The new biology of estrogen-induced apoptosis applied to treat and prevent breast cancer

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Abstract
The successful use of high-dose synthetic estrogens to treat postmenopausal metastatic breast cancer is the first effective ‘chemical therapy’ proven in clinical trial to treat any cancer. This review documents the clinical use of estrogen for breast cancer treatment or estrogen replacement therapy (ERT) in postmenopausal hysterectomized women, which can either result in breast cancer cell growth or breast cancer regression. This has remained a paradox since the 1950s until the discovery of the new biology of estrogen-induced apoptosis at the end of the 20th century. The key to triggering apoptosis with estrogen is the selection of breast cancer cell populations that are resistant to long-term estrogen deprivation. However, estrogen-independent growth occurs through trial and error. At the cellular level, estrogen-induced apoptosis is dependent upon the presence of the estrogen receptor (ER), which can be blocked by nonsteroidal or steroidal antiestrogens. The shape of an estrogenic ligand programs the conformation of the ER complex, which, in turn, can modulate estrogen-induced apoptosis: class I planar estrogens (e.g., estradiol) trigger apoptosis after 24 h, whereas class II angular estrogens (e.g., bisphenol triphenylethylene) delay the process until after 72 h. This contrasts with paclitaxel, which causes G2 blockade with immediate apoptosis. The process is complete within 24 h. Estrogen-induced apoptosis is modulated by glucocorticoids and cSrc inhibitors, but the target mechanism for estrogen action is genomic and not through a nongenomic pathway. The process is stepwise through the creation of endoplasmic reticulum stress and inflammatory responses, which then initiate an unfolded protein response. This, in turn, initiates apoptosis through the intrinsic pathway (mitochondrial) with the subsequent recruitment of the extrinsic pathway (death receptor) to complete the process. The symmetry of the clinical and laboratory studies now permits the creation of rules for the future clinical application of ERT or phytoestrogen supplements: a 5-year gap is necessary after menopause to permit the selection of estrogen-deprived breast cancer cell populations to cause them to become vulnerable to apoptotic cell death. Earlier treatment with estrogen around menopause encourages growth of ER-positive tumor cells, as the cells are still dependent on estrogen to maintain replication within the expanding population. An awareness of the evidence that the molecular events associated with estrogen-induced apoptosis can be orchestrated in the laboratory in estrogen-deprived breast cancers now supports the clinical findings regarding the treatment of metastatic breast cancer following estrogen deprivation, decreases in mortality following

Key Words
- acquired resistance
- selective estrogen receptor modulators
- tamoxifen
- raloxifene
- aromatase inhibitors
long-term antihormonal adjuvant therapy, and the results of treatment with ERT and ERT plus progesterin in the Women's Health Initiative for women over the age of 60. Principles have emerged for understanding and applying physiological estrogen therapy appropriately by targeting the correct patient populations.

Observational clinical trials with high-dose estrogen that became the standard of care for the treatment of postmenopausal metastatic breast cancer appear to be outside the structure before and after tamoxifen (Haddow et al. 1944, Kennedy 1965, Ingle et al. 1981). Tamoxifen conformed to the structure as an antiestrogen, hence estrogen therapy was discarded and the search for mechanisms abandoned. The rediscovery of the phenomenon where physiological estrogen ‘melted away’ tamoxifen-resistant breast cancers under laboratory conditions (Wolf & Jordan 1993, Yao et al. 2000) resurrected the concept but resistance to the reintroduction of estrogen to treat breast cancer patients was fierce during the 1990s.

Following rediscovery of the antitumor action of estrogen, a new dimension to the fascinating story of estrogen action was added to the multiplicity of estrogen’s actions around a woman’s body. However, the new biology of estrogen-induced apoptosis is dependent not upon the estrogenic steroid itself or the ER but on the irrepressible adaptability of ER-positive breast cancer cells to survive any therapeutic intervention. In this case, the cancer cell adapts to the withdrawal of ‘the fuel for the fire’ to evolve, through incessant replication, trial and error to find a new successful population for estrogen-independent growth. The sacrifice that the cancer makes is that the population which has now evolved has a vulnerability – estrogen-induced apoptosis.

This review of progress in understanding the new structure for selective actions of estrogen with applications in women’s health is not only about estrogen and a newly found therapeutic potential in cancer, but also about the general principle of the essence of cancer that first evades therapy and then kills the host through selection pressure. The story will first be placed into historical context, as the key to success for therapy in the future is, as it has always been, selective toxicity.

**Historical introduction**

The selective killing of infectious diseases to cure the patient is a noble goal. At the dawn of the 20th century, Prof. Paul Ehrlich created the systematic method used
to this day, for the synthesis and testing of selectively toxic organic molecules that would kill the disease, but not the patient (Baumler 1984). He reasoned that arsenic, an accumulated and fatal poison, could be ‘emasculated’ through synthetic incorporation into organic molecules that would preferentially target the disease organism. The key to translation across the ‘valley of death’ to effective medicines in patients was the creation of appropriate animal models to predict success in patients without fatal consequences (Jordan 2014a). In the spring of 1909, Dr Sahachiro Hata from Japan joined Ehrlich’s team in Frankfurt and created animal models infected with trypanosomes or spirochetes to identify test compounds for treatment of syphilis. This was a major killer, with a long and distressing course. The discovery of Salvarsan (or compound 606) first reported by Ehrlich at the Congress of Internal Medicine at Wiesbaden on April 19, 1910, its production by Hoechst, and the successful cure of syphilis changed the approach to the treatment of human disease forever. From that time until the present day, pharmacology and therapeutics became a rational and evidence-based science. Ehrlich then turned his attention to the treatment of cancer.

Cancer therapeutics did not exist in the early years of the 20th century; only surgery was available. In Germany, Schinzinger (1889) had suggested that oophorectomy might be used to treat breast cancer, but this does not seem to have been adopted. In contrast, Beatson (1896) reported the favorable response of a premenopausal case of metastatic breast cancer in 1896. Boyd (1900) assembled all known cases of oophorectomy in the UK and reported a 30% response rate. This was perhaps the first clinical trial for the treatment of breast cancer and, remarkably, a 30% response rate has remained a ‘magical’ biological response rate for endocrine therapy ever since. However, responses were transient and not everyone responded. A different approach was required and perhaps Ehrlich could find a drug?

The problem was that there were no suitable animal models for cancer in the early 1900s in which to test any compounds. The ovarian dependence of animal models of spontaneous mouse mammary cancer was to be described by Lathrop & Loeb (1915a, 1916) and Lacassagne (1936a,b) would link estrogen with carcinogenesis in the mouse mammary gland in the mid-1930s. In contrast, Ehrlich found himself at the dawn of the new science of cancer research. In the year before he died in 1916, Ehrlich declared ‘I have wasted 15 years of my life in experimental cancer research’ (Schrek 1959).

The situation remained static until Haddow et al. (1944) reported that high doses of synthetic estrogens were able to produce a 30% response rate in women with metastatic breast cancer. High-dose estrogen therapy remained as the standard of care until the introduction of tamoxifen, a nonsteroidal antiestrogen, for the treatment of breast cancer in the 1970s (Jordan 2003a). But this has remained as a paradox. All the laboratory and clinical evidence indicated that breast cancer was dependent on estrogen for growth, but Haddow taught us that estrogen causes tumor regression!

In 1970, during the inaugural Karnofsky Lecture, Haddow (1970) expressed dismay that there were no laboratory predictive tests available to determine whether a cancer ‘chemical therapy’ or chemotherapy, as Ehrlich suggested, would be effective for appropriate treatment of a cancer. It was trial and error. He was also skeptical that a truly selective drug could be developed for cancer, as cancer was ‘self’. He did, however, offer one positive statement:

‘...the extraordinary extent of tumour regression in perhaps 1% of postmenopausal cases (with oestrogen) has always be regarded as of major theoretical importance and it is a matter for some disappointment that so much of the underlying mechanism continues to elude us...’ (Haddow 1970)

In this review, the clinical facts about the historical use of high-dose estrogen therapy will first be summarized as they provide a clinical insight into the new biology of estrogen-induced apoptosis. It is perhaps to be expected that interest in the antitumor effects of estrogen should have been abandoned once tamoxifen arrived as the antihormone therapy of choice for all stages of breast cancer, ductal carcinoma in situ, and male breast cancer, and as a preventive measure for breast cancer in high-risk pre- and postmenopausal women (1980s–2000). Serious side effects with high-dose estrogen would not permit development as was possible with tamoxifen. It is therefore somewhat ironic that the solution to ‘Haddow’s Paradox’ should come initially from an understanding of acquired resistance to tamoxifen (Wolf & Jordan 1993, Yao et al. 2000). Laboratory models have been developed from that understanding that could be used to decipher the mechanisms. For this reason, I have dedicated this article to the memory of Sir Alexander Haddow FRS.

**Facts about high-dose estrogen therapy**

The discovery and availability of the synthetic estrogens, diethylstilbestrol (DES; Dodds et al. 1938) and the longer acting triphenylethylenes (Robson 1937, Robson & Schonberg 1938, 1942), created opportunities...
for applications in patient care. Following studies in animal models, Haddow & Robinson (1939) noted the antitumor properties of polycyclic hydrocarbons. However, the compounds were themselves classified as carcinogens, hence Haddow examined synthetic estrogens (Fig. 1), because he reasoned that the multiple phenyl rings had structural similarities (lucky logic; frightening rationale!). The estrogens also had antitumor properties.

The results of the first clinical trial published in 1944 (Haddow et al. 1944) are summarized in Table 1. Responses were consistent at approximately 30% but for less than a year. These preliminary results indicated that both breast and prostate cancers were responsive but no other tumor types responded. Haddow went on to organize a larger multicentric study at the Royal Society of Medicine where he was the President of the Section of Oncology. Haddow (1970) described his discovery during his 1970 Karnofsky lecture:

> When the various reports were assembled at the end of that time, it was fascinating to discover that rather general impression, not sufficiently strong from the relatively small numbers in any single group, became reinforced to the point of certainty; namely, the beneficial responses were three times more frequent in women over the age of 60 years than in those under that age: that oestrogen may, on the contrary accelerate the course of cancer in younger women and that their therapeutic use should be restricted in cases 5 years beyond the menopause. Here was an early and satisfying example of the advantages which accrue from cooperative clinical trials' (Haddow 1970)

Walpole & Paterson (1949) (the latter had previously worked with Haddow) followed up Haddow’s study at the Christie Hospital in Manchester. The goal was to understand why some patient’s tumors responded but others did not. They were unsuccessful, but did confirm Haddow’s observation that older patients were more likely to respond than younger patients. Subsequently, Stoll (1977) in London reviewed response rates vs time of treatment after menopause for all breast cancer patients in his practice (Table 2). The results were clear; a period of 5 years after menopause was necessary for optimal antitumor action of high-dose estrogen for breast cancer treatment. All clinical results were, therefore, consistent – a period of time after the menopause was necessary to expose the effectiveness of high-dose estrogen as an anticancer agent for metastatic breast cancer.

The reason why some breast tumors, more than 5 years after menopause, responded to high-dose estrogen therapy had to wait for the discovery of the cellular mechanism of estrogen-stimulated growth and regression. The answer would come initially from DES itself. Stilbene can be hydrogenated with tritium across the double bond to produce a high-specific-activity [3H] hexestrol. Hexestrol is a potent estrogen and the administration of [3H] hexestrol to sheep and goats showed selective binding in estrogen target tissues (Glascock & Hoekstra 1959). The idea that radioactive synthetic estrogens could identify an estrogen-responsive tissue was subsequently translated to a clinical trial (Folca et al. 1961) to identify breast tumors more likely to respond to endocrine ablation. These preliminary encouraging results were refine by Jensen & Jacobson (1962), but using the natural hormone [3H] estradiol E2 to demonstrate that E2 bound to and was retained by

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**Table 1** Results of the first clinical trial of synthetic estrogens for treatment of cancers. *Haddow et al. (1944)*

<table>
<thead>
<tr>
<th>Responses/numbers of patients</th>
<th>Breast</th>
<th>Prostate</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triphenylchlorethylene</td>
<td>10/22</td>
<td>2/2</td>
<td>0/28</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>5/14</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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*Haddow et al. (1944)*
Table 2  Objective response rates in postmenopausal women with metastatic breast cancer using high-dose estrogen therapy. A total of 407 patients were classified based on the time from menopause (Stoll 1977)

<table>
<thead>
<tr>
<th>Age since menopause</th>
<th>Number of patients</th>
<th>Percentage responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postmenopausal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–5 years</td>
<td>63</td>
<td>9</td>
</tr>
<tr>
<td>&gt; 5 years</td>
<td>344</td>
<td>35</td>
</tr>
</tbody>
</table>

estrogen target tissues (i.e., uterus, vagina, and pituitary gland) of the immature rat. Gorski’s group (Toft & Gorski 1966, Toft et al. 1967) subsequently made two important findings: the ER was identified as an extractable complex with E2 and that the receptor protein could be extracted and then labeled with [3H] E2 for quantitation and identification. This property for a putative receptor was unique as all other pharmacological receptors to this point were membrane-bound. Subsequently, breast cancers were found to contain various levels of ER but some had no ER (Johansson et al. 1970, Feherty et al. 1971, Maass et al. 1972, Leclercq et al. 1973).

The validation of the clinical ER assay

On July 18th and 19th, 1974, an international workshop was held in Bethesda, Maryland, to link the biochemical measurement of tumor ER with responsiveness to endocrine therapy (McGuire et al. 1975a). All responses were subjected to an extramural review and the simple question posed: does the ER in the breast tumor predict the response to endocrine therapy, i.e., no ER no response? Conversely, if an ER-positive tumor regressed in response to endocrine ablation therapy, then estrogen must be stimulating and maintaining tumor growth. It should be stressed that the principal goal of the conference was to validate a test to predict the responsiveness of metastatic breast cancer to endocrine ablation (hysterectomy, adrenalectomy, or oophorectomy). This was important because a patient would then not have to go through significant life-threatening surgery, in the case of adrenalectomy and hysterectomy, if there was little chance of a response. The extramurally reviewed clinical data indicated that endocrine ablation did not cause tumor regression in ER-negative disease (McGuire et al. 1975b). The goal was achieved and all women with breast cancer were subsequently mandated to have an ER assay on a biopsy of their breast tumor upon diagnosis. The ER laboratory was born as an essential component of a woman’s breast cancer care (Jordan et al. 1988). However, this strategy was limited.

Antiestrogens were not generally available and tamoxifen would not be available for the treatment of metastatic breast cancer until FDA approval on December 29, 1977 (Jordan 2003a). DES was the standard of care for the treatment of metastatic breast cancer in postmenopausal patients during the 1960s and early 1970s at the time of the Bethesda Conference (Kennedy 1965). Most importantly for the current topic of estrogen-induced apoptosis, DES caused tumor regression in ER-positive breast cancer at about the same frequency (Table 3) as oophorectomy in premenopausal women. Neither treatment strategy was effective if the tumor was ER-negative. Thus, inhibition of the action of estrogen through ablation to remove the circulating effects of estrogen was as effective as using high-dose estrogen therapy for more than 5 years after menopause. The ER controlled both the growth and the death of breast tumor cells. However, the development of the antiestrogen tamoxifen throughout the late 1970s–2000, would result from the definition, in the laboratory, of the initial clinical strategies to target tamoxifen to the tumor ER, using long-term adjuvant tamoxifen therapy as the appropriate strategy to treat breast cancer (Jordan 2008a, 2014b), and the use of tamoxifen as a preventive measure for breast cancer (Jordan 2008a, 2014b). Tamoxifen took precedence. High-dose estrogen therapy and the mechanism of the antitumor effects of estrogen were relegated to the history of medical oncology.

Transition to tamoxifen

Early clinical trials of the treatment of metastatic breast cancer with tamoxifen in postmenopausal women showed similar response rates and durations of responsiveness to DES, but with fewer side effects than high doses of estrogen (Cole et al. 1971, Ingle et al. 1981). However, it was noted that reanalysis of a randomized

Table 3  Objective breast cancer regression according to ER assay and the type of therapy as decided by extramural review. Oophorectomy of premenopausal women or high-dose estrogen therapy for postmenopausal women with metastatic breast cancer (McGuire et al. 1975b)

<table>
<thead>
<tr>
<th>Therapy</th>
<th>ER−</th>
<th>ER+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oophorectomy</td>
<td>5/53 (10%)</td>
<td>25/35 (76%)</td>
</tr>
<tr>
<td>Estrogen</td>
<td>5/56 (10%)</td>
<td>37/57 (65%)</td>
</tr>
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trial of DES vs tamoxifen (Ingle et al. 1981) demonstrated that patients treated with DES had a more prolonged survival when compared with those treated with tamoxifen (Peethambaram et al. 1999). DES was different; nevertheless, this was of no significance for the future of estrogen therapy, as tamoxifen was being developed globally. Only retrospectively, was this clinical observation of biological significance. Laboratory animal model (Jordan & Dowse 1976, Jordan & Jaspan 1976, Jordan & Koerner 1976, Jordan 1976a,b, Jordan & Prestwich 1977, Jordan et al. 1977) and clinical studies (Jordan & Koerner 1975, Morgan et al. 1976, Kiang & Kennedy 1977) all yielded results indicating essential role of the tumor ER as the target for tamoxifen action as an antiestrogen. Tamoxifen became the endocrine therapy standard of care for all stages of breast cancer (Fisher et al. 1987, Scottish Cancer Trials Office (MRC) 1987) and was subsequently successfully tested in the 1990s as the first chemopreventive agent for breast cancer in high-risk pre- and post-menopausal women (Fisher et al. 1998, 2005, Cuzick et al. 2007, Powles et al. 2007).

The overview of all the world’s randomized adjuvant clinical trials at Oxford defined the benefits of tamoxifen in lives saved, serious side effects, and human cancer biology (Early Breast Cancer Trialists’ Collaborative Group 1998, 2005). On balance, the risk-to-benefit ratio for tamoxifen was strongly in the benefit direction and the major clinical side effect of a small but significant increase in endometrial cancer was quantified and appropriate steps were taken to minimize the risks of death. Gynecologists were included in the breast cancer treatment team after 1990. The significant and sustained survival benefits of tamoxifen were, however, unanticipated and shown to increase not only with the duration of adjuvant tamoxifen therapy but also following the cessation of tamoxifen treatment. The 2011 overview analysis (Davies et al. 2011) effectively summarizes the state of knowledge regarding 5 years of adjuvant tamoxifen therapy (then the standard duration of treatment and of care), but the recent results of the Adjuvant Tamoxifen Longer Against Shorter (ATLAS) indicated that 10 years of tamoxifen has a greater effect on decreasing mortality than 5 years of tamoxifen (Davies et al. 2013). The decreases in mortality were actually found to be greater in the decade after tamoxifen treatment was stopped. This was unexpected, as an antiestrogenic drug should only control estrogen-stimulated tumor growth as long as it is taken. Some other factors were involved but elusive.

The translation from animal models of adjuvant therapy and chemoprevention (Jordan 1976b, 1983, Jordan & Allen 1980) to clinical practice was counterintuitive, but, in reality, it turned out that the concepts found in the laboratory were correct. In the sense of the ‘Ehrlich dictum’ that an appropriate animal model should be used to translate findings to treat human disease (Baumler 1984), the carcinogen-induced rat mammary carcinoma model turned out to be the appropriate animal model for laboratory testing and translation to clinical trials. The value of long-term adjuvant tamoxifen clinical trials (5 or more years (Davies et al. 2013)) was again counterintuitive as tamoxifen only controlled metastatic breast cancer for 1–2 years (Ingle et al. 1981). Clearly, something was different about micrometastatic breast cancer that resulted in the control of very small cell populations, which was unlike the inability of tamoxifen to control the established bulky tumors. It is a general principle in oncology that low tumor bulk predicts therapeutic success, but this was not the real explanation for tamoxifen, a medicine classified as a cytostatic and not a cytotoxic agent (Osborne et al. 1987, Gottardis et al. 1988). Where did the cytotoxicity come from? The answer again was unanticipated.

If long-term adjuvant tamoxifen therapy was effective in clinical trials during the 1980s, it would have been naive to believe that acquired resistance would not develop eventually. This is true for all anticancer drugs, but, at that time, there were no animal models of acquired resistance to tamoxifen. The questions to be addressed urgently in the 1980s were, ‘could an appropriate animal model of human acquired resistance to tamoxifen be created, what form would resistance take, and could second line therapies be developed predictably?’

**Acquired resistance to tamoxifen**

The discovery of ER in some breast cancers and the general observation that there was a range of ER concentrations (femtomoles/mg cytosol protein; Jordan et al. 1988) meant that breast cancer could be a mix of ER-positive and -negative cells. In the 1970s, it was believed that endocrine therapy would hold the growth of ER-positive cells, but the ER-negative cells would eventually gain a growth advantage. Resistance would occur as the tumor transitioned from being ER-positive to become ER-negative by cellular population shifts. However, this conceptual model did not fit with clinical experience with ‘the endocrine cascade’. Experience taught physicians that an excellent response to one endocrine therapy would herald a good
response to a second-line agent and so on until tumor bulk overwhelmed the patient. Stoll (1977) documented response to a second-line agent and so on until tumor therapy can also be used for months (Osborne estrogen-stimulated breast tumor growth. Long-term mouse (Harper & Walpole 1967, Terenius 1971), blocks Tamoxifen, despite being classified as an estrogen in the mice (Shafie & Grantham 1981, Huseby et al. 1984). Tamoxifen, despite being classified as an estrogen in the mouse (Harper & Walpole 1967, Terenius 1971), blocks estrogen-stimulated breast tumor growth. Long-term therapy can also be used for months (Osborne et al. 1985), hence this was viewed as a suitable model for studying acquired resistance. Osborne et al. (1987) first demonstrated that MCF-7 tumors would eventually grow despite long-term tamoxifen treatment. However, the unusual feature about acquired resistance to tamoxifen is that the tumors grow upon re-transplantation because of tamoxifen or physiological estrogen. The finding (Gottardis & Jordan 1988) that a tumor became dependent on the treatment was a unique observation in oncology.

The fact that tamoxifen is an estrogen in the mouse naturally raised the question of metabolic conversion of tamoxifen to estrogenic metabolites during long-term therapy in mice or indeed the development of induced enzyme systems in humans that could create estrogenic metabolites over time. Studies using the same MCF-7 tumors with acquired tamoxifen resistance developed in mice, but now implanted into athymic rats, a species where tamoxifen is predominately antiestrogenic, produced the same tamoxifen-stimulated tumor growth (Gottardis et al. 1989a). Additionally, studies on the metabolic stability of tamoxifen in patients treated for up to 10 years with adjuvant tamoxifen demonstrated the stability of antiestrogenic metabolite levels over the whole time period (Langan-Fahey et al. 1990).

The results obtained from the athymic animal/MCF-7 tumor model, which have been reported previously (Gottardis & Jordan 1988), are consistent with those obtained from human studies on acquired resistance to tamoxifen in the patient with metastatic breast cancer. The therapy for tumors failed within 1–2 years (Ingle et al. 1981) and a tamoxifen ‘withdrawal response’ has been noted (Howell et al. 1992). The fact that the experimental tumor with acquired resistance to tamoxifen (Gottardis & Jordan 1988) would subsequently grow with either tamoxifen or E2 provided clues to subsequent treatment strategies. Based on the similarities of the MCF-7 breast cancer/athymic mouse model and metastatic breast cancer, results from therapeutic studies indicated that the lead compound ICI 164 384 for a new class of ER downregulators (referred to as pure antiestrogens) or no estrogen at all, i.e., an AI, was a reasonable second-line therapy to evaluate the subsequent acquired tamoxifen resistance (Gottardis et al. 1989b). A decade later, clinical trials confirmed that an AI and fulvestrant, the clinically available pure antiestrogen, were both acceptable second-line therapies (Howell et al. 2002, Osborne et al. 2002). Nevertheless, there was a translational flaw in the laboratory model when applied to the adjuvant therapy for breast cancer for 5–10 years. If metastatic ER-positive breast cancer cells are converted to acquired resistance to tamoxifen within 2 years, why is it that adjuvant therapy does not fail universally at the 2-year treatment mark? The answer is tumor bulk, genetic variation, and the ability to grow through trial and error.

The few micrometastatic cells exposed to tamoxifen during adjuvant therapy obviously are not recapitulated by the 10 million MCF-7 cells initially inoculated into athymic mice to create tumors that grow in the presence of estrogen or tamoxifen to become a 100 times the size. In other words, there is greater genetic diversity during selection pressure with tamoxifen for 1–2 years in the athymic mouse model.

However, transplantation of the MCF-7 tumor into subsequent generations of athymic mice for up to 5 years to maintain the phenotype of acquired tamoxifen resistance ultimately exposes a vulnerability in breast cancer through expansion and differential growth of favored populations. Long-term antiestrogen therapy with tamoxifen now creates a selected cell population that responds to physiological estrogen once tamoxifen is stopped, not as a growth signal but as an apoptotic trigger (Wolf & Jordan 1993, Yao et al. 2000). The evolution of cell populations to create acquired resistance to tamoxifen is illustrated in Fig. 2.
The evolution of drug resistance to SERMs. Acquired resistance occurs during long-term treatment with an SERM and is evidenced by SERM-stimulated breast tumor growth. Tumors also continue to exploit estrogen for growth when the SERM is stopped, so a dual-signal transduction process develops. The AIs prevent tumor growth in SERM-resistant disease and fulvestrant that destroys the ER is also effective. This phase of drug resistance is referred to as phase I resistance. Continued exposure to an SERM results in continued SERM-stimulated growth, but eventually autonomous growth (phase III) occurs that is unresponsive to fulvestrant or AIs. This is the original concept that was proposed in the mid-2000s and emphasized the switching mechanism that distinguishes phase I from phase II resistance. These distinct phases of laboratory drug resistance (Yao et al. 2000, Lewis et al. 2005b) have their clinical parallels and this new knowledge is being integrated into the treatment plan. Reproduced with permission from Jordan VC 2004 Selective estrogen receptor modulation: concept and consequences in cancer. Cancer Cell 5 207–213, with permission from Elsevier. Copyright 2004 Cell Press. The evolutionary concept has evolved in the past decade.

The antitumor actions of physiological estrogen in vivo

Cell culture models of tamoxifen resistance during the 1980s were focused on mechanistic changes and not biological changes in cellular populations. The fact that tumors with acquired resistance to tamoxifen could only be passaged into athymic mice and maintained in successive generations of animals for years was fortuitous for the chance discovery that physiological estrogen administration (Wolf & Jordan 1993) could cause tumors to undergo ‘the extraordinary extent of tumor regression’ (the words of Haddow (1970) about the response of a few breast tumors to high-dose estrogen treatment for metastatic breast cancer). The evolution of acquired resistance to tamoxifen in vivo was replicated (Yao et al. 2000) and the time course of the antitumor sensitivity to the antitumor action of physiological estrogen over 5 years of estrogen treatment was documented (Yao et al. 2000). The finding that acquired resistance to tamoxifen passes through phases of cellular sensitivity to estrogen is both intriguing and now clinically relevant. The animal transplantation studies revealed two major phases of acquired tamoxifen resistance (Fig. 2): phase 1 occurs in about a year and the new cell population can use either E₂ or tamoxifen to stimulate growth. Phase II resistance occurs over the next 3–4 years of continuous tamoxifen treatment, but there is increasing vulnerability of the cell population to the apoptotic effects of estrogen; the process evolves or intensifies through selection pressure over a 5-year period.

The development of populations of MCF-7 cells vulnerable to estrogen-induced apoptosis is not unique to the selective ER modulator (SERM) tamoxifen. Cells incubated with raloxifene in an estrogen-free environment in vitro (Liu et al. 2003) can be inoculated into ovariectomized athymic mice and shown to grow with raloxifene. Physiological estrogen causes tumor regression once raloxifene is stopped. Similarly, long-term transplantation of MCF-7 tumors over a decade into raloxifene-treated athymic mice can replicate the cyclical sensitivity of an SERM and estrogen to shift tumor cell population sensitivity from SERMs stimulating tumor growth to SERMs inhibiting estrogen-stimulated growth (Balaburski et al. 2010). The cell populations seem to drift very much as Stoll (1977) had observed with DES in elderly women being titrated for tumor bulk.

Early studies of the mechanism of estrogen-induced apoptosis in vivo produced some interesting findings. Estrogen-induced apoptosis causes an increase in Fas receptor associated with the extrinsic pathway of apoptosis and a simultaneous decrease in NFκB (Osipo et al. 2003, Lewis-Wambi & Jordan 2009). Most interesting are the observations that the pure antiestrogen fulvestrant plus physiological estrogen can reverse apoptosis and cause robust growth of tumors (Osipo et al. 2003). This raised the possibility that a combination of fulvestrant and AIs might be a superior therapeutic strategy for the treatment of metastatic disease. Regrettably, clinical results are conflicting (Bergh et al. 2012, Mehta et al. 2012).

The major advances in understanding estrogen-induced apoptosis, however, have come not from studies on animals, but rather mechanisms that have been systematically interrogated using estrogen-deprived cells in vitro. The advent of AIs as the long-term adjuvant therapies of choice for postmenopausal patients (Jordan & Brodie 2007, Dowsett et al. 2010) mandated a strategy for studying the mechanism of acquired resistance to estrogen deprivation. However, in the 1970s and 1980s, the understanding of estrogen-stimulated cell growth was not at all straightforward.
The few ER-positive breast cancer cell lines

Despite the fact that there are very few available ER-positive cell lines, remarkable progress has been made in understanding the basics of hormone and antihormone action that clearly translates to clinical care (Sweeney et al. 2012). Four ER-positive cell lines, MCF-7, T47D, ZR75-1 and BT474, are used routinely in the laboratory. But it is the ER-positive MCF-7 cell line (Brooks et al. 1973) that has perhaps provided the majority of data in translational research. The cells originally were derived from a plural effusion where the patient had been treated with high-dose estrogen therapy and the treatment had failed (Soule et al. 1973). Culture of the cells in estrogen-containing or -free conditions using charcoal stripping of serum to remove estrogenic steroids did not alter growth but tamoxifen could block spontaneous growth which could be reversed by addition of estrogen (Lippman & Bolan 1975)! Transplantation into athymic mice, however, required estrogen supplementation for growth of tumors (Soule & McGrath 1980). The hypothesis was raised that a second messenger might have to be stimulated by estrogen to cause estrogen-stimulated growth in vivo (Shaﬁe 1980). The problem was resolved with the subsequent ﬁnding that the redox indicator in a culture medium, phenol red, contains a contaminant that is an estrogen (Berthois et al. 1986, Bindal & Katzenellenbogen 1988). Up until that time in 1987, MCF-7 cells, it seems, had always been grown in an estrogenic environment. Now it was time to see what estrogen deprivation really did to breast cancer cell populations, not unlike what could be happening at meno pause.

There were two independent reports of the effects of immediate estrogen deprivation on the MCF-7 cell line (Katzenellenbogen et al. 1987, Welshons & Jordan 1987). Both noted a ‘crisis period’ at about a month after estrogen withdrawal with a catastrophic decrease in cell numbers. However, surviving cells grew back over a period of months with an elevation in ER levels and estrogen-independent growth. Several clonal populations were subsequently created; MCF-7:SC cells were refractory to the actions of a nonsteroidal antiestrogen to prevent growth or estrogen to stimulate growth or initiate PgR synthesis (Jiang et al. 1992). In contrast, the MCF-7:2A cells were responsive to stimulation of PgR synthesis by estrogen; antiestrogen decreased growth but estrogen did not affect growth in the 1-week growth assay (Pink et al. 1995). Of interest was the finding that MCF-7:2A cells also had a high-molecular-weight ER (ESR1) protein with a 6/7 exon repeat in the ligand-binding domain as well as the WT ER (Pink et al. 1995, 1996a, 1997). This was a unique biological finding concerning the translation and processing of a steroid receptor protein.

A similar approach to estrogen deprivation in MCF-7 cells was taken by the Santen group but without cloning. The long-term estrogen-deprived MCF-7 cell (LTED) populations went through interesting adaptations to estrogen deprivation. Initially, the cell population experienced ‘adaptive hypersensitivity’ (Masamura et al. 1995, Jeng et al. 1998), i.e., the cells scavenged very low concentrations of estrogen to enhance growth. This observation was offered as an explanation for aromatase resistance, i.e., the estrogen-deprived ER-positive cells would subvert growth control by exploiting the growth potential of any ligands that could activate the enhanced concentration of ER in cells.

However, Song et al. (2001) reported the apoptotic role of estrogen in vitro and proposed this as the mechanism of high-dose estrogen therapy employed by Haddow et al. (1944) 60 years before to treat postmenopausal women with breast cancer. But it was clear from the concentration–response curve presented that low concentrations were able to decrease cell numbers through triggering apoptosis (Jordan et al. 2002); as had been noted with physiological estrogen causing tumor regression in the MCF-7 tamoxifen-resistant tumor in vivo (Wolf & Jordan 1993, Yao et al. 2000). The Song et al. (2001) study in vitro identified an increase in FAS ligand as the mechanism of estrogen-induced apoptosis (via the extrinsic or ‘death receptor’ pathways), but no studies up to that point, nor the subsequent animal studies with tamoxifen-resistant tumors (Liu et al. 2003, Osipo et al. 2003, 2005, 2007), identiﬁed a sequence of events that triggered estrogen-induced apoptosis.

Lewis et al. conducted a series of studies with MCF-7:5C cells and MCF-7:2A cells in vitro. The MCF-7:5C cells (Jiang et al. 1992) were initially noted to be ER-positive, PgR-negative, and nonresponsive to estrogen. However, alteration of the culture conditions dramatically changed estrogen responsiveness (Lewis et al. 2005a): the MCF-7:5C cells were now able to rapidly undergo E2-induced apoptosis, within a few days, in a concentration-related manner. The MCF-7:5C cells (Lewis et al. 2005b) could also be inoculated into athymic mice and grew spontaneously, but the tumors would stop growing with fulvestrant therapy and remain static, whereas physiological E2 administration would result in complete tumor regression. This observation was reminiscent of the effects of physiological estrogen noted a decade earlier with MCF-7 tamoxifen-resistant tumors in athymic mice (Wolf & Jordan 1993). Lewis et al. (2005b)
identified the intrinsic mitochondrial pathway as the primary target for estrogen-induced apoptosis with changes in pro-apoptotic markers, and leaking of cytochrome c through the mitochondrial membrane. In a parallel study by Song & Santen (2003) and Song et al. (2005), an inhibitor of bcl2 was shown to enhance estrogen-induced apoptosis in LTED MCF-7 breast cancer cells.

The MCF-7:2A cells (Pink et al. 1995) were initially found to be resistant to estrogen-induced apoptosis, with slow apoptotic changes occurring after 6 days of estrogen treatment (Lewis et al. 2004, Lewis-Wambi & Jordan 2009). These cells apparently can protect themselves from increases in reactive oxygen species (ROS) through enzymatic overproduction of glutathione. Using buthionine sulfoximine (Lewis-Wambi et al. 2008, 2009), which inhibits glutathione biosynthesis, estrogen-induced apoptosis was advanced to occur during the first 6 days of estrogen treatment. Recent studies have built on these original findings (Sweeney et al. 2014a).

With the transition from long-term adjuvant tamoxifen therapy to use of AIs and the knowledge that cell culture media contain estrogen (the ‘phenol red’ story (Berthois et al. 1986, Bindal & Katzenellenbogen 1988)), other breast cancer cell lines were investigated to document changes during estrogen deprivation. Studies using the T47D cell line were initiated 25 years ago to determine the effects of estrogen deprivation on an ER, PgR-positive breast cancer cell line that was, unlike MCF-7 cells, harboring a mutated p53. This is a key regulator in the decision network for DNA repair or apoptotic death.

Unlike the MCF-7 cell line, the T47D cells not only take a different route for cellular survival but also were found to have a reversal of their ER regulatory mechanism (Pink & Jordan 1996). Culture of T47D in estrogen-free media results in downregulation of the ER (Murphy et al. 1989, 1990). Initial long-term treatment in estrogen-free media for months results in the apparent loss of ER, but this can be ‘rescued’ by re-culture for months in estrogen-containing media. This again is an example of shifting cellular populations with the selective pressure of an estrogen-free medium creating an apparently ER-negative cell outgrowth as a survival response, once the ER is no longer synthesized. The selection pressure of new estrogen-containing media reactivates ER synthesis in the minority of contaminating cells, and ER-positive T47D cells again dominate by overgrowth. Only with repeated dilution cloning can a pure line of ER-negative T47D cells be created that is stable when reintroduced into estrogen-containing media (Pink et al. 1996b). These ER-negative T47D cells are referred to as T47D C4:2 cells.

The two ER regulatory systems are illustrated in Fig. 3 and provide an excellent example of how T47D cells can respond to estrogen deprivation in order to survive. The mutant p53 cell that needs estrogen to survive chooses to lose the ER survival system and evolve to an ER-negative state. In contrast, the WT p53 cell (MCF-7) expands the ER system to survive but in so doing must sacrifice survival to the vulnerability to estrogen-induced apoptosis, should the environment again return to being estrogen-rich.

**Figure 3**
The diagrammatic representation of cellular estrogen receptor (ER) regulation in media with or without estradiol (E2). This diagram is based on the general responses to estrogen illustration by western blotting and presented in detail in Pink & Jordan (1996). Model I ER regulation (MCF-7, ZR-75, and BT-474) involves upregulation of ER mRNA and protein in an estrogen-depleted environment, but ER is downregulated at the mRNA and protein levels in the presence of estrogen. Model II ER regulation (T47D) involves upregulation of ER mRNA and protein in an estrogen-containing environment, but ER is not produced in an estrogen-depleted environment. Cells lose ER to become ER-negative.

An alternate route to estrogen-induced apoptosis

There is an inverse relationship between ER status and protein kinase C alpha (PKCα) in breast cancer (Borner et al. 1987), breast cancer cell lines (Tonetti et al. 2000), and endometrial cancer (Fournier et al. 2001). Indeed, PKCα is associated with antiestrogen resistance (Nabha et al. 2005, Assender et al. 2007, Frankel et al. 2007). Tonetti et al. (2000) posed the question of whether the
stable transfection of the PKCa (PRKCA) gene into the ER-positive T47D:A18 estrogen-responsive breast cancer cell line would influence the responsiveness to estrogens and antiestrogens. Although the T47D:A18/PKCa cells are unaffected by estrogen treatment in vitro (Tonetti et al. 2000), the cells grow spontaneously into tumors when implanted into athymic mice (Chisamore et al. 2001, Zhang et al. 2009), but estrogen causes rapid tumor regression via a mechanism that causes nuclear ER to translocate to extranuclear sites in response to either estrogen or raloxifene (Perez White et al. 2013) as well. This unusual pharmacology indicates that raloxifene derivatives may be found as unique therapeutic agents for future development in antihormone-resistant breast cancer. Those first investigations to create a new targeted group of medicines have begun (Molloy et al. 2014).

With the ongoing development of models to decipher mechanisms of estrogen-induced apoptosis during the first decade of the 21st century, it was also time to address translation to clinical relevance.

**Estrogen salvage therapy**

As noted previously, clinical experience with the ‘endocrine cascade’, i.e., the repeated successful use of different endocrine therapies until there is no choice but to employ combination cytotoxic chemotherapy, would evolve in the 1980s and 1990s into cycling tamoxifen, different AIs, fulvestrant, etc. Years of salvage therapy would create populations of long-term estrogen-deprived cells. High-dose estrogen was still part of the armamentarium for European medical oncologists. Lonning et al. (2001) examined a small series of patients to address the questions of whether estrogen salvage (an endocrine therapy) would be effective in tumors following long-term antihormone (endocrine) therapy that had become refractory to further treatment.

The interesting findings are shown in Table 4. Lonning et al. (2001) noted an overall 30% response rate to high-dose DES (15 mg daily, i.e., 5 mg three times per day) and one patient had a remarkable response.

<table>
<thead>
<tr>
<th>Complete</th>
<th>Partial</th>
<th>Stable disease</th>
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<td>4/32</td>
<td>6/32</td>
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*One patient remained disease free 10 years and 6 months after commencing treatment (Lonning 2009).* Ellis et al. (2009) addressed the experimental concept of high-dose estrogen versus low-dose estrogen as a second-line treatment following recurrence during adjuvant AI treatment. Women received either 30 or 6 mg of E2 daily (DES is not available in the USA) and a 29% clinical benefit was noted for both groups. However, the low-dose E2 provided the same clinical benefit as high-dose therapy but with significantly fewer serious side effects.

The therapeutic use of either high- or low-dose estrogen salvage therapy provided the proof of principle that the animal and cell models had veracity in the context of exhausted antihormone therapy by preparing a vulnerable antihormone-resistant breast cancer cell population for execution with exogenous estrogen therapy. A complementary laboratory study using transplanted MCF-7 tumors in athymic mice (Osipo et al. 2005) built on the original observations that low-dose estrogen could reverse exhaustive antihormone therapy (Yao et al. 2000) and permit the reuse of tamoxifen to control estrogen-stimulated tumor growth. The Osipo et al. (2005) study created four different transplantable MCF-7 tumor models: MCF-7:E2 (WT estrogen responsive), MCF-7:TAMST (phase I resistance that is stimulated to grow with E2 or tamoxifen), MCF-7:TAMLT (phase II resistance that is stimulated to grow with tamoxifen, but in which E2 does not promote growth), and MCF-7:TAME (MCF-7:TAMLT that regrew after long-term E2 treatment). The MCF-7:TAME tumors were inhibited by tamoxifen in a dose-dependent manner in vivo. It was interesting to note, however, that ERBB2/NEU and ERBB3 mRNA in TAM-stimulated MCF-7:TAMLT tumors remained high in E2-stimulated MCF-7:TAME tumors, thus indicating the veracity of the clinical findings that overexpression of ERBB2/NEU alone is insufficient to predict resistance to tamoxifen (Osipo et al. 2005).

However, it was the Women’s Health Initiative (WHI) trials of the value of either combination synthetic progesterin and conjugated equine estrogen (CEE) referred...
to as hormone replacement therapy (HRT) or estrogen replacement therapy (ERT) alone to prevent coronary heart disease in postmenopausal women more than a decade after menopause, which was to provide an initial dilemma. The stop rules for the HRT trial created a predictable result with an increase in breast cancer and this trial was published first (Rossouw et al. 2002). In contrast, it was not deemed necessary to stop the ERT trial until several years later. Eventually, the trial was stopped due to increased strokes and not for breast cancer increases (Anderson et al. 2004). A rise in the incidence of breast cancer was not noted. As a supporting and important additional database, the ongoing analysis of the British Million Women’s Study (Beral et al. 2003, 2011) also noted similar paradoxical findings with HRT and ERT. These epidemiological data and the WHI results now demand an expanded discussion. In so doing, an understanding of the essential role of timing of taking HRT/ERT can be examined, paradoxes addressed, and rules established. The questions raised are as follows: i) If estrogen causes breast cancer to grow, why does estrogen alone in these clinical studies not cause an increase in breast cancer? ii) Why does a combination of a synthetic progestin plus estrogen cause a predicted rise in breast cancer incidence?

**HRT in postmenopausal women**

There are two major databases from which to mine information about the role of estrogens and synthetic progestin in the life and death of breast cancer in postmenopausal women. However, based on the established laboratory data on the replication and apoptosis of breast cancer cells and the documented historical record of the actions of high-dose estrogen therapy in treatment of metastatic breast cancer in postmenopausal women, there are no real surprises (Jordan 2008b). However, one important question remains: why does a synthetic progestin reverse the antitumor and chemopreventive properties of ERT administered a decade after the menopause (Anderson et al. 2012)?

The WHI in the USA and the Million Women’s Study in the UK provide the clinical databases, so that laboratory and other clinical studies can be melded to create evidenced-based principles for safer clinical care. The design and conclusions of these two studies will be summarized for completeness and interpretations made based on existing knowledge in the literature.

The WHI ERT alone trial recruited 10 739 hysterectomized postmenopausal women into a randomized trial to receive either CEE (0.625 mg daily; Fig. 4) or placebo. Women were aged between 50 and 79 years. The women’s median age was in their mid 60s. The treatment phase of the trial was a median of 5.9 years, as stop rules for stroke were triggered but follow-up occurred to yield an overall study median of 11.8 years. The first clinical surprise was the finding of a lower incidence of breast cancer at the initial analysis (Anderson et al. 2004), which was reinforced by a second analysis (LaCroix et al. 2011). At the latest analysis of 11.8 years median follow-up (Anderson et al. 2012), there was a lower incidence of invasive breast cancer (151 cases) compared with placebo (199 cases). Fewer women died from breast cancer in the estrogen group (six deaths) compared with the placebo group (16 deaths). Indeed, few women died of any cause in the estrogen group after breast cancer diagnosis (30 deaths) than did those in the placebo group (50 deaths).

By contrast, the WHI of HRT recruited 16 608 postmenopausal women between the ages of 50 and 79 years with an intact uterus. Women were randomized to receive either CEE (0.625 mg daily) and medroxyprogesterone acetate (MPA 2.5 mg daily) (Fig. 5) or placebo. After a mean follow-up of 5.2 years, the WHI data safety-monitoring committee recommended stopping the trial based on breast cancer incidence exceeding the predefined stopping boundary (Rossouw et al. 2002). The HR was 1.24 for invasive breast cancer with a total of 199 cases of breast cancer vs 150 cases in placebo ($P=0.003$).

In the Million Women’s study (Beral et al. 2003), 1 129 025 postmenopausal women were recruited to evaluate the breast cancer risk in hormone therapy users and never users. The study accrued 4.05 million women years of follow-up and 15 750 incident breast cancers with a total of 7107 breast cancer in current users of hormone therapy.
The principal conclusions (Beral et al. 2011) for the Million Women’s Study relevant to our current considerations of timing and hormone type, i.e., combination of estrogen and progestin (HRT) or estrogen alone (ERT), were as follows: the ERT current users had little increase in breast cancer if use was started more than 5 years after menopause (RR 1.05), but, if ERT was begun straight after menopause, there was an increase in breast cancer (RR 1.43). The pattern was similar for current users of HRT with an anticipated increase in breast cancer in users who start 5 years after menopause (RR 1.53), but a further elevation in risk if HRT is started immediately after menopause (RR 2.04).

Thus, both the WHI and the Million Women’s Study provide evidence that combination HRT increases the risk of developing breast cancer compared with ERT, but ERT either causes a fall in breast cancer in the WHI study that persists, whereas, in the Million Women’s Study, there is no major rise in breast cancer in an estrogen-deprived women, i.e., more than 5 years after menopause. Timing relative to menopause is important, i.e., a greater breast cancer risk occurs nearer to menopause. This concept (Jordan 2014c) is summarized in Fig. 6, which now establishes rules for the actions of different estrogens; estrogen deprivation for 5 years creates populations of breast cancer cells vulnerable to estrogen-induced apoptosis.

However, the second question, to be addressed in the future, is why a synthetic progestin in some way enhances breast cancer risk, whereas estrogen does not in estrogen-deprived women. Clearly, an understanding of the mechanism and modulation of estrogen-induced apoptosis will go some way to advance clarity and create safer HRT if the rules of treatment are obeyed. The way forward is to use models to define mechanisms, so that estrogen-induced apoptosis can be modulated predictably and conclusions applied to health care.

**Deciphering a mechanism of estrogen-induced apoptosis**

Previously, in this review, a few tantalizing clues of the early actions of estrogen to cause apoptosis were reported from studies in vivo (Liu et al. 2003, Osipo et al. 2003) with acquired resistance to tamoxifen or in vitro in LTED cell populations or selected clones (Song et al. 2001, Lewis et al. 2005b). This ‘snap shot’ of molecular events is the usual method employed to decipher potential pathways either using a laboratory model or a tumor sample from heterogeneous patient populations at an arbitrary time during an important cellular process. This provides a patchwork approach that does not reproduce the most important dimension in any molecular mechanism – time. Populations of cells respond to treatment or stress with adaptation, by advancing the survival and replication of cell populations that can now thrive in a once hostile environment. Time in days can affect an individual cell’s response to stress but time in months or years reconfigure the population of cells that survive and thrive. Thus, trial and error for cellular survival is the essence of cancer that kills. The original
goal of therapeutics in the time of Ehrlich was to cure infectious diseases, but, in the case of cancers we aim, in the case of solid tumors, to contain tumor growth, through an understanding of the cellular options of cancer. In this way, life can be extended by decades with dramatic decreases in mortality illustrated by adjuvant tamoxifen therapy given for 5–10 years (Davies et al. 2011, 2013).

To understand cancer’s options, the current ‘snapshot’ needs to be amplified and viewed instead like a ‘movie’. The long-term time course of the process to trigger apoptosis, with estrogen in this case, can then be modulated to learn more of the possible population drifts over months or years. Only in this way can true vulnerabilities be discovered. To this end, the intensive evaluation of a few well-described cellular models has been examined to provide the first ‘movie’ of estrogen-induced apoptosis. Unlike cytotoxic chemotherapy that kills rapidly within a few hours, the process of estrogen-induced apoptosis initially is gradual and relentless, but then is committed after days of subcellular preparation (Ariazi et al. 2011, Obiorah et al. 2014a). Based on this foundation, modulation of the mechanism is the next essential step to define, interrogate, and validate an emerging conceptual model for clinical transition.

A ‘movie’ of estrogen-induced apoptosis in the cell

MCF-7:WS8, MCF-7:2A, and MCF-7:5C cells were used to identify genome-wide alterations in E2-regulated gene expression uniquely involved in apoptosis (Ariazi et al. 2011). The cells used are estrogen-stimulated MCF-7:WS8 to define growth, MCF-7:2A that is grown independently of estrogen and the cells are refractory to apoptosis during the first week of estrogen action (Pink et al. 1995), and MCF-7:5C that respond to E2-induced apoptosis in just a few days (Lewis et al. 2005a, b). Time courses of gene changes were compared and contrasted over a 2–96 h period with each cell line hybridized with a no treatment control using Agilent arrays. This approach, however, does not compensate for adaptive changes in the basal level of genes that must occur to permit estrogen-independent growth compared with MCF-7:WS8 in the absence of estrogen. These data are currently being prepared for publication.
Examination of MCF-7:5C-specific genes reveals an attenuation of ER signaling, but enhancement of endoplasmic reticulum stress (ERS) and inflammatory stress, which heralds apoptotic gene regulation. ERS is characterized by accumulation of unfolded or malfolded proteins and the triggering of an unfolded protein response (UPR) with the function and goal of inhibiting the translation of proteins to alleviate the stress. Expression profiles of E₂-treated MCF-7:5C-specific genes related to ERS are consistent with deficiencies in the UPR, protein translation, protein folding, degradation of malfolded proteins, and fatty acid metabolism. With regard to the ERS-induced apoptosis, E₂ selectively induces the pro-apoptotic BCL2 family members BAX and BIM (Lewis et al. 2005b) and the inflammatory caspase CASP4. BAX, in addition to its mitochondrial outer membrane permeabilization activity, binds to, and activates ERN1 (IRE1α), a key endoplasmic reticulum transmembrane kinase and endoribonuclease that initiates the UPR. BIM is upregulated by a variety of ERS activators and is essential in ERS-induced apoptosis in many cell types. CASP4 localizes to the endoplasmic reticulum, autoactivates in response to severe ERS, and is also required in multiple models of ERS-induced apoptosis. The functional importance of BAX, BIM, and CASP4 is demonstrated by depletion of BAX or BIM with specific siRNAs (Lewis et al. 2005b) or inhibition of CASP4 with z-LEVD-fmk (Ariazi et al. 2011, Obiorah et al. 2014b). Each approach blocks estrogen-induced apoptosis.

The time sequence of estrogen-induced apoptosis has been shown to commence through the intrinsic pathway targeting mitochondria and then subsequently recruiting the extrinsic pathway (death receptor) that consolidates and completes the apoptotic sequence (Ariazi et al. 2011, Obiorah et al. 2014a). However, those events are completely different from the catastrophic apoptotic response initiated almost immediately by paclitaxel. This is complete within 24 h, triggered by p53-mediated cell cycle blockade at G2 (Obiorah et al. 2014a). In contrast, E₂-induced apoptosis via the ER in MCF-7:5C can be rescued during the first 24 h by wash out with an excess of the high-affinity triphenylethylenic antiestrogen 4-OHT. Interestingly, both E₂ (Obiorah et al. 2014a) and the nonsteroidal estrogen bisphenol (Sengupta et al. 2013) initially cause an increase in cell growth and apoptosis is not triggered by cell cycle blockade. It is clear that there is a competition between estrogen-stimulated growth for survival and estrogen-induced apoptosis that ultimately results in cell death.

The key signal transduction system for the regulation of breast cancer cell growth and cell death is the ER. As this is a known pharmacological target and acknowledged to be the most important one in cancer therapy as a whole (Sledge et al. 2014), it is therefore appropriate to consider the modulation of estrogen-induced apoptosis via the ER, the role of ligand shape that programs the conformation of the ligand–ER complex, and then the regulation of ER function through the blockade of cell survival signaling via cSrc and glucocorticoids. Estrogen-induced apoptosis is a stress and inflammatory response (Ariazi et al. 2011); therefore, the blocking of these pathways may provide insights into future clinical applications.

The ER as a target for modulating estrogen-induced apoptosis

The ER is extremely promiscuous in its desire to bind with a wide spectrum of phenolic ligands either to switch off or to switch on the ER signal transduction pathway (Jordan 1984, 2003b,c). The steroidal antiestrogen fulvestrant and the triphenyl ethylene hydroxylated metabolite of tamoxifen 4-hydroxytamoxifen (4-OHT) both block estrogen-induced apoptosis (Obiorah & Jordan 2014, Obiorah et al. 2014b), but it is the wide variety of phenolic compounds with estrogenic properties that is of particular interest with regard to triggering estrogen-induced apoptosis.

Estrogens are classified based on their structure that programs the conformation of the estrogen–ER complex (Jordan et al. 2001). Planar (e.g., E₂) estrogens are referred to as class I and angular estrogens (e.g., bisphenolic triphenylethylenes) as class II. The classification is based on the molecular pharmacology of a mutant ER (D351G) with impaired function, as the natural D351 in the ligand-binding domain pocket needs to communicate with amino acid L540 in helix 12 to seal the ligand-binding domain (Fig. 7). The D351G mutant ER, unlike WT ER, is unable to close helix 12 appropriately to activate transcription of an estrogen-regulated gene transforming growth factor alpha (TGFa (TGFA); Schafer et al. 1999), when there is steric hindrance from an angular estrogen in the ligand-binding domain. Leclercq and colleagues (Bourgoin-Voillard et al. 2010) and Gust and colleagues (Łubczyk et al. 2002) have confirmed and extended the ligand classification using other techniques. The molecular pharmacology of estrogen binding to the ER will be described briefly.

X-ray crystallography (Brzozowski et al. 1997, Shiau et al. 1998) of the ligand-binding domain of the ER liganded with a class I estrogen (i.e., E₂ or DES) or with a nonsteroidal antiestrogen (i.e., 4-OHT or raloxifene) provides precise structural data for the extremes of estrogen and antiestrogen action at one stable moment
in time. The X-ray crystallography model resolved had been predicted by structure–function relationships at the PRL target in primary cultures of immature rat pituitary gland cells regulated by the ER more than a decade earlier (Jordan 1984, Jordan et al. 1986). The biological assay is a dynamic analytical model that integrates receptor responses over time. Thus, structural interpretation of the extremes of estrogen/antiestrogen action and the predictable modulation of gene function by a broad range of systematic ER-binding ligands are consistent. The extremes of the ER complex in both cases predicted that closure of helix 12 sealing a class I ligand within the ligand-binding domain programs full estrogen action. In contrast, the antiestrogenic bulky side chain of nonsteroidal antiestrogens prevents the closure of helix 12 and impairs the binding of co-activator molecules essential for gene transcription. However, an engineered cell model of TGFα transcription demonstrated that there were predictable changes to be anticipated due to interactions of the antiestrogenic side chain of 4-OHT or raloxifene with D351 (Levenson & Jordan 1998, Liu et al. 2001, 2002). Modeling demonstrates that the dimethylaminoethoxy side chain of 4-OHT is not long enough to interact with D351 but the piperidine ring system of the antiestrogenic side chain of raloxifene both neutralizes and shields D351 from interactions with helix 12 (Fig. 8). A D351Y mutation restored binding of Y351 with helix 12 liganded with raloxifene, thereby allowing helix 12 to close and TGFα transcription (Levenson & Jordan 1998, Liu et al. 2001, 2002). The basis for the use of the D351G mutation in the engineered cells at the TGFα target (MacGregor Schafer et al. 2000) is that class I planar estrogens still allow helix 12 closure and TGFα transcription, but class II angular estrogens (e.g., bisphenol triphenylethylene) do not allow helix 12 closure and the D351G is now exposed but unsuitable for interaction with L540 in helix 12. As a result, closure of helix 12 does not occur because of steric hindrance from the projecting phenyl group of a triphenylethylene-type angular estrogen. As illustrated in Fig. 9, the class II estrogens tend to favor an antiestrogenic conformation for the complex and therefore do not allow transcription of TGFα. With this background of the molecular pharmacology of estrogen shape that programs the conformation

**Figure 7**
These images show the binding site of ERα co-crystallized with E2 and the H-bond network between E2 and amino acids from the ligand-binding site, also the H-bond between the backbone of L540 and side chain of D351. It appears that this interaction adds some stability to the agonist conformation and helps to keep H12 in place.

**Figure 8**
The comparative analysis of the experimental structures of ERα–LBD co-crystallized with 4-OHT (PDB entry 3ERT) and RAL (PDB entry 1ERR). Both structures superimposed with helix 12 positioned in the same way for both proteins with the amino acids lining the binding pockets, while only the amino acids involved in H-bonds with the ligands in the ‘Leu-crown’. The first noticeable difference is the orientation of H524. In RAL complex, the side chain of H524 is drawn toward the ligand, being involved in a H-bond with the hydroxyl group. This interaction is missing in the 4-OHT complex. Also, L536, L539, and L540 adopt different conformations than the ones they adopt in the 4-OHT complex, being ‘pushed away’ by the piperidine ring of RAL. The side chain conformations of the amino acids surrounding the ring involved in contact with H524, e.g., M343, M421, M423, and I424, are modified.
of the ER complex, this can now be applied to understanding estrogen-induced apoptosis.

The planar class I estrogens trigger estrogen-induced apoptosis within a few days with the MCF-7:5C cells committed to apoptosis after 24 h of E2 exposure. In contrast, the angular triphenylethylene estrogen bisphenol (class II) binds to the ER in MCF-7:5C cells, but does not trigger apoptosis in a 7-day assay. Bisphenol (class II) binds to ER and blocks E2 (class I)-induced apoptosis (Sengupta et al. 2013). This supports the hypothesis that the class II angular estrogen adopts the ‘antiestrogenic’ conformation of the ER complex despite the fact that bisphenol is a full estrogen agonist on the growth of MCF-7 breast cancer cells (Sengupta et al. 2013) and triphenylethylene estrogens trigger vaginal cornification in ovariectomized mice (Robson & Schonberg 1938). This latter assay was the primary methodology used to discover and classify triphenylethylene compounds as long-acting estrogens in vivo more than 60 years ago.

However, despite the veracity of the in vitro ligand conformation assay, there is a disagreement with the results of the first successful clinical trial in metastatic breast cancer by Haddow et al. (1944). They used several of the class I and II (triphenylethylene) estrogens (Fig. 1) and noted tumor regression with both classes (Table 1). Recent investigations in the laboratory demonstrated that the time courses of class I and II estrogens causing apoptosis are actually different and not static and stable over time. The altered conformation of the resulting class II estrogen–ER complex initially retards and then triggers apoptosis. There is a delay to commitment to apoptosis with the class II estrogen bisphenol of 3 days (Obiorah & Jordan 2014), whereas the class I estrogen (e.g., E2) is committed after 24 h (Obiorah et al. 2014a).

Thus, the in vitro ligand conformation ER efficacy assay does in fact comply with a clinical reality in triggering apoptosis and causing tumor regression (Haddow et al. 1944).

A larger screen (Obiorah et al. 2014c) of class I and II estrogens confirms that class II estrogens, whose structures are based on triphenylethylene, not only have a delay in apoptosis but also exhibit an accumulation of ER that is routinely downregulated by class I estrogen through ubiquitination and proteasomal destruction. However, this initial accumulation is not as pronounced as the accumulation of ER complex noted with 4-OHT and

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**Figure 9**

Functional test: putative conformations of the complex with ligand in LBD for type II estrogen to be ‘antiestrogenic’ with regard to helix 12 positioning. The assay discriminates between ligands (A), which allow helix 12 to seal the LBD or not (B and C). Sealing of helix 12 over the LBD is important for the ability of the ligands to trigger apoptosis. Reproduced with permission from Obiorah I, Sengupta S, Curpan R & Jordan VC 2014c Defining the conformation of the estrogen receptor complex that controls estrogen-induced apoptosis in breast cancer. *Molecular Pharmacology* 85:789–99. Copyright 2014 American Society for Pharmacology and Experimental Therapeutics.
endoxifen (Maximov et al. 2014a). What is particularly informative about the dimension of time is the fact that the class II angular estrogens initially accumulate the ER complex and do not downregulate ER mRNA in MCF-7:5C cells. The class II estrogen–ER complex activates downregulation of the complex and mRNA of ER over time, consistent with the delivery of coactivators to the appropriate protein synthetic machinery. This consumes a UPR response and invokes delayed apoptosis. The recent crystallization of the ER with a class II estrogen, the resolution of the structure, and the finding of a novel closed helix 12 conformation (Maximov et al. 2014a) are consistent with this interpretation of changes in the accumulation, evolution, and slow decrease in the level of class II estrogen–ER complex concentrations. This heralds the triggering of apoptosis by the class II estrogen–ER complex.

A summary of simple facts can now be made. The ligand shape can delay or advance estrogen-induced apoptosis in correctly configured LTED cells. The shape of the estrogen–ER complex determines the delivery of the coactivator SRC3 (NCOA3) to the relevant site within the vulnerable cell to initiate apoptosis. A previous study has shown that knock out of NCOA3 by siRNA will blunt estrogen-induced apoptosis (Hu et al. 2011). The question now is whether other logical treatment strategies can modify or modulate estrogen-induced apoptosis in a predictable manner.

Inhibition of cSrc signaling in LTED cells

The human homolog of the oncogene vSRc plays a fundamental linker role in the signal translation pathways from growth factor (GF) receptors on the cell membrane to activate nongenomic growth pathways in cancer. The essential role of vSRc is to act as a protein kinase that phosphorylates tyrosine (Hunter & Sefton 1980).

There is a cell-membrane-bound ER in the GF pathways, which is involved in immediate estrogen-mediated responses via a nongenomic phosphorylation cascade. However, cSrc is also critically involved in the phosphorylation of Y537 that regulates ER turnover and accumulation (Arnold et al. 1995, Chu et al. 2007).

Inhibitors of cSrc have attracted some attention as potential therapeutic agents in breast cancer; however, the unanticipated finding that a cSrc inhibitor stopped E2-induced apoptosis created a new research opportunity (Fan et al. 2012).

A cSrc inhibitor stops E2-regulated decreases in ER complex concentrations and this event (Fan et al. 2013), in turn, is responsible for regulating protein synthesis within the cell. The antiestrogen–ER complex accumulates but does not facilitate transcription of mRNA for protein synthesis; despite the fact that excess ER binds to the promoter region of genes, there is little or no coactivator binding. The binding of a class I estrogen–ER complex to the promoter regional genes with coactivators is required to ‘breathe’ at the promoter with cyclical destruction of DNA-bound complexes to maintain mRNA transcription (Shang et al. 2000). This would not happen in the presence of a cSrc inhibitor, as there is receptor stagnation.

The fact that a cSrc inhibitor blocks estrogen-induced apoptosis in LTED breast cancer cells required broad mechanistic explanation as there is not only ER at the genome but at the cell membrane. The treatment of MCF-7:5C cells with E2 increases the phosphorylation of cSrc that acts as a key adaptor protein for the activation of stress responses before triggering apoptosis. The sensors of UPR, IRE1α, and PERK kinase (that phosphorylates eukaryotic translational initiation factor 2α) are activated by E2 (Fan et al. 2013). There is also a dramatic increase in ROS and hem oxygenase HO-1 (an indicator of oxidative stress) and the central energy sensor kinase AMPK. However, a cSrc inhibitor and siRNA knockdown of cSrc both block estrogen-induced apoptosis. Despite the delay of commitment to apoptosis by 24 h (Obiorah et al. 2014a), the central role of cSrc raises the possibility that the nongenomic membrane ER may be involved in apoptosis. The estrogen–dendrimer conjugate (EDC) will only bind to membrane ER and is not taken up by the cell (Fig. 10) (Harrington et al. 2006). The treatment of cells with EDC neither initiates pS2 synthesis nor triggers apoptosis, both of which are mediated by a genomic mechanism of the nuclear ER. Interestingly enough, cell replication is increased by the EDC (Fan et al. 2013). It is therefore plausible that cSrc modulation can regulate estrogen-induced apoptosis, probably by reducing ER turnover.

However, all the previously discussed studies are conducted for a short term (1 week) and reflect biochemical events, which influence cell replication or death. A more clinically relevant study is to compare and contrast the actions of estrogen or a cSrc inhibitor alone or the combination over a period of 2 months (Fan et al. 2014a). This is used clinically to assess the success or failure of a treatment regimen for patient care. Studies (Fan et al. 2014a) demonstrate that blockade of cSrc is a reversible process and washes out rapidly to restore estrogen-induced apoptosis. In contrast, E2 alone causes catastrophic cell death and then regrowth of a new cell population that is not rapidly growing but achieves
These data also have important implications for the results antihormone resistance plasticity to different agents. This, in turn, illustrates the rapidity of governing the eventual survival of new breast cancer cell resistant cells over only a few months that ultimately acting as selection pressure on MCF-7:5C aromatase-in such a short time.

Glucocorticoid activity and apoptosis

The glucocorticoid dexamethasone is known to antagonize a number of estrogen-mediated events via an AP-1 (cfos/cJun) pathway (Uht et al. 1997). Simply stated, estrogens can stimulate transcription of genes through interaction at the promoter site but glucocorticoids counteract transcription. The respective actions are dependent on the concentrations of the ER and glucocorticoid receptor (GR) complexes at an AP-1 response element in the promoter (Uht et al. 1997). Recent studies have implicated changes in histone remodeling as a possible mechanism (Pradhan et al. 2012, Miranda et al. 2013). Earlier, estrogen-induced apoptosis has been reported to be mediated by an increase in inflammatory responses (Ariazi et al. 2011), hence it was appropriate to evaluate the action of glucocorticoid in model systems.

Dexamethasone reduces the growth rate of spontaneously growing MCF-7:5C cells in a concentration-related manner (Obiorah et al. 2014b). The inhibition of estrogen-induced apoptosis by dexamethasone is reversible with RU486, an antiglucocorticoid. The antiglucocorticoid RU486 is also, actually, a much more potent antiprogestin, in which application it is used as the ‘early abortion pill’. This fact highlights the promiscuous nature for synthetic steroids to interact with both progesterone and GR. This knowledge now opens up new opportunities to understand the results of the two trials from the WHI (Anderson et al. 2012).

There is considerable promiscuity of ligands between the progesterone receptor and the GR. Indeed, a high dose of MPA used as a breast cancer therapy or as a depot injection for contraception causes weight gain as a glucocorticoid-type side effect. The activation of glucocorticoid and progestin by synthetic progestins has been recognized in laboratory studies and a recent study (Sweeney et al. 2014b) addressed the hypothesis that the glucocorticoid properties of MPA could influence and modulate the apoptotic actions of E2 in LTED breast cancer cells.

Although MPA can activate the GR-regulated gene, it is much less potent than dexamethasone. Similarly, MPA only weakly modifies estrogen-induced apoptosis, but is effective in reducing the activation of estrogen-induced apoptotic genes. However, these clues that MPA might modulate estrogen-induced apoptosis over time and create

![Figure 10](image_url)

**Figure 10**

E₂-induced estrogen receptor signaling. (1). The genomic mechanism of ER signaling is by estrogen binding to the nuclear ER and then binding to hormone response elements in the promoters of target genes (classical) or through protein–protein tethering with nuclear DNA-binding transcription factors (nonclassical) to alter gene transcription. (2). E₂ can act through nongenomic signaling by activating cell surface membrane-localized extranuclear ER. (3). Estrogen–dendrimer conjugate (EDC) specifically activates the nongenomic signaling of ER action. Reproduced with permission from Obiorah IE, Fan P, Sengupta S, Jordan VC 2014 Selective estrogen-induced apoptosis in breast cancer. Steroids 90 60–70, with permission from Elsevier. Copyright 2014 Elsevier, Inc.

an equilibrium state between cell growth and cell death. However, the combination of E₂ and a cSrc inhibitor results in the outgrowth of a new cell population that grows vigorously with estrogen treatment and in which growth is stimulated by either 4-OHT or endoxifen (Fan et al. 2014b,c). These cells are driven through membrane-bound IGFIR and recapitulate phase I acquired resistance to tamoxifen noted in athymic animals (Gottardis & Jordan 1988; Fig. 11). This is not only a new model system in vitro to study acquired resistance to SERMs but also illustrates how selection pressure for only 8 weeks can rapidly change the form of acquired resistance to antihormones. This is the challenge for laboratory research to translate to the clinical setting and block tumor adaptation. In this case, it is the rapidity of change in such a short time.

Thus, it is the results of the cSrc inhibitor plus estrogen acting as selection pressure on MCF-7:5C aromatase-resistant cells over only a few months that ultimately governs the eventual survival of new breast cancer cell populations. This, in turn, illustrates the rapidity of antihormone resistance plasticity to different agents. These data also have important implications for the results of HRT/ERT in the WHI raising again the question as to why MPA plus CEE increases, but CEE alone decreases, breast cancer incidence. Perhaps, the answer lies in the ability of synthetic progestins to be promiscuous and interact with other steroid hormone receptors.
new cell populations that grow into tumors are an important finding: MPA could select for surviving breast cancers by modulating estrogen-induced apoptosis over years of therapy in women who are a decade or more from menopause as documented in the WHI (Anderson et al. 2012). Laboratory evidence for modification of apoptosis by estrogen through the glucocorticoid action of MPA was presented by Sweeney et al. (2014b). This new finding, coupled with the knowledge that rapid plasticity of hormone resistance occurs as a response to selection pressure (Fan et al. 2013, 2014a, b, c), indicates that a fundamental principle has now emerged based on selection pressure over years in patients.

Plasticity of populations of antihormone-resistant breast cancer cells

There are few estrogen-responsive ER-positive breast cancer cell lines (Sweeney et al. 2012), but the MCF-7 cell line continues to provide invaluable information (Levenson & Jordan 1997) and approaches to disease control that can be translated to patient care. Principles emerge that can be addressed in the clinic.

The understanding of acquired hormone resistance has been approached in two ways: either the long-term retransplantation of MCF-7 tumor tissue into successive generations of tamoxifen-treated (Yao et al. 2000) or raloxifene-treated (Balaburski et al. 2010) ovariectomized athymic mice or the estrogen deprivation of MCF-7 cells grown in culture over years. Studies in vivo revealed changes in the populations of cells that no longer responded to tamoxifen, displaying blocked estrogen-stimulated tumor growth, but now were stimulated to grow by either tamoxifen or physiological E2 treatment (Gottardis and Jordan 1988, Gottardis et al. 1989a, b). The populations evolved further during years of retransplantation into tamoxifen-treated athymic mice, with tamoxifen continuing to stimulate tumor growth but now...
physiological levels of E₂ caused tumors to regress rapidly (Wolf & Jordan 1993, Yao et al. 2000). Results of studies with MCF-7 under estrogen-deprived conditions indicated that cells develop that are able to grow autonomously in the absence of estrogen, but physiological E₂ could now induce apoptosis in cell populations (Song et al. 2001) or individual clones could respond rapidly to estrogen-triggered apoptosis (MCF-7:5C) or apoptosis could be delayed by a week (MCF-7:2A) (Ariazi et al. 2011). However, the phenotypes of the tamoxifen-stimulated MCF-7 cells in vivo and the estrogen-deprived MCF-7:5C cells in vitro are different. The tamoxifen-stimulated tumor in vivo is ER- and PgR-positive (Gottardis & Jordan 1988), but the selection pressure of estrogen deprivation in vitro creates an autonomously growing tumor when transplanted into athymic mice (Lewis et al. 2005b) that is ER-positive but PgR-negative. Fulvestrant will partially reduce the growth rate of MCF-7:5C tumors, but SERMs are, in the main, ineffective, although high concentrations of bazedoxifene in vitro are capable of causing death of MCF-7:5C cells (Lewis-Wambi et al. 2011). The question therefore arises, within the same MCF-7 cell line – are these very different forms of antihormone resistance phenotypically interchangeable if the appropriate selection pressure is applied?

The finding that a cSrc inhibitor can block estrogen-induced apoptosis in the MCF-7:5C estrogen-deprived cloned cell line (Jiang et al. 1992, Fan et al. 2012) turned out to be an appropriate model of selection pressure to determine the plasticity of the cell population during an 8-week treatment period. This time of treatment was selected as it is consistent with clinical practice for determining tumor progression or regression to endocrine therapy in metastatic breast cancer.

Treatment with estrogen alone for 8 weeks resulted in a much reduced cell population with cells replicating and undergoing apoptosis in equilibrium, but PgR synthesis was restored. In contrast, the cSrc inhibitor plus estrogen increased IGFRβ levels and the new cell population grew vigorously with estrogen treatment alone with increased PgR production. The role of IGFRβ and ER in facilitating vigorous growth was demonstrated using AG1024, an inhibitor of IGFRβ, and fulvestrant to destroy ER; both strategies to block receptor-mediated growth resulted in the complete inhibition of estrogen-mediated growth (Fan et al. 2014a,b,c). The new cell line was designated MCF-7:PF.

The discovery was that, for the first time to this author’s knowledge, a cell line was able to replicate the estrogenicity of individual SERMs to cause cell replication in vitro (Fan et al. 2014b) in much the same way as SERMs cause tumor growth in proportion to their intrinsic estrogenicity in the athymic mouse model in vivo (Wardell et al. 2013). This new cell line was unanticipated, as the new cell population that was created subsequently grew in response to an SERM. This result created by selection pressure through cellular trial and error was not based on a predetermined hypothesis; it was a discovery outside the accepted structure. Therefore, the plasticity of the MCF-7:5C estrogen-deprived cell line, that has no responsiveness to tamoxifen (i.e., SERM resistance), can have SERM-mediated growth expressed through an inhibition of estrogen-induced apoptosis. Thus, the different results with regard to the development of antihormone resistance in vivo and in vitro can now be connected (Fig. 11), through selection pressure on cell populations.

What is, however, particularly informative is the mechanisms of SERM-stimulated breast cancer cell growth that has now been defined (Fan et al. 2014b,c). Previous studies in vivo noted a paradox; tamoxifen-stimulated tumor growth occurred despite the fact that estrogen-regulated genes that are controlled via the ER through the genomic route were all blocked during growth stimulated by tamoxifen (Osipo et al. 2007). Pathway analysis of MCF-7:PF cells indicates similar patterns for tamoxifen- and estrogen-stimulated growth (Fan et al. 2014c), but closer examination of individual genes regulated by the ER via the genomic route revealed that they are blocked. This is consistent with the results of earlier studies by Osipo et al. (2007) in vivo using tamoxifen-stimulated tumors. In contrast, IGFRβ is activated and SERM-stimulated growth is blocked by AG1024 (Fan et al. 2014b). Other investigators have implicated the IGF receptor system in earlier studies (Massarweh et al. 2006, 2008, Becker et al. 2011, Fagan et al. 2012).

**Summary and future clinical perspective**

The chance ‘rediscovery’ of estrogen-induced regression of long-term (over 5 years) estrogen-deprived breast tumors (Wolf & Jordan 1993, Yao et al. 2000) under laboratory conditions opened the door to deciphering mechanisms that now are the evidence-based scientific framework for future clinical applications.

The dramatic improvements in survival noted with increasing years of adjuvant tamoxifen (Early Breast Cancer Trialists’ Collaborative Group 1998, 2005) and the ATLAS trial of 5 vs 10 years of adjuvant tamoxifen (Davies et al. 2013) were unanticipated. This is because tamoxifen is classified as a nonsteroidal antiestrogen (Jordan 1984) that blocks the tumor ER to stop tumor growth. A medicine that is cytostatic but not cytotoxic...
could not decrease mortality. Nevertheless, mortality decreases improved not only following the cessation of adjuvant tamoxifen (Davies et al. 2011, 2013) but also following 5 years of adjuvant AI therapy (Forbes et al. 2008, Cuzick et al. 2010), and a continuing effect of tamoxifen occurs during chemoprevention that is also noted after tamoxifen is stopped (Fisher et al. 2005, Cuzick et al. 2007, Powles et al. 2007). These consistent clinical results have now been linked to selection pressure of antihormone therapy creating a vulnerability in new populations of cells ready to undergo apoptosis if exposed to a woman’s own estrogen (Jordan 2014d). Indeed, the Study of Letrozole Extension (SOLE; Fig. 12) is testing this hypothesis (Wolf & Jordan 1993, Yao et al. 2000) by comparing 5 years of continuous adjuvant letrozole treatment with 5 years of intermittent adjuvant letrozole treatment (a 3-month annual drug holiday) after completing an initial 5 years of adjuvant antihormone therapy. However, it is now possible to incorporate another complication to the long-term adjuvant tamoxifen and estrogen-induced apoptosis decreasing the mortality. Tamoxifen is considered not only to be a competitive inhibitor of estrogen action at the human tumor ER (Jensen & Jordan 2003) but also to be a prodrug that is converted into hydroxylated metabolites 4-hydroxytamoxifen and endoxifen. Endoxifen is formed by the CYP2D6 enzyme system in the patient (Brauch & Schwab 2014), but aberrant genotypes can create extremes of extensive metabolites (EMs) through multiple gene copies or poor metabolizers (PMs) with zero gene function. This has become an extremely controversial topic for clinical application. No prospective studies have been completed, but retrospective studies have revealed a positive correlation, i.e., EM-genotype patients do better than PM-genotype patients. The logic is that the antiestrogenic mix for the EM patients will serve to block the ER better in the tumor and, as a result, stop estrogen reactivating tumor growth. But there is now another way (Fig. 13) to view these data based on the results discussed previously in the section describing the drift in cell populations in vitro, if estrogen-induced apoptosis is blocked by an inhibitor of cSrc. The principle of improving the antiestrogenic mixture of metabolites in both premenopausal and postmenopausal patient environments has recently been modeled in vitro (Maximov et al. 2014b,c). These studies model the immediate effects of tamoxifen and metabolites of estrogen-stimulated growth and gene regulation. They do not, however, model the years of selection pressure that must occur to create vulnerable cell populations for execution with endogenous estrogen once long-term therapy stops. A challenge for the future is to produce an extensive panel of ER-positive breast cancer cell lines from patients and then model years of adjuvant therapy with tamoxifen using cell culture and animal models. In this way, the veracity of the hypothesis (Fig. 13) that endoxifen is an essential component of adjuvant tamoxifen action can be confirmed as justification to revisit clinical investigations. The examination of tumor cell biology under selection pressure must then be melded with the mechanism of estrogen-induced apoptosis in the new cell line panels. Cellular barriers to apoptosis must be identified and tumor responsiveness enhanced methodically through a multifaceted study of mechanisms.

![Figure 12](http://erc.endocrinology-journals.org) Schema for the Study of Letrozole Extension (SOLE; IBCSG 35-07) conducted by the International Breast Cancer Study Group (IBCSG). Upon completion of 4–6 years of previous adjuvant endocrine therapy with SERM(s) and/or Al(s), patients were randomly assigned to continuous or intermittent letrozole (3-month drug holidays/year) for 5 years. The rationale for this approach was that the woman’s own estrogen in the intermittent arm would trigger apoptosis in long-term estrogen-deprived breast cancer and reduce recurrence rates. Adapted from International Breast Cancer Study Group – Study of Letrozole Extension (www.ibcsg.org). Reproduced with permission from Jordan VC & Ford LG 2011 Paradoxical clinical effect of estrogen on breast cancer risk: a ‘new’ biology of estrogen-induced apoptosis. Cancer Prevention Research 4 633–637. Copyright 2011 American Association for Cancer Research.
The mechanism of estrogen-induced apoptosis can be summarized as shown in Fig. 14. Estrogen binds to the ER in the correctly selected breast cancer cell population derived under estrogen-deprived conditions. Growth of cells occurs through a genomic and a nongenomic pathway and the cell triggers an UPR through ERK signaling. Apoptosis occurs first through the mitochondrial (intrinsic pathway) but subsequently the death receptor (extrinsic pathway) is recruited to consolidate the pathways that execute the cell (Obiorah et al. 2014a).

The fact that estrogen-induced apoptosis does not occur in all ER-positive breast cancer cell populations at all times is important. Not only is the influence of estrogen important for creating a cell line dependent on the ER signal transduction pathway for growth, which dominates, but also estrogen-deprived cells do or do not respond to estrogen to trigger estrogen-induced apoptosis. The question for the refractory LTED cells will be, ‘why not?’ Based on the initial findings regarding the treatment of metastatic breast cancer with high-dose estrogen (Haddow et al. 1944, Stoll 1977) discussed earlier, it was clear that a 5 year ‘gap’ must occur between menopause and treatment success (Fig. 6). If patients are treated too soon after menopause, the tumors grow. Today, these same rules apply for CEE (a hormone replacement), bisphenol A (an endocrine disruptor) or phytoestrogen (daidzein or genistein) (Obiorah et al. 2014b), whereas some long-term estrogen-depleted cells will undergo estrogen-induced apoptosis with all three ligands that bind to ER (Sengupta et al. 2013, Obiorah & Jordan 2013, Obiorah et al. 2014b). Although there is no relevant clinical translation to breast cancer for bisphenol A, there is clinical translation for CEE and phytoestrogens. The administration of either ERT or soy around menopause increases tumor cell replication (Beral et al. 2011, Shike et al. 2014). We have previously emphasized the fact that administration of CEE in the Million Women’s Study straight after menopause during the gap period increases breast cancer risk (Beral et al. 2011), but, after the gap period, there is no increase in breast cancer risk (Beral et al. 2011). With regard to soy supplements and phytoestrogens, the situation is less clear because the appropriate studies have not been completed. This is an opportunity for the future.

The recent study by Shike et al. (2014) is the first, to our knowledge, to examine gene activations in mixed population of patients (premenopausal and early postmenopausal women with breast cancer) who elected to take a soy supplement for 7–30 days. Blood levels of daidzein and genistein broadly range from 0 to 400 mg/ml. However, a genistein signature is identified that contains activated replication genes. This illustrates that soy products are not safe to take to protect against breast cancer in the ‘gap’ period to ameliorate menopausal symptoms. The question for the future is whether soy administration in a woman’s 60s would protect some of them against breast cancer in the same way as CEE (Anderson et al. 2012). The hope is that there would be fewer strokes with soy products. This was the reason that the WHI CEE alone study was stopped (Anderson et al. 2004).

Thus, the rules (Fig. 6) to provide selection pressure through estrogen deprivation after menopause have veracity in the laboratory and at least with CEE therapy in the clinical setting. Another approach to controlling menopausal symptoms may be bazedoxifene/conjugated estrogen combination that has been recently approved for reducing menopausal symptoms through estrogen action in the CNS but with an SERM, bazedoxifene, to prevent uterine carcinoma or breast cancer growth from unopposed estrogen action (Komm & Mirkin 2012). A recent publication (Jordan 2014e) has raised the proposal a clinical trial of the 5-year use of bazedoxifene/conjugated estrogen around menopause to drive occult breast cancer cell selection, but followed by a ‘purge’ for a few months with estrogen alone to execute prepared and vulnerable...
LTED breast cancer cells. However, the question remains: ‘If estrogen triggers apoptosis in ERT given to women in their 60’s, and the administration CEE with MPA a synthetic progestin (HRT) increases the risk of breast cancer, because it has associated glucocorticoid activity, can a safer HRT be developed?’

It is known that 19-nortestosterone derivatives that are progestogenic also have estrogenic activity and trigger breast cancer cell proliferation (Catherino et al. 1993, Jeng et al. 1992, Jeng et al. 1993). However, MPA is not a 19-nortestosterone derivative and does not stimulate breast cancer cell proliferation (Catherino et al. 1993). But MPA does have an associated glucocorticoid activity (Koubovec et al. 2005, Courtin et al. 2012), so that there is the potential, during years of therapy, to modify and protect cells from estrogen-induced apoptosis, thereby modulating breast cancer growth. If this were true, then it would seem to be more reasonable to select a 19-nortestosterone derivative such as norethindrone acetate to reinforce estrogen-induced apoptosis but still provide uterine protection from the growth of endometrial cancer.

Thus, the administration of not only CEE at the appropriate time to trigger apoptosis, but also a 19-nor synthetic progestin to protect the uterus and provide backup sustained estrogen action may be a double application of the new science of estrogen-induced apoptosis. This could benefit millions of women who wish to take a safer HRT preparation. The solution for applications in women’s health may lie in a closer look at the synthetic progestin in HRT.

The search for the mechanism of estrogen-induced apoptosis started with the clinical observations of Haddow et al. (1944) FRS who went on to create the database for the first chemical therapy for any cancer validated in a clinical trial. The clinical findings have stood the test of time and through laboratory, clinical, and epidemiological studies have now created a principle based on the response of some breast cancer cell populations to longer term estrogen deprivation rules that can now be applied (Fig. 6). Some surviving cells become vulnerable to estrogen-induced apoptosis through a unique molecular mechanism targeted by the ER. This mechanism is now validated and has the potential to expose future targets,
even in ER-negative cells, for drug discovery. Following the ER in the estrogen-dependent cell has shown us where to look. Cancer in general is ER-negative, so perhaps it is more vulnerable than we think.

The story of the evolution of some ER-positive breast cancer cell populations in an estrogen-deprived environment to become hormone-independent for growth in response to selection pressure may in fact be a general principle in cancer cell therapeutics. The plasticity of cell populations ebb and flows rapidly, but results from clinical trials indicate (Anderson et al. 2012, Davies et al. 2013) that decreases in mortality are possible with low tumor burdens in an adjuvant setting and patient population survival is enhanced. This is a potential start for a new structure to seek the vulnerabilities in not only breast cancer, but also in other solid tumors that respond and subsequently adapt to selection pressure by succeeding in developing new growth mechanisms. This is not a new idea as Darwinian adaptation teaches that each species or variety of a species must adapt to a hostile environment and can only survive if characteristics favor growth of the population. Population growth is only possible if limited resources are only acquired by the fittest by competition. Survival of population with favorable characteristics was described as ‘survival of the fittest’. But the population shifts of species adapting to change took hundreds of millions of years. For cancer, in the human host, no more than a decade or two is required to create a change in adaptive survival; the random power of continuous replication and mutations creates the ‘survival of the fittest’ by mindless trial and error that ends by killing the host. This is the renegade cell usurping the controlled process for short-term gain now as a parasite that will eventually destroy the host.

The conversation between laboratory and clinical research is a two-way conversation. The translational research by Haddow in the 1940s (Haddow & Robinson 1939, Haddow et al. 1944) posed a question that lingered for 60 years, ‘how does estrogen kill breast tumor cells and why does this occur?’ This review article describes the successes so far in documenting the mechanism of how apoptosis is triggered to kill breast cancer. I also offer herein an explanation as to why cell populations become vulnerable to estrogen-induced apoptosis. The mechanism and rules presented do not provide the understanding of all cancers; that is a naive goal. The goal is more success in our understanding than failures. The fact that there are failures in our comprehension must be viewed as an opportunity for future generations of investigators to add more to the expanding mosaic in the quest to control the relentless adaptation of cancer to selection pressure.

‘Success is not final. Failure is not final. It is the courage to continue that counts’

Declaration of interest
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