MOLECULAR MODULATION OF ESTROGEN-INDUCED APOPTOSIS IN LONG-TERM ESTROGEN-DEPRIVED BREAST CANCER CELLS

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ABSTRACT

Estrogen receptor (ER)-positive breast cancer cell lines have been instrumental in modeling breast cancer and providing an opportunity to document the development and evolution of acquired anti-hormone resistance. Models of long-term estrogen-deprived breast cancer cells are utilized in the laboratory to mimic clinical aromatase inhibitor-resistant breast cancer and serve as a tool to discover new therapeutic strategies. The MCF-7:5C and MCF-7:2A subclones were generated through long-term estrogen deprivation of ER-positive MCF-7 cells, and represent anti-hormone resistant breast cancer. MCF-7:5C cells paradoxically undergo estrogen-induced apoptosis within seven days of estrogen (estradiol, E\textsubscript{2}) treatment; MCF-7:2A cells also experience E\textsubscript{2}-induced apoptosis but evade dramatic cell death until approximately 14 days of treatment. Our data suggest that MCF-7:2A cells employ stronger antioxidant defense mechanisms than do MCF-7:5C cells, and that oxidative stress is ultimately required for MCF-7:2A cells to die in response to E\textsubscript{2} treatment. Tumor necrosis factor (TNF) family member activation also correlates with E\textsubscript{2}-induced apoptosis in MCF-7:2A cells; up-regulation of TNF\alpha occurs simultaneously with oxidative stress activation. Additionally, increased insulin-like growth factor receptor beta (IGF-1R\beta) confers a mechanism of growth and anti-apoptotic advantage in MCF-7:2A cells.
Hormone replacement therapy (HRT) is widely used to manage menopausal symptoms in women, and can comprise an estrogen alone or an estrogen combined with a progestin. The Women’s Health Initiative demonstrated in their randomized trials that estrogen alone HRT decreases the risk of breast cancer in post-menopausal women, while combined estrogen plus a progestin (medroxyprogesterone acetate, MPA) HRT increases this risk. We sought to elucidate the mechanism through which these opposing effects occur. The data suggests that MPA acts as a glucocorticoid which blocks estrogen-induced apoptosis in long-term estrogen-deprived breast cancer cells thereby increasing cancer risk.
This work is dedicated to my loving family and friends, who have shown me unconditional support.

It is also dedicated to all those affected by breast cancer who find hope in scientific research.
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INTRODUCTION

This works seeks to uncover strategies toward improving women’s health and breast cancer risk by describing cellular behavior investigated in the laboratory. Chapter I introduces the notion of clinical resistance to endocrine therapy for breast cancer, and reviews the cellular models available to interrogate this concept in the laboratory. Chapter II delves deeper into two particular cell lines derived from MCF-7 breast cancer cells that paradoxically undergo estrogen-induced apoptosis. Important mechanisms involving oxidative stress and growth pathway signaling are described. Chapter III expands this concept of estrogen-induced apoptosis into how it impacts women taking hormone replacement therapy (HRT). Though HRT has been shown effective in alleviating unpleasant menopausal symptoms and preventing osteoporosis, studies have also suggested its risk in increasing heart disease, stroke, and breast cancer. This work will explain a molecular foundation for increased breast cancer risk in women taking combined estrogen plus progestin HRT.
CHAPTER I:
MODELS AND MECHANISMS OF ACQUIRED ANTI-HORMONE RESISTANCE IN BREAST CANCER:
SIGNIFICANT CLINICAL PROGRESS DESPITE LIMITATIONS

The past four decades have witnessed the successful evolution of effective breast cancer therapies as scientific research has translated into clinical practice. Breast cancer therapy began its story with combination cytotoxic chemotherapy. Chemotherapy, though able to create complete responses in some cases of breast cancer, works non-specifically, causing harmful and sometimes intolerable, life-threatening side effects. Anti-estrogen therapies, by contrast, provide significant therapeutic improvement by focusing on a target, the tumor estrogen receptor (ER) [1]. It is important to point out that the ER was initially used not as a therapeutic target, but as a predictor of response to endocrine ablation, such as oophorectomy [2]. The innovation of targeting the tumor ER specifically using the non-steroidal anti-estrogen tamoxifen (Figure 1) [3] ultimately changed the prognosis of women with breast cancer by proposing two new treatment strategies: a new approach to therapy with long-term early adjuvant tamoxifen treatment following surgery and subsequently the possibility of using tamoxifen for chemoprevention [1,2]. In both cases the target would be the ER, to be blocked by tamoxifen.
Tamoxifen is approved by the Food and Drug Administration (FDA) to treat node-positive and node-negative breast cancer patients with long-term adjuvant therapy, and is

![Chemical structures of 17β-Estradiol, raloxifene, tamoxifen, and tamoxifen’s metabolites n-desmethyl tamoxifen, 4-hydroxytamoxifen, and endoxifen.](image)

**Figure 1.** Chemical structures of 17β-Estradiol, raloxifene, tamoxifen, and tamoxifen’s metabolites n-desmethyl tamoxifen, 4-hydroxytamoxifen, and endoxifen.
approved to lower the incidence of breast cancer in high-risk pre- and post-menopausal women. In both applications, clinical trials established and confirmed that patients with ER-positive breast cancer are the ones who benefit. Tumors that are ER-negative do not respond to tamoxifen. In addition to blocking estrogen’s binding to its receptor, another means of limiting estrogenic activity in breast tissue is by blocking the synthesis of estrogen. Aromatase inhibitors block estrogen’s conversion from its androgen precursor thereby limiting the production of estrogen [4,5]. This approach has proven beneficial clinically with fewer side effects than tamoxifen and improvements in recurrence rates and survival for post-menopausal patients [6-9].

The benefit of anti-hormone therapy (data primarily from tamoxifen trials) targeted to the ER is impressive in terms of both recurrence-free survival and decreases in mortality [7,10]. Millions of women now live longer, healthier lives based on the application of translational research [1]. Women of any age with ER-positive tumors experience an approximately 30% mortality reduction when treated with long-term (5 year) adjuvant tamoxifen [7,10]. Post-menopausal women, however, receive greater clinical benefit with aromatase inhibitors rather than tamoxifen, in terms of lower breast cancer recurrence rates and fewer side effects [6]. Aromatase inhibitors can be used instead of tamoxifen for five years, after tamoxifen for five years, or by switching to an aromatase inhibitor after a year or two of tamoxifen. The important principle is to ensure compliance so that at least five years of anti-hormone treatment is used.

Breast cancer prevention trials built on the previous clinical experience with tamoxifen to demonstrate tamoxifen’s efficacy in preventing ER-positive invasive breast cancer in women at high-risk [11]. However, few high-risk women benefit from population-based chemoprevention with tamoxifen while many are exposed to side effects such as endometrial cancer and
thromboembolic events [12]. As a result, a paradigm shift occurred with the finding that non-steroidal anti-estrogens are, in fact, selective ER modulators (SERMs). The laboratory discovery that SERMs can maintain bone density but prevent mammary carcinogenesis led to the idea of treating osteoporosis while preventing breast cancer at the same time [13-15]. It is fair to say that the laboratory finding [16] that tamoxifen increases the growth of human endometrial cancer but stops the growth of breast cancer, and its subsequent clinical confirmation [16,17], really stressed the need to find a new chemopreventive medicine. Raloxifene is a drug similar in structure to tamoxifen (Figure 1) which is now prescribed indefinitely as a medicine to prevent osteoporosis, offering a beneficial side effect of breast cancer prevention in post-menopausal women [18,19]. Additionally, raloxifene is FDA-approved as a prevention strategy to reduce the incidence of ER-positive breast cancer in at-risk post-menopausal women without increasing the incidence of endometrial cancer as occurs with tamoxifen [20,21]. Figure 1 illustrates the structures of estradiol, raloxifene, tamoxifen, and related metabolites.

With this brief clinical background of progress in the quality of life and survivorship for women with breast cancer, and the practical progress in reducing the incidence of breast cancer, several principles emerge to focus laboratory efforts to enhance further advances. Long-term therapy is the key to successful increases in survivorship and only ER-positive tumors are responsive to anti-hormone therapy. However, because of the finding that five or more years of therapy can control recurrences of the growth of micro-metastatic primary breast cancer, it is acquired resistance to anti-hormone therapy that must be addressed. Models must replicate clinical experience with the ER-positive tumor. The surviving cells whose growth is not blocked
by anti-hormones have the plasticity to respond to treatment in a Darwinian model of continued growth and replication.

This chapter will describe the limited types of ER-positive breast cancer cells available to the scientific community and the strategies used in the laboratory to create models to mimic clinical experience i.e. years of anti-hormone therapy. Through the creation of reproducible models, mechanisms can be deciphered to apply to new clinical treatment strategies.

**Cell lines as platforms for modeling acquired anti-hormone resistance**

The Early Breast Cancer Trialists’ Collaborative Group recently showed that after about 5 years of tamoxifen therapy for women with ER-positive breast tumors (10,645 women), yearly breast cancer mortality rate was reduced by 30% for 15 years after treatment initiation [7]. If we estimate that ER-positive breast cancer, the most prevalent type, accounts for 75% of all breast cancer, it follows that about half of the breast cancers may have or acquire resistance to anti-hormone therapy. This, combined with the fact that over 200,000 new cases of breast cancer [22] are expected to occur each year, makes acquired resistance a critical issue in breast cancer research and women’s health. Prevention of primary breast cancer or the maintenance of patients to prevent recurrence of the disease is an important advance in translational research that continues to reduce healthcare costs and improve survivorship for millions of patients worldwide. Although it is fair to say that few women at high risk for breast cancer elect the chemoprevention option, there are more than half a million women using raloxifene to prevent osteoporosis and prevent breast cancer at the same time [18]. However, tumors that form during long-term raloxifene treatment [19] have acquired resistance to this SERM.
It is currently impossible to analyze the cell biology of every patient’s individual breast tumor and predict outcomes, both practically and financially. The actual relationship of the cancer cell with supporting stroma of an individual tumor cannot yet be reconstructed under laboratory conditions, but what can be achieved at this stage is the interrogation of available cell lines to focus on a specific group of ER-positive tumors and obtain general principles with which to plan treatments. In other words, laboratory models *in vitro* and *in vivo* represent the medium for a conversation between the laboratory and the clinic. These models represent important subgroups of breast tumors in patients.

Breast cancer cell lines that are ER-positive are of specific value to conduct translational research to understand the mechanisms by which hormone-responsive breast tumors may develop acquired anti-hormone resistance. The ER-positive models to be discussed here are: ZR-75-1, BT-474, T47D, and MCF-7. Each cell line is available from the American Type Culture Collection (ATCC) but there are individual variants maintained in specific laboratories. The current ER statuses (Figure 2), ER protein regulation (Figure 2), hormone responsiveness to the principal steroidal estrogens estradiol and estrone (Figure 3), and the relative ability of tamoxifen and its metabolites to block combined circulating levels of estrone and estradiol (Figure 4) are illustrated.
Figure 2. A. ERα expression levels in different ER positive cells. Cell lysates of MCF-7, T47D, ZR-75-1, BT474, MCF-7:5C, and MCF-7:2A were harvested. MCF-7, T47D, ZR-75-1 and BT474 cells were cultured under conditions with estrogen (10% fetal bovine serum, FBS), while MCF-7:5C and MCF-7:2A cells were cultured under estrogen-free conditions (10% charcoal stripped fetal bovine serum, SFS). ERα expression levels were examined by immunoblotting with primary antibody. Immunoblotting for β-actin was determined for loading control. B. Modulation of ERα expression in the absence of estrogen. Wild-type ER positive MCF-7, T47D, ZR-75-1, and BT474 cells were cultured under conditions with estrogen (10% FBS) or without estrogen (10% SFS) for 3 days, respectively. Cell lysates were harvested. ERα expression levels were examined by immunoblotting with primary antibody. Immunoblotting for β-actin was determined for loading control.
Figure 3. Proliferative responses of different ER-positive breast cancer cell lines to treatments with estradiol (E₂, blue) and estrone (E₁, red). Growth of cells was determined by measuring DNA per well after 7 day treatments. A. MCF-7:WS8 cells, hypersensitive clones of MCF-7 cell line; B. T47D:A18 cells, hypersensitive clone of T47D cell line; C. BT474 ER-positive breast cancer cells (ATCC); D. ZR-75-1 ER-positive breast cancer cells (ATCC). Estradiol is the most potent of the natural estrogens in a woman’s body, and estrone, with the 17β hydroxyl oxidized to a ketone, is less potent. It does, however, significantly contribute to breast cancer cell growth.
Figure 4. Biological response of MCF-7 cells after 7 day treatment with premenopausal levels of estrone (E₁, 8nM) and estradiol (E₂, 4nM) found in plasma of pre-menopausal women during follicular phase of menstrual cycle [174] and tamoxifen metabolites 4-OHT (6.3 nM), N-desmethyl-Tam (558 nM), tamoxifen (386 nM) and endoxifen (35.6 nM) at concentrations found in plasma of extensive metabolizers of tamoxifen [175]. Combining E₁/E₂ induces cell growth and treatment, while tamoxifen metabolites have a minor effect on cells. Combination treatment of E₁/E₂ and tamoxifen metabolites does not ablate the proliferation of the cells. However, addition of another tamoxifen metabolite endoxifen at concentrations found in plasma of extensive metabolizers of tamoxifen (35.6 nM) produces almost complete inhibitory effect on cell growth. Treatment with the tamoxifen metabolites plus endoxifen does not have any major biological effect.
The ZR-75-1 breast cancer cell line

The ZR-75-1 human breast cancer cell line was derived in the late 1970s from a 63-year-old post-menopausal female patient with metastatic ductal carcinoma of the breast. The cells were taken from the ascites three months after initiation of tamoxifen treatment and exhibit estrogen and insulin responsiveness [23]. As ZR-75-1 cells are passaged they retain their epithelial morphology, remaining similar in appearance to their original source biopsy, though their chromosome count decreases from approximately 75 to 72 after 38 passages [23]. ZR-75-1 cells are ER-positive, glucocorticoid receptor (GR)-positive, androgen receptor (AR)-positive, and progesterone receptor (PR)-positive [23]. Tamoxifen (10⁻⁶ M) causes growth inhibition and the cells die [24]. Also, the cells are specifically growth-stimulated by insulin, and inhibited by androgens and glucocorticoids [23].

The BT-474 breast cancer cell line

The BT-474 cell line comprises ER-positive, PR-positive epithelial cancer cells derived from invasive ductal breast carcinoma of a 60-year-old female patient [25]. Notably, these cells also express the nuclear receptor human epidermal growth factor receptor 2 (HER2) [26]. With 55 chromosomes, they grow in adherent patches in tissue culture, and are tumorigenic [25]. BT-474 cells grow in response to estradiol, via their ER (see Figure 3).

The T47D breast cancer cell line

The T47D cell line originates from a pleural effusion of a 54-year-old female patient with infiltrating ductal breast carcinoma. The cells have approximately 60 to 70 chromosomes, multiple mitochondria, and irregular nuclei and nucleoli [27]. They maintain their epithelial morphology after several years of passage, can produce casein, and can be grown in a monolayer
in vitro [27]. First described as an ER-positive, PR-positive, AR-positive, GR-positive, epithelial cell carcinoma model, it has since been established that the nuclear receptor levels and hormone responsiveness depend on the culture conditions [28]. T47D cells express ER and PR in estrogen-rich media, but lose most PR and ER expression when grown in the absence of estrogen [28].

Classically, estradiol stimulates proliferation of the T47D cell line through the ER, and stimulates estrogen-regulated proteins such as PR, while tamoxifen inhibits this growth [29]. The stimulatory action of physiologic estrogens and the inhibition caused by tamoxifen and its principal metabolites are shown in Figures 3 and 4, respectively. Without the nuclear receptors, however, neither estradiol nor tamoxifen can influence growth since their mechanism of action through ER is eliminated [28].

The MCF-7 breast cancer cell line

The majority of investigations into acquired anti-estrogen drug resistance have utilized the MCF-7 cell line so prevalent in breast cancer laboratories. The MCF-7 cell line has been the topic of an earlier review [30]. MCF-7 cells are used ubiquitously in research for ER-positive breast cancer cell experiments and many subclones have been established, representing different classes of ER-positive tumors with varying nuclear receptor expression levels.

The MCF-7 cell line was derived from the pleural effusion of a 69-year-old female patient with a diagnosis of adenocarcinoma of the breast [31]. This particular patient had undergone three years of radiotherapy and hormone therapy, most likely high-dose diethylstilbestrol (DES), a synthetic estrogen (the cell line was created before tamoxifen was available for clinical use). The cells were noted to be ER- and PR-positive [32]. In the mid-
1970s Lippman [33,34] demonstrated that non-steroidal anti-estrogens in general, and tamoxifen in particular, could stop the growth of MCF-7 cells in culture, and this could be reversed with the administration of exogenous estradiol.

In the early 1980s, MCF-7 cells were shown to form tumors in vivo [35] with estrogen administration, but estrogen did not significantly stimulate growth of the same cells in vitro [36]. At the time, it was proposed that a factor existing in the animal but not in culture, be it a second messenger system or peptide growth factor, was required for the profound growth influence of estrogen on MCF-7 cells [36]. However, a landmark discovery occurred in 1986 identifying a contaminant of phenol red (phenolsulfonphthalein) (Figure 5), the pH indicator in media, as estrogenic [37,38]. The media was therefore causing cells to grow [37]. All previous studies measuring estrogen’s impact on the cells were undermined since the effects were confounded by additional estrogen in the media. The discovery allowed complete withdrawal of estrogen from the cells and the subsequent ability to document the real impact of estrogen on various cell functions including proliferation and apoptosis of MCF-7 cells [39-43].

Figure 5. Phenolsulfonphthalein (phenol red), the pH indicator in cell culture media, is structurally similar to the natural estrogen estradiol (Figure 1) and synthetic estrogens. Unlike normal chemical titration analyses that use a pH indicator at very low concentrations, phenol red is incorporated at µM levels in culture media. The estrogenicity was found to vary from batch to batch [176]. However, a potent estrogenic contaminant (right) exerts growth stimulatory effects on breast cancer cells [38].
Being ER-positive, the MCF-7 cell line grows and proliferates with estrogens, in concentrations as low as $10^{-11}$ M estradiol (Figure 3) [30]. Tamoxifen competitively inhibits DNA synthesis in MCF-7 cells, binding to the same ER as do estrogens, though with a 1000-fold lower affinity than estradiol [30]. When added to the cells simultaneously, estradiol can reverse this inhibition at a concentration 100-fold lower than tamoxifen ($10^{-7}$ M vs. $10^{-8}$ M) causing cell growth (Figure 4) [30]. The actions of tamoxifen and its metabolites on estrogen-stimulated proliferation are shown in Figure 4. Pure anti-estrogens, such as fulvestrant, that destroy ER, also inhibit growth of MCF-7 cells [44].

ER regulation in ER-positive breast cancer cell lines

Figure 2 illustrates ER expression in the four described ER-positive breast cancer cell lines in different media conditions. ZR-75-1, BT-474, and MCF-7 cells increase expression of ER in the absence of estrogens, represented here by phenol red-free media supplemented with charcoal-stripped fetal bovine serum (SFS). Estrogen exposure to these cells causes decreased ER mRNA and protein levels [45]. T47D cells, by contrast, express more ER in an estrogenic environment, shown here as red media with fetal bovine serum (FBS) [45]. As previously stated, T47D ER expression is lost in an estrogen-free environment. Tamoxifen causes increased ER protein levels in MCF-7 and T47D cells, while fulvestrant causes decreased protein levels in both cell lines [45]. The alternate models of ER regulation in the cell lines has previously been summarized [45] and is now updated and illustrated in Figure 6 for convenience. The consistent model (Model I) of ER regulation is an upregulation of ER in the absence of estrogen. However, T47D does not conform and requires estrogen for ER synthesis (Model II).
Figure 6. The diagrammatic representation of cellular estrogen receptor (ER) regulation in media with or without estradiol (E$_2$). This diagram is based on the general responses to estrogen illustration by Western blotting in Figure 2 and presented in detail in [45]. Model I ER regulation (MCF-7, ZR-75, BT-474) has an upregulation of ER message and protein in an estrogen-depleted environment, but ER is downregulated at the mRNA and protein level in the presence of estrogen. Model II ER regulation (T47D) has upregulation of ER message and protein in an estrogen-containing environment; ER is not produced in an estrogen-depleted environment. T47D cells lose ER to become ER-negative.
Models of acquired anti-hormone resistance in vitro

ER-negative breast cancer cells, such as the MDA-MB-231 and SKBr3 cell lines, do not respond to anti-hormone treatment. There are some ER-positive cell lines that also exhibit intrinsic resistance; that is, anti-hormones do not create a subpopulation of these cells that are resistant over time. They simply do not respond initially, perhaps via growth factor receptor overexpression allowing other mechanisms of growth stimulation. Osborne’s group showed in 1992 [46] that when ER-positive MCF-7 cells are transfected with HER2, the cells are intrinsically resistant to anti-hormones such as tamoxifen, presenting HER2 as a potentially important factor for tamoxifen sensitivity and drug resistance.

To investigate the properties of acquired anti-hormone-resistant breast cancer cells, populations of MCF-7 cells have been created that are adapted to various anti-hormone environments. MCF-7 cells, more than the other three ER-positive cell lines T47D, BT-474, and ZR-75-1, are well-suited for anti-hormone resistance studies since they are easily cultured and retain ER expression when treated with anti-hormones; they are routinely used in the laboratory and have produced more data of practical knowledge for patient care than any other breast cancer cell line (see final section). Figure 7 illustrates the lineages of different subtypes of MCF-7 cells maintained in the laboratory.
One such in vitro model illustrating the varied attributes of tamoxifen-resistant cells are the MCF-7/LCC subclones (Figure 7). The MCF-7/LCC1 variant represents an estrogen-
independent breast cancer cell line obtained from \textit{in vivo} selection in oophorectomized nude mice and re-cultured \textit{in vitro} to become a stable cell line [47,48]. Though estrogen-independent, the cells are still tamoxifen-sensitive [47]. When this cell line was selected for tamoxifen resistance \textit{in vitro}, the MCF-7/LCC2 clone was created. MCF-7/LCC2 cells are stable, ER-positive, and respond to the pure anti-estrogen, fulvestrant [49]. Along the same lineage, MCF-7/LCC9 cells were derived by selecting \textit{in vitro} MCF-7/LCC1 cells for fulvestrant resistance, and subsequently, these cells exhibit cross-resistance to tamoxifen [50].

Another early anti-estrogen-resistant variant of MCF-7 cells is the LY2 line. MCF-7:LY2 cells are resistant to LY117018, a potent anti-estrogen related to raloxifene [51]. The LY2 cells also exhibit cross-resistance to tamoxifen and continue to be responsive to estrogen but with lower ER levels than MCF-7. The cell line was created by selection with increasing the concentration of LY117018 up to 1 µM as MCF-7 cells became resistant [51]. A related MCF-7 raloxifene-resistant line MCF-7/RAL was created by growing MCF-7 cells in estrogen-free culture with 1 µM raloxifene for over a year [52]. These cells grow in response to estradiol and raloxifene, and are growth-inhibited by fulvestrant [53]. Most importantly the cells exhibit an unusual apoptotic response to estradiol \textit{in vivo} (see next section). The MCF-7/F cell line was established by culturing the parental MCF-7 cells in fulvestrant-containing estrogen-free media for 18 months. ER expression was lost, and the cells became resistant to all anti-hormone therapies [54].

Short-term estrogen deprivation causes distinct responses of MCF-7 cells in comparison to long-term (over six months) estrogen deprivation. These studies are important to mimic the early response of ER-positive breast cancer to aromatase inhibition. Culture of MCF-7 cells in
media that is phenol red-free with charcoal-stripped serum (estrogen-free) causes immediate proliferation inhibition [39,43]. Slowed proliferation continues for about a month after estrogen removal, indicating the cells have not yet found adaptive or compensatory growth mechanisms. When stimulated with estradiol, the proliferation rate of these short-term estrogen-deprived cells increases, and anti-estrogens again inhibit growth [39,40]. Over time, MCF-7 cells deprived of estrogen eventually adapt their growth in estrogen-free media, losing their estrogen sensitivity, but anti-estrogens continue to inhibit growth [40]. The ER is retained and expanded.

In 1995, Santen’s group hypothesized [55] MCF-7 cells develop hypersensitivity to minute concentrations of estradiol (or indeed any available estrogen) after estradiol deprivation as a means of adapting to estrogen withdrawal and spontaneous growth. They noted that when MCF-7 cells are deprived of estrogen for 1-6 months, a $10^4$-fold lower concentration of estradiol is needed for maximal growth, when compared to normally cultured MCF-7 cells. This model suggests an explanation for spontaneous growth that occurs after estrogen withdrawal; that is, the breast cancer cells are hypersensitive to minute environmental concentrations of estrogen [55]. Indeed this is a valid hypothesis as the estrogen-deprived cell population adapts by selecting any available cell to grow in the environment: a Darwinian model.

Long-term estrogen deprived (LTED) MCF-7 cells form a stable cell line that has been used to investigate estrogen’s effect on breast cancer cells over varied exposures and lengths of time. MCF-7:LTED cells, in contrast to their short-term estrogen-deprived counterparts, are able to grow despite lack of estrogen in the media, and are growth-inhibited by estradiol [40].

MCF-7:5C cells were developed by long-term estrogen withdrawal from the parental wild-type MCF-7 breast cancer cells [56,57]. The ER in MCF-7:5C cells is wild-type and
expression levels are similar to MCF-7 [56] (Figure 2). This hormone-independent, ER-positive, PR-negative clonal population proved useful in representing the behavior of long-term estrogen-deprived breast cancer cells; that is, those of post-menopausal women decades after menopause, or patients who have undergone long-term anti-hormone therapy, e.g. 5-year aromatase inhibitor treatment [57]. MCF-7:5C cells are unresponsive to 4-hydroxytamoxifen, and estradiol does not enhance growth [56,57] but triggers estradiol-induced apoptosis [41].

The MCF-7:2A cell line is similar to the MCF-7:5C cell line and was generated from long-term estrogen withdrawal from MCF-7 cells. Uniquely, MCF-7:2A cells express two forms of the ER, a 66 kDa wild-type and a 77 kDa mutant (Figure 2) [45,58]. The wild-type ER, expressed 4- to 10-fold higher than the mutant, is still functional, whereas the mutant ER, containing a repeat of exons 6 and 7 in the ER gene [59], can no longer bind estrogens nor anti-estrogens. MCF-7:2A cells grow in estrogen-free media since they are estrogen-independent. In contrast to its parental cell line, the MCF-7:2A cells show no response to estradiol during the first seven days of treatment, and then begin to die via apoptosis during week two. Both tamoxifen and pure anti-estrogens block growth in these cells [45,58].

In search of other in vitro models illustrating anti-hormone-resistant breast cancer cells, the T47D cell line can offer additional information. T47D cells differ from MCF-7 cells in that their tumor suppressor protein p53 is mutated on one allele of the gene (194 Leu-->Phe) [60]. Also, MCF-7 cells continually express ER whereas T47D lose ER expression when estrogen is withdrawn for extended periods of time [61]. The T47D:A18 variant is ER-positive and PR-positive, derived from culturing the T47D cell line in estrogen-rich media [61]. They grow in response to estrogen and are inhibited by 4-hydroxytamoxifen [61]. T47D:C4 cells, in contrast,
were established by culturing T47D cells in estrogen-free media [28,61]. The parental cells are transformed into ER-negative, PR-negative cells which are unresponsive to anti-hormone therapy [62].

To address mechanistic issues of anti-hormone resistance, T47D-r cells, also derived from the parental T47D line, were created to be resistant to fulvestrant [63]. Proteomic analysis was used to compare T47D versus T47D-r cells to identify 38 proteins with significantly (2-fold up- or down-regulation) different expression [63]. Furthermore, mRNA expression differed for 11 of the proteins. These data are evidence supporting the molecular and mechanistic changes that occur to T47D breast cancer cells as they become increasingly resistant to anti-estrogens [63]. The T47Dco subclone is estrogen- and anti-estrogen-resistant, and expresses PR regardless of estrogen stimulation. Progestins inhibit proliferation of T47Dco cells [64]. Initially described as ER-negative [64], it was subsequently shown that the cells express three mutant ERs that have no ability to bind ligand [65]. This cell line allows for extensive study on progestins’ effect on breast cancer independently of estrogen, as well as on ER mutations as a mechanism of hormone resistance.

When ZR-75-1 cells are treated with tamoxifen for six months, both ER and PR levels decrease, but the anti-hormone is still able to impede the cancer growth. Tamoxifen resistance occurs after a year of tamoxifen treatment, as evidenced by the tamoxifen-resistant subclone ZR-75-9a1, a distinct ER-negative, PR-negative cell line [66]. Table 1 summarizes the discussed cell lines’ subclones used for modeling ER-positive breast cancer cells in vitro.
### Table 1.

Various subclones generated from different ER-positive breast cancer cell lines. To simulate different scenarios of therapy and development of resistance to SERMs, cells were cultured in different environments to create stable cell lines. Fulv: fulvestrant, Tam: tamoxifen, Ral: raloxifene, Ref: reference number

<table>
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<tr>
<th>Parental Line</th>
<th>Subclone</th>
<th>How subclone was generated</th>
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### Models of acquired anti-hormone resistance in vivo

Laboratory studies of endometrial cancer in vivo aided in the understanding of acquired resistance to tamoxifen. Estradiol significantly increases the growth rate of human ER-positive endometrial cancer transplanted into ovariectomized nude mice, while the growth rate of ER-negative endometrial cancer in this model is unaffected by estradiol treatment [67]. However, ER-positive endometrial tumors implanted in nude mice also grew more quickly in response to tamoxifen or estradiol treatment than the control-treated mice [68]. When medroxyprogesterone acetate (MPA) (a standard therapy for endometrial cancer) was administered to the tamoxifen-treated animals implanted with endometrial tumors, inhibition of growth was increased in
comparison to the tamoxifen-treated tumors alone. In contrast, the growth of ER-negative endometrial cancer injected into athymic mice was unaffected by all treatments [68].

Subsequently, the human endometrial tumor EnCa101 was pivotal in enhancing knowledge of the target site specificity of tamoxifen, as well as by other similar triphenylethylene anti-estrogens (e.g. clomiphene, trioxifene, nafoxidine) [69]. Athymic mice transplanted with both MCF-7 breast and EnCa101endometrial tumors, and treated with either estradiol, tamoxifen or the combination, demonstrated that estradiol increases the growth in both tumors. Tamoxifen, however, blocks breast cancer growth while enhancing the growth of endometrial cancer [16]. These data were rapidly translated to patient care [17], with breast cancer patients being given routine gynecological examinations to detect endometrial cancer that was slightly but significantly increased during adjuvant tamoxifen therapy. The target site specific action of tamoxifen in breast and endometrium was hypothesized to be dependent on differential modulation of the estrogenic actions of tamoxifen in different target tissues [70]. The concept was supported by studies of anti-estrogens with reduced estrogenic action. Keoxifene (subsequently called raloxifene) and LY117018 are less estrogenic in the rodent uterus and have less of an effect on EnCa101 growth stimulation [69,71]. Further, ICI 164,384, since it is a pure anti-estrogen with no intrinsic estrogenicity, did not stimulate EnCa101 tumor growth, and was able to block tamoxifen-induced growth [15]. Clinical studies demonstrate that unlike tamoxifen, raloxifene [18] and fulvestrant [72] have no estrogen-like action in the human uterus.

MCF-7 models *in vitro* eventually evolved one step further toward clinical practice when they were adapted into models *in vivo* which mirror more closely clinical care. Models *in vivo*
create a new dimension to assess the importance of a functioning physiologic interaction between cancer cells, the interaction of angiogenesis, cellular metabolism, and respiration that are not created in cell culture. The first studies of MCF-7 cells implanted into nude mice were published in the 1980s. MCF-7 cells implanted into mice with intact ovaries, or simultaneously with estrogen into ovariectomized mice, grew in an estrogen-dependent manner [35].

In the 1980s, transplanted models of MCF-7 human breast cancer into athymic mice were used to investigate the unique aspects of acquired resistance to SERMs. Tamoxifen acts as a competitive inhibitor of estradiol-stimulated growth, i.e. the action of tamoxifen as an antitumor agent is reversed by increasing the dose of estradiol [73]. Similarly, months of tamoxifen therapy do not destroy implanted MCF-7 tumors [74,75], as estrogen can reactivate tumor growth. Eventually acquired resistance to tamoxifen occurred after four months of treatment, wherein neither tamoxifen nor estrogen deprivation could produce significant tumor regression [76]. Breast tumors then grew despite tamoxifen treatment demonstrating that acquired resistance to anti-hormone therapy had developed.

However, a similar study came to a different conclusion; MCF-7 tumors grew in the athymic mouse not despite tamoxifen therapy but because of tamoxifen therapy [77]. When the MCF-7 tumors resistant to tamoxifen were transplanted into new athymic animals, these ER-positive, PR-positive tumors were found to grow in response to either estradiol or tamoxifen treatment. It is also noteworthy that the tamoxifen-stimulated tumors expressed twice the level of ER when compared to their estradiol-stimulated counterparts [77]. A survey of other steroidal and non-steroidal anti-estrogens demonstrated that tamoxifen-stimulated growth is dependent on the estrogen-like actions of tamoxifen. Less estrogenic agents do not increase the growth of
acquired tamoxifen resistance in MCF-7 tumors [78]. There is cross-resistance with other anti-
estrogens e.g. toremifene or raloxifene [79,80] but not fulvestrant. Overall, this model mimics
the development of acquired resistance to tamoxifen during the treatment of metastatic breast
cancer. The tumors become resistant to therapy in about two years.

Many of the previously discussed MCF-7 subclones have been examined in animal
models. When the MCF-7/RAL cells are transplanted into athymic ovariectomized mice, they
are able to form tumors when treated with either estradiol or raloxifene. Eventually, after about
eight months of re-transplantation, the tumors grow only in response to raloxifene, and are
inhibited by estradiol [53].

MCF-7/LCC1 cells are estrogen-responsive and tamoxifen-sensitive in vivo. MCF-
7/LCC2 cells, on the other hand, behave estrogen-independently in vivo. They continue to
exhibit tamoxifen resistance in vivo as they do in vitro [49]. The MCF-7/LCC9 cell line,
consistent with its in vitro action, can form tumors in the athymic ovariectomized mouse, and are
unresponsive to fulvestrant [50].

Similarly, MCF-7 cells with acquired resistance to tamoxifen (MCF-7:Tam) in vivo
implanted in athymic ovariectomized mice grow in response to tamoxifen or estradiol but the
steroidal anti-estrogen RU 39,411 or ICI 164,384 inhibit growth [78]. However, long-term
transplantation of MCF-7:Tam tumors into athymic mice eventually results in a change in
response to physiologic estradiol with rapid tumor regression [81,82]. Similarly, MCF-7:5C
cells injected into athymic ovariectomized mice undergo apoptosis when treated with estradiol,
causing complete tumor regression [41]. This unusual change in the biology of the tumors will
be revisited in the next section.
T47D cells have also been examined in vivo to evaluate the role of SERMs to create acquired anti-hormone resistance. T47D cells transplanted into athymic ovariectomized mice can generate tumors in response to estradiol, and tamoxifen can inhibit this estrogen-stimulated growth. However, after high-dose (1.5 mg daily) tamoxifen treatment, the tumor cells become tamoxifen-resistant after about eight weeks, wherein tamoxifen begins to stimulate tumor growth [83]. The T47D cells giving rise to tamoxifen-stimulated tumors produce a subtype of T47D cell named T47D: Tam. Other SERMs, Arzoxifene and LY117018, did not increase growth of T47D: Tam tumors in vivo; likewise, Arzoxifene and LY117018 did not increase the growth of estradiol-stimulated T47D tumors either. This indicates a lack of cross-resistance between tamoxifen and the other anti-estrogens in T47D cells in vivo [84].

In addition to SERM studies, models in vivo also examined the effect of aromatase inhibition on ER-positive cell lines. In 1994, nude mice were injected with MCF-7 cells transfected with the human aromatase gene to study the action of aromatase inhibitors in vivo for the treatment of breast cancer [85]. In the normal nude mouse, tumors grew in response to ovarian estrogen and were inhibited by aromatase inhibitors and tamoxifen. The aromatase substrate, androstenedione, was administered to the ovariectomized mice in order to model human disease since mice express no androgen precursor. Ovariectomized nude mice injected with aromatase-transfected MCF-7 cells grew tumors utilizing estrogen produced through the aromatization of androstenedione via the aromatase pathway. Aromatase inhibitors (4-hydroxyadrostenedione and CGS 16949A) and tamoxifen were able to block the tumor growth. This latter model represents post-menopausal women whose tumors grow not in response to ovarian estrogen, but estrogen generated through the aromatization of androgens found primarily
in the adipose tissue. MCF-7 cells transfected with the aromatase gene and injected into ovariectomized mice were inhibited better with the combination treatment of fulvestrant and anastrozole than either agent alone. This suggests the targeting of both aromatase and the ER for better treatment of post-menopausal breast cancer patients [86]. These studies provide a rationale behind aromatase inhibitors’ efficacy in the clinical setting [85].

Laboratory models set the stage for intense evaluation of anti-hormone-resistant breast cancer cells. By continuing investigation of mechanisms of resistance, many unique and sometimes paradoxical effects of hormones and anti-hormones on ER-positive breast tumors have been discovered. The finding that an estrogen and an anti-estrogen could eventually stimulate breast cancer growth demonstrated the unique qualities of acquired resistance to SERMs [77]. The aforementioned individual findings now began to form models for the evolution of acquired resistance that can not only be interrogated in the laboratory but applied to clinical care.

**Evolution of acquired anti-hormone resistance**

Based on laboratory evidence from both individual reports and studies of up to a decade, the evolution of acquired resistance to SERMs can now be described in distinct phases following long-term SERM treatment and long-term experiments *in vitro* and *in vivo* [87,88]. The evolution of acquired resistance (Figure 8) occurs after an initial period of therapeutic success where anti-estrogenic activity predominates and the SERMs are competitive inhibitors of estrogen-stimulated tumor growth in athymic mice [73,74]. The therapeutic phase of SERM action can be maintained for a year or two (at most) but eventually tumors start to grow despite
continued tamoxifen [76]. However, these tumors can be re-transplanted into other tamoxifen-treated ovariectomized athymic mice [77]. Paradoxically, both physiologic estradiol and tamoxifen (there is cross-resistance with raloxifene and toremifene) [79] can then cause growth, indicating Phase I resistance. The pure anti-estrogens ICI 164,382 and fulvestrant block Phase I growth with either tamoxifen or estradiol. A similar form of acquired resistance to tamoxifen occurs with the T47D breast cancer cell line [83,84]. This type of acquired resistance is characteristic of resistance to tamoxifen during the treatment of metastatic ER-positive breast cancer and is why either fulvestrant or an aromatase inhibitor is effective second-line therapeutic agents in the clinic [89,90]. The laboratory principles are illustrated in Figure 8.
NEW CONCEPT
EVOLUTION OF SERM RESISTANCE

![Evolution of acquired SERM resistance](image)

**Figure 8.** Evolution of acquired SERM resistance. After long-term treatment with SERMs (1-2 years *in vivo*), initially responsive ER-positive tumors become resistant to treatment and are stimulated by SERMs (Phase I of resistance) as well as by $E_2$. After long-term transplantation into SERM-treated animal (5+ years), breast tumor growth is inhibited by $E_2$, though still stimulated by SERMs (Phase II of resistance). A stylized representation of MCF-7 tumor growth is illustrated in Figure 9. This process with SERMs *in vivo* is replicated with estrogen deprivation with MCF-7 breast cancer cells *in vitro*; cells initially start to grow spontaneously but estrogen still induces growth (Phase I). Long-term estrogen deprivation causes spontaneous growth in culture but apoptosis with physiologic estrogens both *in vitro* and *in vivo* (Phase II).

However, these laboratory data are inconsistent with the successful adjuvant treatment of node-positive and node-negative ER-positive breast cancer with five years of tamoxifen [7]. In fact, not only is tamoxifen effective during adjuvant therapy but it is also effective at maintaining recurrence-free survival and reducing mortality by 30% during the 10 years following tamoxifen treatment. Laboratory studies have now provided an insight into this clinical advance.
Repeated transplantation of tamoxifen-resistant tumors into subsequent generations of tamoxifen-treated athymic mice results in a change in the clonal selection of tumor cells. Not only do the tumors remain tamoxifen-dependent for growth over a five-year period but the constant exposure to tamoxifen changes the tumor response to estradiol from being a survival signal to an apoptotic trigger. Tumor regression occurs in response to physiologic estrogen and this has been proposed as a mechanism to explain the decreasing mortality of tamoxifen-treated patients following adjuvant tamoxifen [81,82]. In other words, short-term adjuvant tamoxifen only pushes acquired resistance into Phase I resistance where estradiol is still a growth stimulator once tamoxifen is stopped. In contrast, longer tamoxifen forces clonal selection into Phase II resistance where apoptosis occurs upon exposure to a woman’s own estrogen. This is illustrated when a comparison between Figures 9A and 9B is made. Indeed it was proposed that since tumors that regress and subsequently regrow in response to physiological estrogen can again respond to subsequent anti-hormone treatments, and then this could be applied in the clinic [82]. This experiment has recently been reported in a clinical study by Ellis [91].
The evolution of cell populations to long-term anti-hormone therapies has been replicated with raloxifene in a 10-year study in vivo [53]. The reason for doing this is because raloxifene will be used indefinitely to prevent osteoporosis [19] and breast cancer [21]. The same evolution of acquired resistance occurs with the development of Phase I and Phase II raloxifene resistance characterized by Phase I resistance with estradiol- or raloxifene-stimulated tumor growth and Phase II resistance characterized with estradiol-induced tumor regression. It is perhaps relevant

**Figure 9.** Diagram of the growth rates of MCF-7 tumors during the evolution of drug resistance to selective estrogen receptor modulators (SERMs). A. During Phase I SERM resistance, tumors transplanted into athymic mice grow in response to either a SERM, tamoxifen (Tam) or raloxifene (Ral), or estrogen, but no estrogen (equivalent to the use of an aromatase inhibitor used clinically after Tam resistance occurs) or fulvestrant does not support growth (fulvestrant in used in this indication as a second-line therapy). B. During Phase II SERM resistance, tumors transplanted into athymic mice treated with SERMs now grow with a SERM (Tam or Ral). No treatment (equivalent to an aromatase inhibitor clinically) causes growth to slow, as does administering fulvestrant, but physiologic estradiol (E₂) causes dramatic apoptosis and tumor regression. Paradoxically, physiologic E₂ plus fulvestrant actually causes tumor growth. The low concentration of fulvestrant cancels out the apoptotic effect of E₂ thereby redirecting E₂ as a growth signal, but higher concentrations of fulvestrant now have effective antitumor effects. This is now noted clinically [92].
to point out that MCF-7 cells exposed to both raloxifene and estrogen deprivation \textit{in vitro} rapidly advance to Phase II resistance with estradiol-induced apoptosis \textit{in vivo} [52].

Additionally, there are a couple of other clinically relevant points that can be made about acquired SERM resistance in the laboratory. The T47D cell line advances to Phase I tamoxifen resistance but does not progress to Phase II. The fact that T47D cells have mutant p53 may be relevant as estrogen-induced apoptosis does not develop.

The pure anti-estrogen fulvestrant is an excellent anti-estrogen/anti-tumor agent in the laboratory but results have been disappointing clinically until the recent successful use of twice the recommended dose [92]. Laboratory studies with Phase II tamoxifen-resistant tumors grown in athymic mice suggest that the second-line use of fulvestrant in an environment of physiologic estrogen is destined to fail and, in fact, cause enhanced tumor growth [93]. The reason for this is unknown.

The fact that aromatase inhibitors are now the adjuvant treatment of choice for post-menopausal patients with ER-positive breast cancer makes an examination of acquired resistance mandatory. Suffice to say that the principles first described for SERMs are true for aromatase inhibitors and the development of acquired resistance to estrogen deprivation \textit{in vivo} [94-96] and \textit{in vitro} [30,39-41,97].

**Mechanisms of acquired anti-hormone resistance**

Breast cancer can be resistant to anti-hormones in varied ways. As previously noted, intrinsic resistance can occur \textit{de novo} wherein anti-hormone therapy generates no disease regression. This occurs in ER-negative tumors, as well as in some subgroups of ER-positive
tumors. However, we will focus on the mechanisms involved in the evolution of acquired anti-hormone resistance. Acquired resistance to anti-hormone therapy can be caused by three main mechanisms to be discussed here: loss of ER function, aberrant growth factor signaling, and estrogen-induced apoptosis.

Loss of ER function as a mechanism of acquired anti-hormone resistance

Experiments in vitro provide an initial platform for studying the mechanisms of acquired anti-hormone resistance. Firstly, if the ER in breast cancer cells is altered, the effects of anti-hormones will be altered accordingly. If ER expression is lost, the whole mechanism of endocrine therapy will be undermined; ER-mediated actions will no longer contribute to proliferation or apoptosis. Similarly, if ER is mutated in such a way that no longer binds its ligands, resistance will occur. Nonetheless, ER mutation is not a major factor in drug resistance but one example that has provided insight into ER modulation of anti-estrogen action [98-101].

If the promoter regions of ER target genes are hyper-methylated during acquired resistance, transcription of ER target genes is again blocked, abrogating anti-hormone efficacy in vitro [102]. Coupling of ubiquitin conjugation to ER degradation (CUE) domains are approximately 50 amino acids long and bind monoubiquitin molecules used in trafficking and ubiquitylation [103]. CUE domain-containing protein-2 (CUEDC2) is shown to have an inverse correlation with ER protein expression in breast cancer cells in vitro. High levels of CUEDC2 protein expression correlate with tamoxifen resistance, probably due to loss of ER via the ubiquitin/proteasome pathway [104].

If the ER is inactivated because of histone methylation or deacetylation, treating breast cancer cells that have acquired resistance to anti-hormones with a histone deacetylase (HDAC)
inhibitor can re-activate the ER. This concept has been illustrated using ER-negative MDA-MB-231 wherein an HDAC inhibitor generates both ER and aromatase expression. Letrozole can then be used as effective treatment [105], suggesting a potential treatment mechanism for ER-positive cells that have lost ER expression during acquired resistance. Loss or reduction of ER as a primary cell survival pathway can also be replaced by an increase in the mosaic of growth factor signaling pathways. These pathways can modulate and subvert steroid hormone receptor synthesis and action [106,107]  

*Growth factor signaling as a compensatory mechanism of survival*

Growth factor signaling and ER crosstalk are consistent mechanisms by which acquired resistance to anti-hormones develops; they provide the breast cancer cells a means of escape from suppressive signaling and a way to continue proliferation. Growth factors may be able to contribute enough proliferative signal to drive ER-target gene transcription even without normal ER ligand [108]. Growth factor signaling contributes indirectly to ER function, both genomically, through transcriptional regulation, and non-genomically, by means of other signaling pathways [108].

An important mechanism for bypassing anti-hormone-induced apoptosis is through increased expression of membrane receptor tyrosine kinases, including epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), fibroblast growth factor receptor (FGFR) and HER2. These membrane receptors can activate not only the ER signaling pathway [109], but also the MAPK and AKT signal transduction pathways through increased phosphorylation of p42/44. This is demonstrated *in vitro* through inhibiting MCF-7:LTED cell
growth by IGFR knockdown [110]. OSI-906, an IGFR tyrosine kinase inhibitor, prevents MCF-7:LTED growth both \textit{in vitro} and \textit{in vivo} [110].

When EGFR is transfected into ZR-75-1 cells, the cells become estrogen-independent. These cells become ER-negative when tamoxifen is introduced and continued to grow using EGF and its receptor, indicating a possible growth mechanism for anti-hormone resistant breast cancer cells [111]. Further, ZR-75-1 cells treated with a 5-azacytidine (a DNA methylation inhibitor used to study influence of epigenetic changes on acquired estrogen independence) develop estrogen independence when grown in estrogen-free media, increasing their HER2 and EGFR expression. Growth of these anti-hormone-resistant cells can be slowed by an anti-EGFR antibody, indicating a crucial role of EGFR and growth factor signaling in the progression of anti-hormone resistance in ZR-75-1 cells [112]. When EGF-stimulated growth was measured in MCF-7 cells, it was not able to be blocked by tamoxifen, 4-hydroxytamoxifen, nor ICI 164,384, suggesting an important growth factor influence on their proliferation [113]. Further, breast cancer cells with amplified FGFR show increased resistance to 4-hydroxytamoxifen \textit{in vitro}, reversible with FGFR-targeted siRNA, indicating a mechanism driving endocrine resistance [114].

If cancer cells are using downstream signaling pathways to continue their growth independent of ER, then blocking key signaling molecules could reveal additional mechanisms of escape. Antagonists of downstream ER signaling pathway proteins, such as mammalian target of rapamycin (mTOR) and phosphoinositide 3-kinase (PI3K), provide potential targets to prevent breast cancer growth after anti-hormone resistance occurs. The combination of tamoxifen and the mTOR inhibitor RAD001 have an additive effect on MCF-7 cells, together blocking tumor
growth *in vitro* better than either agent alone [115], identifying mTOR as an important target to delay the development of anti-hormone resistance.

Breast cancer cells that have acquired letrozole resistance highly overexpress the growth factor progranulin when compared to their letrozole-sensitive counterparts *in vitro* [116]. Progranulin is shown in the laboratory to cause breast cancer cells to acquire letrozole resistance, and knocking down this growth factor can confer letrozole-sensitivity to cells that had acquired letrozole resistance, thereby blocking their proliferation [116]. This example again demonstrates the complexity and flexibility of breast cancer cells to utilize growth factor signaling for survival after long-term anti-hormone therapy [116].

Long-term estrogen-deprived ER-positive breast cancer cells transfected with the human aromatase gene were studied in ovariectomized athymic nude mice to elucidate mechanisms of acquired resistance to aromatase inhibitors *in vivo*. Similar concepts emerge *in vivo* as have been described *in vitro*. Letrozole-resistant tumors express decreased levels of ER compared with letrozole-sensitive tumors *in vivo*, and an increase in HER2 (6-fold) and IGFR tyrosine kinase receptors and their downstream signaling proteins (e.g. MAPK), suggesting a shift in signaling pathways away from ER [96,117-120]. Inhibiting these tumors with the anti-HER2 antibody trastuzumab restores letrozole sensitivity [120,121] by down-regulating HER2 and restoring ER expression [105]. This indicates that letrozole-resistant ER-positive tumors utilize HER2 signaling to survive despite therapy. HER2 and ER expression were shown *in vivo* to correlate inversely with one another; that is, when HER2 is inactivated by trastuzumab or herceptin, ER expression increases and the cells become re-sensitized to anti-hormones and aromatase inhibition [96,118]. EGFR inhibitors are also able to restore letrozole sensitivity [119].
Proteins involved in the MAPK signaling pathway, p-Raf, p-Mek1/2, and p-MAPK, are increased in tumors \textit{in vivo} that have acquired resistance to letrozole \cite{119,120,122}, suggesting the activation of aberrant signaling for compensatory proliferation after long-term aromatase inhibition. Blocking ER with fulvestrant simultaneously with the PI3K inhibitor wortmannin is more effective than anti-hormone alone, suggesting that the pathway involving PI3K provides a means of growth escape to long-term anti-hormone-treated breast cancers \cite{123}.

Growth factors, e.g. the nuclear coactivator Amplified in Breast Cancer-1 (AIB1, also SRC-3, NCoA-3) can activate the ER pathway during anti-hormone treatment. In the clinical setting, high levels of AIB1 expression in tamoxifen-treated tumors are associated with worse disease-free survival for breast cancer patients, illustrating the importance of AIB1 in the resistance pathway \cite{124}. AIB1 exerts control over many of the growth factor signaling pathways relevant to acquired anti-hormone resistance, such as EGFR, HER2, PI3K, and mTOR, and interacts with many proteins associated with transcription, cell cycle regulation, and protein degradation \cite{125,126}.

\textit{Estrogen-induced apoptosis mechanisms during acquired Phase II resistance}

The most significant aspect of the evolution of anti-hormone resistance is the drift toward reconfiguring signaling networks to make the cell survive with no estrogen, but this creates a vulnerability to estrogen-induced apoptosis. After five years of treatment with anti-hormones, the sophisticated growth pathways become sensitive and paradoxically collapsed by estrogen, once a growth and survival signal. Clinically in the past, women with breast cancer have been successfully treated with high-dose estrogen therapy \cite{127,128}. This was the first effective chemical therapy for any cancer and was the standard-of-care before tamoxifen \cite{129}.  

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Investigation has sought to uncover mechanisms by which apoptosis occurs in Phase II acquired resistance, and how estrogen makes this switch in signaling.

B-cell lymphoma 2 (Bcl-2) is a signaling molecule expressed in 40-80% of primary breast cancers that functions to prevent apoptosis [130], thereby contributing to malignancy and resistance. It acts as an anti-apoptotic signal in long-term estrogen-deprived ER-positive breast cancer cells [131] to subvert estrogen-induced apoptosis. Inhibition of Bcl-2 via siRNA *in vitro* confers caspase-7 and caspase-9 activation and causes the cells to be synergistically sensitive to estrogen-induced apoptosis [131], making Bcl-2 an interesting therapeutic target. Bcl-2-interacting killer (BIK) regulates calcium release from the endoplasmic reticulum that triggers downstream mitochondria-mediated apoptosis, also inhibiting Bcl-2. High levels of BIK’s inhibitory chaperone, GRP78, in ER-positive breast cancer cells, prevents apoptosis and causes endocrine resistance, [132], thereby asserting itself as another potential therapeutic target.

Studies of varied ER-positive breast cancer cells began to investigate the unique properties of physiologic estrogen that causes tumor regression in postmenopausal women [40]. Santen’s group showed in 2001 [40] estrogen-independent growth of MCF-7:LTED cells, and significant reduction of tumor growth when treated with estradiol. Using annexin V staining and Western blot analysis, the experiments demonstrated induction of FasL, a death receptor ligand associated with the apoptosis cascade, when cells were treated with estradiol [40]. This finding established the notion of estrogen-inducing Fas-mediated apoptosis in LTED breast cancer cells. Apoptosis via the Fas/FasL pathway was increased seven-fold in the estradiol-treated LTED breast cancer cells when compared to the vehicle-treated LTED cells [40]. Fas mRNA and protein were also increased in MCF-7: Tam tumors *in vivo*, correlated with decreases in NF-κB.
expression. The laboratory experiment showed that increased Fas signaling and simultaneous suppression of NF-κB’s anti-apoptotic signaling may be characteristic of estradiol-induced apoptosis [93].

Estrogen-induced apoptosis can also originate through the intrinsic mitochondrial apoptosis pathway, when cytochrome C is released from the mitochondria [41]. This is shown in the laboratory using MCF-7:5C cells in vivo [41]. MCF-7:5C cells injected into ovariectomized athymic mice exhibited increased apoptotic protein (e.g. Bax, Bim, p53) expression and tumor regression when treated with estradiol [41].

In tamoxifen-stimulated (Phase II resistant) MCF-7 xenografts, fulvestrant can reverse estrogen-induced apoptosis, stimulating growth and expression of phosphorylated HER2, HER3, p-ERK1/2, and p-GSK3α and β proteins [133]. Pertuzumab blocks the interaction of p-HER2 and HER3 and is able to decrease tumor growth in this model in vivo, suggesting that fulvestrant stimulation of anti-hormone-resistant ER-positive breast cancers depend not on ER or ER target genes, but on the HER2/HER3 signaling pathway [133].

Additionally, AIB1 is required for estrogen-induced apoptosis in MCF-7:5C cells in vitro. The Wellstein group found that AIB1 is involved in signaling pathways that encourage apoptosis in this context, most prominently through associations with G-protein-coupled receptors, PI3K, Wnt, and Notch signaling pathways [126]. MCF-7 gene expression was examined for the MCF-7:WS8 (wild-type), MCF-7:5C, and MCF-7:2A derived cell lines to examine differences in gene regulation during Phase II estrogen-induced apoptosis [97]. For the cell line most sensitive to estrogen-induced apoptosis (MCF-7:5C), genes associated with estrogen signaling, endoplasmic reticulum stress, and inflammation were up-regulated, along
with apoptotic genes such as BIM and caspase-4, in comparison to MCF-7:WS8 and MCF-7:2A cells. Analysis of the gene regulation and protein expression indicates that estrogen-induced apoptosis is induced through an inflammatory response in the breast cancer cells, inducing pro-inflammatory genes (e.g. IL, IFN, arachidonic acid) [97]. The aforementioned examples allow translational research to apply laboratory-revealed mechanisms of acquired resistance to anti-hormones toward treatment strategies for overcoming or preventing such resistance in ER-positive breast cancer.

**Clinical translation via cell models of ER-positive breast cancer**

Laboratory models *in vitro* and *in vivo* are the invaluable link to clinical translation and enhanced patient survivorship. During the past three decades, the ER-positive breast cancer cell line MCF-7 has been indispensable in this process not only to test therapeutic strategies but also to advance our understanding of hormone-dependent cancer growth [30]. The MCF-7 cell line was the first hormone-responsive breast cancer cell line used effectively to decipher hormone action in breast cancer [30]. Additionally, the ER from MCF-7 cells was prepared on an “industrial scale” to prepare the first monoclonal antibodies [134,135]. These antibodies are now used ubiquitously to determine the ER status of a patient’s tumor by immunohistochemistry [136-139] or flow cytometry [140-142]. However, it was the acquisition of monoclonal antibodies that permitted the cloning and sequencing of the human ER [143-145]. This advance has had a major impact on our understanding of the structure-function relationships of ER-mediated cell regulation.
The availability of ER-positive breast cancer cells and the development of models to test therapeutic strategies continue to play an essential part in the development of clinical trials. By way of example, we will close by considering the role of the MCF-7 cell line in the context of current clinical practice. There are two therapeutic scenarios to consider: disease in the pre-menopausal patient and disease in the post-menopausal patient.

Pre-menopausal women who present with ER-positive breast tumors are generally prescribed combination cytotoxic chemotherapy with five years of adjuvant tamoxifen treatment, while post-menopausal women with ER-positive breast cancer are likely to receive an aromatase inhibitor. If these anti-estrogenic approaches fail to prevent recurrence, fulvestrant is used as a second-line anti-hormone treatment [146].

The strategy of targeting the ER in the tumor micrometastases with long-term adjuvant tamoxifen was created using the 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary carcinoma model [1,3,147]. The first specific aromatase inhibitor, 4-hydroxyandrostenedione (formestane) was compared and contrasted to tamoxifen in the DMBA-induced rat mammary carcinoma model [148-150], but with the development of the model of estrogen-simulated MCF-7 tumors grown in athymic mice in the early 1980s [35,36], the DMBA model was discarded. Initial studies in the athymic mouse model [74] only served to confirm the previous results in the DMBA model, but the breakthrough with the MCF-7 model really occurred with the discovery of the evolution of drug resistance to either tamoxifen (or indeed any SERMs) or aromatase inhibitors. We will consider several examples of progress using models of resistance in available breast cancer cell lines that are changing patient care.
The discovery that in vivo acquired tamoxifen resistance is unique, as the tumors grow with either tamoxifen or physiologic estrogen [77], recreated a new dimension to consider in therapeutics: the tumor was amplifying the weak estrogen-like properties of tamoxifen by cell selection. An anti-estrogenic strategy of no estrogen (an aromatase inhibitor) or an anti-estrogen with no estrogen-like properties was required. The genesis and development of fulvestrant, the injectable long-acting pure steroidal anti-estrogen, began in the mid-1970s, but only now is the clinical community able to apply the drug optimally for appropriate patient care [92].

The idea for studying the therapeutic value of 6,7-substituted estradiol analogs was started through a joint research scheme between ICI pharmaceutical division and Leeds University. The idea was to develop a cytotoxic carrier molecule based on the binding of estradiol to ER that would invariably target and destroy ER-positive metastases [151]. The last compound tested in the series was a 7-substituted (-CH₂)₁₀ chain with the alkylating function on the end. This was based on the knowledge from Roussel Uclaf chemists who had made resin columns to extract and purify the ER [152]. The 7-substitution was an appropriate substitution to retain ER binding. The project to discover ER-targeted cytotoxic agents was abandoned but subsequently, and independently, scientists at ICI pharmaceuticals discovered the merits of this class of molecules to create a “pure” anti-estrogen [153]. The lead compound, ICI 164,384, first tested successfully in the tamoxifen-stimulated MCF-7 tumor athymic mouse model [78], provided the reassurance necessary for the clinical development of fulvestrant [44] or an aromatase inhibitor as a second-line agent following the failure of tamoxifen [89,90]. The clinical results mimicked the animal data.
Osborne’s group made the important discovery that transfection of the HER2/neu gene would enhance and accelerate the development of resistance in MCF-7 cells to tamoxifen [46]. This has had important implications for the selection of breast cancer patients for tamoxifen treatment. Indeed, it is the important interplay and interaction of the ER and growth factor receptor pathways that is currently a major focus of translational research. The question has become, “what are the mechanisms and changes that occur in breast cancer cell populations that cause acquired resistance?” Once this question is answered, it will be followed by a different question of, “how do we use the knowledge to delay the process and improve survivorship?” A clinical trial was launched in 2009 comparing lapatinib, a HER2 tyrosine kinase inhibitor, with letrozole versus letrozole alone in post-menopausal hormone receptor-positive patients who have acquired tamoxifen resistance [154]. Lapatinib increases progression-free survival in these patients better than the aromatase inhibitor alone, illustrating a compensatory mechanism of anti-hormone-resistant cells via HER2 after tamoxifen failure [154]. There are ongoing pre-clinical and clinical trials investigating the EGFR pathway as a growth mechanism after acquired resistance, comparing anti-hormone treatments, such as tamoxifen and aromatase inhibitors, with and without EGFR inhibitors, such as gefitinib and erlotinib [155,156].

Breast cancer cells that have acquired resistance to anti-estrogen therapy are shown to remain sensitive to therapies targeted against the PI3K pathway [157]. Signaling molecules in the PI3K pathway are frequently mutated in anti-hormone-resistant ER-positive breast cancer, and comprise a targetable pathway to inhibit for effective therapy [157]. Multiple Phase I and Phase II prospective randomized trials focused on combinations of PI3K pathway inhibitors (e.g. everolimus, trastuzumab, lapatinib, gefitinib, enzastaurin, tipifarnib, BMS-754807, IMCA12,
AMG479) and anti-hormone treatments (e.g. letrozole, exemestane, tamoxifen, anastrozole, fulvestrant) are underway [157] and predicted to provide valuable information.

The encouraging study of mTOR inhibitors in anti-hormone resistance has advanced to a successful Phase II trial comparing the effectiveness of letrozole, an aromatase inhibitor, treatment alone versus letrozole plus the mTOR inhibitor, everolimus, in patients with ER-positive breast cancer. The results [158] demonstrate increased response rates for the combination arm, which has prompted the initiation of a Phase III clinical trial comparing everolimus in combination with exemestane, a different aromatase inhibitor, for postmenopausal women with ER-positive breast cancer resistant to other aromatase inhibitors [159,160].

Brodie’s group has advanced knowledge of the development of acquired resistance to aromatase inhibitors. Fulvestrant (to destroy the ER) plus an aromatase inhibitor is superior to either strategy alone [86] and trastuzumab reverses letrozole resistance and amplifies the sensitivity of breast cancer cells to estrogen [161]. Each of these strategies have been addressed in clinical trials [162-164] recruiting patients with ER-positive tumors in late-stage breast cancer, but it will be in the adjuvant setting that most gains may occur for patient survivorship. Osborne’s group [155,165] has independently pioneered the strategy of using multiple inhibitors of the growth factor receptor family in combination with either estrogen deprivation or tamoxifen therapy and these strategies are moving into clinical trial.

However, it is the laboratory knowledge derived from the evolution of acquired resistance to long-term anti-hormone therapy that is providing an insight into past clinical research and future opportunities. All MCF-7 or T47D laboratory models for SERM resistance in vivo develop acquired resistance within a year or two. This is consistent with the endocrine
treatment of metastatic breast cancer but does not explain the remarkable success of five years
adjuvant tamoxifen to create a 30% decrease in mortality, not only during therapy but sustained
for ten years after therapy stops [7]. The treatment of micrometastatic disease with tamoxifen is
clearly different than treatment of established tumors. A breakthrough occurred in the early
1990s with the finding that three repeated transplantations of small MCF-7 tumor pieces into
subsequent generations of tamoxifen-treated athymic mice for more than five years exposes a
vulnerability to the tumor cells that rapidly die during physiologic estrogen treatment [81,82].
This phenomenon was originally advanced [81] to explain the sustained anti-tumor action of
tamoxifen when adjuvant treatment is stopped. It was suggested that women’s own estrogen
causes apoptosis in micrometastases during Phase II of acquired resistance. Subsequent studies
in vitro with estrogen-deprived MCF-7 breast cancer cells demonstrated estradiol-induced
apoptosis [40,41].

Based on these studies with MCF-7 cells alone, clinical trials have demonstrated the
effectiveness of both high- and low-dose estrogen therapies to treat breast cancer following the
development of acquired resistance to anti-hormone therapy in metastatic disease [91,166]. The
approach [81,167] is now being applied indirectly to adjuvant clinical trials of long-term
adjuvant therapy (Study of Letrozole Extension), where it is anticipated that a three-month drug
holiday per year for five years may reduce recurrence rates during letrozole adjuvant therapy.
This is the same principle that is now applied to explain [168] the efficacy of low-dose estrogen
replacement alone to reduce the incidence of breast cancer in women with a median of 20 years
past their menopause (i.e. long-term estrogen deprivation) [169].
For the future of research in cellular models of breast cancer and acquired resistance to anti-hormone therapy there are four new developments. Firstly, new primary breast cancer cell lines are being developed and tested both in vivo and in vitro for drug sensitivity. Secondly, a huge pool of human breast cancer cell lines has been interrogated for drug sensitivity and pathway analysis completed to procure new clinical strategies for treatment [170,171]. Thirdly, signatures have been created to define acquired drug resistance to tamoxifen in existing breast cancer cell lines [114,172] that can be applied to clinical trial. Finally, new methodologies are now available to enrich for breast cancer stem cells and expanding this populations for drug sensitivity testing [173]. Should the future of the “many” new cell systems from primary tumors deliver the promise achieved by the “few” cell lines in the past then there is every reason to believe that enormous progress will occur in the successful treatment and prevention of breast cancer in the coming decades.
Aromatase inhibitor-resistant breast cancer cells are modeled in vitro by long-term estrogen-deprived breast cancer cell lines. The MCF-7:WS8 cell line represents a clone of the estrogen receptor (ER)-positive cell line MCF-7 that is highly sensitive to estrogen-stimulated growth [1]. The MCF-7:5C and MCF-7:2A subclones are derived from the parental MCF-7 cell line through long-term estrogen deprivation [1,56-58]. MCF-7:5C cells express wild-type ER at a higher level than the parental line, and are progesterone receptor (PR)-negative [57]. These cells grow in the absence of estrogen, and do not respond to 4-hydroxytamoxifen (4-OHT) [56,57]. MCF-7:2A cells can induce expression of PR and express both wild-type (66 kDa) and mutant (77 kDa) ER [45,58]. The mutant ER contains a repeat of exons 6 and 7 and cannot bind estrogens nor anti-estrogens; it is expressed 4- to 10-fold lower than the wild-type ER [59]. MCF-7:2A cells’ total ER level is higher than in parental MCF-7 cells, and they also grow in estrogen-free media. 4-OHT and pure anti-estrogens are able to block their growth [45,58].

In addition to the different responses to anti-estrogens observed in MCF-7:5C versus MCF-7:2A cells, they also have different apoptotic responses to estrogen (estradiol, E_2). The MCF-7:5C cells undergo apoptosis and die during the first week of E_2 treatment, whereas the MCF-7:2A cells die later, after two weeks of E_2 treatment [41]. MCF-7:5C cell response to estrogens and anti-estrogens has been extensively studied in our lab; the data shows that these cells undergo E_2-induced apoptosis through mechanisms associated with endoplasmic reticulum
stress (ERS) and oxidative stress [97,178]. Thus far, there has been less focus on the classification and mechanisms of the MCF-7:2A response.

Network enrichment analyses done using gene arrays in timecourse experiments show overexpression of apoptotic- and stress-related pathways in the MCF-7:5C cells after 24-96 hours of E₂ treatment; however, these analyses show the MCF-7:2A cells expressing more genes associated with glutathione metabolism during this time period of E₂ exposure (Fig. 1). This suggests that the two cell lines respond to E₂ treatment using different signaling pathways. The MCF-7:5C cells respond by quickly inducing apoptosis, while the antioxidant pathway may be more relevant to the MCF-7:2A cells. Experiments were designed to interrogate the apoptotic, stress, and antioxidant pathways in both cell lines to distinguish signaling mechanisms in response to E₂.
The concept of estrogen-induced death is important because of its clinical relevance. A clinical study published in 2009 [91] compared two doses of estrogen for second-line treatment after breast cancer patients had failed aromatase inhibitor therapy. The authors showed that after long-term anti-hormone therapy, no response is lost with the lower dose of estrogen; overall
about 30% of women responded to estrogen treatment. The goal of this work is to uncover the mechanisms preventing the other 70% of patients from responding, and perhaps find ways to circumvent their resistance. To this end, MCF-7:2A cells were used as a model for estrogen-deprived breast tumors with the ability to evade estrogen-induced apoptosis in the clinic.
MATERIALS AND METHODS

Cell culture
All cell lines were cultured in phenol red-free Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% charcoal-stripped fetal bovine serum (SFS). Media and treatments were replaced every three days. Estradiol (E\textsubscript{2}) (Sigma-Aldrich, St. Louis, MO), buthionine sulfoximine (BSO) (Sigma-Aldrich, St. Louis, MO), and combinations were dissolved in ethanol and then in media. AG1024 (CalBiolchem, San Diego, CA) was dissolved in DMSO and then in media.

DNA assays
MCF-7:WS8, MCF-7:5C, and MCF-7:2A cells were harvested after 7 or 14 days treatment with vehicle (0.1% ethanol), E\textsubscript{2} (10\textsuperscript{-9} mol/liter, 1 nM), BSO (10\textsuperscript{-4} mol/liter, 100 µM), or E\textsubscript{2} (1 nM) + BSO (100 µM). DNA content was measured as using the Fluorescent DNA Quantitation Kit (Bio-Rad, Hercules, CA).

Western blots
Total MAPK (#9102), phosphorylated MAPK (#9101), total AKT (#9272), phosphorylated AKT (#4051L), total eIF2α (#9722S), phosphorylated eIF2α (#9721S), and IRE1α (#3294S) antibodies were all purchased from Cell Signaling Technology (Beverly, MA). IGF-1Rβ antibody (sc-713) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin loading control antibody (A5441) was purchased from Sigma-Aldrich (St. Louis, MO). Proteins were harvested from cells using cell lysis buffer (Cell Signaling Technology, Beverly, MA)
supplemented with Protease Inhibitor Cocktail Set I and Phosphatase Inhibitor Cocktail Set II (Calbiochem, San Diego, CA). Bicinchoninic acid (BCA) assay was used to quantify total protein content (Rio-Rad Laboratories, Hercules, CA). Fifty micrograms of protein were probed run on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel, and transferred to a nitrocellulose membrane which was probed with primary antibodies overnight. The membrane was then washed and incubated with secondary antibody conjugated with HRP. To visualize the proteins, the membranes were incubated with Western Lighting™ plus-ECL enhanced chemiluminescent substrate (PerkinElmer Inc., Waltham MA), and then exposed to x-ray film.

*Cell cycle analysis*

MCF-7:2A cells were cultured in dishes and treated with vehicle (0.1% ethanol) or E₂ (10⁻⁹ mol/liter, 1 nM). Cells were harvested after 24 hours, fixed in 75% ethanol on ice, stained with propidium iodide, and sorted using FACS flow cytometry (Becton Dickinson, San Jose, CA). Results were analyzed using CellQuest software.

*RT-PCR*

Cells were harvested using TRIzol, and RNA was isolated using RNeasy Micro kit (Qiagen, Valencia, CA). RNA was reverse transcribed to cDNA using a kit (Applied Biosystem, Foster City, CA). SYBR green (Applied Biosystems, Foster City, CA) was used for quantitative real-time polymerase chain reaction (RT-PCR) in a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA).
Glutathione assay

Cells were harvested and de-proteinized with 5% 5-sulfosalicylic acid solution (SSA) (Sigma-Aldrich, St. Louis, MO). Total glutathione (reduced glutathione (GSH) plus glutathione disulfide (GSSG)) was measured spectroscopically at 412 nm using a Glutathione Assay Kit (CS0260, Sigma-Aldrich, St. Louis, MO) and the manufacturer’s instructions.

ROS assay

MCF-7:2A cells were harvested, stained with $10^{-6}$ mol/liter (1 µM) CM-H2DCFDA (Invitrogen, Eugene, OR), and analyzed for ROS fluorescence using flow cytometry.

Statistical analysis

Values reported are means +/- standard deviation (SD). Significant differences were found by Student’s t test. P values $<$0.05 were considered statistically significant.
**RESULTS**

*MCF-7:2A initial response to E₂*

The MCF-7:WS8, MCF-7:5C, and MCF-7:2A cell lines respond differently to $10^{-9}$ mol/liter (1 nM) E₂. In the presence of 1 nM E₂, MCF-7:WS8 cells are stimulated to proliferate over seven days, whereas MCF-7:5C cells are killed by this timepoint (Fig. 2A). MCF-7:2A cell growth is unaffected by the presence of E₂ after one week, but their DNA is reduced by 50% after the second week of treatment (Fig. 2A). Interestingly, MCF-7:2A cells are initially stimulated to proliferate in response to E₂. After 24 hours treatment with 1 nM E₂, both the mitogen-activated protein kinase (MAPK) and serine/threonine protein kinase Akt (AKT) pathways are activated, as shown by an increase in phosphorylated MAPK (p-MAPK) and phosphorylated AKT (p-AKT) proteins, respectively (Fig. 2B). Further, MCF-7:2A cells treated with E₂ for 24 hours show an increase in the percentage of dividing cells compared with vehicle treatment (34.78% versus 20.17%), illustrated by S-phase in cell cycle analysis (Fig. 2C).
Figure 2. MCF-7:2A growth response to E\textsubscript{2}. A. DNA was measured from MCF-7:WS8, MCF-7:5C, and MCF-7:2A cells after 7 or 14 days treatment with vehicle or 1 nM E\textsubscript{2}. Values are normalized to vehicle-treated cells. Means represent samples in triplicate. B. MAPK and AKT growth pathway protein levels were measured by Western blot after 24 h vehicle or 1 nM E\textsubscript{2} treatment. β-actin was used as a loading control. C. Cell cycle analysis was performed after 24 h vehicle or 1 nM E\textsubscript{2} treatment.
MCF-7:5C and MCF-7:2A UPR

To determine whether the different biological effects observed in MCF-7:5C and MCF-7:2A cells is due to different patterns of the unfolded protein response (UPR), proteins associated with the UPR were measured over a 72 hour timecourse. Two markers of the UPR, phosphorylated eIF2α (p-eIF2α) and IRE1α, were visualized by Western blot in MCF-7:5C and MCF-7:2A cells in the presence of vehicle and 1 nM E2 (Fig. 3). p-eIF2α is directly downstream of protein kinase RNA-like endoplasmic reticulum kinase (PERK), a sensor which initiates UPR. Both cell lines show an increase in the protein expression of p-eIF2α and IRE1α by 72 hours of E2 treatment, indicating activated UPR. Though MCF-7:2A cells show a slightly higher basal p-eIF2α level, no differences in UPR activation can be seen between the two cell lines.

<table>
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<tr>
<th>MCF-7:5C</th>
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<tr>
<td><strong>1 nM E2</strong></td>
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**Figure 3. MCF-7:5C and MCF-7:2A UPR.** Cell lines were probed for UPR-related proteins after treatment with vehicle or 1 nM E2 for 24, 48, and 72 h. β-actin was used as a loading control.
MCF-7:5C and MCF-7:2A estrogen-induced apoptosis

To determine whether MCF-7:2A cells experience apoptosis through the same pathway as MCF-7:5C cells, RT-PCR was used to quantify mRNA levels of apoptosis-related genes. MCF-7:5C cells noticeably up-regulate LTA (4.19 ± 1.92 fold change), LTB (5.39 ± 1.82), TNFα (9.40 ± 3.86), and BCL2L11 (6.06 ± 0.87) after 72 hours of E2 treatment, while MCF-7:2A cells show no major changes during this time period (Fig. 4A). MCF-7:2A cells were then treated with E2 for a longer time period to measure apoptosis-related genes during the time when they appear to die. MCF-7:2A cells increase both TNFα (33.55 ± 12.09 fold change) and BCL2L11 (3.71 ± 0.35 fold change) after 12 days of 1 nM E2 treatment (Fig. 4B). The up-regulated apoptosis-related genes correspond to the time when cell death is most apparent in both cell lines, during week one in MCF-7:5C cells, and during week two in MCF-7:2A cells.
Heme oxygenase 1 (HMOX1) was used as an indicator to illustrate when MCF-7:5C and MCF-7:2A cells experience oxidative stress. After 72 hours of 1 nM E\textsubscript{2} treatment, HMOX1 mRNA was increased 4.61-fold in MCF-7:5C cells (Fig. 5A), suggesting this cell line undergoes oxidative stress at this time point. MCF-7:2A cells did not increase HMOX1 mRNA expression until 12 days of 1 nM E\textsubscript{2} treatment when it increased 10.03-fold (Fig. 5B), suggesting an earlier protective mechanism inherent in these cells to prevent oxidative stress longer than MCF-7:5C cells.

**Fig 4. Apoptosis-related genes in MCF-7:5C and MCF-7:2A cells.** A. MCF-7:5C and MCF-7:2A cells were treated with vehicle or 1 nM E\textsubscript{2} for 24, 48, and 72 h. LTA, LTB, TNF\textalpha, and BCL2L11 mRNA levels were measured using RT-PCR. 36B4 was used as an internal control. B. MCF-7:2A cells were treated with vehicle or 1 nM E\textsubscript{2} for 3, 6, 9, and 12 days. TNF\textalpha and BCL2L11 mRNA levels were then measured using RT-PCR. 36B4 was used as an internal control. Means represent at 7 to 18 replicates.

MCF-7:5C and MCF-7:2A oxidative stress

Heme oxygenase 1 (HMOX1) was used as an indicator to illustrate when MCF-7:5C and MCF-7:2A cells experience oxidative stress. After 72 hours of 1 nM E\textsubscript{2} treatment, HMOX1 mRNA was increased 4.61-fold in MCF-7:5C cells (Fig. 5A), suggesting this cell line undergoes oxidative stress at this time point. MCF-7:2A cells did not increase HMOX1 mRNA expression until 12 days of 1 nM E\textsubscript{2} treatment when it increased 10.03-fold (Fig. 5B), suggesting an earlier protective mechanism inherent in these cells to prevent oxidative stress longer than MCF-7:5C cells.
Figure 5. MCF-7:5C and MCF-7:2A HMOX1 regulation. A. MCF-7:5C and MCF-7:2A cells were treated with vehicle or 1 nM E$_2$ for 24, 48, and 72 h; HMOX1 mRNA was measured using RT-PCR. 36B4 was used as an internal control. Mean represents 18 replicates. B. MCF-7:2A cells were treated with vehicle or 1 nM E$_2$ for 3, 6, 9, and 12 days; HMOX1 mRNA was measured using RT-PCR. 36B4 was used as an internal control. Means represent at least 8 replicates.
Glutathione is a potent antioxidant and was quantified in MCF-7:5C and MCF-7:2A cells to illustrate a potential protective mechanism in MCF-7:2A cells against oxidative stress (Fig. 6A). In fact, MCF-7:2A cells have significantly more basal glutathione than do MCF-7:WS8 and MCF-7:5C cells (Fig. 6A). Buthionine sulfoximine (BSO) is a synthetic amino acid that blocks glutathione synthesis by inhibiting gamma-glutamylcysteine synthetase. One hundred µM BSO dramatically decreases glutathione levels in both MCF-7:5C and MCF-7:2A cells (Fig. 6B). To ask the question of whether glutathione is protecting MCF-7:2A cells from oxidative stress and E₂-induced apoptosis, HMOX1 was measured following treatment with vehicle, 1 nM E₂ alone, 100 µM BSO alone, and 1 nM E₂ + 100 µM BSO after 24, 48, and 72 hours (Fig. 6C). MCF-7:2A cells show increased HMOX1 mRNA at 72 hours after treatment with 100 µM BSO and 1 nM E₂ + 100 µM BSO (3.57 ± 0.36 and 2.60 ± 0.70 fold changes, respectively), suggesting a protective role of glutathione in these cells. Reactive oxygen species (ROS) increased 634% over vehicle in MCF-7:2A cells after 12 days of the combination treatment (Fig. 6D). Furthermore, 1 nM E₂ + 100 µM BSO treatment caused a significant decrease in DNA after 14 days treatment (Fig. 6E), suggesting that oxidative stress is a key factor in determining E₂-induced MCF-7:2A cell death.
Figure 6. MCF-7:2A oxidative stress and glutathione. A. Total basal glutathione (GSSG+GSH) levels were measured in MCF-7:WS8, MCF-7:5C, and MCF-7:2A cells. Means represent samples in triplicate. B. Total glutathione in MCF-7:5C and MCF-7:2A cells were quantified after 72 h treatment of vehicle or 100 µM BSO. Means represent samples in triplicate. C. MCF-7:2A cells were treated for 24, 48, and 72 h with either vehicle, 1 nM E\textsubscript{2}, 100 µM BSO, or 1 nM E\textsubscript{2} + 100 µM BSO; HMOX1 mRNA was measured using RT-PCR. 36B4 was used as an internal control. Means represent at least 8 replicates. D. MCF-7:2A were subjected to the aforementioned treatments for 5, 7, 9, and 12 days, and ROS levels were measured. Data is normalized to vehicle treatment. E. MCF-7:2A cells were treated likewise, and DNA was harvested and quantified after two weeks. Means with error bars represent samples in triplicate. **p<0.01, ***p<0.001
**MCF-7:5C and MCF-7:2A IGFR**

Insulin-like growth factor receptor beta (IGF-1Rβ) up-regulation is another mechanism through which MCF-7:2A cells could receive anti-apoptotic advantage over MCF-7:5C cells. MCF-7:2A cells exhibit 2.71-fold greater basal IGF-1Rβ mRNA than MCF-7:5C cells (Fig. 7A). This is consistent at the protein level as shown by Western blot, where MCF-7:2A cells exhibit more IGF-1Rβ protein expression than MCF-7:5C cells (Fig. 7B). When treated with an IGF-1Rβ inhibitor (10 µM AG1024) for 7 days, MCF-7:2A cells show significantly decreased DNA content when compared to vehicle and 1 nM E2 treatments (Fig. 7C). Combination treatment of 1 nM E2 + 10 µM AG1024 decreased DNA content significantly more than either treatment alone (Fig. 7C), suggesting an integral role of IGF-1Rβ in MCF-7:2A cells evading E2-induced apoptosis. To interrogate this further, growth pathway proteins were measured in response to 10 µM AG1024 treatment. MAPK and AKT pathways are both blocked by the IGF-1Rβ inhibitor after 72 hours as shown by decreased p-MAPK and p-AKT levels when compared to vehicle-treated MCF-7:2A cells (Fig. 7D).
Figure 7. MCF-7:2A IGF-1Rβ. A. Basal IGF-1Rβ mRNA was measured in MCF-7:5C cells and MCF-7:2A cells via RT-PCR. MCF-7:2A values are normalized to MCF-7:5C. 36B4 was used as an internal control. Means represent samples in triplicate. B. Basal IGF-1Rβ protein levels were measured in MCF-7:5C and MCF-7:2A cells by Western blot. β-actin was used as a loading control. C. MCF-7:2A cells were treated with vehicle, 1 nM E2, 10 µM AG1024, or 1 nM E2 + 10 µM AG1024. DNA was harvested and quantified after seven days. Means represent samples in triplicate. D. MCF-7:2A cells were treated for 72 h with vehicle or 10 µM AG1024. Growth pathway protein levels were visualized via Western blot. Total MAPK, total AKT, and β-actin were used as loading controls. *p<0.05, ***p<0.001
**DISCUSSION**

This study investigated the mechanisms through which MCF-7:2A cells evade E₂-induced apoptosis *in vitro* as a means to understand resistant breast cancer cells after long-term anti-hormone therapy in the clinic. After failure on an aromatase inhibitor, approximately 30 percent of breast cancer patients will respond to treatment with estrogen [91]; their nascent or remaining breast tumors will become cytostatic or disappear with physiological levels of estrogen. Further, estrogen replacement therapy (ERT) has been shown to reduce the risk of breast cancer in hysterectomized post-menopausal women [129], perhaps due to estrogen-deprived breast cancer cells undergoing estrogen-induced apoptosis before resulting in clinically apparent disease. This study sought to discriminate between estrogen-deprived breast tumors that will quickly respond to treatment with E₂ versus those that will respond more slowly and less dramatically. We modeled these different scenarios with MCF-7:5C and MCF-7:2A cell lines, respectively.

Previously published literature shows that the UPR, associated with endoplasmic reticulum stress (ERS), is a fundamental element in E₂-induced MCF-7:5C cell apoptosis [97]. In this setting, E₂ triggers UPR and rapidly causes apoptosis within one week of treatment. Two main sensors of the UPR, IRE1α and PERK, are activated in both cell lines similarly. PERK activation is confirmed by p-eIF2α expression, since eIF2α is phosphorylated by activated PERK. In MCF-7:2A cells, the same sensors are activated as in MCF-7:5C cells (Fig. 3), but significant cell death is not apparent at the same timepoint (Fig. 2A). Despite similar signaling
patterns, the biological responses between the two cell lines differ. Our data suggested that another mechanism was preventing cell death after E2-induced UPR in MCF-7:2A cells.

Oxidative stress is a critical pathway for MCF-7:2A cells to undergo E2-induced apoptosis. MCF-7:2A cells inherently exhibit stronger survival and antioxidant mechanisms than MCF-7:5C cells (Figs. 4-6). This relationship is consistent with previously published data showing that MCF-7 cells with higher levels of glutathione peroxidase 1 (GSHPx-1) can survive better under oxidative stress conditions, such as hydrogen peroxide treatment [180], and that MCF-7 cells can increase antioxidant enzymes (i.e. manganese superoxide dismutase, MnSOD) to prevent TNF-mediated apoptosis [181]. Activation of E2-induced apoptosis in MCF-7:2A cells also correlates with TNF family member up-regulation (Figs. 4A and 4B). Oxidative stress occurs concurrently with up-regulation of apoptosis-related genes in the TNF family. Whether increased TNFα causes oxidative stress or oxidative stress causes increased TNFα is not yet documented in this setting.

Additionally, B-cell lymphoma 2 (BCL2) plays a role in preventing cell death caused by oxidative stress [182]. In fact, MCF-7:2A cells exhibit 3.76-fold and 3.02-fold higher basal BCL2 and B-cell lymphoma extra-large (BCL-xL, BCL2L1) mRNA levels than MCF-7:5C cells, respectively (Table I), providing support for the idea of a stronger survival signal. Other data from our lab shows that MCF-7:2A cells exhibit 6.19-fold higher glutathione peroxidase 2 gene (GPX2) over MCF-7:5C cells (Table II), illustrating more evidence in favor of increased protection from E2-induced oxidative stress and apoptosis in this context.
Increased IGFR promotes anti-hormone resistance in breast cancer, likely through growth factor receptor crosstalk and aberrant ER, MAPK, and AKT signal transduction pathway activation [96,109-110]. Our data correlate with these findings in that higher IGF-1Rβ mRNA and protein expression confer a growth advantage and apoptotic resistance in MCF-7:2A cells despite treatment with E$_2$ (Fig. 7). This suggests an IGF-1Rβ signaling pathway that can circumvent normal ER signaling in long-term estrogen-deprived breast cancer cells. Studies using hepatocellular carcinoma cells (HCC) have demonstrated that IGF-1R overexpression can potentially cause increased glutathione transferase (GST) and protection from oxidative stress [183]. Although this mechanism is shown in liver cancer cells, it may apply to our models of breast cancer as well. Perhaps the higher level of IGF-1Rβ in MCF-7:2A cells generates the increased glutathione levels necessary to escape cell death in the presence of E$_2$.

The evidence thus far shows that TNF family member gene expression, protection against oxidative stress, and growth factor signaling are major mechanisms underlying the different biological responses to E$_2$ seen in MCF-7:2A cells versus MCF-7:5C cells. Despite similar UPR signaling patterns, MCF-7:2A cells resist ERS-induced death longer and stronger than MCF-7:5C cells. Additional studies may provide further insight into the connection between IGF-1Rβ and glutathione in MCF-7:2A cells, and how this relationship functions in the presence and absence of a stressor such as E$_2$. In order to effectively treat breast cancer patients who have undergone exhaustive anti-hormone treatment, and to explain why ERT can prevent breast cancer in some post-menopausal women, the examination of breast cancer cell models of estrogen deprivation is proving invaluable. By understanding mechanisms that prevent apoptosis in these breast cancer cells, we can translate key findings into clinical practice.
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**Table I. Basal apoptosis gene expression in MCF-7:2A cells versus MCF-7:5C.** RT-PCR gene arrays of apoptosis-related genes were performed using MCF-7:5C and MCF-7:2A cells. Fold change represents gene expression of basal MCF-7:2A levels over basal MCF-7:5C levels. Only genes over-expressed in MCF-7:2A cells are shown.
### Table II. Top 10 over-and under-expressed oxidative stress-related genes in MCF-7:2A versus MCF-7:5C.

Global gene expression analyses were performed, and oxidative stress-related genes were ranked by fold change of MCF-7:2A expression over MCF-7:5C expression.

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Chapter III:
A molecular mechanistic approach to deciphering cellular selection pressure and breast cancer risk in the Women’s Health Initiative

Conjugated equine estrogen (CEE) was first introduced into clinical practice in 1941 as Premarin (pregnant mares’ urine) for the treatment of menopausal symptoms and related conditions. Despite the widespread use of estrogen replacement therapy (ERT) there were concerns with unopposed estrogen use. Reports of an increased risk of endometrial cancer in women taking ERT [184,185] resulted in package label warnings about the risks of cancer and thrombosis. A solution was required for the use of ERT to treat osteoporosis in post-menopausal women without risking endometrial cancer. Histological examination of biopsies from women taking ERT showed proliferation of the endometrial lining, but the addition of a progestin prevented this action [186]. The Food and Drug Administration approved the use of a combination of estrogen and a progestin for the treatment and prevention of osteoporosis in 1986. A combination of CEE and a progestin, here medroxyprogesterone acetate (MPA), is referred to as hormone replacement therapy (HRT).

Observational clinical studies [187] and laboratory studies [188] supported the view that HRT users had a reduced risk of coronary heart disease (CHD) and reduced atherosclerosis in animal models. These encouraging data led to randomized clinical trials, the largest of which is the Women’s Health Initiative (WHI). There are two parallel studies that enrolled a total of 27,000 subjects: women with an intact uterus were randomized to placebo or HRT (CEE 0.625 mg and MPA 2.5 mg daily), and hysterectomized women were randomized to placebo or CEE alone. The trial was stopped when the HRT arm exceeded the pre-defined safety limit for breast
cancer, i.e. a hazard ratio of 1.26. However, the unanticipated result was the decrease in breast cancer incidence and mortality in the CEE alone group [189].

Laboratory studies [190,191] over the past decade have documented and deciphered a new biology of estrogen-induced apoptosis that occurs in long-term estrogen-deprived breast cancer cells. These studies translate to benefit in clinical trials of estrogen therapy in anti-hormone-resistant breast cancer [88,91]. Therefore, if the molecular mechanism of estrogen-induced apoptosis is defined in well-documented laboratory models of estrogen-deprived breast cancer cells [97,178,192,193] why does a combination of MPA plus CEE in the parallel trial in the WHI increase the risk of breast cancer [189]? Although there is a large body of experimental evidence that the female sex hormones estrogen and progesterone are responsible for breast cancer growth [194], the paradox that estrogen induces apoptosis in estrogen-deprived breast cancer cells has created a new dimension in our understanding of physiologic estrogen action in a woman’s body.

The central question to be addressed here is whether a synthetic progestin, MPA, can modulate estrogen-induced apoptosis and cause a growth of new populations of breast cancer cells. We have noted previously that estrogen-induced apoptosis is accompanied by an increase in the activation of inflammatory genes [97]. Here we demonstrate that synthetic progestins have a range of pharmacologic actions that exert different selection pressures during long-term treatment in culture. We report for the first time that the glucocorticoid properties of MPA [195] are responsible for blunting the apoptotic actions of estrogen resulting in robust growth of a new breast cancer cell population that is better able to survive.
**Materials and Methods**

*Cell culture*

All cell lines were cultured in phenol red-free Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% charcoal-stripped fetal bovine serum (SFS). Media and treatments were replaced every three days. Estradiol (E₂, Sigma-Aldrich, St. Louis, MO), dexamethasone (Dex, Sigma-Aldrich, St. Louis, MO), medroxyprogesterone acetate (MPA, Sigma-Aldrich, St. Louis, MO), norethindrone acetate (NETA, Sigma-Aldrich, St. Louis, MO), R5020 (Sigma-Aldrich, St. Louis, MO), RU486 (Sigma-Aldrich, St. Louis, MO), 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich, St. Louis, MO), and combinations were dissolved in ethanol and then in media.

*Chemical structures*

![Structures](dexamethasone.png, MPA.png, NETA.png)

Dexamethasone    MPA    NETA
**DNA assays**

MCF-7:5C cells were harvested after treatment with vehicle (0.1% ethanol), E$_2$ (10$^{-9}$ mol/liter, 1 nM), Dex (10$^{-6}$ mol/liter, 1 µM), MPA (10$^{-6}$ mol/liter, 1 µM), NETA (10$^{-6}$ mol/liter, 1 µM), R5020 (10$^{-6}$ mol/liter, 1 µM), RU486 (10$^{-6}$ mol/liter, 1 µM), 4-OHT (10$^{-6}$ mol/liter, 1 µM), or combinations, for specified time. DNA content was measured as using the Fluorescent DNA Quantitation Kit (Bio-Rad, Hercules, CA).

**Western blots**

ERα (sc-544), GR (sc-8892), and PR (sc-810) antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PARP (#9532S) antibody was purchased from Cell Signaling Technology (Beverly, MA). β-actin loading control antibody (A5441) was purchased from Sigma-Aldrich (St. Louis, MO). Proteins were harvested from cells using cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with Protease Inhibitor Cocktail Set I and Phosphatase Inhibitor Cocktail Set II (Calbiochem, San Diego, CA). Bicinchoninic acid (BCA) assay was used to quantify total protein content (Rio-Rad Laboratories, Hercules, CA). Fifty micrograms of protein were run on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel, and transferred to a nitrocellulose membrane which was probed with primary antibodies overnight. The membrane was then washed and incubated with secondary antibody conjugated with HRP. To visualize the proteins, the membranes were incubated with Western Lighting™ plus-ECL enhanced chemiluminescent substrate (PerkinElmer Inc., Waltham MA), and then exposed to x-ray film.
Annexin V

Apoptosis was quantified by flow cytometry using the FITC Annexin V Detection Kit I (BD Pharmingen) according to the manufacturer's instructions. MCF-7:5C cells were treated with different compounds, suspended in 1× binding buffer, and stained simultaneously with fluorescein isothiocyanate (FITC)-labeled Annexin V (FL1-H) and PI (FL2-H). Cells were analyzed using FACSort flow cytometer (Becton Dickinson).

RT-PCR

Cells were harvested using TRIzol, and RNA was isolated using RNeasy Micro kit (Qiagen, Valencia, CA). RNA was reverse transcribed to cDNA using a kit (Applied Biosystem, Foster City, CA). SYBR green (Applied Biosystems, Foster City, CA) was used for quantitative real-time polymerase chain reaction (RT-PCR) in a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA).

GRE activity

Transient transfection assay was conducted using a dual-luciferase system (Promega). To determine GR transcriptional activity, cells were transfected with a glucocorticoid response element (GRE)-regulated dual-luciferase reporter gene set (Qiagen). The cells were treated with the specified compounds for 24 hours following the transfection. The cells were then harvested and processed for dual-luciferase reporter activity, in which the firefly luciferase activity was normalized by Renilla luciferase activity.
Statistical analysis

Values reported are means +/- standard error (SEM). Significant differences were found by Student’s t test. P values <0.05 were considered statistically significant.
RESULTS

In order to discover the mechanism through which MPA blocks the beneficial effect of E₂ on breast cancer cells, systematic experiments were performed in this study. MCF-7:5C cells represent a stable cell line derived from parental MCF-7 cells by long-term estrogen deprivation, and are the relevant cells used in the following studies. MCF-7:5C cells were initially treated for eight days with a vehicle or 1µM R5020, Dex, MPA, or NETA, to demonstrate how the cells respond to the compounds compared with a control. R5020 was used as a positive control for a pure progestin. The cells were harvested every two days to create a growth curve over time (Fig. 1A). One µM concentrations of Dex and MPA cause a decrease in MCF-7:5C cell DNA of 28.7% and 21.6%, respectively, compared with vehicle treatment after eight days. The cells responded the same to R5020 as to vehicle treatment. One µM NETA, however, caused a 93.6% decrease in MCF-7:5C cells after 8 days.

MCF-7:5C cells were then treated for a longer time period with vehicle, 1nM E₂, or 1µM Dex, MPA, or NETA (Fig. 1B). Again, cells treated with Dex and MPA do not grow as quickly as the control cells. NETA and E₂ both cause MCF-7:5C cell death; minimal DNA is sustained for six weeks of treatment. To mimic HRT, MCF-7:5C cells were treated with combinations of E₂+MPA and E₂+NETA for this same time period, and compared with vehicle, E₂ alone, and E₂+Dex (Fig. 1C). Cells treated with 1nM E₂ die during the first week of treatment, but Dex and MPA seem to reverse this effect; cells are able to grow. E₂+NETA triggers the same death response in these cells as E₂ alone, suggesting NETA’s role as an estrogen.
Figure 1. MCF-7:5C growth over time. A. MCF-7:5C cells were treated with vehicle or 1μM concentrations of R5020, Dex, MPA, or NETA. Cells were harvested every two days for eight days, and DNA was quantified. B. MCF-7:5C cells were treated with vehicle, 1nM E₂, or 1μM Dex, MPA, or NETA. Cells were harvested and DNA was quantified every 3 days until the cells reached confluency, up until 42 days. C. MCF-7:5C cells were treated with vehicle, 1nM E₂, or combinations of 1nM E₂ plus 1μM Dex, MPA, or NETA. Cells were harvested and DNA was quantified every 3 days until the cells reached confluency, up until 42 days. Means represent samples in triplicate.
MCF-7:5C cells underwent these same treatments for 72 hours, and annexin V was stained and measured by flow cytometry to indicate apoptosis (Fig. 2). One nM E$_2$ causes 29.03 (±1.44) % of MCF-7:5C cells to undergo apoptosis after 72 hours of treatment. Dex is able to block this apoptosis as shown by E$_2$+Dex causing only 5.37 (±0.35) % of cells to stain positive for annexin V. NETA and NETA+E$_2$ both show similar annexin V staining as E$_2$ alone; both treatments cause apoptosis (30.17 ±0.65 and 33.23 ±0.97%, respectively). Although Figure 1 showed MCF-7:5C cells were able to grow under E$_2$+MPA treatment, MPA is not able to block E$_2$-induced apoptosis at 72 hours; more time is required for MPA to exert its affect. When Poly (ADP-ribose) polymerase (PARP) cleavage is investigated by Western blot to indicate apoptosis, again we show that E$_2$ alone, and in combination with 1µM MPA, causes apoptosis after 72 hour treatments. However, MPA can block E$_2$-induced apoptosis after 6, 9 and 12 days of combination treatment, as shown by decreased cleaved PARP protein expression. This confirms the biological effect already seen, as MCF-7:5C cells can grow in the presence of E$_2$+MPA treatment.
Figure 2. Dex and MPA block E₂-induced apoptosis. A. MCF-7:5C cells were treated with vehicle, 1nM E₂, 1µM Dex, 1µM MPA, 1µM NETA, or combinations, for 72 hours. Cells were harvested and apoptosis was measured by annexin V staining and flow cytometry. Means represent samples in triplicate ± SEM. B. MCF-7:5C cells were treated with vehicle or 1nM E₂ for 3 days, or 1nM E₂ + 1µM MPA for 3, 6, 9 and 12 days. Proteins were harvested and probed for PARP cleavage to indicate apoptosis. β-actin was used as a loading control. ***p < 0.001
To begin investigating the mechanism by which Dex and MPA can block E$_2$-induced apoptosis, nuclear receptor protein levels were measured by Western blot. Basal MCF-7:5C cells express estrogen receptor (ER$_\alpha$) and glucocorticoid receptor (GR), but not progesterone receptor (PR). One nM E$_2$ treatment causes a decrease in both ER$_\alpha$ and GR after 24 and 72 hours (Fig. 3A). MCF-7:WS8 cells, as a positive control, can induce PR expression after 72 hours of E$_2$ treatment. After two months of treatment with E$_2$, ER$_\alpha$ is dramatically reduced in MCF-7:5C cells compared with vehicle treatment. MCF-7:5C cells treated with Dex or MPA alone sustain ER$_\alpha$ expression after two months treatment, but ER$_\alpha$ is reduced when the cells are treated in combination with E$_2$. NETA and E$_2$+NETA both generate undetectable ER$_\alpha$ protein, similar to E$_2$ treatment. GR levels are sustained by MCF-7:5C cells after all two month treatments, but Dex appears to induce GR protein reduction at this time point (Fig. 3B).
Figure 3. MCF-7:5C nuclear receptor status. A. MCF-7:WS8 and MCF-7:5C cells were treated with either vehicle for 72 hours or 1nM E$_2$ for 24 or 72 hours. Cells were then harvested, and ER$\alpha$, PR, and GR proteins were measured by Western blot. MCF-7:WS8 cells were used as a positive control for PR expression. B. MCF-7:5C cells were treated with vehicle, 1nM E$_2$, 1µM Dex, MPA, or NETA, or combinations of 1nM E$_2$ plus 1µM Dex, MPA, or NETA for two months. Cells were harvested, proteins were extracted, and Western blots for ER$\alpha$ and GR protein levels were performed. β-actin was used as a loading control.
After this two-month treatment, microscopy photographs were taken to illustrate the dramatic effects Dex and MPA have on E\textsubscript{2}-treated cells. Cells treated with both E\textsubscript{2} and NETA+E\textsubscript{2} show a vast reduction in MCF-7:5C cell number after two months of treatment. E\textsubscript{2}+Dex and E\textsubscript{2}+MPA treatments both allow MCF-7:5C cells to grow during the two months of treatment as demonstrated by increased cell number seen in the photographs (Fig. 4).

Figure 4. Dex and MPA allow MCF-7:5C cells to grow in the presence of E\textsubscript{2}. High-contrast microscopy photographs were taken after MCF-7:5C cells were treated with vehicle, 1nM E\textsubscript{2}, or 1nM E\textsubscript{2} plus 1\mu M Dex, MPA, or NETA for two months. 10x magnification, exposure time: 1/1000s.
To classify NETA as an estrogen, and Dex and MPA as glucocorticoids, MCF-7:5C cell DNA was measured after seven days of treatment with the drugs alone and in combination with an anti-estrogen or anti-glucocorticoid, respectively. As a positive control, the data confirms that 1µM 4-OHT is able to reverse E2’s apoptotic action as shown by significantly increased DNA when MCF-7:5C cells are treated with combination treatment compared to E2 alone. 4-OHT also has the ability to reverse NETA’s decrease in MCF-7:5C DNA after seven days of treatment, further suggesting its role as an estrogen (Fig. 5A). Dex and MPA alone cause a reduction in MCF-7:5C cell DNA after seven days of treatment. However, when treated in combination with the anti-glucocorticoid RU486, Dex and MPA generate significantly higher MCF-7:5C cell DNA after seven days, when compared with treatment alone (Fig. 5B). This data suggests MPA could be working as a glucocorticoid in these cells.
Figure 5. 4-OHT and RU486 reversal ability in MCF-7:5C cells. A. MCF-7:5C cells were treated with vehicle, 1µM 4-OHT, 1nM E₂, 1µM NETA, or combinations for seven days, and DNA was quantified. B. MCF-7:5C cells were treated with vehicle, 1µM RU486, 1µM Dex, 1µM MPA, or combinations for seven days, and DNA was quantified. Means represent samples in triplicate. *p < 0.05, ***p < 0.001
Furthering the evidence toward classifying NETA as an estrogen, estrogen target gene mRNA expression was measured in MCF-7:WS8 cells by RT-PCR after 24 treatments with vehicle, 1nM E$_2$, or varied concentrations of Dex, MPA, and NETA. Whereas Dex and MPA generate no increase in estrogen target genes PgR or pS2 at any concentration, NETA elicits significant upregulation of both PgR (30.6- and 81.0-fold) and pS2 (23.6- and 46.9-fold) at 100nM and 1µM concentrations, respectively. This result is similar to that of 1nM E$_2$, which increases PgR 58.9-fold and pS2 64.9-fold over vehicle (Fig. 6A and 6B) in MCF-7:WS8 cells after 24 hour treatment.
Figure 6. NETA increases estrogen target gene expression similarly to E$_2$. MCF-7:WS8 cells were treated with vehicle, 1nM E$_2$, or 10nM, 100nM or 1µM concentrations of Dex, MPA, or NETA for 24 hours. PgR (A) and pS2 (B) mRNA expression was quantified by RT-PCR. 36B4 was used as an internal control. Means represent three samples in triplicate.
To test glucocorticoid behavior of MPA, glucocorticoid target gene SGK1 mRNA was quantified by RT-PCR after 72 hour treatments with vehicle, 1nM E$_2$, 1µM Dex, MPA, or NETA, or combinations of Dex or MPA with 1µM RU486. Signifying the glucocorticoid nature of Dex and MPA, both drugs alone cause SGK1 upregulation (411.4- and 81.4-fold over vehicle, respectively), and RU486 is able to decrease this induction (Fig. 7A). Neither E$_2$ nor NETA causes a dramatic increase in SGK1 mRNA expression. Further, GRE activity was detected by transiently transfecting MCF-7:5C cells with a GRE-luciferase reporter vector for 24 hours. The cells were then treated for 24 hours with a vehicle, 1nM E$_2$, or 1µM Dex, MPA, or NETA. Cells treated with Dex induced 5.39-fold (±0.26) GRE activity over vehicle-treated cells, confirming the validity of the assay. Importantly, MPA also caused 2.16-fold (±0.05) increased GRE activity compared to control, indicating its ability to function like a glucocorticoid.
To uncover the mechanism through which Dex and MPA can block E\textsubscript{2}-induced apoptosis, RT-PCR was performed using primers for genes associated with E\textsubscript{2}-induced apoptosis. Estradiol triggers apoptosis in MCF-7:5C cells by increasing levels of TNF\textalpha, HMOX1, LTA, and LTB after 72 hours of treatment. Both Dex and MPA are able to significantly decrease upregulation of these genes in response to E\textsubscript{2} when treated in combination (Fig. 8). This suggests that MPA can work in a similar way as Dex by blocking genes necessary for E\textsubscript{2} to initiate apoptosis in this setting.

**Figure 7. Dex and MPA increase SGK1 mRNA expression and GRE activity.** A. MCF-7:5C cells were treated for 72 hours with vehicle, 1nM E\textsubscript{2}, 1\muM Dex, MPA, or NETA, or combinations with 1\muM RU486. SGK1 mRNA expression was quantified by RT-PCR. 36B4 was used as an internal control. Means represent three samples in triplicate. B. MCF-7:5C cells were transfected with a GRE-luciferase reporter construct for 24 hours, then treated with vehicle, 1nM E\textsubscript{2}, or 1\muM, Dex, MPA, or NETA for 24 hours. GRE activity was measured by luciferase assay and normalized to vehicle control. Means represent samples in triplicate. ***p < 0.001
Figure 8. Dex and MPA block genes associated with E2-induced apoptosis. MCF-7:5C cells were treated with vehicle, 1nM E2, 1µM Dex, 1µM MPA or combinations for 72 hours. RT-PCR was performed using primers for TNFα, HMOX1, LTA, and LTB. 36B4 was used as an internal control. Means represent three samples in triplicate. ***p < 0.001
DISCUSSION

The hypothesis addressed in this study is whether synthetic progestins in HRT can interfere with estrogen-induced apoptosis in breast cancer cells. The results of the WHI [189] indicate an increase in the risk of breast cancer in women taking CEE+MPA; MPA was therefore expected to increase the growth of breast cancer cells in the presence of E₂ in our cellular model.

Evidence is presented here that dexamethasone, a glucocorticoid, can inhibit E₂-induced apoptosis. This finding is predictable and consistent with reports in the literature [196-202]. Studies show glucocorticoids to be effective in blocking apoptosis in glomerular endothelial cells [196,197]. Others have shown glucocorticoids effectively inhibiting apoptosis in fibrosacroma development [198], serum depletion-induced apoptosis [199], and apoptosis in neutrophils [200]. The proposition that glucocorticoids can block apoptosis in breast cancer has been investigated previously [201,202], but not in the context of estrogen-induced apoptosis in breast cancer, as this study examines.

There is also evidence in the literature demonstrating the potential of hormones to bind and activate other nuclear receptors than their own cognate receptors. NETA and other 19-nortestosterone derivatives, norethynodrel and norgestrel, have been shown to activate the ER and stimulate the growth of estrogen-responsive MCF-7 and T47D cells [203]. The estrogenic activity of NETA is again confirmed in this work through its ability to increase estrogen target genes, generate apoptosis in MCF-7:5C cells, and be blocked by 4-OHT.
Importantly, reports have indicated that MPA can bind to and activate not only the PR, but also the androgen receptor (AR) and the GR in breast cancer [204]. MPA cannot, however, bind to the ER. This finding is consistent with a more recent report showing that MPA has high affinity for the GR and can compete with the natural glucocorticoid, cortisol, in the body [205]. Others have recently demonstrated MPA increasing glucocorticoid activity in MCF-7 cells [206].

The data presented in this study integrate the previously published findings and demonstrate that MPA functions as a glucocorticoid in long-term estrogen-deprived breast cancer cells, blocking E\(_2\)-induced apoptosis and allowing cells to grow. Although others have demonstrated MPA’s function as a glucocorticoid, it is illustrated here as an explanation for the increased risk of breast cancer observed in the CEE+MPA arm of the WHI clinical trials by using a cell line appropriate to represent this population. By modeling the environment of the post-menopausal breast cancer cell using long-term estrogen-deprived MCF-7:5C cells, we can model clinical responses through laboratory experiments.

Critical to the understanding of how these effects are occurring in the MCF-7:5C model, literature has shown us the interplay between ER and GR, and how AP-1 can integrate their transcriptional responses [207]. Recent reports also show that the ER and GR can interact and cause differential activation by reconfiguring the chromatin structure at GRE or ERE sites in the DNA [208]. Further, it is also shown that GR can inhibit ER transcriptional activity and ER-mediated proliferation in breast cancer [209]. We can speculate with an extrapolation of these data into a potential model governing glucocorticoids’ and MPA’s ability to modulate estrogen-induced apoptosis.
Figure 9A proposes a mechanistic model that when MPA binds to the GR, the complex binds to GREs in the DNA which then impacts the ability of ligand-bound ER to functionally transcribe ER-target genes. In order for E₂-induced apoptosis to occur, E₂ binds to ER, which results in transcription of particular genes (e.g. HMOX1, TNFα, etc.) necessary for apoptosis. When this process is inhibited, possibly by the chromatin remodeling actions of MPA-bound GR, apoptosis is decreased even in an estrogenic setting.

Figure 9B expands on the proposed mechanism of MPA’s ability to inhibit E₂-induced apoptosis. Inflammation is critical for MCF-7:5C cells to undergo apoptosis; it has been shown that key inflammatory genes are up-regulated by E₂ in these cells and not in MCF-7:WS8 cells [97]. Glucocorticoids function to block inflammation, and are used in the clinic as anti-inflammatory drugs. Perhaps the action of MPA (and other glucocorticoids) binding to GR to down-regulate pro-inflammatory genes (e.g. IFNL1, BCL10, IL4R, FADS1, etc.) causes the decrease in E₂-induced apoptosis in breast cancer.
Figure 9. Proposed model for the mechanism of MPA inhibiting E₂-induced apoptosis. A. MPA binds to GR, which then binds to glucocorticoid response elements in the DNA. This generates a change in the transcriptional ability of ER, which may cause a decrease in genes necessary for E₂ to trigger apoptosis. B. Glucocorticoids, and perhaps MPA, work as anti-inflammatory agents by blocking transcription of pro-inflammatory genes. Since inflammation is involved in E₂ generating apoptosis, this may be a reason MPA can modulate apoptosis in the presence of E₂.

It is important to consider timing when considering the implications of this work. First of all, the data suggest that short-term experiments can be deceptive, and that long-term studies can give a better insight into the population changes occurring in the cellular models. For example,
the short-term studies showed that MPA did not readily modulate E\textsubscript{2}-induced apoptosis when compared with Dex, but after the cells were grown under these conditions for an extended period of time, the biological result emerged; MPA supports survival of these estrogen-deprived cells in an estrogenic environment.

Furthermore, the timing of HRT can make a dramatic difference in the response to treatment. MCF-7:5C cells represent long-term estrogen deprived cells; this is the biological context required in the patient as well. Previous studies have examined how a “gap” is needed after menopause to deprive women’s nascent breast cancer cells of estrogen sufficiently [210]. Five to ten years post-menopause is the current suggestion to ensure effective apoptosis when HRT is introduced to the woman [210]. Without this delay, introducing estrogen to the body may stimulate breast cancer growth rather than regression, resulting in an increased risk of breast cancer rather than the desired decreased risk. A model is proposed in Figure 10 to summarize our findings and the proposed clinical implications.

Nascent ER-positive breast tumors that have not been sufficiently deprived of estrogen will respond to CEE treatment (either alone or in combination with a progestin) by proliferating. On the other hand, after 5-10 years post-menopause, CEE alone or in combination with the estrogenic progestin NETA will cause nascent tumor regression. When the cells have been adequately deprived of estrogen or CEE+MPA will inhibit this apoptotic effect of CEE and cause these tumor cells to grow
This finding can have clinical impact as CEE+MPA is frequently taken as HRT by post-menopausal women in an effort to alleviate menopausal symptoms. Combinations with MPA can be explained by MPA blunting estrogen-induced apoptosis. This would occur over long periods and be consistent with the known fact that CEE+MPA increases the incidence of breast cancer in women over the age of 60. Patients perhaps should be advised then to choose a different progestin for their combination HRT, and also to delay HRT until they are appropriately past menopause. Since NETA provides the beneficial effects of progestins (preventing endometrial cancer) and also acts as an estrogen causing apoptosis, it appears to be a logical choice. In light
of our findings, post-menopausal women with breast cancer who have undergone exhaustive anti-hormone therapy may be reasonable candidates for estrogen salvage therapy in combination with an anti-glucocorticoid in future clinical trials. Such a trial would investigate whether an anti-glucocorticoid could prevent inference of endogenous glucocorticoids with E₂-induced apoptosis in this context.

Despite the positive results with MCF-7:5C cells, there are limitations in the interpretations that are mandated by a single cell line. As described in the introductory chapter, there are very few cell lines available to create definitive answers. Nevertheless, the small pool of ER-positive breast cancer cell models is a valuable resource that has aided understanding of breast cancer growth regulation. However, what is needed now is a pool of long-term estrogen deprived ER-positive breast cancer cell lines. Since the ER-positive cell lines generate different responses to estrogen deprivation (e.g. T47D cells become ER-negative), we predict a wide spectrum of cellular responses to exogenous E₂ after long-term deprivation. The hope is that this spectrum of responses will mirror those seen clinically; that is, we have seen that roughly 30% of women respond to E₂ treatment after aromatase inhibitor resistance is acquired [91]. A panel of long-term estrogen-deprived cell lines could allow us to investigate mechanisms preventing the other 70% of patients from responding, and perhaps find ways to generate better responses. The development of new cell lines to represent our biological context is a goal for the future. Further validation in other cell lines and clinical trials is necessary to confirm the conclusions made here.
CONCLUSIONS

Practical application of estrogen-induced apoptosis dates back to the 1940’s when high-dose diethylstilbestrol (DES) was effectively used in the clinic to treat breast cancer [127,211,212]. It has been a phenomenon exploited by clinicians, although its mechanisms are yet to be fully described. DES was commonly used to treat breast cancer until 1977 when tamoxifen, a selective estrogen receptor modulator (SERM), was approved by the Food and Drug Administration (FDA) for the treatment of breast cancer with fewer side effects than DES [128]. Long-term tamoxifen then became the standard of care for adjuvant treatment and prevention of ER-positive breast cancer [1,2].

Although tamoxifen is proven to be effective, patients can acquire resistance to anti-hormone therapy. Studies in vitro and in vivo have been able to describe the evolution of anti-hormone resistance in ER-positive breast cancer [87,88]. The first phase of resistance occurs when tamoxifen, or another SERM, can no longer prevent tumor growth, and both estrogen and the SERM allow for cancer cell survival and replication [76,79]. This phase is followed by the paradoxical phase wherein the surviving breast cancer cells can respond to physiological estrogen by undergoing apoptosis; this idea has been proposed to explain the decrease in mortality of tamoxifen-treated patients after they have stopped taking tamoxifen [81,82]. Estrogen causing breast cancer tumor regression seems counter-intuitive since estrogen is known to stimulate ER-positive breast cancer growth [87]. It is consistent, however, with the early treatment of breast cancer with high-dose DES [127]. Further, clinical trials have shown that
approximately 30% of women who have failed aromatase inhibitor therapy benefit from treatment with estradiol [91].

More recent studies expand on estrogen-induced apoptosis and its relevance in determining the appropriate timing of estrogen replacement therapy in terms of years after menopause [210]. It is imperative that breast cancer cells are adequately deprived of estrogen in order for estrogen-induced apoptosis to occur. Therefore, to receive the benefit of estrogen killing breast tumors, women should wait at least five years after menopause to begin estrogen therapy [210] to ensure their nascent breast tumors have been hormonally deprived for a long enough time period.

Estrogen-induced apoptosis can also help explain the paradoxical results of the WHI clinical trials. Contrary to the hypothesis, hysterectomized post-menopausal women randomized to CEE demonstrated a decrease in the incidence of breast cancer [WHI] when compared to their placebo-treated control group. Because these women were appropriately deprived of estrogen, introducing CEE to manage menopausal symptoms actually prevented breast cancer from developing or progressing. Though cellular models of this biological context are limited [177], clinical trials [91,189] have provided us a wealth of information corroborating our laboratory data.

Part of this dissertation confirmed and expanded the molecular model of E2-induced apoptosis by considering the timecourses of two long-term estrogen-deprived cell lines, MCF-7:5C and MCF-7:2A. The MCF-7:2A cell line was of interest because of its delay in triggering apoptosis in response to E2 compared with the MCF-7:5C cells (though both cell lines were
derived in the same manner). By modulating E$_2$-induced apoptosis with various agents such as AG1024 (IGF-1R antagonist) and BSO (blocks glutathione synthesis), pathways were proposed to be involved in this process. When AG1024 was added in combination with E$_2$ to MCF-7:2A cells, the cells were much more vulnerable to apoptosis, suggesting an integral role of IGF-1R in MCF-7:2A cell survival in an estrogenic environment [213].

In addition, inhibiting glutathione synthesis with BSO generated much more oxidative stress in these cells in response to E$_2$, and allowed for increased cell death [213]. This implied a protective role of glutathione in MCF-7:2A cells in the context of E$_2$ treatment. BSO was first proposed to be a supplement to enhance the actions of chemotherapeutic agents [214-216], but it is not commonly prescribed for the treatment of breast cancer. In correctly primed estrogen-deprived patients, i.e. those represented by MCF-7:2A cells, it is plausible BSO could have clinical benefit. By depleting glutathione in the appropriate patients, E$_2$ could then be introduced to prevent or diminish breast tumors. Clinical trials should be considered.

The final section of this dissertation explored the effect of HRT on breast cancer cell models. Particularly, the experiments sought to describe the mechanism through which combination CEE + MPA HRT is able to increase risk of breast cancer in post-menopausal women. The data suggest that MPA may block the beneficial apoptotic effect of CEE in this context by modulating apoptosis through the GR. Dex has been established in the literature to block apoptosis [196-200], and it is confirmed here in its inhibition of E$_2$-induced apoptosis. MPA has a similar structure to Dex (page 75) and is suggested in this study to function as a glucocorticoid through the GR.
It may be reasonable to consider clinical trials for breast cancer patients who are more than five years past menopause using estrogen treatment in combination with an anti-glucocorticoid such as RU486. Recent reports suggest patients with triple-negative breast cancer (ER-, PR-, HER2-negative) may receive therapeutic benefit from combining chemotherapy with RU486 [202]. The concept here can be applied to other breast cancer phenotypes; that is, perhaps glucocorticoids are blocking E$_2$-induced apoptosis in other subgroups of patients, and that adding an anti-glucocorticoid could then allow for the apoptotic trigger when physiological E$_2$ is re-introduced.

Overall, HRT presents many difficulties for menopausal women. Although its efficacy in alleviating menopausal symptoms is appealing, it has thus far been controversial if the benefits outweigh the risks. It is vital to elucidate which populations of women are prone to the dangerous side effects such as breast cancer and endometrial cancer. The findings of the WHI clinical trials [189] began to delve into correlating the type of HRT with overall risk, but recent data show it may be more complicated. Hysterectomized post-menopausal women taking CEE HRT are predicted to benefit in the context of breast cancer, but only if they have waited more than five years after menopause. Women with an intact uterus who need a progestin to prevent endometrial cancer growth from unopposed estrogen should possibly consider the detrimental consequences of using a progestin with glucocorticoid-like actions. Instead, a progestin with estrogenic functions, such as NETA, could be a safe alternative. By scheduling HRT appropriately, and prescribing the correct drugs for the environment of each woman, HRT still can have clinical benefit by reducing side effects, protecting the uterus, and protecting from breast cancer.
These findings require further laboratory investigation and methodical clinical trials. First, since NETA is proposed here as a promising replacement for MPA in HRT, more experiments are necessary. It is important to characterize the how NETA, a progestin, can function as an estrogen through the ER in long-term estrogen-deprived breast cancer cells. Although NETA, like E\textsubscript{2}, clearly up regulates ER-target genes and triggers apoptosis in MCF-7:5C cells, the exact method through which this occurs is not fully established. To investigate whether NETA binds directly to the ER, a future experiment would be to use an ER siRNA to deplete ER expression in MCF-7:5C cells. If NETA is still able to generate apoptosis in this context, it is unlikely to bind to ER and assert its effects through the ER.

Another interesting aspect of the estrogenicity of NETA is the fact that it has no aromatic A ring, a characteristic implicit in the definition of an estrogen [217]. Perhaps aromatase is functioning to aromatize NETA into an estrogen in this context, just as it works to aromatize testosterone into estrogen. To test this hypothesis, a logical experiment is to treat MCF-7:WS8 and MCF-7:5C cells with letrozole, an aromatase inhibitor, in combination with NETA, and compare its growth response. If NETA fails to generate growth in MCF-7:WS8 cells, or fails to trigger apoptosis in MCF-7:5C cells, it is likely that aromatase is actually aromatizing NETA into an estrogen. This finding would offer a more mechanistic view of NETA’s actions in the body.

Overall, this dissertation advances various aspects of estrogen-induced apoptosis and how the cellular behavior observed in the laboratory can explain clinically relevant phenomena. This work shows that long-term estrogen-deprived breast cancer cells can undergo estrogen-induced
apoptosis to varying degrees depending on factors including glutathione levels, IGFR expression, and TNF family member activation. Furthermore, estrogen-induced apoptosis of long-term estrogen-deprived breast cancer cells can help to explain the decreased risk of breast cancer in post-menopausal women taking CEE. Conversely, the increased risk of breast cancer in post-menopausal women taking combined HRT is shown here to result from the glucocorticoid activity of MPA blocking estrogen’s apoptotic effect. These laboratory findings may be able to begin translation into the clinic by advising patients that HRT has potentially harmful consequences when MPA is used, and suggesting NETA instead. Women who have undergone exhaustive anti-hormone therapy, or are years past menopause, should be wary of the risks associated with taking glucocorticoids. Future experiments and clinical trials continuing this work may further improve women’s health and decrease the risk and incidence of breast cancer.
**Appendix A: Characterizing anti-hormone resistance and ERα level in a panel of breast cancer cell lines**

Fourteen cell lines were chosen as a panel to interrogate estrogen receptor (ERα) status and growth ability under varied hormone and anti-hormone treatments. MCF-7 [1], MCF-7/LCC1 [2], MCF-7/LCC2 [3], MCF-7/LCC9 [4], MCF-7:WS8 [5], MCF-7:5C [5], MCF-7:2A [6], MCF-7:PF [7], MCF-7:ICI [8], MCF-7:RAL [9], T47D:ATCC [10], T47D:C4:2 [11], T47D:A18 [12], and T47D:A18:4-OHT [13] cell lines (Fig. 1) were probed for ERα protein levels after 24 hour treatments with vehicle, 1nM E2, 1µM 4-OHT, 1nM E2 + 1µM 4-OHT, 1µM ICI 182,780, or 1nM E2 + 1µM ICI 182,780 (Fig. 2) (in phenol red-free RPMI media supplemented with 10% charcoal-stripped fetal bovine serum). The cell lines were also grown under the same treatment conditions and DNA was quantified after seven days (Fig. 2). Figure 1 illustrates the cell lines chosen for the study, their lineage, and their ERα and progesterone receptor (PR) status.
Figure 1
Figure 2

MCF-7

DNA (ng/well)

MCF-7/LCC1

DNA (ng/well)

MCF-7/LCC2

DNA (ng/well)

MCF-7/LCC9

DNA (ng/well)

ERα

B-actin

ERα

B-actin

ERα

B-actin

ERα

B-actin
APPENDIX A REFERENCES


APPENDIX B: GLOSSARY OF TERMS AND ABBREVIATIONS

4-hydroxyandrostenedione (Foremstane): Type I steroidal aromatase inhibitor

4-hydroxytamoxifen (4-OHT): Metabolite of tamoxifen

Buthionine sulfoximine (BSO): Inhibits synthesis of glutathione

CGS 16949A (Fadrozole Hydrochloride): Non-steroidal aromatase inhibitor

Dexamethasone (Dex): Synthetic glucocorticoid, anti-inflammatory

Endoxifen: Metabolite of tamoxifen

Estradiol (E\textsubscript{2}): Primary estrogen in a woman’s body

Estrone (E\textsubscript{1}): Naturally occurring estrogen hormone

Everolimus (RAD-001): mTOR inhibitor

Exemestane: Aromatase inhibitor

Fulvestrant: Selective estrogen-receptor down-regulator (SERD)

Heme oxygenase 1 (HMOX1): Converts heme to biliverdin, known to be an indicator of oxidative stress

Hormone replacement therapy (HRT): Treatment to alleviate menopausal symptoms in women that comprises estrogens alone or estrogens plus a synthetic progestin.

ICI 164,384: Pure anti-estrogen

Keoxifene: see Raloxifene

Letrozole: Non-steroidal aromatase inhibitor

Lymphotoxin A (LTA): Cytokine produced by lymphocytes and member of the TNF family

Lymphotoxin B (LTB): Cytokine produced by lymphocytes and member of the TNF family
LY117018: Raloxifene analog

Medroxyprogesterone acetate (MPA): Synthetic progestin used in HRT

N-desmethyltamoxifen: Metabolite of tamoxifen

Norethindrone acetate (NETA): Synthetic progestin used in HRT

R5020: Synthetic progestin and progesterone receptor (PR) agonist

Raloxifene: Selective estrogen receptor modulator (SERM) used to prevent osteoporosis and breast cancer

RU 39,411: Steroidal anti-estrogen

Tamoxifen: SERM used in the treatment and prevention of estrogen receptor (ER)-positive breast cancer

Tumor necrosis factor (TNF) family: Cytokines involved in apoptosis

Trastuzumab: Targeted HER2 monoclonal antibody

Toremifene: SERM used in metastatic breast cancer
## Appendix C: Cell line characteristics

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**Appendix C. Estrogen receptor (ER) and progesterone receptor (PR) status for seven cell lines of interest.** Basal ER and PR protein expression is qualified as positive (+) or negative (-).
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