MINIREVIEW

The Consequences of Exhaustive Antiestrogen Therapy in Breast Cancer: Estrogen-Induced Tumor Cell Death

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Forty years ago, the endocrine treatment for breast cancer was a last resort at palliation before the disease overwhelmed the patient (1). Ovarian ablation was the treatment of choice for the premenopausal patient, whereas either adrenalectomy or, paradoxically, high-dose synthetic estrogen therapy were used for treatment in postmenopausal patients. A reduction or an excess of estrogen provoked objective responses in one out of three women. Unfortunately, there was no way of predicting who would respond to endocrine ablation, and because so few patients responded there was no enthusiasm for developing new endocrine agents. All hopes for a cure for breast cancer turned to appropriate combinations of cytotoxic chemotherapy.

Today tamoxifen, a nonsteroidal antiestrogen (2), has proven to be effective in all stages of premenopausal and postmenopausal breast cancer, and several new endocrine strategies, including aromatase inhibitors, luteinizing-hormone releasing hormone (LHRH) superagonists, and a pure antiestrogen (fulvestrant), are now available for breast cancer treatment. Additionally, tamoxifen and raloxifene, a related compound, are used to reduce the risk of breast cancer and osteoporosis, respectively, in high-risk groups (3). Hormonal modulation and strategies to prevent the actions of estrogen in the breast are ubiquitous. However, with successful changes in treatment strategies comes the consequence of change.

This minireview will describe the current strategies for the treatment and prevention of breast cancer and present emerging new concepts about the consequences of exhaustive antiestrogen treatment on therapeutic resistance. Exp Biol Med 229:722–731, 2004

Key words: raloxifene; tamoxifen; HER2/neu; apoptosis; selective estrogen receptor modulator; aromatase inhibitor; phytoestrogens

The Strategic Use of Endocrine Therapy

The discovery of the estrogen receptor (ER), the mechanism that directs estrogen action in target tissues, proved to be an invaluable breakthrough that heralded the era of molecular endocrinology (4). The translation of the basic knowledge of estrogen action to breast cancer therapy also helped to explain why only one in three women with advanced breast cancer responded to endocrine ablation. This concept was reviewed at an international meeting in Bethesda, Maryland, in September 1974, and the results were used to establish the ER assay as a predictive test for endocrine therapy (5).

Prior to the 1970s, there was only modest interest in developing antiestrogenic treatments for breast cancer because of known or suspected toxicities (2). Indeed, the position was taken that endocrine agents were palliative, therefore, any advances would be modest compared with the potential of cytotoxic chemotherapy to cure.

Nonsteroidal antiestrogens were initially targeted for the modulation of the sexual cycle because compounds such as ICI 46,474 were known to be postcoital contraceptives in the laboratory (6) but inducers of ovulation in subfertile women (7).

The reinvention of ICI 46,474 as tamoxifen for palliative therapy for breast cancer was an important step forward (8). However, the real advance occurred through viewing the ER as a target for drug action and developing a new strategy for treatment. Tamoxifen blocks binding of estrogen to the human and rat tumor ER and causes the

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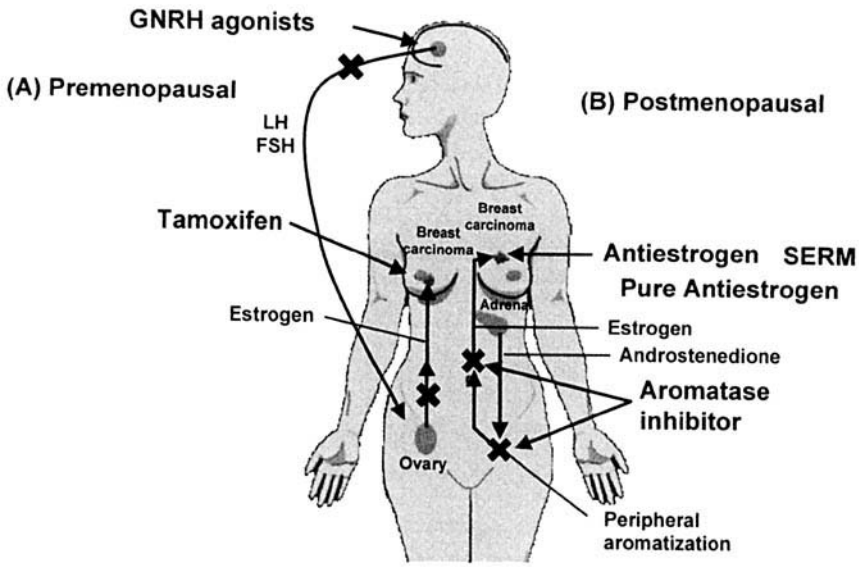

Figure 1. The potential endocrine strategies to control the growth of ER-positive breast cancer in premenopausal and postmenopausal patients. Tamoxifen and gonadotropin-releasing hormone (GNRH) agonists are useful in premenopausal women, whereas selective ER modulators (tamoxifen or toremifene) and the pure antiestrogen fulvestrant are useful in postmenopausal women. The third-generation aromatase inhibitors are only useful therapeutic agents in postmenopausal women or in women under 50 following complete ovarian ablation.

regression of ER-positive rat mammary tumors (9–11). Tamoxifen is ineffective in ER-negative tumors so the targeting of tamoxifen to the patient with an ER-positive tumor was a logical strategy. However, even the extensive treatment of advanced disease was unlikely to contribute more than palliation.

During the 1970s, a laboratory strategy was developed to use tamoxifen to its maximal effectiveness and target the disease at its earliest stages. Tamoxifen was found to prevent the development of ER-positive tumors if the drug was given for long periods (12). Tamoxifen was classified as a tumoristatic rather than a tumoricidal drug, so continuous therapy was anticipated to prevent reactivation of occult tumor growth by endogenous estrogen. However, emerging data clearly demonstrate that tamoxifen also induces apoptosis and, therefore, tumor regression through epigenetic alterations (13). For example, tamoxifen modulates signaling proteins such as protein kinase C, calmodulin, transforming growth factor beta, and the proto-oncogene c-*myc* (13). In addition, recent studies implicate a role for caspases, mitogen-activated protein kinases, including c-Jun N-terminal kinase and p38, in tamoxifen-induced apoptosis.

The strategy for using adjuvant therapy following surgery to destroy micrometastatic disease initially started to use only 1 year of tamoxifen. The reason for the selection of this treatment regimen was that tamoxifen was only effective for about 1 year in the treatment of advanced breast cancer and there was a sincere concern about the development of premature drug resistance if longer schedules of adjuvant therapy were used. Today, the laboratory principles of targeting only ER-positive tumors with long-term tamoxifen treatment are proven in clinical trials. Five years of adjuvant tamoxifen is effective at reducing the death rate from breast cancer in ER-positive premenopausal and postmenopausal patients whether they are node positive or negative (14). One year of adjuvant tamoxifen is ineffective in preventing recurrence in ER-positive premenopausal patients and ineffective in controlling contralateral breast cancer (14).

Long-term tamoxifen therapy prevents rat mammary carcinogenesis more effectively than short-term therapy (12, 15). These data translate to the clinical application of 5 years of tamoxifen, which is effective at reducing the incidence of ER-positive breast cancer in premenopausal and postmenopausal women at high risk (16, 17).

The results of three decades of translational research in breast cancer are that long-term endocrine therapy targeted to the ER can enhance the survival of breast cancer patients and long-term endocrine therapy has produced a pioneering advance in chemoprevention.

Long-Term Endocrine Modulation

There are currently multiple therapeutic approaches to target the ER and restrict the access of estrogen to the breast cancer cell. These therapeutic strategies (Figure 1) have all shown promise in clinical trials, but the key to success remains a long-term intervention. Tamoxifen remains the cheapest and most extensively used antiestrogen approach in both premenopausal and postmenopausal ER-positive breast cancer patients. Be that as it may, valuable advances in efficacy and improvements in reducing the side effects profile are being documented by fine tuning antiestrogen therapy. Several innovations can be used for illustration. Although long-term adjuvant tamoxifen is an effective treatment in premenopausal patients, an increase of up to

twofold in levels of circulating estrogen has been a worrisome consequence of tamoxifen use (18). Clearly, if tamoxifen is a competitive inhibitor of estrogen action at the ER, then less estrogen in the local environment will enhance tamoxifen action. The recent combined use of LHRH superagonists with tamoxifen to prevent the secretion of gonadotropins and cause a medical oophorectomy demonstrates that less circulating estrogen can prevent local recurrence by almost twofold compared with chemotherapy (19). Similarly, the evaluation of anastrozole, a third-generation aromatase inhibitor, against tamoxifen in the adjuvant treatment of postmenopausal breast cancer demonstrates a significant reduction in side effects of venous thromboembolism (VTE) and endometrial cancer for the "no estrogen" approach for postmenopausal patients (20). Based on these findings, it will not be long before combinations of aromatase inhibitors and LHRH superagonists will be used routinely to treat premenopausal women who present with an ER-positive breast tumor.

Attempts to extend the value of tamoxifen beyond 5 years of treatment have been disappointing (21), since there are increases in side effects and, in fact, a decrease in disease-free survival. In contrast, switching to letrozole, a noncross-resistant aromatase inhibitor, after 5 years of tamoxifen reduces recurrence rates and contralateral breast cancer by more than 40% (22). These data support the laboratory concept (12) that long-term antiestrogen therapy, first with tamoxifen and then with further reductions in circulating estrogen, that is, the use of an aromatase inhibitor, is an appropriate adjuvant strategy to control breast cancer recurrence.

In parallel, the laboratory study of tamoxifen has also created novel therapeutic opportunities for the application of medicines as preventives for several diseases in otherwise well women (23, 24). Tamoxifen is not a complete or pure antiestrogen but has estrogenic and antiestrogenic actions at different sites around the body. Studies in animals and postmenopausal patients demonstrate that tamoxifen is estrogenic in bone, antiestrogenic in breast cancer, and has mixed estrogenic/antiestrogenic actions in the uterus. These observations led to the concept of selective estrogen receptor modulation and promoted the testing of tamoxifen as a preventive in high-risk women (25). Tamoxifen would potentially prevent breast cancer without increasing the risk of osteoporosis or coronary heart disease (26). Five years of tamoxifen treatment is now successfully used to reduce the incidence of breast cancer in premenopausal and postmenopausal women at high risk (16). The clinical prevention studies show a reduction in fracture rate, but unfortunately tamoxifen treatment is associated with a modest, but significant, increase in endometrial cancer incidence in postmenopausal women (16, 27). As a result of these concerns, a change in therapeutic strategy for chemoprevention occurred in the 1990s (7).

The recognition of selective estrogen receptor modulator (SERM) action, in the group of drugs then known as nonsteroidal antiestrogens, suggested that a compound that had fewer uterine side effects than tamoxifen could be targeted, not to breast cancer, but to prevent osteoporosis with breast and endometrial safety (7). As a result of this new concept, raloxifene is now available to treat and prevent osteoporosis but with breast and endometrial safety (28, 29). These encouraging preliminary data prompted the testing of tamoxifen against raloxifene in the study of tamoxifen and raloxifene (STAR) trial. Additionally, since raloxifene also reduces circulating cholesterol (30) and there are suggestions that this could result in reductions in coronary heart disease (CHD) in women at high risk (31), raloxifene is currently being tested as an agent to reduce fatal CHD and at the same time protect against osteoporosis and breast and endometrial cancer.

Despite the positive advances in therapeutics, the widespread use of antiestrogen therapies will have consequences for the natural history of breast cancer as it adapts and evolves in its new environment.

The Evolution of Drug Resistance to SERMs

There are two forms of drug resistance. Intrinsic resistance occurs in 50% of patients with ER-positive tumors, and these individuals do not respond to antiestrogen therapy. Recent studies demonstrate that one mechanism that might be responsible for intrinsic resistant to tamoxifen is overexpression of *HER*2/neu (32) and/or overexpression of both *HER*2/neu and amplified in breast cancer1 (*AIB*1) genes (33). In contrast, acquired resistance occurs when the patient with an ER-positive tumor initially responds to a SERM but then SERM-induced tumor growth develops.

Acquired resistance to SERMs is particularly interesting because the SERM ER complex stimulates tumor growth as efficiently as the estradiol ER complex. Laboratory models of tamoxifen-stimulated breast or endometrial cancer illustrate the concept. ER-positive breast or endometrial cell lines (34, 35) or tumors (36) grow in response to estradiol in ovariectomized athymic mice. Tamoxifen or raloxifene inhibits estradiol-stimulated growth (34, 35), but continuous treatment with SERMs results in SERM-stimulated tumors that are transplantable to new generations of athymic mice (37–39). Cross-resistance between tamoxifen and raloxifene occurs with both breast and endometrial resistant models, but estradiol also supports tumor growth (40). However, growth of tumors is not spontaneous. In other words, a SERM or an estrogen must activate the ER signal transduction pathway; no treatment or treatment with the pure antiestrogen fulvestrant (ICI182,780 or Faslodex), which causes rapid ubiquitination and destruction of the fulvestrant ER complex (41), results in growth inhibition (42, 43).

The laboratory studies of acquired resistance provide a mechanistic understanding for the often serendipitously successful application of endocrine therapies following the failure of tamoxifen treatment. The treatment of advanced

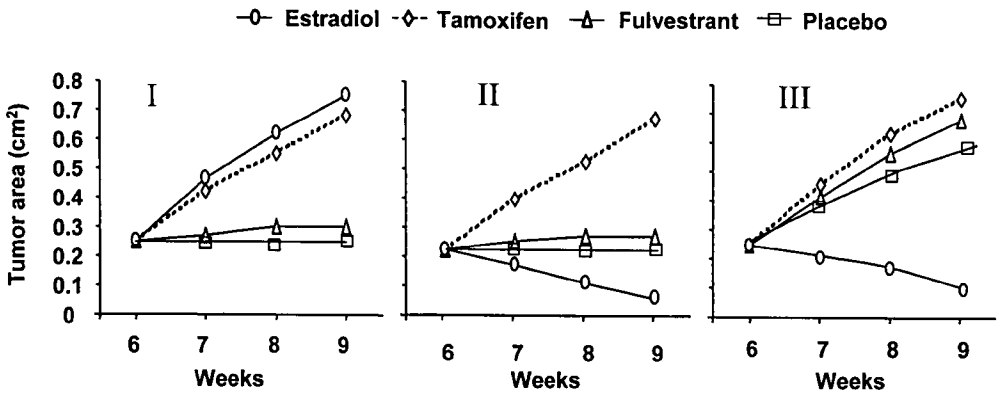

Figure 2. This is a schematic diagram to demonstrate the response of breast tumors transplanted into athymic mice to no treatment, estradiol, tamoxifen, or the pure antiestrogen, fulvestrant. The breast tumor growth is initially completely dependent on estrogen for growth, but then SERM resistance develops and tumors evolve to be SERM dependent and ultimately SERM independent. In contrast, estradiol changes from being a growth stimulant to an apoptotic agent.

(8) breast cancer with tamoxifen was standard first line therapy for ER-positive disease throughout the 1980s and 1990s. Disease progression during tamoxifen treatment can be identified as tamoxifen stimulated by the documentation of withdrawal inducing regression starting several weeks after discontinuation of the drug (44). The weak estrogen-like actions of tamoxifen ultimately appear to promote drug resistance in select tumors, so treatment with agents without any estrogenic properties would appear to be logical second line therapies. Aromatase inhibitors prevent the synthesis of potent estrogens from androgen precursors in postmenopausal patients, so a "no estrogen" environment is a logical second line therapy. Competitive (45) and suicide (46) inhibitors of the aromatase enzyme are proving to be effective at treating tamoxifen-resistant breast cancer (47). Similarly, fulvestrant is as effective as anastrozole at treating tamoxifen-resistant breast cancer (48, 49).

The previously mentioned laboratory models (37) of SERM resistance replicate the clinical situation (44). However, there is an inconsistency. Adjuvant tamoxifen therapy applied to premenopausal and postmenopausal patients with ER-positive breast cancer exposes the entire breast cancer population to 5 years of treatment to prevent recurrence of disease. Until recently, there were no laboratory models that replicated the exposure of hormone-dependent cancer to long-term tamoxifen treatment.

The serial transplantation of mammary adenocarcinoma from Caucasian female-7 (MCF-7) tumors into generations of athymic mice for 5 years might more closely replicate the exposure of a few micrometastatic breast cancer cells to 5 years of adjuvant tamoxifen. The undetected tamoxifen-stimulated cells would be exposed subsequently to years of adjuvant tamoxifen. An initial examination of the long-term tamoxifen-exposed MCF-7 breast tumor model paradoxically demonstrated that although tamoxifen was still required to cause tumor growth, that is, the SERM receptor complex was necessary for growth, estradiol now caused rapid regression and apoptosis in small tumors (50, 51). Continuous estradiol treatment results in the resensitization to estradiol and growth of some tumors, which upon retransplantation into athymic mice are again responsive to tamoxifen as an antitumor agent. Therefore, lower physiologic doses of estradiol can reverse tamoxifen resistance and reestablish the effectiveness of antiestrogen therapy (51).

Overall, these laboratory observations create an insight into the evolution of antiestrogen resistance and open the door to future treatment strategies and, potentially, to the identification of novel targets for new drug development.

Stages of Antiestrogen Resistance

There are now numerous biological properties that can be used to characterize the evolution of SERM resistance in breast and endometrial cancers. The phases of SERM resistance are identified by the response of the ER-positive tumor to tamoxifen, estradiol, or the pure antiestrogen fulvestrant. The schematic growth characteristics of both breast and endometrial ER-positive tumors transplanted into athymic mice are summarized in Figures 2 and 3. Tumor resistance to SERMs is evidenced by growth stimulation with either estradiol or SERMs. This established form of resistance is now classified as Phase I resistance. Both breast (37, 38) and endometrial (52) cancers exhibit Phase I resistance with estradiol, tamoxifen, or raloxifene-stimulating tumor growth. Fulvestrant or no treatment (which could be viewed as treatment with aromatase inhibitors) does not induce tumor growth when Phase I resistance is established. Interestingly, the continuous treatment of ER-positive T47D breast cancer cells can cause the rapid induction of Phase I drug resistance to tamoxifen (38), but, unlike tamoxifen-resistant MCF-7 breast cancer cells (40), raloxifene like SERMs are not cross resistant (53). Phase I drug resistance, however, can evolve by continuous treatment with SERMs, causing increases in survival pathways and a significant change in the biological response to estradiol and fulvestrant. Estradiol, at physiologic levels, causes the rapid decrease in tumor growth (51), whereas, paradoxically, a combination of estradiol and fulvestrant causes robust tumor

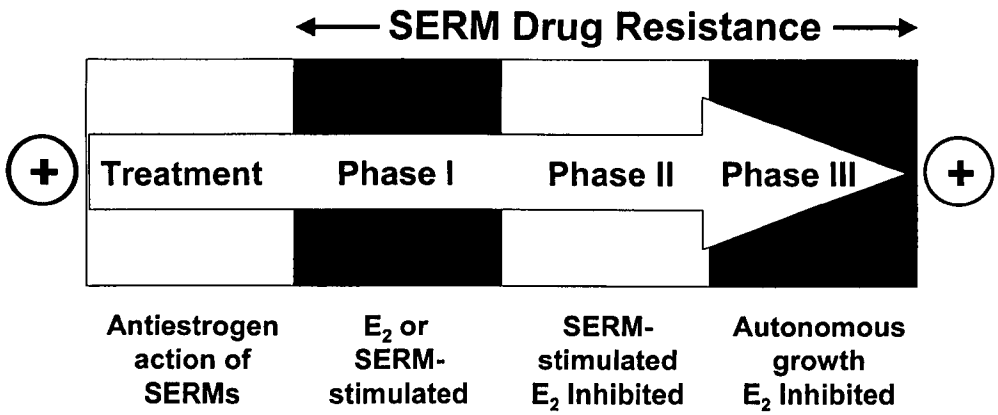

Figure 3. The evolution of breast cancer treatment and tamoxifen-induced drug resistance. The ER-positive tumor will eventually become stimulated by either tamoxifen or estrogen during Phase I drug resistance, but after about 5 years Phase II resistance develops where estrogen alone now kills tumor cells. In the final phase of SERM resistance (Phase III), growth is autonomous and all ER targeted therapies fail except estrogen. Eventually, either the tumor burden becomes too great or the ER is lost and chemotherapy is the only option remaining.

growth (54). This is now referred to as Phase II resistance. To date, there are no reports of Phase II resistance with T47D cells treated indefinitely with SERMs, although stable transfection of T47D cells with protein kinase C alpha causes hormone independent growth in athymic mice (55). Estradiol provokes rapid tumor regression, but fulvestrant blocks estradiol-induced tumor apoptosis (55). This type of resistance defines Phase II resistance and suggests that T47D cells can evolve in much the same way as MCF-7 cells.

Ultimately, SERM-resistant MCF-7 tumors grow spontaneously (56). This is classified as Phase III resistance and shown in Figure 3, which is a schematic illustration of the concept. However, low circulating concentrations of estradiol completely inhibit tumor growth (56). Growth is not controlled by letrozole, fulvestrant, tamoxifen, or raloxifene. Finally, it is interesting to note that endometrial cancer cells can also evolve to become Phase III SERM resistant. This classification is appropriate because endometrial cancer cells exposed to raloxifene, first *in vitro* and then through several transplantation generations in athymic mice, grow spontaneously *in vivo*. (57). The Phase III endometrial tumors grown *in vivo* are unaffected by fulvestrant, but estradiol prevents tumor growth.

Overall, SERMs appear to cause a consistent alteration in the biology of breast and endometrial cancer. However, the current fashion in the treatment of breast cancer is changing from the exhaustive use of adjuvant tamoxifen to the extended use of aromatase inhibitors. The question to be asked is whether aromatase inhibitors will also cause similar changes in the sensitivity of breast cancer cells to estrogen action and induce tumor cell death.

Resistance to Aromatase Inhibitors

One of the first studies that observed acquired resistance to estrogen deprivation was done with premenopausal women after surgical oophorectomy (58). The investigators noted that women that had previously responded to removal of their ovaries later relapsed with tumor recurrence (58). Interestingly, the first generation aromatase inhibitor, aminoglutethimide, was effective in promoting tumor regression of these secondary tumors, suggesting that local activity of the aromatase enzyme in the tumor microenvironment also plays an important role for tumor growth. Based on the current understanding of estrogen withdrawal from breast cancer cells, it is now hypothesized that cells initiate a short period of growth reduction followed by a crisis period and then, for the majority, cell death. However, a proportion of cells do survive and continue to replicate rapidly in the estrogen-free environment (59, 60). There are numbers of cell lines available that grow under estrogen-free conditions that have no response to estradiol or antiestrogens or, alternatively, for which antiestrogens are still effective as second line therapy (61, 62).

Song and coworkers (63) first noted that some estrogen-deprived cell lines can become supersensitized to the cidal effects of low concentrations of estradiol. Song and coworkers also noted (63) that the Fas/Fas ligand (death receptor) system was activated by estradiol to induce apoptosis. These workers used their results to suggest a mechanism to explain the pharmacological actions of high-dose estrogen therapy previously used as standard breast cancer therapy during the 1940s to 1970s (1). However, the emerging new data on the central role of the ER to kill cancer cells selectively now creates new opportunities to integrate low-dose estrogen therapy in the breast cancer treatment plan.

A Role for Estrogen Therapy

The paradoxical antitumor action of high doses of synthetic estrogens in the treatment of breast cancer has been known for nearly 60 years (64). Rigorous clinical studies during the 1950s established that response rates to estrogen are higher in older rather than younger postmenopausal patients (1). Indeed, these observations established

estrogen therapy as an alternative to the surgical approach of adrenalectomy and glucocorticoid treatment. However, tamoxifen, with fewer serious side effects than high-dose estrogen treatment, emerged as the standard of care during the 1970s (65), and estrogen was all but abandoned as a breast cancer treatment.

Nevertheless, the changing fashion of therapeutics with long-term antiestrogen therapies has resulted in renewed interest in high-dose estrogen therapy prior to chemotherapy. There are anecdotal reports (66) and an interesting Phase II study of diethylstilbestrol treatment (5 mg, three times daily) following exhaustive antiestrogen therapy (67) that illustrate the potential value of a new strategy incorporating estrogen. A total of 32 postmenopausal patients who were refractory to antiestrogen treatments were challenged with diethylstilbestrol (5 mg, three times daily). Response rates were significant with 4/32 complete responses, 6/32 partial responses, and 2/32 stable disease. Clearly, these are important new data. However, the use of high-dose estrogen therapy is associated with the serious side effect of VTE. This is particularly of concern for patients with extensive metastatic disease, since there is already a higher incidence of VTE in these cases. The goal of current translational research is to leverage emerging laboratory and clinical observations to design a logical strategy for enhancing the effectiveness of endocrine maintenance. Much new work has been completed on the molecular actions of estrogen, antiestrogens, and SERMs (23, 24), and a summary of progress will be presented to illustrate opportunities for targeted therapy.

The Evolution of Estrogen Action

Estrogens are traditionally recognized as hormones that stimulate growth and activate transcription of genes in target tissues. Estradiol binds to the ER in the nucleus of breast cancer cells and initiates a series of conformational changes in protein structure. The planar steroid is buried within a hydrophobic pocket, formed from the ligand-binding domain, and subsequently a specific part of the ER referred to as helix 12 seals the steroid within the pocket. These structural changes cause the exposure of two areas on the external surface of the protein complex activating functions (AFs) 1 and 2, responsible for binding coactivator molecules necessary for gene transcription. The formation of the estradiol (E_2) ER complex also exposes a DNA binding domain and a dimerization domain. Thus, the conformational changes induced in the ER by estradiol set into motion a series of events that result in the dimerization and binding of ER complexes at the appropriate estrogen response elements (EREs) in the promoter region of estrogen responsive genes. The coactivator molecules, binding to the synergistic sites AF-1 and AF-2, then link with the transcription machinery for the initiation of mRNA synthesis.

The events above describe, in simple terms, estrogen-induced gene activation, but there are multiple layers of complexity before cell replication can occur. The ER complex not only interacts directly with DNA, but also can bind to other proteins at AP-1 and Sp-1 sites. Thus, the simultaneous interaction of the E_2 ER receptor complex at a select sequence of intracellular targets programs the breast cancer cell for DNA replication.

The converse of cell survival and DNA replication in response to estrogen is quiescence and cell death induced in breast cancer cells by either blocking the ER with SERMs or the withdrawal of a ligand through estrogen deprivation. Much work has been completed on the interaction of raloxifene and 4-hydroxytamoxifen (the active metabolite of tamoxifen) with the ER. Essentially, both ligands bind in the hydrophobic ligand-binding domain, but the alkylamino-ethoxyphenyl side chain of both antiestrogenic ligands prevents helix 12 from sealing the hydrophobic pocket. Helix 12 is repositioned to inactivate AF-2. The two ligands are, however, different in their basic pharmacology; 4-hydroxytamoxifen is more promiscuous than raloxifene and possesses more estrogen-like actions in target tissues such as the uterus (68, 69). The differences in the SERM ER complexes are illustrated at the subcellular and molecular levels because the 4-hydroxytamoxifen ER complex is much more estrogenic at gene sites than the raloxifene ER complex. The key to understanding the modulation of the SERM ER complex is to understand the relationship between the antiestrogenic side chains of SERMs and the external surface of the ER complex. By changing the SERM side chain and the surface amino acids, the ER complex can be interrogated to predict precisely the estrogenic or antiestrogenic actions of the complex. Essentially, the charge on amino acid D351 must be neutralized and shielded to prevent the allosteric activation of AF-1. The position of the side chain of 4-hydroxytamoxifen in the SERM ER complex is unable to neutralize D351, so the complex can activate AF-1 but the ligand still inactivates AF-2 (70). In contrast, raloxifene inactivates both AF-1 and AF-2 because the side chain completely neutralizes D351 (71).

However, these simple models of stimulation by estrogen and blockade by SERMs ultimately result in changes that can be either genomic or epigenetic, resulting in SERM resistance. Although the majority of cells either become static or die, cell survival pathways are initiated that ultimately result in the reactivation of the SERM-ER complex. In particular, there is an increase in HER2/neu signaling through an increase in *HER2*/neu mRNA synthesis (72). The stable transfection of the *HER2*/neu gene into MCF-7 breast cancer cells causes resistance to tamoxifen and promotes spontaneous growth (32). Thus, one hypothesis is that an increase in cell surface signaling will enhance phosphorylation of the ER and coactivator molecules to produce SERM-stimulated growth. Although the precise mechanism is at present unknown, it is possible that the nuclear effects of the ER are modified by the redistribution of some of the ER toward the cell membrane. This

membrane ER could then control (nongenomically) an enhanced phosphorylation network that ultimately subverts the dependence on the ER and produces SERM-independent growth. This type of growth response *in vivo* has been noted with stable transfectants of T47D cells using the *PKC* alpha gene (55).

Together, studies now suggest that enhancement of survival pathways occur in breast cancer cells exhaustively treated with antiestrogens. However, estradiol, which initially enhances cell growth and survival, now appears to collapse the enhanced survival systems, for example, HER2/neu and NFκB (54, 73). Simultaneously, estradiol initiates the synthesis of the Fas receptor and the activation of caspase-8 from procaspase-8 (54, 73) to provoke apoptosis. The result is that tumors previously exposed to exhaustive antiestrogen therapies rapidly regress *in vivo* in response to low concentrations of estradiol (83–96 pg/ml) in the postmenopausal range.

One unanticipated observation is the paradoxical action of fulvestrant, which not only reverses the apoptotic action of estradiol during Phase II resistance but also induces a robust growth response in tumors treated with low-dose estradiol plus fulvestrant (54). Remarkably, fulvestrant continues to function as a recognized antiestrogen at nuclear sites, that is, at the transforming growth factor alpha gene where it blocks estradiol-induced transcription. In contrast, fulvestrant causes a dramatic increase in *HER2*/neu that completely reverses the effect of estradiol to downregulate the gene (54). These data obviously have important implications for the second or third line use of fulvestrant as an antiestrogen treatment in women with significant levels of circulating estradiol.

Clearly, there is much to do to understand how the estradiol ER complex can distinguish between a cell that grows and a cell that dies. These basic pathways could potentially be deciphered using combinations of proteomics and genomics. However, if estradiol action again becomes the focus of cancer therapeutics it will be important to incorporate emerging new data on the molecular aspects of estrogen action. In this way, new approaches to therapy could be designed that could enhance target specificity and also be used to identify new targets for the regulation of apoptosis.

Estrogenic Ligands

There is general agreement that antiestrogens bind to the ER and produce a series of perturbations that prevent full estrogen action (74), but until recently some have thought that all estrogens bind to the receptor to cause activation of AF-1 and AF-2. This view has recently been modified with the subclassification of differently shaped estrogens into Class I and Class II (75–77). The evidence to support the subclassification system comes from two sources: the use of a functional assay employing a comparison of the transforming growth factor alpha gene with either wild type or mutated ER used to identify different shaped estrogenic complexes (75, 77) or an engineered MCF-7 cell stably transfected with an *ERE* luciferene gene to classify a series of novel weak estrogens (76). Both approaches employ ligand docking into the known ligand-binding domain structures of estradiol or 4-hydroxytamoxifen.

The idea that there are multiple conformations possible for the estrogen-ER complex explains why the ER is promiscuous and can bind to ligands with very different structures (78). However, the subclassification of estrogens now has important ramifications for biology, and the knowledge can potentially be applied to therapeutics.

Environmental estrogens can all stimulate the growth of breast cancer cells in culture. Planar phytoestrogens such as genistein and coumestrol are Type I estrogens that activate transcriptions through an AF-1 and AF-2 synergy. In contrast, the nonplanar three-dimensional estrogens activate transcription via the complex interdependent site AF-2b (79) that allosterically activates AF-1 (75, 77). Thus, there is potential for different shaped environmental estrogens either to enhance or to block carcinogenesis at different sites around the body. Additionally, there is potential to exploit the molecular knowledge of different shaped estrogen complexes and apply it to the new knowledge of the evolution of resistance to antiestrogen therapy. It may be possible to discover whether select estrogens could be employed as unique, highly active apoptotic agents. Studies are currently ongoing to establish whether phytoestrogens or dietary changes could induce apoptosis in breast cancer cells *in vitro* and *in vivo* respectively following the development of Phase II resistance.

Conclusion and Clinical Applications

The concept that the ER would be a valuable therapeutic target for the treatment of breast cancer (4) is now proven. However, and somewhat unexpectedly, there are consequences for the strategy of extended antiestrogen therapy. The enhanced survival mechanisms present in breast cancer cells accumulate and may lead to autonomous growth. This final phase of resistance (Phase III) is unaffected by all antiestrogen modalities. However, the cells retain the ER and the tumors respond to estrogen both in clinical practice and in the laboratory with apoptosis and regression. The clinical reintroduction of high-dose estrogen therapy will undoubtedly provide palliation for some breast cancer patients with metastatic disease, but there are now new opportunities to translate laboratory findings to both enhance the duration of successful antiestrogen therapy and increase the proportion of responses to estrogen therapy.

We propose a systematic laboratory and clinical evaluation of the targeted estrogen approach to breast cancer treatment. Studies are already under way to confirm and extend the findings of the Lonning *et al.* (67) report, but

several questions need to be answered in a methodical series of clinical trials:

1. Can the standard therapeutic dose of estrogen be lowered to produce the same response rates in patients following exhaustive antiestrogen therapy?

2. Does the response to therapeutic estrogen reactivate responsiveness to antiestrogen therapy so treatments can be cycled?

3. Do a variety of estrogens, for example, conjugated estrogens, phytoestrogens, or dietary changes, increase apoptosis in patients who have received exhaustive antiestrogen therapy?

4. If each of the concepts 1–3 prove to be true, can the targeted estrogen approach enhance the apoptotic action of select chemotherapeutic regimens?

Development of a series of successful treatment protocols could then be exported to consider the idea of an "estrogen purge" for patients with disease that is estimated to be increasingly resistant to antiestrogen therapy. Thus there could be indefinite maintenance for select patients on cycles of noncross-resistant antiestrogen therapy with short preemptive estrogen treatment periods to kill Phase II and Phase III resistant tumor cells. As a result, the patient's disease could be maintained for decades.

More than half of ER-positive breast cancers already have intrinsic resistance to antiestrogen therapy, so there is now a potential opportunity for applying the current concepts to broaden the responses to endocrine therapy. The fact that antiestrogen therapy is undermined in acquired resistance by the development of sophisticated cell surface survival mechanisms suggests a possible treatment strategy to apply to intrinsic resistance. The blockade of multiple survival pathways by antibodies to cell surface receptors, tyrosine kinase inhibitors, and, potentially, specific inhibitors of subcellular phosphorylation could significantly reduce cellular survival, but the estrogen-activated ER may now have no alternative but to provoke apoptosis. Clearly, laboratory models are needed to test these hypotheses, but the expanding list of agents that target cell survival will soon be available for rational combination therapy followed by an estrogen challenge. The question to be answered will be whether intrinsic resistance can be remodeled with estrogen to resensitize the tumor to antiestrogens.

Studies of the mechanism of estradiol-induced apoptosis will provide an invaluable insight into how the estradiol ER complex interprets its natural environment to decide upon survival or cell death. It would clearly be important to discover the events that allow the ER to identify a super surviving cell as an alien environment so that apoptosis is initiated. We suggest that clues are already available from earlier reports to address the question. ER-negative cells can be stably transfected with the cDNA for human ER (80). A consistent observation has been that estrogen causes a decrease in cell growth (81). These observations could now be interpreted as the ER-reducing proliferation in cells that are completely independent of estrogen for survival. The molecular trick will be to use the ectopic ER to discover a new molecular target for drug discovery. The research goal will be to establish whether the potential molecular target for apoptosis is cancer cell specific so that a new era of cancer therapeutics can emerge. Cancer control through targeted apoptosis can then become a novel, tumor specific treatment and preventive.

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