenotype, with a switch to an antiestrogen-stimulated growth being a minor phenotype. Since antiestrogens compete with estrogens for binding to ER, clinical response to antiestrogens may be affected by exogenous estrogenic exposures. Such exposures include estrogenic hormone replacement therapies and dietary and environmental exposures that directly or indirectly increase a tumor's estrogenic environment. Whether antiestrogen resistance can be conferred by a switch from predominantly ERα to ERβ expression remains unanswered, but predicting response to antiestrogen therapy requires only measurement of ERα expression. The role of altered receptor coactivator or corepressor expression in antiestrogen resistance also is unclear, and understanding their roles may be confounded by their ubiquitous expression and functional redundancy. We have proposed a gene network approach to exploring the mechanistic aspects of antiestrogen resistance. Using transcriptome and proteome analyses, we have begun to identify candidate genes that comprise one component of a larger, putative gene network. These candidate genes include NFκB, interferon regulatory factor-1, nucleophosmin, and the X-box binding protein-1. The network also may involve signaling through ras and MAPK, implicating crosstalk with growth factors and cytokines. Ultimately, signaling affects the expression/function of the proliferation and/or apoptotic machineries.
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Introduction

Antiestrogens primarily act by competing with estrogens for binding to the estrogen receptor (ER) and are the most widely administered endocrine agents for the management of ER-expressing breast cancers. The first antiestrogens were generated in the mid-1950s as fertility agents and included ethamoxytriphetol (MER-25) and clomiphene. The ability of these compounds to induce responses in some breast cancer patients soon became apparent (Kistner and Smith, 1960), but the compounds induced significant toxicity (Herbst *et al*., 1964). In the early 1970s, the first study in breast cancer patients was published with a new antiestrogen tamoxifen (TAM, ICI 46474) (Cole *et al*., 1971). Over the next 17 years, the total exposure to TAM reached 1.5 million patient years (Litherland and Jackson, 1988) and other selective estrogen receptor modulators (SERMs) are being developed and studied. TAM is now the most frequently prescribed antiestrogen, and compelling data have demonstrated a significant overall survival benefit with the administration of this agent in breast cancer patients with endocrine responsive disease (EBCTCG, 1992, 1998).

When compared with cytotoxic chemotherapy, antiestrogens are well tolerated and are associated with mostly minor toxicities (Love, 1989). Common side effects associated with TAM therapy include vasomotor symptoms, gastrointestinal disturbance, atrophic vaginitis, and changes in sexual functioning (Day *et al*., 1999). While the frequency and severity of hot flashes and other toxicities can be particularly unpleasant for some women, remarkably few discontinue TAM because of these side effects. Medical indications for the

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prompt discontinuation of therapy include associated venous thromboembolic disease and endometrial cancer (typically invasive adenocarcinoma, although uterine sarcomas have been reported). The incidence of these events is very low, and screening methods for both deep vein thrombosis and endometrial abnormalities exist. However, these increased risks must be considered in the light of the potential benefits—particularly in the case of healthy women considering TAM in the setting of chemoprevention as opposed to active treatment. The development of both venous thromboembolic disease and endometrial cancer is attributed to the estrogenic effects of TAM and may be abrogated by the development of more SERMs (e.g., raloxifene) or of pure ER antagonists (e.g., ICI 182,780; fulvestrant) (Robertson, 2001).

Some antiestrogens produce beneficial effects beyond their ability to inhibit existing breast cancers. The most convincing evidence supports an association between TAM treatment and a marked reduction in the risk of developing a contralateral breast cancer (EBCTCG, 1992) and a significant reduction in the incidence and severity of osteoporosis in postmenopausal women (Freedman *et al*., 2001; Kinsinger *et al*., 2002). Several early studies suggested a reduction in the risk of cardiovascular disease with TAM therapy, but this is not consistently reported (EBCTCG, 1998; Fisher *et al*., 1998). When observed, the cardiovascular benefit was usually attributed to the estrogenic effects of TAM; both estrogens and TAM produce apparently beneficial changes in serum triglyceride and cholesterol concentrations (Joensuu *et al*., 2000), perhaps through effects mediated by apolipoprotein E (Liberopoulos *et al*., 2002). However, these findings must be considered in the light of recent large studies of estrogenic hormone replacement therapy (HRT) that either failed to identify an HRT-induced reduction in coronary heart disease (Hulley *et al*., 1998; Grady *et al*., 2002; WHI, 2002) and stroke (Viscoli *et al*., 2001; WHI, 2002), or demonstrated an increase in the risk of these diseases.

An overview of antiestrogen resistance

Despite the relative safety and significant antineoplastic and chemopreventive activities of antiestrogens, most initially responsive breast tumors acquire resistance (Clarke *et al*., 2001b). It is unlikely that any single mechanism or single gene confers antiestrogen resistance. Rather, several mechanisms likely exist that encompass pharmacologic, immunological, and molecular events. These mechanisms, none of which are fully understood, likely vary within tumors. Intratumor variability in antiestrogen responsiveness will reflect the presence of multiple cell subpopulations (Clarke *et al*., 1990a). Since breast cancers appear highly plastic and adaptable to selective pressures, the intratumor diversity in antiestrogen responsive subpopulations also likely changes over time. Tumors appear capable of dynamically remodeling their cell populations in response to changes in host immunity or endocrinology, or the administration of local or systemic therapies. This plasticity is probably both cellular (some existing populations die out/back while other populations become dominant) and molecular (new cell populations emerge as individual cells/populations adapt their phenotypes by modifying their transcriptomes/proteomes).

Since the major pharmacologic and immunologic mechanisms of antiestrogen resistance have been previously reviewed (Clarke *et al*., 2001b), we will focus on the role of molecular signaling through ER-mediated activities in antiestrogen responsiveness. Antiestrogen resistance can be either *de novo* or acquired. The most common and best defined mechanism of *de novo* resistance is the absence of both ER and progesterone receptor (PR) expressions. However, we fail to predict response to antiestrogens in approximately 25% of ER+/PR+, 66% of ER+/PR−, and 55% of ER−/PR+ breast tumors (Honig, 1996). Many ER+ and/or PR+ breast tumors are already resistant by the time of diagnosis and the resistance mechanism in these tumors is unknown.

Overall, a loss of antiestrogen responsiveness by initially responsive tumors is likely to be the most common acquired resistance phenotype. Most initially antiestrogen responsive tumors retain levels of ER expression at recurrence on antiestrogen therapy that would still define them as being ER+ (Encarnacion *et al*., 1993; Kuukasjarvi *et al*., 1996; Bachleitner-Hofmann *et al*., 2002). Most data are for TAM treatment; ICI 182780, which causes degradation of ER (Dauvois *et al*., 1992), may have a greater potential for producing ER− tumors (Kuukasjarvi *et al*., 1996). From our *in vitro* studies, loss of ER is not required to achieve resistance to either ICI 182,780 or TAM (Brünner *et al*., 1993b, 1997). The loss of ER expression upon recurrence despite adjuvant TAM therapy has been reported in less than 25% of tumors (Kuukasjarvi *et al*., 1996; Bachleitner-Hofmann *et al*., 2002). Overall, a loss of ER expression does not seem to be the major mechanism driving acquired antiestrogen resistance.

A different resistance phenotype has been described in human breast cancer xenografts that exhibit a switch to a TAM-stimulated phenotype. This mechanism of clinical but not pharmacologic resistance may not be the dominant antiestrogen resistance phenotype. If the prevalence of acquired resistance phenotypes in ER+ tumors broadly reflects what is seen in *de novo* resistance, then the dominant resistance phenotype is a loss of antiestrogen responsiveness.

Whether the continued expression of ER is required for antiestrogen-resistant tumor growth or survival is not known. However, responses to aromatase inhibitors after an initial response and then failure on TAM are common (Buzdar and Howell, 2001) and strongly suggest that some TAM-resistant tumors retain a degree of estrogen responsiveness. Where durations of responses to second-line endocrine manipulations are short, truly estrogen-independent cell populations are either already present at the time of recurrence and/or many cells in the tumor are able to adapt rapidly to further changes in their endocrine environment. Very

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short response durations or disease stabilization may reflect the withdrawal of a mitogenic stimulus that is not required for the survival or basal proliferation of most cells in the tumor.

Antiestrogens

TAM is a triphenylethylene and its triaryl structure has been widely copied in the design of new compounds. Several TAM derivatives are already available, including toremifene (chloro-tamoxifen) and droloxifene (3-hydroxytamoxifen). Not surprisingly, both drugs are essentially equivalent to TAM in terms of their antitumor activities and toxicities (Roos *et al*., 1983; Pyrhonen *et al*., 1999), so neither is widely used in clinical practice.

The characteristic of raloxifene that has attracted the most interest is its apparent lack of estrogenic effects in the uterus, resulting in great interest in this drug's potential role in breast cancer chemoprevention. Subgroup analysis of the data from the Multiple Outcomes of Raloxifene (MORE) trial revealed that administration of raloxifene was associated with a 75% reduction in the incidence of invasive breast cancer without a concurrent increase in the incidence of endometrial cancers (Cummings *et al*., 1999). This finding has led to the ongoing randomized study of TAM and raloxifene (STAR) in breast cancer prevention. Raloxifene still acts as an antiestrogen in the brain, increasing the incidence of hot flashes (Davies *et al*., 1999). A high incidence of severe hot flashes is problematic for a drug to be administered for approximately 5 years to otherwise apparently healthy women. Raloxifene was recently approved by the Food and Drug Administration for the treatment and prevention of osteoporosis in postmenopausal women. While a benzothiophene, raloxifene (keoxifene; LY 156,758) has a three-dimensional structure broadly similar to the triphenylethylenes.

ICI 182,780 (Faslodex; Fulvestrant) is among the more promising new antiestrogens. Unlike TAM, ICI 182,780 is a steroidal ER inhibitor that is often described as a 'pure' antagonist with no estrogenic activity. This is in comparison to the triphenylethylene and benzothiophene antiestrogens, which are nonsteroidal, competitive ER inhibitors with partial agonist activity. The pure antagonist is characterized by antineoplastic activity in breast cancer and is devoid of uterotropic effects. However, the lack of agonist activity limits beneficial effects in bone. Whether ICI 182,780 also will increase hot flashes depends on whether it reaches adequate concentrations in the brain. Unlike TAM (Clarke *et al*., 1992), ICI 182,780 appears to be a substrate for the P-glycoprotein efflux pump (De Vincenzo *et al*., 1996), a major contributor to the blood–brain barrier (Cordon-Cardo *et al*., 1989). Consistent with this observation, initial studies suggest that this antiestrogen does not enter the brain in high concentrations (Howell *et al*., 1996). Pure antagonists may further exacerbate bone loss, a concern that also applies to aromatase inhibitors (Dowsett, 1997), but this issue may be addressed with the concurrent use of bisphosphonates or other therapies for osteoporosis. Clinical experience with ICI 182,780 has been reviewed by Howell (2001).

Antiestrogens and breast cancer treatment

Antiestrogens are effective in the adjuvant, metastatic, and chemopreventive settings and clearly induce significant increases in overall survival in some breast cancer patients (EBCTCG, 1992, 1998). Unlike aromatase inhibitors (inhibit estradiol biosynthesis), which are administered as single agents only to women with nonfunctioning ovaries, TAM can be given irrespective of menopausal status. In the adjuvant setting, TAM is administered at a daily oral dose of 20 mg, and several studies have now shown that the optimal duration of treatment is 5 years. While shorter (2 years) and longer (10 years) treatment durations produce notable responses, the risk : benefit ratios are strongly in favor of 5 years of treatment (Stewart *et al*., 1996; EBCTCG, 1998).

While molecular predictors of tumor responsiveness are rare for most breast cancer treatments, expressions of ER and PR strongly predict for a response to antiestrogens. Up to 75% of breast tumors expressing both receptors (ER+/PR+) respond to TAM. Response rates are somewhat lower in ER+/PR− tumors (~34%) and ER−/PR+ tumors (45%). The response rate in ER−/PR+ may be an overestimate; relatively few tumors with this phenotype have been evaluated and the ER− assessment may include false-negative ER measurements. Only a small proportion of ER−/PR− tumors respond to antiestrogens (<10%), perhaps also reflecting false-negative ER measurements. Indeed, the most recent meta-analysis from the Early Breast Cancer Trialists Collaborative Group (EBCTCG) found no significant reduction in recurrence rates in patients with ER-poor tumors who received adjuvant TAM (EBCTCG, 1998).

Results of the 1998 EBCTCG meta-analysis found limited evidence for a TAM-induced increase in the risk of death from any cause in women with ER-poor tumors. Why TAM might be detrimental to some women is unclear. However, ER− tumors are known to exhibit a more aggressive phenotype associated with lower rates of overall survival (Aamdal *et al*., 1984) and would be expected to recur earlier and more frequently. Estrogenic effects of TAM in these women also could have increased the number of deaths from cardiovascular disease and stroke, reflecting the data noted above from recent studies of estrogenic HRT use (Viscoli *et al*., 2001; WHI, 2002).

Antiestrogens and breast cancer chemoprevention

TAM's ability to inhibit contralateral breast cancers and relatively low incidence of serious side effects led to studies into its potential use as a chemopreventive agent for patients with a high breast cancer risk. Three large, randomized, chemoprevention studies with TAM have

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been performed to date: the NSABP P-1 trial ($n = 13\,388$ participants) (Fisher *et al*., 1998), the Royal Marsden Trial ($n = 2471$ participants) (Powles *et al*., 1998), and the Italian Chemoprevention Trial ($n = 5408$ participants) (Veronesi *et al*., 1998). Outcomes have been mixed: no significant reduction in risk was seen in the initial reports of either the UK or Italian trials, whereas the P-1 trial reported significant reductions in the incidence of both noninvasive (50%) and invasive (49%) breast cancers. A recent update on the Italian Trial reports an 82% TAM-induced reduction in the breast cancer risk among women at high risk for ER + breast cancer (Veronesi *et al*., 2003). In the NSABP trial, reductions in breast tumor incidence were seen only in the incidences of ER+ tumors (Fisher *et al*., 1998). Reasons for the disparities among the trials have been widely discussed; these tend to focus on differences in patient populations, subject eligibility criteria, and study size. Results from the NSABP P-1 trial, which are broadly consistent with the 39% reduction in contralateral breast cancer incidence reported for TAM use (EBCTCG, 1992), are usually considered the more definitive. These data contributed to the decision by the Federal Drug Administration (USA) in October 1998 to allow the use of TAM as a chemopreventive agent for breast cancer. More recently, NSABP has reported TAM-induced reductions in the risks of adenosis, fibrocystic disease, hyperplasia, metaplasia, fibroadenoma, and fibrosis in the P-1 trial (Tan-Chiu *et al*., 2003).

Estrogens and breast cancer

Since antiestrogen action and resistance are intimately affected by estrogen exposure, we briefly address the role of estrogens in breast cancer. An association between parity and breast cancer risk was observed by the 16th century Italian physician Bernadino Ramazzini (1633–1714) in his '*De Morbis Artificium*' published in 1700. The ability of ovariectomy to induce remissions in premenopausal breast cancer patients was shown by the Scottish physician George Beatson, the first clear evidence of an effective endocrine therapy for this disease (Beatson, 1896). More recent epidemiologic data show clear associations of early age at menarche, late age at menopause (Nishizuka, 1992), pregnancy (Hsieh *et al*., 1994), obesity (Hulka and Stark, 1995), serum estrogen concentrations (EHBCCG, 2002), and use of estrogenic HRTs (Magnusson *et al*., 1999; Schairer *et al*., 1999, 2000) or oral contraceptives (Berger *et al*., 2000) with an increase in the risk of developing breast cancer. Risk appears related to the timing of exposure and whether the cancer develops during the premenopause or postmenopause (Hilakivi-Clarke *et al*., 2002).

Precisely how estrogens affect breast cancer risk remains controversial and outcome may be dependent upon the timing and duration of exposure. During the postmenopausal years, estrogenic stimuli are more closely associated with an increased breast cancer risk. However, we have recently reviewed evidence consistent with the hypothesis that, depending on the timing of exposure, increased estrogenic exposure can be associated with a reduced risk of breast cancer (Hilakivi-Clarke *et al*., 2002). For example, estrogenic stimuli during childhood or the premenopausal years may affect breast development such that the breast is less susceptible to transformation. Estrogens may reduce breast cancer incidence in some women by altering mammary gland development and inducing the expression of genes involved in DNA repair (Hilakivi-Clarke *et al*., 1999a; Hilakivi-Clarke, 2000).

For the purposes of this review, we will focus on the aspects of estrogen exposure that are associated with increased breast cancer risk and the survival/proliferation of established neoplastic breast cells. Hence, estrogens can be considered to act either as promoters (factors that stimulate the growth and/or survival of existing transformed cells) or as initiators (factors that induce the genetic damage that leads to cellular transformation). Evidence that estrogens are tumor promoters is well established from both experimental and clinical observations. For example, the growth of several human breast cancer cell lines *in vitro* and *in vivo* is stimulated by estrogenic supplementation. Indeed, such estrogenic supplementation is effective whether administered as classical estrogens (e.g., estradiol, estrone, or estriol) or plant-derived phytoestrogens such as the isoflavone genistein (Hsieh *et al*., 1998). In addition, antiestrogens, aromatase inhibitors, leutinizing hormone releasing hormone agonists/antagonists, and ovariectomy are effective in the treatment of some breast cancer patients, all of which limit the interaction between a promotional (estrogenic) stimulus and cancer cells.

As tumor promoters, the effects of estrogens are related to the duration and timing of exposure. Withdrawal of an estrogenic stimulus that acts as a promoter could produce an eventual reduction in risk because it no longer promotes the growth or survival of existing cancer cells. Pregnancy produces a natural and significant increase in circulating estrogens, but only a transitory increase in breast cancer risk in young women. Indeed, if the first pregnancy was at a young age, the short-term increase may eventually translate into a lifetime reduction in breast cancer risk (Hsieh *et al*., 1994). The increased breast cancer risk associated with either oral contraceptive or estrogenic HRT use is also related to the recency of use. Risk begins to reduce with the cessation of use and is highest in current users (CGHFBC, 1996; Schairer *et al*., 2000).

Evidence that estrogens act as chemical initiators is more controversial. Estrogens can exhibit carcinogenic activity in some animal models; perhaps the best-known example is the ability of estrogens to induce renal cancers in Syrian hamsters (Kirkman, 1972). However, compelling evidence that estrogens initiate mammary cancer in animals is hard to find. In the 1930s, Lacassagne (1932) performed several studies in male mice and showed that administration of large doses of estrone can induce mammary tumors. While consistent

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with an estrogen-mediated initiation of mammary cancer, it is possible that the mice were infected with the mouse mammary tumor virus (MMTV). Other than some transgenic/null mouse models, only in the ACI rat does estrogen administration reproducibly produce a high incidence of mammary tumors (Cavalieri and Rogan, 2002).

Reactive estrogen semiquinone/quinone intermediates, produced by the redox cycling of estrogen metabolites hydroxylated at the C3 and C4 positions of the aromatic A-ring, are the most likely estrogen initiators (Cavalieri *et al*., 1997; Bishop and Tipping, 1998; Cavalieri and Rogan, 2002). These reactive species can generate a substantial intracellular oxidative stress and directly damage DNA through the production of DNA adducts. Such events could define reactive estrogen metabolites as initiators, rather than as merely promoters of carcinogenesis. Recently, the National Toxicology Program (2003) listed, for the first time, steroidal estrogens as carcinogens.

Estrogen independence and antiestrogen resistance

Estrogen independence and antiestrogen resistance are often considered to be synonymous, which is not surprising since ER− tumors are definitively estrogen-independent and very rarely respond to antiestrogens, ovariectomy, or aromatase inhibitors. Nonetheless, several observations suggest that various forms of both estrogen independence and antiestrogen resistance exist and that these may be biologically and clinically very different. For example, second-line responses to aromatase inhibitors after response and recurrence on TAM are common (Goss *et al*., 1995; Buzdar *et al*., 1996). Crossover between more similar compounds, such as other nonsteroidal antiestrogens, rarely produces secondary responses (Johnston, 2001), although crossover to structurally different antiestrogens can produce secondary responses in patients. Tumors that respond first to TAM (triphenylethylene) show a marked response to ICI 182,780 (steroidal) administered upon failure of the TAM therapy (Howell *et al*., 1995). Similar patterns of responses were seen previously in experimental models (Brünner *et al*., 1993b). For example, MCF-7 human breast cancer cells were selected for the ability to grow in the absence of estrogens (Clarke *et al*., 1989a). The selected cells are estrogen-independent because they no longer require estrogens for growth either in cell culture or as xenografts in athymic nude mice. However, when exposed to either 4-hydroxytamoxifen or ICI 182,780, the cells are growth inhibited both *in vitro* and *in vivo* (Clarke *et al*., 1989a; Brünner *et al*., 1993a, b).

These observations strongly imply that the ability of breast cancer cells to grow in a low or nonestrogenic environment is not always synonymous with antiestrogen resistance. Four antiestrogen resistance phenotypes have been defined (Clarke and Brünner, 1995) and are shown in Table 1. The clinical applicability of these phenotypes remains to be determined but they are useful for defining resistance phenotypes in experimental models.

Intratumor estrogens and antiestrogens and exogenous estrogenic exposures

Antiestrogens act within cells, primarily to compete with available estrogens for binding to ER. Thus, the antiestrogenic potency of any compound is related to its affinity for ER relative to that of any estrogens present and the concentrations of both the antiestrogens and estrogens. The data in Table 2 show the relative affinities of the primary estrogens, antiestrogens and their major metabolites, and selected environmental estrogens and phytoestrogens. Intratumor estrogen concentrations are affected by several factors including serum estrogen concentrations and local estrogen production within the breast. Serum estrogen concentrations are affected by the presence or absence of functional ovaries and exogenous estrogen use such as HRT, some oral contraceptives, and various dietary components.

Passive diffusion into cells across the plasma membrane appears to be TAM's and estradiols's primary method of entry into cells. However, both TAM and estrogens are extensively bound to serum proteins and probably also to cellular proteins in tumor/nontumor cells within the breast (Clarke *et al*., 2001b). Release from serum proteins likely occurs within the tumor vasculature, with both estrogens and antiestrogens being subsequently sequestered within tumor/nontumor cells by intracellular proteins. The lipophilicity of both hormone and drug, and the significant amount of adipose tissue in the breast, may produce a local reservoir for both estrogens and antiestrogens. However, the concentration of free drug/hormone within cells and serum may be relatively low. Intracellular sequestration of drug/hormone in tumor and stromal cells could produce a concentration gradient favoring

Table 1 Antiestrogen resistance phenotypes

Antiestrogen resistance	*Phenotype*
Type 1	Fully responsive to antiestrogens and aromatase inhibitors
Type 2	Resistant[a] to nonsteroidal antiestrogens but responsive to ICI 182,780 and aromatase inhibitors (or resistant to ICI 182,780 but responsive to nonsteroidal antiestrogens and aromatase inhibitors)
Type 3	Resistant to all antiestrogens but potentially responsive to aromatase inhibitors
Type 4	Multihormone-resistant (resistant to all endocrine therapies and includes ER− and PR− tumors)

[a]Resistance can be considered as unresponsiveness and antiestrogen-stimulated phenotypes

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