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Estrogen-Induced Apoptosis in Breast Cancer Cells: Translation to Clinical Relevance

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1. Introduction

The first example of hormonal dependency of breast cancer can be dated back as far as 1896, when Dr. G.T. Beatson observed and described the reduction of breast cancer progression in a premenopausal patient after bilateral oophorectomy (Beatson 1896). It was an indication that the ovaries produced something in a woman’s body that fueled breast cancer growth. This phenomenon was reconfirmed in a collected series of patients with advanced breast cancer following oophorectomy (Boyd 1900), however there was only a 30% percent response. In 1916 Lathrop and Loeb demonstrated in mice, that ovarian function has an influence on the growth of mammary glands and tumorigenesis, and that castration of immature female mice has delayed the evolution of mammary tumors (Lathrop 1916). However, the chemical control mechanisms of breast cancer progression and the relevance of ovarian function remained uncertain, until the first animal models were introduced to test the effects of oophorectomy and estrogenic properties of different chemical compounds under precise laboratory conditions (Allen 1923). This model allowed the identification the ovarian hormone, which induced estrus in oophorectomized mice, estrogen.

In subsequent years during the 1930s and 1940s many other compounds, including diethylstilbestrol, and triphenylethylene derivatives would be identified as estrogens utilizing the ovariectomized mouse model (Robson 1937; Dodds 1938). The connection between the beneficial effects of oophorectomy as a treatment for advanced breast cancer provoked questions about the actual role of estrogen and other estrogenic compounds in breast cancer growth. High dose estrogen therapy was the first chemical therapy (“chemotherapy”) to treat any cancer successfully. In 1944 Haddow (Haddow 1944) published the results of his clinical trial with the synthetic estrogens triphenylchlorethylene, triphenylmethylethylene, and diethylstilbestrol. He found that 10 out of 22 post-menopausal patients with advanced mammary carcinomas, who were treated with triphenylchlorethylene, had significant regression of tumor growth. Five patients out of 14 who were treated with high dose stilbestrol produced similar responses. The finding that high doses of synthetic estrogens induced regression of tumor growth in some, but not all postmenopausal patients with breast cancer (50% of patients responded to therapy favorably) was similar to the random responsiveness of oophorectomy in premenopausal patients with metastatic breast cancer (Boyd 1900). However, Haddow (Haddow 1944) noted that the first successful use of a chemical therapy to treat breast and prostate cancers

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was affiliated with significant systemic side effects, such as nausea, areola pigmentation, uterine bleeding, and edema of the lower extremities. At approximately same time Walpole was investigating the role of diethylstilbestrol and dienestrol in breast cancer (Walpole 1948). He confirmed the results obtained by Haddow that estrogens are effective in the treatment of breast cancer and can be of benefit for patients, but also noticed that older women, and women who received higher doses of estrogens had a better response to hormonal therapy (Walpole 1948; Haddow 1950). However, the mechanisms were again undefined.

The first successful attempt to decipher the biochemistry of estrogens in mammals occurred a decade later. Tritium-labeled hexestrol was found to accumulate in reproductive organs, including mammary glands, in female goats and sheep (Glascock and Hoekstra 1959). This finding was a crucial observation to understand the role of estrogens in processes involving target tissues, such as the mammary gland. Subsequently this research was translated to the clinic with the finding that tritium-labeled hexestrol accumulated at a higher rate in patients that favorably respond to adrenalectomy and oophorectomy, comparing to patients that do not (Folca et al. 1961). This indicated that patients who would accumulate estrogens better in target breast tissue would respond better to surgical castration. However, this technical approach was not pursued further.

During the 1950’s Kennedy (Kennedy and Nathanson 1953) systematically investigated the efficacy of synthetic estrogens for the treatment of advanced breast cancer. Kennedy examined a variety of different estrogens, however he found no significant differences and diethylstilbestrol became the standard drug. However, side effects still remained a concern and responses lasted for only about a year in the majority of patients. By the 1960’s, the standards for the hormonal treatment of breast cancer were established. Premenopausal women were to be treated with ovarian irradiation therapy or bilateral oophorectomy. However, based on data from the clinical trials, postmenopausal patients with advanced breast cancer were to be treated with high dose of the most potent synthetic estrogenic compound diethylstilboestrol (Kennedy 1965). Overall, one could anticipate that 36% of patients would respond favorably to high dose estrogen therapy (Kennedy 1965). However, the molecular mechanisms of the anticancer action of estrogen remained elusive. In 1970 Haddow (Haddow 1970) was not enthusiastic about the overall prospects of chemical therapy of breast cancer, he felt that it was important that safer less toxic “estrogens” were developed that might extend therapeutic use. There were clues that deciphering the mysteries of endocrine therapy, such as unknown mechanisms of tumor regression after high-dose estrogen therapy, which could be of major benefit for patient’s treatment. Haddow stated: “In spite of the extremely limited practicality of such measure [high dose estrogen], the extraordinary extent of tumor regression observed in perhaps 1% of post-menopausal cases has always been regarded as of major theoretical importance, and it is a matter of some disappointment that so much of the underlying mechanisms continues to elude us”. However, as noted previously, high dose estrogen therapy was more successful as a treatment for breast cancer the farther the woman was from the menopause. Estrogen withdrawal somehow played a role in sensitizing tumors to the antitumor actions of estrogen, but this fact was not appreciated at that time. We will return to this concept.

Elwood Jensen predicted the existence of estrogen receptor (ER) in 1962 (Jensen 1962), and the isolation and identification of the ER protein by Toft and Gorski occurred in 1966 (Toft and Gorski 1966). The mediating role of the ER in the estrogen responsiveness of breast
cancer was established, and eventually the ER became the molecular target for targeted therapy and prevention of ER-positive breast cancer (Jensen and Jordan 2003). It was suggested (Lacassagne 1936) in 1936 that a therapeutic agent to block estrogen action would be useful in breast cancer prevention, but there were no clues. Potential candidate antiestrogens were only discovered 20 years later in the late 1950s, but these agents were identified and screened as contraceptive drugs in laboratory animals. MER25 (Lerner et al. 1958), which was first reported as a non-steroidal antiestrogen and subsequently found to be a post-coital contraceptive in animals (Lerner and Jordan 1990). But the drug was too toxic. The first clinically useful compound MRL41 or clomiphene was tested in women; however, it was not a contraceptive, but actually induced ovulation. Nevertheless, clinical trials of clomiphene in the early 1960’s did move forward to evaluate its activity in the treatment of breast cancer, but were terminated because of concerns about the drug’s potential to cause cataracts (Jordan 2003). In parallel studies stimulated by the initial reports of the non-steroidal antiestrogens, ICI 46,474, the pure trans-isomer of a substituted triphenylethylene, was discovered at Imperial Chemicals Industry (ICI) Pharmaceuticals (now Astra Zeneca) and was described as a postcoital contraceptive in the rat (Harper and Walpole 1967). The Head of the Fertility Control program, Arthur Walpole, earlier in his career was interested in why only some postmenopausal women with metastatic breast cancer respond favorably to high dose estrogen therapy (Walpole 1948). Later Walpole ensured that ICI 46,474 was tested in the clinic and placed on the market as an orphan drug while ICI invested in the scientific research by others in academia to conduct a systematic study of the anticancer actions of tamoxifen and its metabolites (Jordan 2008). This investment reinvented tamoxifen as the first anticancer agent specifically targeted to the ER in the tumor and created the scientific principles to ultimately establish tamoxifen as the “gold standard” for the adjuvant therapy of breast cancer and as the first chemopreventative agent that reduces the incidence of breast cancer in women with elevated risk (Fisher et al. 1999; EBCTCG 2005).

2. Development and clinical application of antihormonal therapy

Since the clinical application of the laboratory principle of targeting the ER with long-term antihormonal therapy (Jordan 2008) to treat breast cancer has become the standard of care, two different approaches to adjuvant antihormonal therapy have been developed in the past 30 years: first, is the blockade of estrogen-stimulated growth (Jensen and Jordan 2003) at the tumor ERs with antiestrogens, and the second one, is the use of aromatase inhibitors to block estrogen biosynthesis in postmenopausal patients (Jordan and Brodie 2007). Tamoxifen was originally referred to as a non-steroidal antiestrogen (Harper and Walpole 1967). However, as more has become known about its molecular pharmacology (Jordan 2001) it has become the pioneering Selective Estrogen Receptor Modulator (SERM). The concept of SERM action was defined by four main pieces of laboratory evidence: 1) ER-positive breast cancer cells inoculated into athymic mice grew into tumors in response to estradiol, but not to tamoxifen (antiestrogenic action), however both estradiol and tamoxifen induced uterine weight increase in mice (estrogen action) (Jordan and Robinson 1987); 2) raloxifene (another non-steroidal antiestrogen), which is less estrogenic in rat uterus, maintained the bone density in ovariectomized rats (estrogen action), as did tamoxifen (Jordan et al. 1987), and prevented mammary carcinogenesis (antiestrogenic action) (Gottardis and Jordan 1987); 3) tamoxifen blocked estradiol-induced growth of ER-positive breast cancer cells in athymic mice.
(antiestrogenic action), but induced rapid growth of ER-positive endometrial carcinomas (estrogenic action) (Gottardis et al. 1988); 4) raloxifene was less effective in promoting endometrial cancer growth than tamoxifen (less estrogenic action in uterine tissue) (Gottardis et al. 1990). These laboratory results all translated into clinical practice where it was shown that tamoxifen effectively can reduce the incidence of breast cancer in high-risk pre- and postmenopausal women, however increases the incidence of blood clots and endometrial cancer, which is linked to estrogen-like actions of tamoxifen in these tissues in postmenopausal women, who have a low-estrogen environment (Fisher et al. 1998).

Aromatase inhibitors have an advantage in the therapy of postmenopausal patients over tamoxifen, firstly, because there are fewer side effects, such as blood clots or endometrial cancer, and aromatase inhibitors have a small, but still significant efficacy in increasing disease free survival (Howell et al. 2005). However, most postmenopausal patients worldwide continue treatment with tamoxifen, either for economic reasons or because they were hysterectomized and also have a low risk of developing blood clots (low body mass index and are athletically active). In premenopausal women, long term tamoxifen is the antihormonal therapy of choice for the treatment of ductal carcinoma in situ (DCIS) (Fisher et al. 1999), ER-positive breast cancer treatment (EBCTCG 2005) and the reduction of breast cancer incidence in those premenopausal women at elevated risk (Fisher et al. 1998). It is important to stress that premenopausal women treated with tamoxifen do not have elevations in endometrial cancer and blood clots, thus risk: benefit ratio is in favor of tamoxifen treatment (Gail et al. 1999).

The development of raloxifene from a laboratory concept (Jordan 2007) to a clinically effective drug to prevent both osteoporosis and breast cancer (Cummings et al. 1999; Vogel et al. 2006) has created new opportunities for clinical applications of SERMs. Raloxifene is the result. However, the biggest advantage of raloxifene is that it does not increase the incidence of endometrial cancer (Vogel et al. 2006), which was noted in postmenopausal women taking tamoxifen (Fisher et al. 1998). Raloxifene is used primarily for the prevention of osteoporosis and for the prevention of breast cancer in high risk postmenopausal women. The current clinical trend for the use of antihormonal therapy for the treatment and prevention of breast cancer is to employ long-term treatment durations. Currently aromatase inhibitors are used for a full 5 years after 5 years of tamoxifen (Goss et al. 2005). Though, the clinical application of the SERM concept has proven itself to be successful for the prevention of osteoporosis and 50% of breast cancers (Vogel et al. 2006; Vogel et al. 2010), drug resistance remains an important issue arising from long-term SERM treatment. Studies have shown that after long-term SERM treatment, the pharmacology of the SERMs changes from an inhibitory antiestrogenic state to a stimulatory estrogen-like response (Gottardis and Jordan 1988).

3. Evolution of SERM resistance as deciphered by the laboratory models

Clinical and laboratory studies have identified possible mechanisms for the acquired resistance to SERMs, and tamoxifen. Acquired resistance to SERMs is unique as the tumors are SERM stimulated for growth (Howell et al. 1992). The first laboratory model (Gottardis and Jordan 1988; Gottardis et al. 1988; Gottardis et al. 1990) of transplantable tamoxifen resistant cells demonstrated that 1) tamoxifen or estrogen can cause tumors to grow, 2) tumors require a liganded receptor to grow, 3) an aromatase inhibitors (estrogen deprivation) or a pure antiestrogen that causes ER degradation would be useful second line
agents, 4) there was cross resistance with other SERMs (O'Regan et al. 2002). Currently, numerous model systems exist to study SERM resistance. Some are engineered to increase the likelihood of resistance (Osborne et al. 2003) and others are engineered by transfection of the aromatase gene to study resistance to aromatase inhibitors and compare them with tamoxifen (Brodie et al. 2003). In contrast, others have chosen to develop models naturally through selective pressure either in vivo or in vitro. The natural selection approach is to either continuously transplant the resulting SERM resistant breast cancer into SERM-treated athymic animals (Wolf and Jordan 1993; Lee et al. 2000) or to employ strategies in vitro that use continuous SERM treatment (Herman and Katzenellenbogen 1996; Liu et al. 2003; Park et al. 2005) or long term estrogen deprivation in culture (Song et al. 2001; Lewis et al. 2005). Distinct phases of resistance were elucidated with the use of unique models of tamoxifen-resistant breast cancer developed in vivo, in order to better understand the biological consequences of extended antiestrogen treatment on the survival of breast cancer. The model for the treatment phase was developed by injecting ERα-positive MCF-7 cells into athymic mice and supplementing them with post-menopausal doses of estradiol (E2) (86–93 pg/ml) (Robinson and Jordan 1989), which were estradiol-stimulated and tamoxifen (TAM)-inhibited (Figure 1).

Fig. 1. Evolution of SERM resistance as observed in animal models.

With short term treatment (<2 years) with tamoxifen Phase I TAM-resistant breast tumors developed, which were stimulated to grow by both E2 and tamoxifen (Figure 1) (Gottardis and Jordan 1988; Osborne et al. 1991). The novel model of Phase II resistance to tamoxifen was developed by long-term treatment (>5 years) of breast tumors with tamoxifen (MCF-7TAMLt). These MCF-7TAMLt tumors were stimulated to grow with tamoxifen, but paradoxically were inhibited by estradiol (Figure 1) (Wolf and Jordan 1993; Yao et al. 2000; Osipo et al. 2003). The phase when all known therapies fail and only E2-inhibit the growth is referred to as phase III resistance (Figure 1) (Jordan 2004). Interestingly, during the progression from the treatment phase to Phase III resistance, a cyclic phenomenon was observed where initially estradiol-inhibited growth of Phase II TAM-resistant tumors followed by re-sensitization to estradiol as a growth stimulant (Yao et al. 2000). These new estradiol-stimulated MCF-7 tumors from Phase II tamoxifen-resistant tumors were inhibited by treatment with either TAM or fulvestrant demonstrating complete reversal of drug resistance to tamoxifen (Yao et al. 2000). A similar phenomenon was observed with
raloxifen-resistance (Balaburski et al. 2010). In addition to SERM-resistant tumors, estradiol, at physiologic concentrations, has also been shown to induce apoptosis in long term estrogen deprived (LTED) breast cancer cells in vitro and in vivo. We noted previously, that in the past, pharmacologic estrogen was employed in therapy of advanced breast cancer that resulted in favorable responses with regression of disease (Haddow 1944). Estrogen therapy yields as high as 40% response rate as first-line treatment in patients with hormonally sensitive breast cancer with metastatic disease (Ingle et al. 1981) and approximately 31% in patients heavily pre-treated with previous endocrine therapies (Lonning et al. 2001). The unique aspect of current laboratory findings is that physiologic estrogen can induce tumor regression in long-term anti-hormone drug resistance (Wolf and Jordan 1993; Yao et al. 2000; Song et al. 2001; Jordan and Ford 2011). But what are the mechanisms?

Known mechanisms of estrogen-induced apoptosis in LTE D breast cancer cells

Fig. 2. Mechanisms of estrogen-induced apoptosis in Long-Term Estrogen Deprived (LTED) breast cancer cells. Both FasR/FasL death-signaling and mitochondrial pathways are involved.

4. Mechanism of estrogen-induced apoptosis

To investigate the mechanisms of estradiol-induced apoptosis SERM-stimulated models (Liu et al. 2003; Osipo et al. 2003) or long-term estrogen deprived MCF-7 breast cancer cell lines (Song et al. 2001; Lewis et al. 2005; Lewis et al. 2005) have been interrogated. A link between estradiol-induced apoptosis and activation of the FasR/FasL death-signaling pathway was demonstrated in tamoxifen-stimulated breast cancer tumors by inducing the death receptor
Fas with physiologic levels of estradiol and suppressing the antiapoptotic/prosurvival factors NF-κB and HER2/neu (Osipo et al. 2003; Lewis et al. 2005). A similar finding was reported (Liu et al. 2003) for raloxifene-resistant tumor cells where the growth of raloxifene-resistant MCF-7/Ral cells in vitro and in vivo was repressed by estradiol via mechanism involving increased Fas expression and decreased NF-κB activity. Furthermore, MCF-7 cells deprived of estrogen for up to 24 months (MCF-7/LTED) in vitro expressed high levels of Fas compared to the parental MCF-7 cells, which do not express Fas and treatment of the MCF-7/LTED cells with estradiol resulted in a marked increase in Fas ligand (FasL) in these cells (Song et al. 2001). It was also noted that mitochondrial pathway could play a role in mediating estrogen induced apoptosis as the basal expression levels of Bcl-2 were higher in these cells than in the parental MCF-7 cells. Estradiol induced apoptosis occurs in a LTED breast cancer cell line named MCF-7:5C by neutralization of the Bcl-2/Bcl-XL proteins, and upregulation of proapoptotic proteins such as Bax, Bak and Bim, which proves the role of intrinsic mitochondrial pathway (Lewis et al. 2005) (Figure 2).

In MCF-7:5C cells the expression of several pro-apoptotic proteins—including Bax, Bak, Noxa, Puma, and p53—are markedly increased with estradiol treatment and blockade of Bax and Bim expression using siRNAs almost completely reversed the apoptotic effect of estradiol. Estradiol treatment also led to a loss of mitochondrial potential and a dramatic increase in the release of cytochrome c from the mitochondria, which resulted in activation of caspases and cleavage of PARP. Furthermore, overexpression of anti-apoptotic Bcl-xL was able to protect MCF-7:5C cells from estradiol-induced apoptosis. This particular study was the first to show a link between estradiol-induced cell death and activation of the mitochondrial apoptotic pathway using a breast cancer cell model resistant to estrogen withdrawal (Lewis et al. 2005). Besides the action on the mitochondrial pathway, Bcl-2 overexpression increases cellular glutathione (GSH) level which is associated with increased resistance to chemotherapy-induced apoptosis (Voehringer 1999). GSH is a water-soluble tripeptide composed of glutamine, cysteine, and glycine. It is the most abundant intracellular small molecule thiol present in mammalian cells and it serves as a potent intracellular antioxidant protecting cells from toxins such as free radicals (Schroder et al. 1996; Anderson et al. 1999). Changes in GSH homeostasis have been implicated in the etiology and progression of some diseases and breast cancer (Townsend et al. 2003) and studies have shown that elevated levels of GSH prevent apoptotic cell death whereas depletion of GSH facilitates apoptosis (Anderson et al. 1999). Our laboratory has found evidence which suggests that GSH participates in retarding apoptosis in antihormone-resistant MCF-7:2A human breast cancer cells, which have ~60% elevated levels of GSH compared to wild-type MCF-7 cells and unable to undergo estrogen-induced apoptosis within 1 week unlike MCF-7:5C cells, and that depletion of GSH by 100 μM of L-buthionine sulfoximine (BSO), a potent inhibitor of glutathione biosynthesis, sensitizes these resistant cells to estradiol-induced apoptosis (Lewis-Wambi et al. 2008). However, the question arises as to the actual mechanism of the apoptotic trigger mediated by the ER complex.

5. Structure-function relationship studies for deciphering estrogen-induced apoptosis

The fact that SERMs do not affect the spontaneous growth of MCF-7:5C cells, but can completely block estradiol-induced apoptosis, was an important clue that the shape of the
ER can be modulated to prevent apoptosis. Extensive structure-function relationship studies were initially used to develop a molecular model of estrogen and antiestrogen action (Lieberman et al. 1983; Jordan et al. 1984; Jordan et al. 1986). The hypothetical model presumed the envelopment of a planar estrogen within the ligand-binding domain (LBD) of the ER complex. In contrast, the three-dimensional triphenylethylene binding in the LBD cavity prevents full ER’s activation by keeping the LBD open. This structural perturbation of the ER complex is achieved by a correctly positioned bulky side chain on the SERM. This model was enhanced by the subsequent studies to solve the X-ray crystallography of the LBD ER’s bound with an estrogen or an antiestrogen (Brzozowski et al. 1997; Shiau et al. 1998). The LBD of ERα is formed by H2-H11 helices and the hairpin β-sheet, while H12, in the agonist bound conformation closes over the LBD cavity filled with E2. E2 is aligned in the cavity by hydrogen bonds at both ends of the ligand, particularly the 3-OH group at the A-ring end of E2. This allows hydrophobic van der Waals contacts along the lipophilic rings of E2, in particular between Phe404 and E2’s A-ring, to promote a low energy conformation (Brzozowski et al. 1997). This results in sealing of the ligand-binding cavity by H12, and exposes the AF-2 motif at the surface of the receptor for interaction with coactivators to promote transcriptional transactivation. In contrast, 4-hydroxytamoxifen binds to ER’s LBD to block the closure of the cavity by relocating H12 away from the binding pocket, thus preventing coactivator molecules from binding to the appropriate site on the external surface of the complex, which produces an antiestrogenic effect (Shiau et al. 1998). Therefore, it is the external shape of the ERs that is being modulated by the ligand which dictates the binding of coactivator molecules. In other words, the shape of the ligand actually causes the receptor to change shape and programs the ER complex to be able to bind coregulator molecules. However, the simple model of a coregulator controlling the biology of an ER complex is not that simple. The modulation of the estrogen target gene is in fact, regulated by a dynamic process of assembly and destruction of transcription complex at the promoter site of a target gene. After ER is bound to an agonist ligand, its conformation changes allowing coregulator molecules to bind to the complex, for example, SRC-3. SRC-3 is a core coactivator that also attracts other coregulators that do not directly bind to ER, such as p300/CBP histone acetyltransferase, CARM1 methyltransferase, and ubiquitin ligases Ubc and UbL. All of these coregulators perform specific subreactions within the protein complex of ER and DNA necessary for transcription of target genes, such as chromatin remodeling through methylation and acetylation modifications, and also direct their enzymatic activity towards adjacent factors, which promote dissociation of the coactivator complex and subsequent ubiquitinilation of select components for proteosomal degradation. As a result, this allows the next cycle of coactivator-receptor-DNA interactions to proceed and the binding and degradation of transcription complexes sustaining the gene transcription (Lonard et al. 2000). However, although AF-2 is deactivated by 4OH TAM, the 4OH TAM:ERα complex has estrogen-like activity (Levenson et al. 1998), whereas raloxifene does not (Levenson et al. 1997). This is believed to be because the side chain of raloxifene shields and neutralizes asp351 to block estrogen action (Levenson and Jordan 1998). In contrast the side chain of tamoxifen is too short. It appears that when helix 12 is not positioned correctly the exposed asp351 can interact with AF-1 to produce estrogen action. This estrogen-like activity can be inhibited by substituting asp351 for glycine an uncharged amino acid (MacGregor Schafer et al. 2000). However, knowledge of the structure of the
4OHTAM: ER LBD complex (Shiau et al. 1998) led to the idea that all estrogens may not be the same in their interactions with ER (Jordan et al. 2001). Previous studies suggest that non-planar TPEs with a bulky phenyl substituent prevents helix-12 from completely sealing the LBD pocket (Jordan et al. 2001). This physical event creates a putative ‘anti-estrogen like’ configuration within the complex. However, the complex is not anti-estrogenic because Asp351 is exposed to communicate with AF-1 thus causing estrogen-like action. Therefore, there are putative Class I (planar) and Class II (non-planar) estrogens (Jordan et al. 2001). A similar classification and conclusion has been proposed (Gust et al. 2001), but the biological consequences of this classification were unknown until recently.

To further address the hypothesis that the shape of the ER complex can be controlled by the shape of an estrogen, and thereby altering its functional properties, such as induction of apoptosis, a range of hydroxylated TPEs was synthesized (Figure 3) to establish new tools to investigate the relationship of shape with estrogenic activity through the exposure of asp351 (Maximov et al. 2010).

![Synthesized non-steroidal estrogens](image)

Fig. 3. Synthesized class II non-steroidal estrogens. All estrogens are hydroxylated derivatives of triphenylethylene; 1 – 3-hyroxytriphenylethylene (3OHTPE), 2- bisphenoltripenylethylene, 3 – E-dihydroxytriphenylethylene, 4- Z-dihydroxytriphenylethylene, 5- ethoxytripenylethylene, and Endoxifen (a metabolite of the antiestrogenic triphenylethylene tamoxifen with high affinity for the estrogen receptor).

We compared and contrasted the estrogen-like properties of the hydroxylated TPEs to promote proliferation in the ERα-positive human breast cancer cell line MCF-7:WS8 cells (Figure 4A), which are hypersensitive to the proliferative actions of E2. Compounds were compared with the tamoxifen metabolites 4-OHT and endoxifen. Results show that our
MCF-7:WS8 human breast cancer cells were exquisitely sensitive to E2 which produced a concentration-dependent increase in growth, and all of the TPEs were potent agonists with the ability to stimulate MCF-7:WS8 breast cancer cell growth, however, their agonist potency was less compared to E2. The metabolites, 4-OHT and endoxifen, had no significant agonist effect in MCF-7:WS8 cells, however, these compounds at 1 µM were able to completely inhibit estradiol-stimulated MCF-7:WS8 breast cancer cell growth, thus confirming their role as antiestrogens (data not shown). To determine the ability of the test TPEs to activate the ER, MCF-7:WS8 cells were transiently transfected with an ERE-luciferase reporter gene encoding the firefly reporter gene with 5 consecutive Estrogen Responsive Elements (EREs) under the control of a TATA promoter. The binding of ligand-activated ER complex at the EREs in the promoter of the luciferase gene activates transcription. The measurement of the luciferase expression levels permits a determination of agonist activity of the TPE:ER complex. All the phenolic TPEs were estrogenic and induced the increase of ERE-luciferase activity, but were less potent compared to E2. To confirm and advance the hypothesis that the shape of the estrogen ER complex was different for planar and nonplanar (TPE-like) estrogens, series of tested phenolic TPEs were evaluated in the ER-negative breast cancer cell line T47D:C42 (Pink et al. 1996) which was transiently transfected with an ERE luciferase plasmid and either the wild-type ER or the D351G mutant ER plasmids. Previously it was found that the mutant D351G ER completely suppressed estrogen-like properties of 4-OHT at an endogenous TGFα target gene (MacGregor Schafer et al. 2000). We established that in the presence of the wild-type ER all of the tested TPE compounds were potent agonists with the ability to significantly enhance ERE luciferase activity (Figure 4C). In contrast, when the D351G mutant ER gene was transfected with the ERE luciferase reporter only the planar E2 was estrogenic whereas the TPEs did not activate the ERE reporter gene (Figure 4D). These results confirm the importance of Asp351 in ER activation by TPE ligands to trigger estrogen action. To further confirm the hypothesis, the best “fits” of the tested TPEs and endoxifen, obtained from docking simulations ran against the antagonist conformation of the ER, were superimposed on the experimental agonist conformation of the ER. Overall the TPEs are unlikely to be accommodated in the agonist conformation of the ER due to the sterical clashes between “Leu crown”, mostly Leu525 and Leu540, helix 12 and ligands, indicating that these ligands most likely bind to ER’s conformation more closely related with the antagonist form. X-ray crystallography of ER-4OHTAM and ER-Raloxifene complexes, demonstrating that the presence of the alkylaminoethoxy sidechain of 4OHTAM is crucial for the ER to gain an antagonistic conformation by displacing the H12 of the receptor by 4OHTAM’s bulky sidechain, thus preventing the binding of the coactivators (Shiau et al. 1998). The absence of the alkylaminoethoxy sidechain on the tested TPEs does not allow these compounds to act as antiestrogens, like 4-OHT or endoxifen, which possesses the alkylaminoethoxy sidechain (Shiau et al. 1998). However, the fact that these TPEs were able to significantly induce growth and ERE activation in MCF-7:WS8 cells demonstrated that they are still full agonists, despite the changes in biological potencies of the tested TPEs, due to repositioning of the hydroxyl groups and addition of the ethoxy group. Thus cell growth is a very sensitive property of the ligand:ER complex and can occur minimally with an AF-1 function alone in the case of TPEs but also with the possibility for interacting with a perturbated LBD. 4OHT does not stimulate growth so possibly a corepressor binds in the case of a SERM:ER complex. An interesting aspect of the study (Maximov et al. 2010) is the importance of Asp351 in activation of the ER thereby acting as a molecular test for the presumed structure.
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Fig. 4. A: Agonist activity in MCF-7:WS8 cells of synthesized TPEs and E2 and anti-estrogens 4-OHT and Endoxifen; B: E2 induces apoptosis in long-term estrogen deprived MCF-7:5C cells and synthesized TPEs are unable to act as full agonists resembling more anti-estrogens 4-OHT and Endoxifen; C: E2 and all TPEs are able to increase the activity of luciferase in T47D:C4:2 cells transiently transfected with wild-type ER DNA construct; D: E2 is the only agonist in D351G ER mutant T47D:C4:2 cells, as TPEs are unable to increase the luciferase activity in cells expressing the mutant form of ER, indicating the importance of Asp351 of the ER for activation with non-planar TPEs.

of the TPE:ER complex. Based on the X-ray crystallography of the ER in complex with 4OHTAM (Shiau et al. 1998) and raloxifene (Brzozowski et al. 1997), it was determined that the basic side chains of these antiestrogens are in proximity of Asp351 in the ER. It was hypothesized that this interaction with raloxifene actually neutralizes and shields Asp351 preventing it from interacting with ligand-independent activating function 1 (AF-1). In contrast, 4OHTAM possesses some estrogenic activity, because the side chain is too short (Shiau et al. 1998). Substitution of Asp351 with Glycine which is a non-charged aminoacid, leads to loss of estrogenic activity of the ER bound with 4OHTAM (MacGregor Schafer et al. 2000; Levenson et al. 2001). Results from ERE luciferase assays in T47:C4:2 cells transiently transfected with wild type and D351G mutant ER expression plasmids demonstrated that wild type ER was activated by all of the tested TPEs, however substitution of Asp351 by Gly prevented the increase of ERE luciferase activity by all TPEs and only planar E2, which does
not interact with Asp351 at all, or exposes it on the surface of the complex, was able to activate ERE in D351G ER transfected cells. This confirms and expands the classification of estrogens, where planar estrogens such as E2 are classified as class I and all TPE-related estrogens are classified as class II estrogens based on the mechanism of activation of the ER (Jordan et al. 2001).

Further we tested the hypothesis that, the shape of the ER complex with either planar estrogens (Class I) or angular estrogens (Class II), can modulate the apoptotic actions of estrogen through the shape of the resulting complex. In this study MCF-7:5C cells were employed to investigate the actions of 4-OHT and our model TPEs on estradiol-induced apoptosis. As estrogen-induced apoptosis can be reversed in a concentration related manner by the nonsteroidal antiestrogen 4-OHT, paradoxically, all tested TPEs were able to reverse the apoptotic effect of estradiol in MCF-7:5C cells, at the same time the tested TPEs alone were not able to induce apoptosis in these cells significantly (Figure 4B). However, the tested TPEs have still retained their ability to induce ERE-luciferase activity in MCF-7:5C cells, indicating that these compounds are still agonists of the ER in these cells, but biologically acted as antagonists. Besides differences in biological effects of TPEs in MCF-7 cells and MCF-7:5C cells, biochemical effects of tested TPEs on ER complex similar to those with 4-OHT were studied. 4-OHT is known to retard the destruction of the 4-OHT ER complex (Pink and Jordan 1996; Wijayaratne and McDonnell 2001). Similarly, the TPEs do not facilitate the rapid destruction of the TPE:ER complex, as it was shown via Western blotting that the TPE:ER levels are analogous to 4-OHT:ER levels rather than estradiol ER-like, where ER is rapidly degraded. As it was noted previously, ER degradation plays a crucial role in estrogen-mediated gene expression. It was previously shown that ER protein degradation is proteosome mediated (Lonard et al. 2000; Reid et al. 2003), and ER coactivator SRC3/AIB1 links the transcriptional activity of the receptor and its proteosome degradation (Shao et al. 2004). Our results indicate that the transcriptional activity of ER, based on qRT-PCR results, is similar on the pS2 gene in both MCF-7:WS8 cells and MCF-7:5C cells with the tested TPE compounds, and based on our ChIP assay results for evaluating the ER’s recruitment on the pS2 gene promoter, the E2:ER complex has robust binding in the promoter region and SRC-3 is detected presumably bound to the ER complex, however, 4-OHT:ER complexes only have modest binding of ERs and virtually no SRC-3 in the promoter region, at the same time, the TPEs permit some binding of the TPE:ER complexes in the promoter region but there are lower levels of SRC-3 and a reduced ability to stimulate PS2 mRNA synthesis (Figure 5).

We believe that the changed conformation of the TPE:ER complex, prevents the complete closure of H12 over the ligand-binding cavity and thus does not allow co-activators to bind to the incompletely open AF-2 motif on the ER’s surface. Indeed, LeClercq’s group (Bourgoin-Voillard et al. 2010) have recently confirmed and extended our molecular classifications of estrogens, with a larger series of compounds and have also shown that an angular TPE does not cause the destruction of the ER complex in a manner analogous to estradiol when MCF-7 cells are examined by immunohistochemistry for the ER, and that the putative Class II estrogens that do not permit the appropriate sealing of the LBD with helix 12 do not efficiently bind co-activators, therefore our respective studies are in agreement.

In summary, the proposed hypothesis that the TPE-ER complex significantly changes the shape of the ER to adopt a conformation that mimics that adopted by 4-OHT when it binds to the ER. A co-activator now has difficulty in binding to the TPE-ER complex.
appropriately, but whereas this does affect cell replication, it dramatically impairs the events that must be triggered to cause apoptosis. Future studies will confirm or refute our hypothesis based upon the known intrinsic activity of mutant ERs and their capacity to investigate estrogen-target genes.

Fig. 5. A&B: ChIP analysis performed in MCF-7:WS8 cells with pS2 promoter region was pulled down via anti-ERα antibody (A) and anti-SRC3/AIB1 antibody (B); C&D: ChIP analysis performed in MCF-7:5C cells with pS2 promoter region pulled down via anti-ERα antibody (C) and anti-SRC3/AIB1 antibody (D). All results indicate that in both cell lines tested TPEs and E2 recruit ERα complex to the pS2 promoter region, but interestingly, class II estrogens are unable to co-recruit sufficient amount of SRC-3 co-activator, unlike E2.

6. Relevance to current clinical research

Laboratory studies show that low concentrations of estrogen can cause apoptotic death of breast tumor cells, following estrogen deprivation with antihormonal treatment. This has translated very well into the clinic, and recent clinical trials have demonstrated that low-dose estrogen treatment can effectively be utilized after the formation of resistance to antihormonal treatment. Ellis and colleagues (Ellis et al. 2009) have shown, that a daily dose of 6 mg of estradiol could stop the growth of tumors or even cause them to shrink in about 25% of women with metastatic breast cancer that had developed resistance to antihormonal therapy. At the same time, these results correlate with earlier results obtained by Lonning and coworkers (Lonning et al. 2001), who have studied the efficacy of high dose of DES on the responsiveness of metastatic breast cancer following exhaustive antihormonal treatment.
with tamoxifen, aromatase inhibitors and etc. 4 out of 32 patients had complete responses (Lonning et al. 2001) and 1 patient after 5 year treatment with DES had no recurrence for a following 6 years (Lonning 2009). The question at that moment remains whether estrogen at physiologic concentrations can be efficient as antitumor agent in estrogen-deprived breast tumors. As mentioned previously, Ellis and coworkers have demonstrated that an equivalent clinical benefit for high (30 mg daily) and low (6 mg daily) dose of estradiol in metastatic breast cancer patients who had failed aromatase inhibitor therapy, which is long-term estrogen deprivation. Overall, the results demonstrate that low dose estrogen therapy has fewer systemic side effects, but the same efficacy as a treatment for long-term antihormone resistant breast cancer as high dose estrogen therapy. This can be seen as “replacement with” physiologic estrogen to premenopausal levels. The benefit-risk ratio is in favor of low-dose estrogen therapy. These results correlate well with results from WHI trial of estrogen-replacement therapy (ERT) in hysterectomized postmenopausal women (LaCroix et al. 2011). The WHI results show a sustained reduction in the incidence of breast cancer in postmenopausal women up to 5 years after the intervention with conjugated equine estrogens for 5 years prior. It was demonstrated that the group of patients receiving conjugated equine estrogens had incidence of breast cancer 0.27% in comparison to the control group of patients the incidence was 0.35%. The idea that woman’s own estrogen can act as an antitumor agent after estrogen-deprivation to prevent metastization and tumor growth (Wolf and Jordan 1993) has lead to incorporation into the Study of Letrozole Extension (SOLE) trial. This trial is addressing the question whether regular drug holydays can decrease recurrence of breast cancer by physiologic estrogen after deprivation with aromatase inhibitor letrozole. Subsequent trials may have to use ERT for a few weeks to trigger apoptosis.

7. Conclusion
Taken together, the demonstrations of the apoptotic actions of estrogen as a potential anticancer agent in postmenopausal breast cancer patients, now provides a rationale to further explore and decipher mechanisms of estrogen-induced apoptosis. There is a possibility that future studies on the molecular mechanism of estrogen-induced apoptosis will help to indentify new more safer and specific agents for breast cancer therapy.

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9. References


This book presents novel interesting findings by multiple accomplished investigators in breast cancer. These chapters elucidate new mechanisms of breast cancer cell death as well as discuss new pathways for therapeutic targeting.

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