THE MODULATION OF ESTROGEN INDUCED APOPTOSIS IN BREAST CANCER

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ABSTRACT

Development of acquired antihormone resistance exposes a vulnerability in estrogen receptor (ER) positive breast cancer: estrogen induced apoptosis. Estrogen (E2) induces apoptosis in long-term E2-deprived MCF7 cells (MCF7:5C). Triphenylethylenes (TPEs) which are structurally similar to 4-hydroxytamoxifen (4OHT) were used for mechanistic studies of estrogen induced apoptosis. These TPEs all stimulate growth in MCF-7 cells but unlike the planar estrogens they block estrogen induced apoptosis in the long term estrogen deprived MCF7:5C cells. The TPE:ER complexes did not readily recruit the coactivator SRC3 or ER to the PS2 promoter in MCF-7 and MCF7:5C cells and molecular modeling showed that they prefer to bind to the ER in an antagonistic fashion i.e.: helix 12 not sealing the ligand binding domain (LBD) effectively and therefore reduces critical SRC3 binding. The fully activated ER complex with helix 12 sealing the LBD is suggested to be the appropriate trigger to initiate rapid estrogen induced apoptosis. We determined that E2- induced apoptosis is a delayed process, while paclitaxel immediately causes a G2/M blockade and induces death of MCF7:5C cells. We are first to show that the cellular commitment for E2-triggered apoptosis occur after 24 h. A sequential activation of the intrinsic pathway occurs at 36h of E2 treatment with subsequent induction of the extrinsic apoptotic pathway by 48h. Unlike the trigger by E2 that occurs after 24h the trigger of apoptosis for BP occurs at 4 days with quantifiable apoptotic changes noted at 6 days. A prolonged
induction of endoplasmic reticulum stress (ERS) and inflammatory stress (IS) response genes was observed with subsequent activation of apoptosis related genes in the second week of treatment with BP. Phytoestrogens which are planar compounds act like steroidal estrogens and induce apoptosis in MCF7:5C cells through the ERα and induces ERS, IS and apoptosis related genes as the steroidal estrogens. These data indicate that estrogen-induced apoptosis involves a novel, multidynamic process that is distinctly different from that of a classic cytotoxic chemotherapeutic drug used in breast cancer.
Acknowledgement

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Many Thanks

IFEYINWA OBIORAH
DEDICATION

THIS DISSERTATION IS DEDICATED TO MY FAMILY, WHOSE ENDURING LOVE AND ENCOURAGEMENT HAVE STRENGTHENED MY BELIEF IN MYSELF AND COMMITMENT TO ACHIEVING MY GOALS. I WILL LIKE TO SPECIALLY THANK MY HUSBAND, IKECHUKWU OBIORAH FOR HIS LOVE AND SUPPORT THROUGHOUT THIS PROCESS. TO THE MOST WONDERFUL KIDS IN THE WORLD, SOLU, KAMSI, NULIA AND TIBE, YOU ARE LIGHT OF MY WORLD AND YOU ALL WILL EVER REMAIN MY BEST CHEERLEADERS. TO MY PARENTS, YOUR WORDS OF ENCOURAGEMENT AND PUSH FOR TENACITY RING IN MY EARS. I WILL ALWAYS STRIVE TO MAKE YOU PROUD. TO ALMIGHTY GOD, YOU ARE THE REASON WHY I BREATHE. THIS THESIS IS FOR YOU.
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CHAPTER I

INTRODUCTION

Selective Estrogen-Induced Apoptosis in Breast Cancer

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I.1. Summary

Ovarian estrogen in premenopausal patients or estrogens produced by peripheral aromatization of adrenal androgenic precursors in postmenopausal patients support the growth of breast cancer. As a result of this knowledge, treatment practices evolved throughout the 20th century to either remove the source of estrogen synthesis by ablative surgery (oophorectomy, adrenalectomy or hypophysectomy) or block the actions of estrogen which stimulates tumor growth through the breast tumor estrogen receptor (ER)-signal transduction system(1). Two clinical approaches to breast cancer therapy have proved to be successful(2): either the development of nonsteroidal antiestrogens that block estrogen binding to the ER or the development of aromatase inhibitors which block the peripheral aromatase enzyme system that convert steroidal precursors from the adrenals to estrogens. Both therapeutic advances have resulted in dramatic increases in patient survival if the nonsteroidal antiestrogen tamoxifen or an aromatase inhibitor is given for extended periods (5-10 years) as an adjuvant therapy(3-5).

There is compelling support for the proposition that estrogen is an essential component for the development of breast cancer and is essential for the promotion and replication of breast cancer cells. The first evidence that there was a link between estrogen and the development of breast cancer was presented at the annual meeting of the American Association for Cancer Research in Boston in 1936. Professor Antoine Lacassagne (6) presented his vision of the prevention of breast cancer in the future based on the results he had obtained in laboratory animals by either administering estrogens to develop mammary cancer (7) or removing estrogen through ovariectomy to prevent mammary cancer in high incident strains: “if one accepts the consideration of adenocarcinoma of the breast as the consequences of a special hereditary sensibility to the proliferative actions of oestrone, one is led to imagine a therapeutic preventive
for subjects predisposed by their heredity to this cancer. It would consist- in the near future when knowledge and use of hormones will be better understood- in the suitable use of a hormone antagonist to prevent the stagnation of estrone in the ducts of the breast.” This visionary strategy became a reality with the development of the nonsteroidal antiestrogen, tamoxifen for the treatment of breast cancer (8) and the successful testing of its worth in high risk women to reduce the incidence of breast cancer(9, 10). Thus, the use of an “antiestrogen” to prevent the development of breast cancer was further proof of the critical role of estrogen in the process of breast carcinogenesis. Today, two selective ER modulators (SERMs) tamoxifen and raloxifene are available for the chemoprevention of breast cancer in both the United States and United Kingdom (11, 12).

The final proof of the direct role of estrogen to stimulate breast cancer cell proliferation came from the laboratory. Initially it was difficult to demonstrate that estrogen directly caused the replication of breast cancer cells in vitro but growth of ER positive tumors could be demonstrated in vivo if the same ER positive MCF7 breast cancer cells were inoculated into athymic (immune deficient mice) and treated with estrogen (13). It was to remain a mystery why exogenous estrogen could not stimulate MCF-7 breast cancer cells to grow in vitro until Berthois and colleagues (14) made the landmark discovery that MCF-7 cells had been routinely maintained for more than a decade in media containing high concentrations of phenol red as a pH indicator. Phenol red or a contaminant was actually an estrogen, so cells were already growing maximally. Removal of the phenol red from the media stopped cell replication and the cells now had a robust proliferation in response to exogenous estrogen. The evidence of the relevance of the critical role of endogenous estrogen being necessary for breast cancer development and growth was overwhelming but there was an unexplained paradox lurking in the historical record
The first successful therapy used to treat any cancer was the use of high dose estrogen to treat metastatic breast cancer in postmenopausal patients (15). The encouraging initial trial was the result of laboratory studies, so the treatment strategy was based on translational research. However, the clinical research went a step further. Haddow used this preliminary data (15) to conduct a multicentric clinical trial through the Royal Society of Medicine. He made a discovery: “When the various reports were assembled at the end of that time, it was fascinating to discover that rather general impression, not sufficiently strong from the relatively small numbers in any single group, became reinforced to the point of certainty; namely, the beneficial responses were three times more frequent in women over the age of 60 years than in those under that age; that estrogens may, on the contrary, accelerate the course of mammary cancer in younger women, and that their therapeutic use should be restricted to cases 5 years beyond the menopause. Here was an early and satisfying example of the advantages which may accrue from cooperative clinical trial” (16). Dr Basil Stoll (17) was able to quantify this finding within his own clinical practice and demonstrated that patients more than 5 years beyond menopause had a high probability of a response to high dose estrogen therapy but those less than five years from menopause were unlikely to have a responsive tumor (Table 1).

High dose estrogen therapy for the treatment of metastatic breast cancer in postmenopausal women became the standard of care for 30 years (18-20) until the advent of tamoxifen. Response rates to tamoxifen for postmenopausal patients with metastatic breast cancer were similar at 30% (21, 22), but side effects with tamoxifen were much less severe. This allowed tamoxifen to advance as long term adjuvant therapy and ultimately be shown to save lives. However, in 1970 at the dawn of interest ICI 46, 474 (to become tamoxifen) as an
experimental antiestrogen for the treatment of breast cancer, Sir Alexander Haddow was selected to present the inaugural Karnofsky lecture at the American Society of Clinical Oncology (16). His article paints a gloomy picture for the future of targeted cancer therapies and the remote prospects of success for anticancer agents as had been achieved with the selective toxicity of antibiotics for the cure of infectious disease. He did however highlight a potential glimmer of hope with his statement that reflected upon the pioneering success he had achieved with his discovery of high dose estrogen treatment as the first “chemical therapy” in cancer. “The extraordinary extent of tumour regression observed in perhaps 1% of post-menopausal cases (with estrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanism continues to elude us……” (16)

However, resolution of the paradox of the antitumor actions of estrogen was to be discarded and dismissed with the refocusing on the accepted paradigm of the obvious understanding of the antitumor action of antiestrogen, tamoxifen. It is therefore ironic that through the clinical development of tamoxifen as a long term adjuvant therapy and the necessity to examine the evolution of acquired resistance to long term tamoxifen, that the veil should be lifted on Haddow’s paradox and the new biology of estrogen-induced apoptosis be discovered.
<table>
<thead>
<tr>
<th>Age Since Menopause</th>
<th>Patient number</th>
<th>% Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postmenopausal 0-5 Years</td>
<td>63</td>
<td>9%</td>
</tr>
<tr>
<td>Postmenopausal &gt; 5 Years</td>
<td>344</td>
<td>35%</td>
</tr>
</tbody>
</table>

Table 1. **Objective response rates in postmenopausal women with metastatic breast cancer using high dose estrogen therapy.** The 407 patients are divided in relation to menopausal status (17). The objective remission rate of breast cancer tumors was higher in women more than 5 years postmenopausal. Source: Obiorah I and Jordan VC. Menopause 2013; 20:372-382.

I.2. The evolution of acquired resistance to SERMs

The first clinically relevant models of acquired resistance to tamoxifen were developed by inoculating MCF-7 cells into ovarectomised athymic mice and initially treating with estrogen for a short time to establish palpable tumors. Continuous tamoxifen treatment of the tumor bearing mice resulted in the growth of tumors despite tamoxifen treatment(23). However, the finding through retransplantation studies that tumors grew because of tamoxifen treatment and also continued to respond to estrogen for growth (24, 25), recapitulated acquired resistance to tamoxifen therapy in the treatment of metastatic breast cancer. Tamoxifen treatment fails in a year or two (21, 22), tumors exhibit a “withdrawal” response from tamoxifen (26, 27) so this is tamoxifen stimulated growth. Finally estrogen can still maintain tumor growth following the cessation of tamoxifen. Second line therapies with either an aromatase inhibitor to prevent
estrogen synthesis or a pure antiestrogen fulvestrant to destroy ER, are effective second line therapies (28, 29). However, the characteristics of the model of acquired resistance did not explain why tamoxifen could be given effectively as a long term adjuvant therapy. If this model was true for all acquired resistance, micrometastatic disease would fail to be controlled for more than two years of adjuvant therapy. It was the retransplantation of tumors into new generations of athymic mice for at least 5 years that was to result in the discovery of a new biology of estrogen action: estrogen induced apoptosis.

I.3. The antitumor action of physiologic estrogen

The finding that acquired resistance to tamoxifen evolves through phases also demonstrates that the cell selection pressure of tamoxifen and its metabolites exposes a vulnerability in cell populations that struggle to survive in a long term tamoxifen (antiestrogen environment) environment. Estrogen triggers apoptosis after 5 years of retransplantation in tamoxifen-treated mice (30, 31). Numerous publications in vivo with SERM (tamoxifen or raloxifene) stimulated tumors (32-34) were of value to document biological control mechanisms. The key to advancing the mechanistic understanding of estrogen induced apoptosis and the reasons for selectivity in breast cancer occurred with the development of long term estrogen deprived cell models to replicate resistance to aromatase inhibitors during adjuvant therapy.

I.4. Cellular models of estrogen deprivation in vitro

To decipher the mechanism involved in antihormone resistance following long term treatment, antiestrogen resistant clonal variants of MCF-7 cells: MCF7:5C and MCF7:2A were established after long-term culture in estrogen free media (35, 36) or long term estrogen deprived (LTED) MCF-7 cell populations examined(37). The variant clone, MCF7:5C cells express wild type ER
but have drastically reduced levels of progesterone receptor (PR) when compared to the parent MCF-7 cells. Using DNA quantification assays 17β estradiol (E$_2$) drastically reduced the growth of the MCF7:5C cells in a time dependent manner that resulted in 90% inhibition after six days of treatment (38). The observed E$_2$ induced inhibition in cell proliferation was confirmed to be apoptosis using annexin v-FITC and DNA binding dye, DAPI staining methods.(38, 39) Although fulvestrant partially inhibited the growth of the MCF7:5C cells, this biological effect was not due to apoptosis. On the other hand, these cells are resistant to 4-hydroxytamoxifen (4OHT), while fulvestrant caused 40% growth inhibition. The induction of E$_2$ induced apoptosis in vitro raised the question of its ability to induce tumor regression in vivo. MCF7:5C cells injected into athymic nude mice resulted in detectable spontaneously growing tumors within 4 weeks. Treatment of the MCF7:5C tumors with E$_2$ resulted in complete regression after 4 weeks of therapy. Involvement of apoptosis in the E$_2$ induced tumor reduction was determined using TUNEL assay.

Another clone, MCF7:2A was identified and characterized from long term estrogen deprived MCF-7 breast cancer cells (36). Significant growth inhibition is observed in the second week of treatment with E$_2$ (40). Similar to the MCF7:5C cells, the MCF7:2A cells grow maximally in the absence of estrogens (36, 40). However, the MCF7:2A are inhibited by both antiestrogens 4OHT and fulvestrant and these cells are both ER/PR positive(36). Because the MCF7:2A cells were initially resistant to E$_2$ induced apoptosis with proapoptotic genes activated much later than in MCF7:5C cells(41), potential mechanisms of action for this resistance were explored. Glutathione (GSH), a tripeptide has been implicated in the tumorigenesis and progression of breast cancer (42). Elevated levels of GSH were observed in MCF7:2A cells and microarray studies show high levels of glutathione synthetase and glutathione peroxidase 2 (40,
Both enzymes are involved in GSH synthesis. Depletion of the cells of GSH using L-buthionine sulfoximine (44), a GSH inhibitor, sensitized the MCF7:2A cells to E2 induced apoptosis(41, 43, 45). Therefore, utilization of BSO with estrogen in patients with ER positive metastatic breast cancer in the context of a clinical trial could potentially inhibit disease progression in patients with exhaustive anti-hormone resistance.

I.5. The modulation of c-Src on estrogen induced apoptosis

c-Src is a non-receptor tyrosine kinase that plays a crucial role in signaling cascades that control cell growth, angiogenesis, invasion adhesion and metastasis and act as an adaptor protein in the crosstalk between the ER and growth factors such as the EGFR family(46, 47). Many of the proliferative actions of estrogen are dependent on c-Src (47). The multiple involvement of c-Src in many intracellular signaling pathways, such as the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways makes it a potential therapeutic target in breast cancer cells. Elevated c-Src activity has been noted in tamoxifen resistant breast cancer cells and treatment of these cells with a c-Src inhibitor suppressed growth, invasion and motility of the endocrine resistant cells (48, 49). However, treatment with therapeutic c-Src inhibitors shows either modest or limited activity in patients with advanced breast cancer (50-52). Because c-Src alone is not sufficient to cause oncogenic transformation, improvement in the value of a c-Src inhibitor could be achieved in combination with other targeted therapies. Due to the fact that we have previously shown that E2 induces apoptosis in the MCF7:5C cells, we reasoned that combination of PP2 and E2 will potentiate the apoptotic effect of E2. Surprisingly, although PP2 was able to block E2 induction of c-Src, PP2 failed to induce apoptosis but rather blocked E2 apoptosis (53), which was confirmed using siRNA to knockdown c-Src in MCF7:5C cells which resulted in a reduction of E2 induced apoptosis (54). These data indicate that E2 may trigger
apoptosis via the non-genomic c-Src signaling pathway. The long term combination treatment of the MCF7:5C cells with PP2 and E2 resulted a new cell line, MCF7:PF cells(55). Interestingly, the MCF7:PF cells grow in response to E2 and contain elevated levels of insulin growth factor-1 receptor beta (IGF-1Rβ)(55). Inhibition of IGF-1Rβ completely prevented E2 stimulated growth in the MCF7: PF cells. Furthermore an increase in regulators of epithelial- mesenchymal transition (EMT) was noted in the MCF7: PF cells suggesting that the antihormone resistant breast cancer cell line has undergone some reprogramming to generate a more aggressive phenotype. However the fact that c-Src can inhibit estrogen induced apoptosis, and that c-Src is part of the non-genomic signal transduction pathway linking growth factor receptors to AKT activation, one could argue the activation of ER at the membrane could result in apoptosis. This hypothesis, though unlikely due to the long delay in estrogen induced apoptosis observed previously (56), was addressed using a novel reagent supplied by Dr John Katzenellenbogen(57).

I.6. E2 induces apoptosis through the genomic pathway in MCF7:5C cells

Estrogens can exert their effects by either classically binding to the nuclear ER and hormone response elements to alter gene transcription (genomic pathway) or by acting through nongenomic signaling via cell surface membrane localized extranuclear ER. The role of the nongenomic pathway in ER signaling was evaluated in E2 induced apoptosis. Estrogen dendrimer conjugate (EDC), a synthetic ligand that only interacts with the extra-nuclear ER to induce nongenomic signaling was used to activate the nongenomic pathway(57). EDC was neither able to activate the estrogen responsive gene, pS2 nor induce apoptosis in the MCF7:5C cells and PP2 blocked the EDC activated nongenomic pathway. Nevertheless, EDC did activate the nongenomic pathway via induction of phosphorylated c-Src, MAPK and AKT in the MCF7:5C cells. This suggests that the non-genomic signaling pathway is not crucial for E2
induced apoptosis but that E₂ immediately activates the non-genomic pathway within minutes with subsequent activation of the genomic pathway. On the other hand, E₂ induces ERE activity which can be blocked by 4OHT but not by PP2. Inhibition of c-Src increased expression of classic ER targeted genes such as pS2, by increasing the accumulation of ER. C-Src is important for phosphorylation and degradation of ER (54) thereby indicating that E₂ triggered apoptosis occurs via the non-classical genomic pathway. Selective induction of AP-1 complexes, consisting of c-Fos, c-Jun and Jun d, were activated by E₂ in MCF7:5C cells suggesting that AP-1 may play an important role in E₂ mediated apoptosis(40).

I.7. Estrogen induced endoplasmic reticulum stress and unfolded protein response

Differential regulation of global gene expression and identification of genes and potential signaling pathways associated with E₂ induced apoptosis was interrogated using Agilent microarray studies. The major groups of MCF7:5C specific genes overrepresented include estrogen signaling, endoplasmic reticulum stress (ERS) and inflammatory response genes and functional testing indicate that ERS and inflammatory stress response led to apoptosis. Endoplasmic reticulum is the key site for the synthesis and folding of proteins. Disturbances of the homeostasis within the endoplasmic reticulum can lead to accumulation of unfolded proteins that result in ERS. In order to overcome, a number of responses occur within the endoplasmic reticulum(58, 59). The first response is the synthesis of new proteins and prevention of accumulation of unfolded proteins(60). Next chaperone proteins, such as BiP/GRP78, trigger an unfolded protein response (UPR) to relieve the ERS. Under normal state, BiP binds to unfolded proteins, PERK, ATF6 and IRE1 and maintains them in an inactive state. Under stress
conditions, BiP dissociates from the UPR proteins and allows their oligomerization and autophosphorylation to initiate a UPR signal that serves to prevent protein synthesis and induce transportation of malfolded proteins to the cytosol for degradation. The UPR signal causes activation of PERK, ATF6 and IRE1. Activation of PERK induces phosphorylation of eIF2α resulting in inhibition of protein synthesis and translocation into the lumen of the endoplasmic reticulum (58, 59). Dissociation of BiP from ATF6 results in its transport to the golgi apparatus where it undergoes cleavage and translocate to the nucleus to induce transcription of UPR genes (58, 61) such as XBP-1. Activated IRE1 induces splicing of XBP-1 which can now efficiently activate UPR (59, 62). Under severe or prolonged ERS, the UPR signal switches from cell survival to apoptosis. The regulation of UPR genes was evaluated in the MCF7:5C cells in response to E2. Significant induction of UPR sensors, IRE1α and PERK/eIF2α by E2 was detected after 24h of treatment which further increased by prolongation of treatment to 72h (54). Treatment of the MCF7:5C cells with a PERK inhibitor blocked phosphorylation of eIF2α and blocked E2 induced apoptosis, thus confirming that ERS is necessary for E2 triggered apoptosis (54).

I.8. Apoptotic pathways mediated by estrogens

We previously reported that MCF7:5C cells respond to E2 by suppressing ERα signaling leading to activation of ERS and inflammatory stress (40). One of the apoptosis pathways associated with ERS mediated apoptosis is via activation of DDIT3/CHOP (58, 63). Studies suggest that PERK/eIF2α and IRE1α/ATF6 signaling are necessary for maximum induction of DDIT3 (64-66). Overexpression of DDIT3 leads to a decrease in bcl-2 protein and translocation of Bax protein from the cytosol to the mitochondria (67, 68). Puthalakath and colleagues (69) reported that ERS induced by diverse stimuli required Bim for initiating apoptosis in a variety of cell lines.
including MCF-7 cells. Knockdown of Bim expression resulted in protection from ERS induced apoptosis. Increased Bim levels noted with ERS induction was dependent on transcriptional activation of DDIT3(69). Therefore prolonged ERS potentially leads to activation of BCL2L11/Bim and Bax. The involvement of the intrinsic pathway in E2 induced apoptosis was first reported by Lewis and colleagues(38) who showed that E2 treatment increased expression of proapoptotic proteins including, Bax, Bak, Bim, Noxa, Puma and p53. Depletion of Bim and Bax using short interfering RNAs (siRNAs) reversed the apoptotic effect of E2. Furthermore mitochondrial pathway activity was determined by loss of mitochondrial potential, increase in cytochrome c release and cleavage of caspase 9 and poly ADP ribose polymerase (PARP) protein. The Fas/Fasl signaling (extrinsic) pathway has been implicated in E2 induced apoptosis. Song and colleagues(37) reported elevated levels of Fas in long term estrogen deprived (LTED)MCF-7 cells and a marked increase of FasL noted with E2 treatment. This correlated with the report from Osipo et al (32) which showed that E2 induced reduction of tamoxifen resistant breast cancer tumors, by activating Fas expression and suppressing NF-kB and HER2/neu activity. A similar observation was noted in raloxifene resistant MCF7 cells (33). The growth of cells *in vitro* and *in vivo* was repressed by E2 by increasing induction of Fas expression and reducing expression of NF-kB. Although there is obvious involvement of both intrinsic and extrinsic pathways in E2 induced apoptosis, none of the previous studies investigated a time course of the sequence of activation of the apoptotic pathways in estrogen induced apoptosis.
I.9. Summary of chapters and projects

The information provided above provides the foundation of studies discussed in chapters III-VIII. Based on the fact that the ER is the major signaling pathway for breast cancer growth and apoptosis, a series of planar and angular estrogens were evaluated on their ability to trigger apoptosis in the MCF7:5C cells. Estrogens can be classified (70) into class I (planar) and class II (angular) estrogens based on the reported crystal structure of the ligand binding domain (LBD) ER with estrogens (E₂, diethylstilbestrol) and antiestrogens (4OHT and raloxifene)(71, 72). The planar estrogens are sealed within the LBD by helix 12 to induce estrogenic action, whereas the bulky side chains of 4OHT and raloxifene prevent helix 12 from sealing the LBD resulting in antiestrogen action. We previously synthesized a range of estrogenic angular triphenylethylenes (TPEs) which are structurally similar to 4OHT. The TPEs (bisphenol (BP), ethoxytriphenylethylene (EtOX) and trihydroxytriphenylethylene (3OHTPE) cause proliferation of MCF-7 cells (73) at higher concentrations when compared to the planar estrogens.

In Chapters III and IV we compare the ability of planar estrogens, to induce apoptosis in the MCF7:5C cells to angular TPEs. The angular TPEs possess antiestrogenic properties similar to those of the 4OHT, which indicate that they make an antiestrogenic like conformation with the ER. The recruitment of ER α or coactivator, SRC3 (AIB1) at the promoter of PS2 (TFF1) gene, an estrogen responsive gene, by the planar and angular estrogens was determined using chromatin immunoprecipitation assays in MCF-7 and MCF7:5C cells. Next we determine the critical point of trigger of E₂ induced apoptosis and compare the apoptotic related genes induced by E₂ to that of a classic cancer chemotherapeutic drug and determined their differential effects on the cell cycle (Chapter V). This also provided the basis of the hypothesis that estrogen induced apoptosis is a slow process and this was further explored using BP an angular TPE to
determine how readily they induce apoptosis and apoptotic related genes (Chapter VI). In addition we elucidated the effects of phytoestrogens on cell growth in fully estrogenised MCF-7 cells, simulating the perimenopausal state, and long term estrogen deprived MCF7:5C cells which simulate the postmenopausal state of a woman after years of estrogen deprivation and compared the effects to that of steroidal estrogens: 17β estradiol (E2) and equilin present in conjugated equine estrogen. We also devised a mechanism of action for their apoptotic and growth inhibitory effects. This was to determine their potential use as chemopreventive agents in breast cancer. These studies provide an excellent means to evaluate the modulation of the effects of various estrogens on breast cancer cells.
CHAPTER II

MATERIALS AND METHODS

II.1. Cell Culture and Reagents

Cell culture media were purchased from Invitrogen Inc. (Grand Island, NY) and fetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT). Compounds 17β estradiol (E\(_2\)), diethylstilbestrol (DES), equilin, estrone, equilenin, equol, genistein and coumestrol, ICI 182,780 and 4-hydroxytamoxifen (4OHT) were obtained from Sigma, St. Louis, MO. Raloxifene (ral) was a gift from Eli Lilly (Indianapolis) and bazedoxifene (baze) was synthesized as previously described (74). The TPEs were synthesized as previously described (73). Caspase 4 inhibitor with the peptide sequence z-LEVD-fmk (z, benzyloxy carbonyl; LEVD -Leu-Glu(OMe)-Val-Asp(OMe),fmk, fluoromethyl ketone) was from Biovision. Dexamethasone was obtained from Tocris Biosciences, Bristol, UK. MCF7:5C were derived from MCF7 cells obtained from the Drs. Bill McGuire and Dean Edwards, San Antonio, Texas as reported previously(35, 39). MCF7:WS8 cells were derived from the original MCF-7 wild type and were maintained in RPMI media supplemented with 10% FCS, 6 ng/ml bovine insulin and penicillin and streptomycin. These have been maintained for > 20 years. The MCF7 cells were cultivated in phenol red-free media containing 10% charcoal dextran treated FCS for 3 days prior to the start of the experiment. MCF7:5C cells were maintained in phenol-red free RPMI media containing 10% charcoal dextran treated FCS, 6ng/ml bovine insulin and penicillin and streptomycin. MDA-MB-231 cells were obtained originally from American type culture collection (ATCC, Rockville,
MC2 (MDA-MB-231 stably transfected with wild type ER) and JM6 (MDA-MB-231 stably transfected with D351G ER) were obtained as previously described (75). MC2 and JM6 cells were maintained in phenol red-free minimal essential medium supplemented with 5% 33 dextran coated, charcoal-treated calf serum, 2 mM glutamine, 6 ng/ml bovine insulin, 100 units/ml penicillin/100 mg/ml streptomycin, nonessential amino acids, and 500 mg/ml G418. The cells were treated with indicated compounds for the specified time and were subsequently harvested for tissue culture experiments.

II.2. Cell growth assay

The cell growth was monitored by measuring the total DNA content per well in 24 well plates. Fifteen thousand cells were plated per well and treatment with either the indicated concentrations of compounds or the control vehicle (0.1% ethanol) were started after 24 hours, in triplicates. Media containing the specific treatments were changed every 48 hours. On day 7, the cells were harvested. The DNA content was assessed using a fluorescent DNA quantitation kit (Cat # 170-2480; Bio-Rad, Hercules, CA, USA). In brief, cells were harvested after the appropriate treatment using hypotonic buffer solution and were subsequently sonicated in 0.1× phosphate-buffered saline (PBS) for 10 seconds, and incubated with a Hoechst dye mixture for 1 hour. For each analysis, three replicate wells were used for three independent experiments.

II.3. Annexin V Analysis of Apoptosis

The annexin V – FITC-labeled Apoptosis Detection Kit I (Pharminen, San Diego, CA) was used to detect and quantify apoptosis by flow cytometry according to the manufacturer’s instructions. In brief, MCF-7:5C cells (1 × 10^6 cells/mL) were seeded in 100-mm dishes and cultured overnight in estrogen-free RPMI 1640 medium containing 10% SFS. The next day, cells
were treated with <0.1% ethanol (control), or the indicated compounds for 72h and then harvested in cold PBS (Invitrogen, Grand Island, NY) and collected by centrifugation for 2 min at 500 × g. Cells were then resuspended and stained simultaneously with either with FITC-labeled annexin V and PI(Pharmingen, San Diego, CA) or DNA binding dye, YO-PRO-1 and propidium iodide (PI) (Life technologies, (Grand Island, NY). Apoptosis was verified based on loss of plasma membrane integrity. Viable cells excluded these dyes, whereas apoptotic cells allowed moderate staining. Cells were analyzed using a fluorescence- activated cell sorter (FACS) flow cytometer (Becton Dickinson, San Jose, CA). The percentage of apoptosis was calculated by adding the percentage of cells stained with either annexin V alone (early apoptosis) and those stained with both PI and annexin V (late apoptosis). Experiments are repeated three times with similar results.

II.4. RNA isolation and real time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and RNAeasy kit according to the manufacturer’s instructions (all cells were treated in triplicates). Briefly, High capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA) was used to generate cDNA was using 1 μg of total RNA in a total volume of 20 μl. The cDNA was subsequently diluted to 500 μl and RT-PCR was performed using ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). In each well 20 ul reaction volume included 10μl SYBR green PCR master mix (Applied Biosystems, Foster City, CA), 125 nM each of forward and reverse primers and 5 μl of diluted cDNA. RT-PCR was performed using specific primers which are shown in Table 2 and the change in expression of transcripts was determined and the ribosomal protein 36B4
mRNA was used as the internal control.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>36B4</td>
<td>GTGTTGCACAATGGCAGCAT</td>
<td>GACACCCCTCCAGGAAAGCGA</td>
</tr>
<tr>
<td>BCL2L11(Bim)</td>
<td>TCGGACTGAGAAACGCAAG</td>
<td>CTCGGTCACCTCAAGAACCCTGAC</td>
</tr>
<tr>
<td>ESR1 (ERα)</td>
<td>GGAGGGCGAGGCTGAAA</td>
<td>GGCCAGGCTGTTCTCTTTAGA</td>
</tr>
<tr>
<td>ESR2 (ERβ)</td>
<td>GGTCGTGTGAGGATGTAAGG</td>
<td>TCTCCACCTTCGTAACACTTTCC</td>
</tr>
<tr>
<td>Caspase 4</td>
<td>CCATAGAAGCCGACTGTCATGAC</td>
<td>GCTGTACTAATGAAGGTTGCTCC</td>
</tr>
<tr>
<td>CEBP β</td>
<td>CCGCCGTTGTTATTTTA</td>
<td>GCAGAGGGGAAGACAGAGGTTTA</td>
</tr>
<tr>
<td>DDIT3</td>
<td>TGACCAGGGAAGTGAAGGC</td>
<td>AGTGAGAGGTTAGTCAGTAGC</td>
</tr>
<tr>
<td>FADD</td>
<td>CCGGACTGACAGGAGGTTCAGC</td>
<td>CTGTGTAGATGCCTGTGGTC</td>
</tr>
<tr>
<td>FAS</td>
<td>AAGCTCTTTCACTTCGAGG</td>
<td>GGGCATTAACACTTTTGGACG</td>
</tr>
<tr>
<td>GREB1</td>
<td>CAAAGAATAACCTGGGCTTCGTA</td>
<td>GACATGCCCTGCGCTCCTACCTTTATTA</td>
</tr>
<tr>
<td>IL6</td>
<td>ACAATACCTCGAGCCACAAC</td>
<td>CTGAGCCCATTGTTGTATTATC</td>
</tr>
<tr>
<td>IRE1α</td>
<td>GCGAAGCGACGTACACACATCAC</td>
<td>ACCAGCCCACCCACATTTG</td>
</tr>
<tr>
<td>LTB</td>
<td>CCTATCAGTCTGCGTGGCTG</td>
<td>CTGAGATCTGGTATTGTGGCTCC</td>
</tr>
<tr>
<td>PS2</td>
<td>CATCGACGTCCCTCCAGAAGG</td>
<td>CTTCTGGGAATACACGGATTGCTGA</td>
</tr>
<tr>
<td>TNF</td>
<td>ACTTGGAGTGTGAGGCC</td>
<td>GCTTGAGGGTTGGCTTACAA</td>
</tr>
<tr>
<td>TGFα</td>
<td>TGTAATCACCTGTGTCAGCCTTT</td>
<td>GTGGTCGCGCTATTTCTTCCT</td>
</tr>
<tr>
<td>LTA</td>
<td>TCTTCTTGGAGCCTTGC</td>
<td>AGACTTGGAGCTGGTGAATG</td>
</tr>
<tr>
<td>RB1</td>
<td>TTGGATCACAGCGATAAAACTT</td>
<td>AGCGACGCGCAATAAGACAT</td>
</tr>
</tbody>
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Table 2. Sequence of primers used for real time PCR of genes in various cell lines.
II.5. Real time profiler assay

RT-PCR profiler assay kits for apoptosis and cell cycle was used from a commercial vendor which uses 384 well plates (Qiagen; SABiosciences Corp, Fredrick, MD; Cat# PAHS-3012E) to profile the expression of 370 apoptosis related human genes and 4 x 96 well plates to profile expression of 84 cell cycle related genes (Qiagen; SABiosciences Corp, Fredrick, MD; Cat# PAHS-020). Briefly, cells were treated with indicated compounds (in triplicates) for the indicated time points. To identify cell cycle or apoptosis related genes, total RNA was isolated using the method mentioned earlier. Two micrograms of total RNA was reverse transcribed and RT PCR was performed using ABI 7900HT. We created a gene signature throughout the indicated time points after comparing them with vehicle treatment. This gene signature was generated by comparing the expression level of all the genes with vehicle treatment and selecting the genes which were at least 2.5 fold over-expressed or under-expressed as compared to vehicle treated cells at a statistical significance of p value of 0.05. The fold change was calculated by ΔΔCt method (Qiagen; SABiosciences Corp, Fredrick, MD).

II.6. Transforming growth factor assay

Three hundred thousand MDA-MB231 cells stably transfected with wild type ER (MC2) or mutant D351ER were seeded in six well plates and treated with either vehicle control (0.1% ethanol) or various concentrations of planar estrogens, triphenylethylenes or antiestrogens after 24h. After 24h, the cells were harvested for mRNA and RT-PCR was performed to quantify
TGFα mRNA levels as mentioned previously. The assay elucidates the putative conformation of ligand-receptor complex in relation to apoptotic inducing action of the ligands.

II.7. Immunoblotting

Proteins were extracted in cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and Phosphatase Inhibitor Cocktail Set I and Set II (Calbiochem, San Diego, CA). Total protein content of the lysate was determined by a standard BCA assay using the reagent from Bio-Rad Laboratories (Hercules, CA). Twenty five micrograms of total protein was separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with primary antibodies followed by incubation with secondary antibody conjugated with HRP and reaction with Western Lighting™ plus-ECL enhanced chemiluminescent substrate (PerkinElmer Inc., Waltham MA). Membranes were probed using ERα and β antibodies from Santa Cruz Biotechnology (Dallas, TX). Phosphorylated eIF2α, total eIF2α, IRE1α and β actin antibodies were from Cell Signaling Technology (Danvers, MA). Protein bands were visualized by exposing the membrane to X-ray film.

II.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously(76). Briefly, cells were treated with indicated compounds for 45 min and cross-linked using 1.25% paraformaldehyde for 15 min and subsequently stopped cross-linking with 2 M glycine. Cells were collected, followed by nuclei isolation by centrifugation. Isolated nuclei were resuspended in SDS-lysis buffer followed by sonication and centrifugation at 14 000× g for 20 min at 4°C. The supernatant were diluted 1:10 with ChIP dilution buffer. Normal rabbit IgG and Magna ChIP protein A magnetic bead (Upstate
Cell Signaling Solutions, Temecula CA, USA) were used to immunoclear the supernatant followed by immunoprecipitation with antibodies against ERα (1:1 mixture of cat# sc-543 and sc-7207; Santa Cruz Biotechnology Inc., Dallas, TX, USA) and steroid receptor coactivator-3 (SRC3) (cat# 13066; Santa Cruz Biotechnology, Inc. Dallas, TX, USA). Immunocomplexes were pulled down using protein A magnetic beads and a magnet. The beads bound to Immunocomplexes were washed using different buffers as described (76). Precipitates were finally extracted twice using freshly made 1% SDS and 0.1 M NaHCO$_3$ followed by de-crosslinking. The DNA fragments were purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA). RT-PCR was performed using 2 μl isolated DNA, using primers specific for PS2 promoter (76). The data are presented as percent input of starting chromatin input after subtracting the percent input pull down of the negative control (normal rabbit IgG).

II.9. Molecular modeling

The molecular modeling study was performed using the available X-ray crystallographic structures of ERα in the agonist and antagonist conformations. The 3D coordinates of ERα co-crystallized with E2 (1GWR) and 4OHT (3ERT) were extracted from RCSB Protein Data Bank (PDB) (77). The ligand was prepared for docking using the LigPrep utility (LigPrep 2.5; Schrodinger, LLC, Portland, OR, 2011). Protein preparation workflow (Schrodinger, LLC, New York, NY, 2011) was employed to prepare the proteins for molecular docking. The residues well-known to be important for biological activity D351 and E353 were kept charged in both receptors, the free rotation of hydroxyl group for T347 was allowed and H524 residue was protonated at the epsilon nitrogen atom based on the available literature data. Glide software
(Glide 5.7; Schrodinger, LLC Portland, OR, 2011) was used for molecular docking and the best docking poses were selected based on the composite score, Emodel, which accounts not only for the binding affinity but also for the energetic terms, such as ligand strain energy and interaction energy.

II.10. Cell cycles analysis

MCF7:5C cells were cultured in dishes and were treated with <0.1% ethanol (control) or the indicated compounds for the indicated times. Cells were harvested and gradually fixed with 75% EtOH on ice. After staining with propidium iodide (PI), cells were analyzed using a fluorescence-activated cell sorter (FACS) flow cytometer (Becton Dickinson, San Jose, CA), and the data were analyzed with ModFit software.

II.11. Small Interfering RNA Transfection

For transient transfections, MCF7:5C cells were seeded at a density of 50-70% in 6 well plates in estrogen free RPMI media containing 10% SFS. The following day, cells were transfected with 100 nM small interfering RNAs (siRNAs) for ER α (Dharmacon, SMART pool: ON-TARGETplus ESR1 siRNA product number L-003401-00-0005) and ERβ (Dharmacon, SMART pool: ON-TARGETplus ESR2 siRNA product number L-003402-00-0005), using DharmaFECT transfection reagent (Dharmacon, product number T-2001-03), according to the manufacturer’s recommended protocol. Non target siRNA was purchased from Dharmacon and was used as a control (Silencer negative control siRNA, product number D-001810-01-20). The cells were harvested 48h posttransfection and analyzed by western blot (as described above). Transfected cells were also treated with vehicle, steroidal estrogens or phytoestrogens for either an additional 72h, or 6 days and apoptotic cells and DNA content were measured using annexinV staining and DNA quantification assays respectively (as described above).
II.12. Statistical analysis

All data are expressed as the mean of at least three determinations, unless otherwise stated. The differences between the treatment groups and the control group were determined by one-factor analysis of variance (ANOVA with Tukey’s posttest and two-way ANOVA with Bonferroni posttest using GraphPad Prism, version 5.00 (GraphPad Software Inc., La Jolla, CA). Results were considered statistically significant if the \( P < 0.05 \)
Defining the conformation of the estrogen receptor complex that controls estrogen induced apoptosis in breast cancer.

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III.1. Introduction

Estradiol (E₂) is a key stimulus of growth for estrogen receptor (ER) positive breast cancer. Endocrine therapy has been the gold standard of treatment in ER positive breast cancer(1) but, acquired antihormone resistance to long-term antihormone therapy is a continuing clinical dilemma. Discovery of the evolution of acquired resistance exposed a vulnerability of cells by paradoxically triggering apoptosis with physiologic E₂ treatment(78). Laboratory evidence demonstrates that E₂ is capable of inducing apoptosis in long term estrogen deprived MCF-7 cells(38, 39). Similarly, tamoxifen-stimulated tumors (23, 24) that develop in athymic nude mice in about a year will undergo regression after 5 years of tamoxifen if exposed to physiologic E₂ (31).

Clinical data support the use of estrogens in the treatment of ER positive postmenopausal breast cancer. Synthetic high dose estrogens induced regression of tumors in postmenopausal women with advanced breast cancer in the first ever reported cancer chemical therapy (chemotherapy) mediated clinical study (15). Clinical trials now exploit the concept for patients with metastatic breast cancer who develop resistance to endocrine therapy which shows that estrogens induce a partial to complete response in about 30 percent of postmenopausal breast cancer patients who had previous exhaustive antihormone therapy(79, 80). A re-analysis (81) of the Women Health Initiative estrogen alone trial(82) which compared conjugated equine estrogen therapy with placebo in hysterectomised postmenopausal women showed a significant decrease in the incidence and mortality from breast cancer in these patients. The success of estrogen therapy in postmenopausal women depends on the menopausal status of the patients. Women who are more than 5 years postmenopausal i.e. long term estrogen deprived, have better
tumor remission rate as well as prevention from breast cancer (83).

The ER is the key signal transduction pathway for breast cancer growth and apoptosis based on studies with competitive inhibition of E\textsubscript{2} action with antiestrogen (76). The question to be addressed is how a series of estrogens with planar or angular structures can reprogram the estrogen-ER complex, to be either a survival signal in breast cancer or triggers apoptosis. We have previously classified estrogens(70) based on reported data on the crystallization of the ligand binding domain (LBD) of the ER with estrogens (E2, DES) and antiestrogens (4OHT and raloxifene) (71, 72) (See Fig 1). The planar estrogens are sealed within the LBD by helix 12, thus activating the AF2 domain which leads to coactivator binding and subsequent interaction of AF1 and AF2 (84) to initiate growth and protein synthesis. In contrast, the bulky side chain of nonsteroidal anti-estrogen causes displacement of helix 12 and prevents coactivator binding to the AF2 resulting in anti-estrogenic action. Tamoxifen, a substituted triphenylethylene (TPE) derivative possesses estrogen-like activity (85-87). We previously discovered that the surface amino acid D351 within the LBD is critical for the estrogenic actions of 4-hydroxytamoxifen (4OHT) (70, 75). Unlike raloxifene, which is less estrogenic and possesses an anti-estrogen side chain that shields and neutralizes D351, the side chain in 4OHT is too short(88).

To interrogate the relationship of structure of an estrogenic ligand to program the conformation of the ER complex, we synthesized a range of estrogenic TPEs (73), which are structurally similar to 4OHT. We and others hypothesize that the structure of the ligand governs the external surface of the ER complex with either planar estrogens or the TPEs(70, 89). As a result of the ligand shape, the estrogens can program the conformation of the estrogen-ER complex to modulate rapid or delayed apoptosis. The growth response of the ER positive breast cancer cells is very sensitive to a wide range of estrogenic ligands. This is to ensure cancer cell
survival in austere estrogen environments. This may not be true for estrogen induced apoptosis and the ligand shape may be required to be more specific to trigger cell death. The estrogen deprived cancer cell is protected.

We investigated the actions of clinically relevant planar estrogens (E₂, diethylstilbestrol, equilin, estrone and equilenin), anti-estrogens (4OHT, endoxifen, raloxifene and bazedoxifene) and model TPEs (bisphenol, trihydroxytriphenylethylene and ethoxytriphenylethylene) on growth in MCF-7 cells and apoptosis in MCF-7:5C cells. In order to understand the biological activity of the TPE:ER, we employed a validated ER engineered assay using induction of the mRNA for the transforming growth factor (TGF-α) gene in situ in MDA-MB231 cell stably transfected with wild type ER or mutant D351G:ER(70) (Fig. 1). We classified the structure of the ligands based on their ability to initiate TGFα mRNA synthesis through the ER complex. The biological assay predicts two extremes of the ligand ER complex based on known X-ray crystallography (71, 72): an “estrogen-like” shape and an “antiestrogen-like” shape. We find that the TPE:ER complex is antiestrogen-like which explains the delayed apoptosis in MCF7:5C cells compared to the estrogenic complex formed by the planar estrogens.
Fig. 1. Functional Test: Putative conformations of the complex with ligand in LBD for Type II estrogen to be “antiestrogenic” with regard to helix 12 positioning. The assay discriminates between ligands (A) which allow helix 12 to seal the LBD or not (B) and (C). Sealing of helix 12 over the LBD is important for the ability of the ligands to trigger apoptosis.
III.2. Results

III.2a. Growth effects of estrogens and anti-estrogens in MCF-7 cells

To study the biological activity of the planar estrogens (Fig. 2A) which include E2, DES, equilin, estrone and equilenin), and triphenylethylenes (Fig. 2B) namely, EtOX (ethoxytriphenylethylene), 3OHTPE (trihydroxytriphenylethylene) and bisphenol, we tested their ability to induce cell proliferation in wild type MCF-7 cells. As controls we used SERMs; 4OHT, endoxifen (endox), raloxifene (ral) and bazedoxifene (baze) (Fig. 2C) which are known anti-estrogens. MCF-7 cells were grown in estrogen free media for 3 days and treated with various concentrations of the indicated compounds and their effects were compared to E2. All planar estrogens (Fig. 3A) were able to induce cell proliferation in a concentration dependent manner to the maximum level as E2. DES, equilin and estrone induced cell proliferation with maximum stimulation occurring at 0.1nM, whereas equilenin reached maximal stimulation at 1nM as compared to 0.01nM for E2. Similarly, the triphenylethylenes tested were able to induce cell growth to the maximum level as E2, although their agonistic potency was less than E2 (Fig. 3B). Bisphenol, EtOX and 3OHTPE all induced cell proliferation in a concentration dependent manner with maximum stimulation at 1-10 nM as compared to 0.01 nM for E2. Nonetheless, the TPEs all were potent estrogen-agonists in this assay. On the other hand, as expected, the SERMs, 4OHT, endox, ral and baze (Fig. 3C) which are antiestrogens did not induce cell growth.
Fig. 2. Chemical structures of the compounds used in the experiments. (A) Planar estrogens (B) Triphenylethylenes (C) Selective estrogen receptor modulators.
Fig. 3. Growth characteristics of planar estrogens and triphenylethylenes in MCF7:WS8 cells. MCF7:WS8 cells were seeded in 24-well plate and treated with (A) planar estrogens over a range of doses for seven days. Cell growth was assessed as DNA content in each well. Induction of cell growth by (B) Triphenylethylenes and (C) SERMs was assessed in comparison to E₂. Each data point is average +/- SD of three replicates.
III.2b. Effects of planar estrogens, TPEs and SERMS on apoptosis in MCF7:5C cells

We tested if TPEs and SERMS were able to induce apoptosis in long term estrogen deprived MCF7:5C breast cancer cells as effectively as E2. All planar estrogens were able to cause growth inhibition as effectively as E2 (Fig.4A). All the planar estrogens achieved maximal growth inhibition in the range of 1 nM as compared to E2 which achieved maximal growth at 0.1 nM. To confirm that the decrease in cell proliferation was due to apoptosis, MCF-7:5C cells were treated with ethanol vehicle (control), E2 (1nM), or DES (1nM), equilin (1nM), estrone (1nM) and equilenin (1nM) for 72 hours, and annexin V – FITC and PI fluorescence was determined by flow cytometry. In the control-treated group, only 5.9% stained positive for apoptosis, whereas, in the E2 treated group (SFig. 1A), cells that stained positive for apoptosis increased by 3 fold. Interestingly, the estrogenic triphenylethylenes did not inhibit the growth of MCF7:5C cells even at higher concentrations (Fig.4B) at the end of a 7 day assay. Compared to E2, bisphenol, 3OHTPE and EtOX did not show any effective apoptosis even at micro-molar concentration (SFig. 1B) and were comparable to that of the SERMs (Fig 4C). Furthermore, the TPEs were able to block E2 mediated apoptosis in a similar manner to the SERMs (Fig. 4D-E). However, the TPEs were able to induce apoptosis after 14 days of treatment (Fig.4F), whereas the SERMs still did not induce apoptosis in the MCF7:5C cells (SFig.1C).
Fig.4. Differential effect of planar estrogens and triphenylethylenes in MCF7:5C cells. Dose dependent effect of (A) planar estrogens (B) Triphenylethylenes and (C) SERMs on apoptosis of MCF7:5C cells treated for 7 days as indicated. Cells were treated with 1nM 17-β estradiol (E2) in presence of increasing concentration of indicated TPEs (D) and SERMs (E). (F) Effect of TPE in MCF7:5C cells after 14 days of treatment. Each data point is average +/- SD of three replicates.
Figure 1. Differential effect of planar and non-planar estrogens and SERMs on apoptosis.

Annexin V staining for apoptosis. MCF7:5C cells were treated with (A) ethanol Vehicle (Veh), planar estrogens (1 nM) (B) TPE (1 μM) and SERMS (1 μM) for 72h, and then cells were stained with FITC– annexin V and propidium iodide (PI) and analyzed by flow cytometry. (C) MCF7:5C cells treated with either Veh or 1μM SERMS for 7 days and apoptosis was quantified by measuring DNA content using a fluorescent DNA quantitation kit.
III.2c. Regulation of transforming growth factor alpha (TGFα) gene by planar and non-planar estrogens in MDA: MB-231 cells stably transfected with wild type ERα or D351G mutant ERα.

The TGFα gene is induced by 4OHT as effectively as E2 in MDA:MB-231 cells stably transfected with wild type ERα (MC2 cells). In contrast, in MDA:MB-231 cells stably transfected with a mutant D351ER (JM6 cells), 4OHT fails to induce expression of the TGFα gene but E2 retains its ability to induce the TGFα gene. We determined if the TPEs (3OHTPE, EtOX and bisphenol) and the planar estrogens (DES, equilin, estrone and equilenin ) resembled E2 or 4OHT in inducing the TGFα gene expression by using the assay system summarized in Fig. 1). As expected, all the planar estrogens were able to induce TGFα gene expression in a concentration dependent manner in both wild type ERα (MC2) (Fig. 5A) and D351G mutant ERα (JM6) cells (Fig. 5D). On the other hand, the TPEs and tamoxifen metabolites; 4OHT and endox were able to induce TGFα gene expression in MC2 cells (Fig. 5B) in a concentration dependent manner, whereas ral and baze do not activate the TGFα gene in this cell line (Fig. 5C). By contrast, the TPEs, 4OHT and endox distinctly failed to induce TGFα gene expression (Fig. 5E) in JM6 cells which expresses D351G mutant form of the ERα, rather they block E2 mediated TGFα induction(Fig. 5F). Similarly, ral and baze are antiestrogenic in the mutant stable transfectant. These findings indicate that the TPEs possess antiestrogenic properties and binds with ERα in a manner which is distinctly different from the planar estrogens but strikingly resembles 4OHT and endox
Fig. 5. The concentration-dependent action of test compounds using wild type (MC2) and mutant D351G ER (JM6) stable transfectants. MC2 cells were treated with (A) planar estrogens (B) TPEs, 4OHT and endox for 24h at indicated concentrations and expression of TGFα RNA was measured using quantitative real time PCR. (C) MC2 cells treated with ral and baze in a dose responsive manner. JM6 cells were treated with (D) planar estrogens (E) E2, TPEs, active metabolites of tamoxifen (4OHT and endox) for 24 h with various concentrations and expression of TGFα RNA was measured using quantitative real time PCR. (F) JM6 cells were treated with 1nM E2 alone or in presence of increasing concentration of indicated TPEs and SERMs. Data are represented as fold difference versus vehicle treated cells. Each data point is average +/- SD of three replicates.
III.2d. Recruitment of ERα (estrogen receptor α) and SRC3 (steroid co-activator-3) at the proximal promoter of PS2 gene after treatment with triphenylethenes

To further understand the ER mediated mechanism involved in the regulation of the model estrogen responsive gene PS2 by the TPEs in MCF7:WS8 and MCF7:5C cells we determined the recruitment of the ERα and SRC-3 protein at the proximal promoter of PS2 gene, which has a classical estrogen responsive element (Fig. 6A), using ChIP (chromatin immunoprecipitation) assay after 45 minutes of treatment with TPEs (1 µM) and compared it with E2 (1nM) and 4OHT (1µM). The whole assay was repeated two further times with similar results occurring in each cell line (Supplemental Figures 4-5). In MCF7:WS8 cells, E2 was able to recruit very high level of ERα at the PS2 promoter (Fig. 6B) where more than 8% of input PS2 promoter region was occupied by ERα. On the other hand TPEs were ~50% as efficient as E2 treatment in terms of recruiting ERα whereas very low level (~20% of E2) of ERα recruitment was observed after 4OHT treatment. Recruitment of the co-activator SRC3, which is critical in inducing the estrogen responsive gene, was not observed at all after 4OHT treatment at the PS2 promoter. All the TPEs tested recruited only about 15-20% of SRC3 as compared to E2 treatment, which showed around 0.9 % of input PS2 promoter region was occupied by SRC3 protein. Interestingly, in MCF7:5C cells treated with E2, around 5% of input PS2 promoter region was occupied by ERα (Fig. 6C). In MCF7:5C cells treated with TPEs had 50% less ERα occupancy and ~80% less SRC3 occupancy was observed as compared to E2 treatment in MCF7:5C cells, whereas no SRC3 recruitment was observed after 4OHT treatment. These ChIP data concurs with the PS2 mRNA induction level in MCF7:WS8 and MCF7:5C cells with their respective treatments (SFig. 2A-B).
Fig. 6. Recruitment of ER alpha and SRC3 (AIB1) at PS2 proximal promoter region containing ERE in MCF7:WS8 and MCF7:5C cells. (A) Depiction of PS2 proximal promoter region and the ERE region relative to TSS (transcription start site). (B) MCF7:WS8 cells treated for 45 minutes with E2 (1nM), 3OHTPE (1μM), EtOX (1μM), bisphenol (1μM) and 4OHT (1μM) and ChIP assay was performed as described in materials and methods. (C) MCF7:5C cells were treated identically as mentioned above and ChIP assay was performed under identical conditions. Data is represented as percent input of the starting chromatin used for the ChIP
SFig. 2. Regulation of PS2 (TFF1) gene by triphenylethylenes, E<sub>2</sub> and 4OHT in MCF7:WS8 and MCF7:5C cells. (A) MCF7:WS8 and (B) MCF7:5C cells were treated E<sub>2</sub> (1 nM), 3OHTPE (1 μM), EtOX (1 μM), bisphenol (1 μM) and 4OHT (1 μM) for 2 h or 6 h. Cells were harvested and RNA extracted. Expression level of PS2 (TFF1) was assessed using RT-PCR. Data is represented as fold difference versus vehicle (veh) treated cells. All data points are average of three replicates ± SD.
III.2e. Induction of ERα expression by planar and non-planar estrogens

To test whether the structure the compounds create with the ER affects the ERα expression levels, 4 breast cancer cell lines, which include MCF-7:WS8, MCF7:5C, MC2 and JM6 cells, were treated with planar estrogens (1nM), TPEs (1µM) and SERMs (1µM) for 24h and ERα levels were determined by western blotting. ICI was included as a positive control. All planar estrogens and ICI caused decrease in the ERα protein levels in MDA-MB231 cells stably transfected with either wild type ER (MC2) (Fig. 7A) or with the mutant receptor (JM6) (Fig. 7B). On the other hand, the TPEs do not decrease the ERα protein levels in the MC2 cells, whereas 4OHT and endox cause accumulation of the receptor, while ral and baze cause moderate down regulation of the ER. In the JM6 cells, all TPEs and SERMs did not dramatically affect the ERα protein expression. As expected, all planar estrogens and ICI cause a decrease of ERα protein levels in MCF7:WS8 (Fig. 7C) and MCF7:5C (Fig. 7D) cells, whereas the tamoxifen metabolites caused increase in ERα protein expression. Interestingly the TPEs cause moderate decrease of ERα in MCF7:WS8 and MF7:5C cells compared to E2, and the reduction is more dramatic in the MCF7:5C cells. In contrast to the tamoxifen metabolites, ral and baze also cause a reduction in the protein levels of ERα in both MCF-7 derived breast cancer cell lines. ER α protein levels of all breast cancer cell lines used in the study are compared in SFig. 3.
Fig. 7. Differential regulation of the ERα protein by planar and non-planar estrogens. (A) MC2 (B) JM6 (C) MCF7:WS8 (D) MCF7:5C cells were treated with E2 (1nM), DES (1nM), equilin (1nM), estrone (1nM), equilenin (1nM), 3OHTPE (1µM), EtOX (1µM), bisphenol (1µM), 4OHT (1µM), endox (1µM), baze (1µM), ral (1µM) and cell lysates were analysed by western blotting by anti ERα antibody. Blot was reprobed by anti actin antibody.
SFig.3. ERα expression levels in breast cancer cell lines used in the study.

MC2, JM6, and MCF7:WS8 and MCF7:5C cells were treated with control (0.1% ethanol) for 24h and lysates were prepared and analysed by Immunoblotting for ERα protein levels. β-actin was used as a loading control.
III.2f. Binding of Bisphenol to the LBD of ER alpha

Next, the binding mode of the TPEs was investigated by the molecular docking of bisphenol to the ligand binding domain (LBD) of ERα. Thus, the flexible docking of bisphenol into the LBD of the receptor co-crystallized with E2 and 4OHT (Fig. 8A-B) were performed. The superimposition of the top ranked docking pose of the ligand onto the E2 co-crystallized with ERα, agonist conformation of the receptor, shows some incompatibility (Fig. 8C). Hence the resulting model revealed steric clashes between bisphenol and “Leu crown”, mostly with the side chains of Leu525 and Leu540. Due to this steric hindrance it is most unlikely for bisphenol to bind in a conformation of ERα that is similar to that of E2. On the other hand, when bisphenol is docked into the binding site of 4OHT co-crystallized with ERα (Fig. 8D), the binding mode is similar to that of 4OHT. Namely the same alignment of the ligand in the binding pocket is noticed having the propensity to form the same hydrophobic contacts with the amino acids lining the binding cavity and to recapitulate the complex H-bond network involving E353, R394 and highly ordered water molecule. Taken together, this data show that bisphenol and extrapolating TPEs would most likely bind to the ERα in the antagonist conformation of the receptor.
**Fig. 8. The binding site of ERα with different ligands.** The ligands are depicted with their corresponding grid molecular surfaces colored in gray. Also, Leu525 and Leu540 are depicted as grid molecular surfaces colored in blue. (A) Agonist conformation of ERα with E₂ (depicted in magenta; PDB code: 1GWR). (B) Antagonist conformation of ERα with 4OHT (colored in green; PDB code: 3ERT). (C) Docking of bisphenol in agonist conformation (colored in cyan; PDB code: 1GWR). (D) Docking of bisphenol in antagonist conformation (colored in cyan; PDB code: 3ERT).
III.3. Discussion

Estrogens are potent mitogens for the proliferation of breast cancer cells. In contrast, planar estrogens (class 1) can induce apoptosis of long term estrogen deprived MCF-7 cells (MCF7:5C) in a paradoxical manner. 4OHT has no effect in the MCF: 5C cells but rather blocks E2 mediated apoptosis (76). TPEs which are structurally similar to 4OHT possess estrogenic properties in the MCF-7 cells at comparable concentrations to the planar estrogens. The TPEs (class II angular estrogens) do not rapidly trigger estrogen induced apoptosis in MCF7:5C cells, but block class 1 planar estrogen induced apoptosis. However, prolonged treatment with the TPEs lead to an eventual induction of apoptosis in the MCF7:5C cells, whereas the cells continue to be resistant to the actions of the SERMs which are known antiestrogens (SFig 1C). As a result of these aforementioned findings we initially proposed a hypothesis(76), that the TPE-ER complex mimics of an anti-estrogen-ER complex and this may be responsible for the delay of apoptosis by the TPEs. We addressed the hypothesis in four ways: utilizing our validated functional assay to classify estrogens using the induction of the TGFα gene (70)(Fig. 1), binding of ER and recruitment of SRC3 to the promoter region of a model estrogen response gene (PS2) (Fig. 6), ligand bound ER accumulation or reduction and putative ER docking experiments(Fig. 8).

We have previously demonstrated the critical importance of D351 in modulating the SERM:ER complex (90) for the estrogen-like actions of the 4OHT by removing the exposed surface charge by engineering a mutant ER D351G, which causes a conversion of the 4OHT:ER from being estrogenic to completely antiestrogenic at the TGFα gene (75, 86). The anchoring role of D351 in the activation of the helix 12 mutated ER has recently (91, 92) been illustrated in tissue from metastatic breast cancer resistant to anti-hormones. Mutations of Y537 in helix 12 are shown to anchor to D351 to accomplish sealing of the unoccupied LBD by helix 12. This
provides evidence of the clinical relevance of our assay system.

To determine whether the conformation of the ER complex determines the triggering of apoptosis in long term estrogen deprived ER positive breast cancer cells, MCF-7:5C, we employed (70) an assay using induction of the mRNA for the TGF-α gene in situ in MDA-MB-231 cells stably transfected with cDNA wild-type(MC2) or D351G ER(JM6). As expected, all planar estrogens cause activation of the TGFα gene in the MC2 and JM6 cells. The planar estrogens are not affected by the mutation on D351 because upon binding to the ER, they are sealed within the LBD by helix 12 allowing for coactivator binding on the surface of helix 12 (AF-1) and gene activation. The TPEs induce TGFα gene at comparable concentrations as the tamoxifen metabolites, 4OHT and endox in the MC2 cells (Fig. 5B), but lose this estrogen-like action in the JM6 cells (Fig. 5E) and block E2 induction of TGFα (Fig. 5F). The results of the TGF assay imply that TPEs adopt a 4OHT-like conformation with the ER with helix 12 pushed back and D351 exposed. By inference the “antiestrogenic conformation” of the TPE: ER complex is responsible for the initial inhibition of E2 induced apoptosis. The short aminoethoxy side chain of the tamoxifen metabolites(75) and the absence of this side chain in the TPEs prevent adequate shielding of the charged D351, whereas the anti-estrogenic side chain of ral and baze provides effective interaction and neutralization of this charge(88) (Fig. 5C). Thus, this prevents the induction of the TGFα gene by ral and baze.

SRC3 has been shown to be extremely important in estradiol induced growth in breast cancer cells (93-95). Additionally, SRC3 knockdown was found to reduce apoptosis induced by E2 in MCF7:5C cells(96). Using ChIP assays we show that TPEs are able to recruit ERα but less efficiently when compared to E2 and this was further observed with SRC3 (Fig. 6).The ER:TPE complex binds to the promoter with about 50% of E2 but SRC3 binding is <25% of E2. This
suggests that treatment with TPEs influences the conformation of the liganded-ER\(\alpha\) complex such that efficiency of ER\(\alpha\) binding to ERE region is moderately inhibited whereas binding of SRC3 is severely inhibited as compared to E\(_2\) treatment which is a planar estrogen. This may also explain why bisphenol is a partial agonist at the prolactin gene and exhibits antiestrogen properties (97, 98). Of notable importance, the magnitude of SRC3 recruitment by the TPEs is far less in MCF7:5C cells (Fig. 6C) when compared to MCF7:WS8 cells (Fig. 6B) and may play a crucial role in manifesting the functional role of the TPEs in these cells. This observation may contribute to the robust cell replication in MCF-7 with TPEs but delayed apoptosis in MCF7:5C.

Estradiol induces downregulation of the ER in breast cancer cells (99-101) and this process is inhibited by 4OHT, thereby causing accumulation of ER-\(\alpha\)(102). Similarly in all our cell lines, the planar estrogens all downregulate the ER while tamoxifen metabolites, 4OHT and endox, do not (Fig. 7). The western blot analysis shows that the TPEs do not readily decrease ER\(\alpha\) protein levels when compared to the planar estrogens. This illustrate the fact that the TPE:ER complex appears to be “antiestrogen-like” when compared to 4OHT and endox (Fig. 7). However in the MCF7:5C cells, their ability to downregulate ER\(\alpha\) protein levels is more apparent. The ER complex resembles the vehicle (control) rather than the extremes of E\(_2\) or 4OHT. Ral and baze also cause moderate decrease in ER\(\alpha\) levels which concurs with previous studies done on these compounds(74). Bourgon-Voillard and colleagues (103) determined that class II ligands such as bisphenol had less tendency to promote recruitment of coactivators containing LxxLL motif and this appeared to be a requirement for the downregulation of the ER in MCF7 cells. Bourgoin-Voillard and colleagues (103) also illustrate the accumulation of the bisphenol:ER complex in MCF-7 cells using immunocytochemistry.

The molecular modeling data (Fig. 8) provide evidence that the TPEs bind to the ER\(\alpha\) in
a manner similar to that observed with 4OHT using x-ray crystallography. The bulky phenyl ring of the TPEs prevent helix 12 from sealing the LBD and will result in an initial steric hindrance when attempting to bind in the $E_2$-ER $\alpha$ conformation, resulting in their blockade of $E_2$ induced apoptosis. However, continuous treatment of the MCF7:5C with the TPEs for 14 days result in induction of apoptosis similar to the planar estrogens. This suggests that the antiestrogenic conformation the TPEs create with the ER prevents immediate coactivator binding, causing a delay in the trigger for apoptosis but this delay disappears with prolonged treatment. This conclusion correlates with the Haddow clinical study(15), where postmenopausal women with advanced breast cancer were treated with TPE- like estrogens leading to about a 30% response rate during breast cancer therapy. The planar estrogens form compact estrogen-ER complex with excellent SRC3 binding and recruitment and it appears that this event is necessary to induce apoptosis in the MCF7:5C cells. On the other hand, angular TPEs form antiestrogen-like-ER complex with less SRC3 binding and recruitment, thereby leading to delayed apoptosis, whereas the SERMS do not recruit SRC3 so this results in no apoptosis.

In conclusion, we have advanced the hypothesis that TPE-ER conformation is initially similar to that of tamoxifen metabolites, 4OHT and endox and our molecular classification assay indicate that helix 12 is pushed back in the TPE-ER complex. The antiestrogenic conformation of the TPE-ER complex appears to be responsible for the initial blocking of apoptosis and reduction in coactivator recruitment observed with the TPEs in the MCF7:5C cells. It is important to stress that the evidence we present suggests that the TPE:ER complex conformation may in fact be in between the extreme structures of $E_2$:ER and 4OHT:ER ligand binding domain (71, 72). Since prolonged treatment with TPEs causes triggering of ER mediated apoptosis
similar to that of the planar estrogens but 4OHT does not, an intermediate conformation of the TPE:ER complex may be responsible for these observations.
CHAPTER IV

Molecular Mechanism of Action of Bisphenol and Bisphenol-A Mediated by Estrogen Receptor alpha in Growth and Apoptosis of Breast Cancer Cells

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IV.1. Introduction

Estrogen receptor alpha (ERα) mediates its action in cells and tissues by binding to its cognate ligands and function as a “ligand-activated” transcription factor (104). Apart from its natural ligands, many different compounds can bind to ERα and thus can function as its ligand (105). However, depending upon the chemical structures of these ligands they can either function as a complete / partial estrogen- agonist or antagonist. Broadly, the estrogenic compounds can be classified as class I and class II depending upon their planar or non-planar chemical structures, respectively(70). Different ligands bind to the same core of the ligand binding domain (LBD) of ERα protein but can evoke distinct three-dimensional conformation of the liganded-ERα complex which can either interact with the co-activators or the co-repressors (collectively known as co-regulators) at the promoters of estrogen responsive genes(104). Consequently, this complex modulates the transcriptional activity of the various estrogen-responsive genes and eventually determines the outcome of the ERα dependent physiological responses of a particular cell or tissue type. The molecular basis of this differential recruitment of the co-regulators has been attributed to the ability of the liganded-ERα to re-orient the helix 12 (H12) of the LBD in such a manner that the complex can interact with the coactivators at the structural interface formed by H3, H4 and H5 helices, when ERα is bound to an agonist (17-β estradiol (E2) or di-ethyl stilbestrol (DES))(71, 72), but this interaction is completely blocked when the ERα is bound to antagonists, such as 4-hydroxy-tamoxifen (4OHT) (71)or raloxifene (RAL) (72). Interestingly, when ERα is liganded with an antagonist, such as 4OHT, an active metabolite of tamoxifen, which is extensively used in treatment and prevention of breast cancers (106), it can now interact with the co-repressors and can inhibit the transcriptional activity from the estrogen responsive genes(107-109). Besides the interaction of co-regulators with the liganded ERα the
levels of co-activators and co-repressors in a given cell can also determine the physiological responses to different ligands of ERα (109). Earlier studies from our laboratory have identified that the amino acid aspartate at 351 (which is in the helix 3 (H3)) of the ERα LBD is critically important for maintaining the integrity of antiestrogenic activity of keoxifene (RAL) and 4OHT (86, 110). Earlier, the mutation of ERα encoding amino acid 351 which substituted the aspartate to tyrosine amino acid was detected in one of the xenograft tumors stimulated by tamoxifen in the athymic mice (111). Further investigations have revealed that changing the amino acid aspartate 351of the ERα to glycine (D351G) abolishes the estrogenic effect of 4OHT but does not affect estradiol action on TGFα gene activation in the ER negative breast cancer cells stably transfected with either wild type ERα or D351G mutated ERα (75). Using these models, estrogens were classified as either type I, which have the planar structures or type II, which have the angular or non-planar structures (70, 112). A recent confirmatory study evaluated the ability of several type-I and II liganded ERα to associate with the specific peptide motif “LXXLL” which co-activators use to interact with the ERα (103). A previous study (76) from our laboratory indicated that the conformation of the ERα complex can govern the estrogen-induced apoptosis in the MCF7:5C breast cancer cells. The present study dissects the ERα mediated effect of two structurally similar estrogenic ligands, namely, bisphenol (BP) and bisphenol-A (BPA) (Fig. 9), on two critical physiological responses, i.e. growth and apoptosis in the breast cancer cells. BP is structurally related to 4OHT with E2-like agonistic properties, whereas BPA has been characterized as an endocrine disruptor with weak estrogenic properties. Using various investigative tools, this study underscore the fact that minor difference in the shape of the ERα-liganded complex has profound modulation on estrogen-induced apoptosis but not on estrogen-induced replication of breast cancer cells.
IV.2. Results

IV.2a. Differential effect of Bisphenol and Bisphenol-A in inducing apoptosis in MCF7:5C cells but not growth in MCF7 cells

BP (Fig. 9) a triphenylethylene (TPE) is a known partial estrogenic ligand which can induce growth of the ERα positive breast cancer cells (73) and can also partially initiate prolactin synthesis from primary culture of cells from immature rat pituitary glands (97). Another compound with similar chemical structure, bisphenol–A (BPA) (Fig. 9) is also a well characterized but weak estrogenic ligand (113). Here we evaluated the ability of these two estrogenic compounds to induce growth and apoptosis in MCF7 and MCF7:5C cells respectively, as both these responses are dependent on estrogen agonistic action. As expected, BP as well as BPA was able to induce the concentration dependent growth in the MCF7 cells (Fig. 10A). BPA was less potent compared to BP as maximal growth was achieved by BP at 1nM concentration as compared to 1µM for BPA. By comparison, E₂ induced maximal growth at 10 pM concentration in the MCF7 cells. In the case of MCF7:5C cells, which undergo apoptosis with E₂ treatment (39, 40), a marked contrast was observed between BP and BPA in the induction of apoptosis. BPA was able to induce apoptosis to the same extent as E₂ in these cells at a higher (1 µM) concentration (Fig. 10B) as compared to E₂ which achieved maximal effect at 10 nM. However, BP failed to induce apoptosis even at 10 µM concentration (Fig. 10B). We further investigated that if BP was actually binding to the ERα in the MCF7:5C cells by treating these cells with BP in combination with 1 nM of E₂. BP was able to block the effect of E₂ in the MCF7:5C cells (Fig. 10C)(SFig. 4) in a concentration dependent manner indicating that the effect of BP was through the ERα, thus inhibiting the E₂ action. On the other hand, BPA was not able to block the effect E₂ action (Fig. 10C). In addition, we also show that the estrogenic effect
of BPA (1µM) in inducing apoptosis in MCFF7:5C cells was completely blocked by BP (1µM) as well as 1µM of 4OHT (Fig. 10D).

Fig.9. Chemical structures of 17β-estradiol (E₂), Diethylstilbestrol (DES), 4-Hydroxytamoxifen (4OHT), Bisphenol (BP) and Bisphenol-A (BPA).

Fig.9. Chemical structures of 17β-estradiol (E₂), Diethylstilbestrol (DES), 4-Hydroxytamoxifen (4OHT), Bisphenol (BP) and Bisphenol-A (BPA).
Fig. 10. Differential effect of bisphenol (BP) and bisphenol-A (BPA) on growth and apoptosis of ERα positive breast cancer cells. A. Dose dependent effects of BP, BPA and (estradiol) E₂ on growth of MCF7 cells treated for six days as indicated. The black bar denotes the level of DNA in vehicle treated cells over a six day period. B. Dose dependent effect of BP, BPA and E₂ on apoptosis of MCF7:5C cells treated for six days as indicated. C. Dose dependent effect of BP and BPA on E₂ (1nM)-induced apoptosis in MCF7:5C cells, treated over a six day period. The growth is measured as amount of DNA present in each well. D. Effect of BP (1 µM)
and 4OHT (1 µM) on BPA (1 µM) induced apoptosis in MCF7:5C cells over six day period. (*p<.05 versus vehicle treatment; # p<.05 versus BPA treatment) The data is presented as percent of growth considering the vehicle treated cells as 100 percent. Each value is average of at least three replicates +/- S.D.
SFig.4. Dose dependent effect of BP (at various concentrations between $10^{-8}$M and $10^{-7}$ M) on E$_2$ (1nM)-induced apoptosis in MCF7:5C cells, treated over a six day period. The growth is measured as percent of DNA present in each well; vehicle treated cells were considered as 100%.
IV.2b. Regulation of estrogen responsive gene trefoil factor 1 (TFF1 or PS2) by bisphenol and bisphenol-A

We next investigated the transcriptional regulation of a well characterized estrogen regulated gene, TFF1 (PS2) (114) by BP and BPA and compared it with E2 and 4OHT. MCF7 cells were treated for 4 hours with the 0.1% ethanol (veh), E2 (1 nM), 4OHT (1 µM), BP (1 µM and 10 µM) or BPA (1 µM and 10 µM) and the transcripts levels of PS2 gene were measured using real-time PCR. Two different concentrations (10 µM and 10 µM) were used for BP and BPA because BPA is a weak estrogen and we wanted to evaluate the concentration dependent regulation of these compounds. As expected, PS2 mRNA was up-regulated around fivefold by E2 (1 nM) compared to vehicle treatment and 4OHT (1 µM) which completely failed to induce the levels of PS2 mRNA (Fig. 11A). On the other hand, BP treatment at 1 µM concentration moderately (~2 fold) up-regulated the PS2 mRNA levels and higher concentration (10 µM) of BP failed to further increase the levels of PS2 (Fig. 11A). Conversely, cells treated with BPA exhibited concentration dependent increase in up-regulation of the PS2 mRNA and the magnitude of up-regulation with high concentration (10 µM) of BP was equivalent to the E2-mediated up-regulation of PS2 mRNA (Fig. 11A).
Fig. 11. Regulation of PS2 (TFF1) gene by bisphenol (BP), bisphenol-A (BPA) compared with 17-beta estradiol (E2) and 4-hydroxy-tamoxifen (4OHT) and recruitment of estrogen receptor alpha (ER alpha) and steroid receptor co-activator-3 (SRC3) at the estrogen responsive element (ERE) of proximal promoter of PS2 gene followed by 45 minutes treatments of bisphenol (BP), bisphenol-A (BPA) compared with 17-beta estradiol (E2) and 4-hydroxy-tamoxifen (4OHT) in MCF7 cells. A. MCF7 cells were treated with indicated treatments for 4hrs and harvested for total RNA. Total RNA was reverse transcribed and assessed for PS2 gene expression levels using real time PCR. B. Schematic representation of the PS2 proximal promoter containing an ERE (grey box) and the black bars represent the primers.
used for RT-PCR. 

C. Recruitment of ERα at the PS2 proximal promoter, by ChIP assay after 45 minutes of indicated treatment. (*p<.05 versus vehicle treatment; *p<.05 versus 1µM BPA and 10µM BP treatment)

D. Recruitment of SRC3 at the PS2 proximal promoter, by ChIP assay after 45 minutes of indicated treatment. All the values are represented as percent input of the starting chromatin material and after subtracting the IgG control for each sample. (*p<.05 versus vehicle treatment; *p<.05 versus 1µM BPA and 10µM BP treatment)

**IV.2c. Recruitment of ERα and SRC3 at the promoter of TFF1 gene after treatment with BP and BPA**

To understand the differences in the molecular mechanism of the transcriptional activation of PS2 gene *in vivo* by BP and BPA in comparison to E2 and 4OHT treatment we performed chromatin immuno-precipitation (ChIP) assay to evaluate the recruitment of ERα and SRC3 at the promoter region of TFF1 (PS2) gene (Fig. 11B) which has a well characterized functional estrogen responsive element (ERE) (108). MCF7 cells were treated with either 0.1% ethanol (veh), E2 (1 nM), 4OHT (1 µM), BP (1 µM or 10 µM) or BPA (1 µM or 10 µM) for 45 minutes and thereafter harvested for ChIP assay. The results (Fig. 11C) reveal that both concentrations of BPA (1 µM and 10 µM) recruited ERα to the PS2 promoter with ERE in a concentration dependent manner which was equivalent to results obtained with E2 treatment. In contrast BP did not show a concentration related effect and the levels of ERα plateaued at 50% of either E2 or BPA (Fig. 11C). Recruitment of the co-activator, SRC3 (AIB1), which plays a key role in transcriptional activation of several estrogen regulated genes including PS2 gene (115, 116), followed the similar pattern as the ERα (Fig. 11D). BPA treatment at both the concentrations (1
µM or 10 µM) recruited SRC3 in a concentration dependent manner to become equivalent to levels observed with E2 treatment whereas BP treatment (both concentration) plateaued at 50% of E2 or BPA recruitment levels (Fig 11D). As expected, 4OHT treatment did not recruit SRC3 and was comparable to vehicle treatment. The ChIP data correlates very well with the observed pattern of transcriptional activation of PS2 gene (Fig. 11A) under same treatment conditions.

**IV.2d. Differential induction of transforming growth factor alpha (TGFα) gene by BP and BPA in MDA: MB-231 cells stably transfected with wild type ERα or D351G mutant ERα.**

Previous studies from our laboratory have established an *in vitro* system to evaluate and differentiate the conformation of liganded ERα induced by planar and non-planar ligands (70). Activation of TGFα gene in MDA: MB 231 cells stably transfected with wild type (wt) ERα (MC2 cells) or mutant ERα (JM6 cells, D351G; which has the aspartate substituted with glycine at amino acid 351), is used as a marker to distinguish the ERα interactions between planar and non-planar estrogen ligands (70). We treated the MC2 and JM6 cells with increasing concentrations of BP and BPA and measured the TGFα induction in these cells. E2 was used as a positive control. In MC2 cells, (wt ERα), all the tested ligands induced TGFα transcripts level to similar levels (Fig. 12A). Induction of TGFα by BPA was observed at higher concentrations whereas BP and E2 had similar effects (Fig. 12A). On the other hand, in JM6 cells (mutant; D351G ERα), BP failed to induce TGFα transcription even at higher concentrations (Fig. 12B), whereas E2 and BPA treatment induced TGFα (Fig. 12B), although the maximal induction with BPA was observed at higher concentration (10 µM) which was less than 50% of E2 treatment. We further confirmed that E2-induced TGFα stimulation in JM6 cells was completely blocked by BP and 4OHT in a dose dependent manner; whereas co-treatment of BPA in presence of E2 failed to inhibit it (Fig. 12C).
Fig. 12. Induction of TGFα mRNA by E₂, BP, and BPA in MDA:MB 231 cells stably transfected with wild type ERα (MC2 cells) or D351G mutant ERα (JM6 cells). A. MC2 cells were treated with 17β estradiol (E₂), bisphenol (BP) or bisphenol-A (BPA) at indicated concentration for 48 hrs and cells were harvested for total RNA. Total RNA was reverse transcribed and real time PCR (RTPCR) was performed to assess the expression of TGFα using 36B4 as an internal control. B. JM6 cells were treated with 17β estradiol (E₂), bisphenol (BP) or bisphenol-A (BPA) at indicated concentrations for 48 hrs. The values are presented as fold difference versus vehicle treated cells. (*p<.05 versus 10 µM BP treatment) C. JM6 cells were treated with E₂ alone or in combination with different concentration of bisphenol (BP),
bisphenol-A (BPA) or 4-hydroxytamoxifen (4OHT) as indicated for 48 hrs. The values are presented as percentage of expression of TGFα mRNA considering the E2-induced levels as 100 percent. (*p<.05 versus 1nM E₂ and 1nM E₂ +1 µM BPA treatment)

IV.2e. Molecular docking of BP and BPA to the LBD of ER alpha

To determine the binding mode of BPA and BP to ERα, the ligands were docked to the agonist and antagonist conformations of the receptor. The experimental structure, 3ERT, was selected from PDB for the antagonist conformation of ERα (Fig. 13A) containing 4OHT, while for the agonist conformation two experimental structures were selected, namely the receptor cocrystallized with E₂, 1GWR (Fig. 13B) and DES, 3ERD (Fig. 13C), respectively. When BPA is docked to the antagonist conformation, 3ERT, it is oriented perpendicular with the binding pocket and in this alignment it has the propensity to form the H-bond network involving E353, R394 and a water molecule (Fig. 13D). Additionally, a hydrogen bond with the hydroxyl group of T347 is formed. In this alignment the binding site is poorly occupied and the hydrophobic contacts with the amino acids lining the bottom of the binding site are missing. In the case of BPA two highly probable binding modes have been identified. The first one has been mostly predicted when the ligand has been docked into the binding sites of ERα cocrystallized with E₂ and DES, the structure 3ERD_ε using the service pack (SP) mode. The ligand is placed across the binding site in a similar orientation with the native ligands, having the two methyl groups involved in hydrophobic contacts with the side chains of amino acids W383, L384, L525, and L540. Also, BPA forms H-bonds with H524 and E353 (Fig. 13E). When docking calculations
have been run in the XP mode of Glide a second alignment of the top tanked poses in the binding site of 3ERD_ε and 3ERD_δ has been noticed. This orientation involves the formation of H-bonds between the hydroxyl groups of BPA and amino acids G521, E353 and R394 (Fig. 13F). Apart from the H-bond formation, the methyl groups are involved in hydrophobic contacts with amino acids L346, F404, and L428. Also, this binding mode has been encountered for 6 out of 10 poses resulted from the docking of BPA into the experimental structure 1GWR. The predicted binding modes of BP to the open and closed conformation of ER are similar, forming the H-bond network between E353, R394 and the highly ordered water molecule and an additional H-bond with the hydroxyl group of T347 (Fig. 13G-I). The composite score, Emodel, shows that BP is better accommodated in the binding site of the open or antagonist conformation of ERα and it is more likely for the ligand to bind at this conformation of ER. Similar results have been obtained using the Induced Fit docking method, which accounts for both the ligand and protein flexibility (73). The comparative analysis of the composite score Emodel for the agonist and antagonist top ranked docking poses of BPA has shown that the binding mode predicted for the antagonist conformation is highly improbable and it is more likely for BPA to bind to a conformation of ERα closely related with the agonist one. Two distinct binding modes of BPA to the agonist conformations of ERα have been predicted with tight Emodel scores and cannot be clearly discriminated which alignment is correct or at least with the highest probability of being right. The docking scores calculated for E_2, DES and BPA shows the binding affinity of BPA to ERα is much lower when compared with the binding affinities of E_2 or DES to ERα.
Fig.13. **Molecular docking of BP and BPA with ERα ligand binding domain.** Crosssectional representations of ERα binding sites in the antagonist (A) with 4OHT and agonist (B, C) with 17β estradiol and DES conformations. The top ranked docking poses of BPA into the binding site of 3ERT (D), 1GWR (E), 3ERD (F) are displayed with C atoms colored in magenta while the best docking solutions of BP computed for 3ERT (G), 1GWR (H), 3ERD (I) are represented with C atoms colored in blue. The amino acids involved in H-bond contacts are depicted as sticks and the rest of the amino acids lining the binding site are shown as lines having the C atoms colored in gray. Only polar hydrogen atoms are shown, for simplicity.
IV.2f. Comparative analysis of regulation of apoptotic genes by BP, BPA, 4OHT and E2 in MCF7:5C cells using apoptotic gene RT-PCR profiler

We thereafter determined the effect of BP and BPA treatment in regulating the apoptosis related genes in MCF7:5C cells and compared it with E2 and 4OHT as a positive and negative inducer of apoptosis respectively. We used the RT-PCR profiler assay kits for apoptosis from a commercial vendor which uses 384 well plates to profile the expression of 370 apoptosis related human genes. To select a single time point of treatment with the ligands we first treated the MCF7:5C cells with E2 (1 nM) for 24, 48 and 72 hrs (in triplicates) and created an apoptotic gene signature throughout these time points after comparing them with vehicle treatment (SFig.5A-C and Table 3). This gene signature was generated by comparing the expression level of all the genes with vehicle treatment and selecting the genes which were at least 2.5 fold over-expressed or under-expressed as compared to vehicle treated cells. The fold change was calculated by delta-delta Ct method using the web based tool, RT2 profile PCR array data analysis version 3.5.
SFig.5. Representation of E₂ (1nM) regulated apoptotic genes in MCF7:5C cells at 24, 48 and 72 hrs of treatment versus vehicle treatment using volcano plots. A, B and C are the volcano plots of E₂-regulated apoptotic genes at 24 hrs, 48 hrs and 72 hrs respectively. Each circle in the plot represents one gene. Genes which are up-regulated at least 2.5 fold over vehicle treatment are denoted as red circles whereas the genes which are down-regulated at least 2.5 fold over vehicle are in green circles. The genes represented by black circles were not considered as differentially regulated. The circles above the blue horizontal line represent the genes which achieves the statistical significance of p value of 0.05. The p values are calculated based on a Student’s t-test of the replicate $2^\Delta(-\text{Delta Ct})$ values for each gene in the control group and treatment groups.
Table 3. Gene list of E2 (1nM) regulated apoptotic genes in MCF7:5C cells at 24, 48 and 72 hrs of treatment versus vehicle treatment. MCF7:5C cells were treated with vehicle or 1nM E2 for 24, 48, and 72 hrs. Total RNA was isolated and reverse transcribed. Subsequently real-time PCR was performed using RT-profiler assay kits for apoptosis and the genes which were at least
2.5 fold up-regulated or down-regulated as compared to vehicle treatment were selected for creating this gene list. All the treatments were performed in triplicate, and the data is presented as average fold regulation. *Gene S: Gene symbol; Fold Reg: Fold regulation.

After carefully analyzing the gene list generated by E2 treatments over the above said time period we selected 48 hrs as the time point to treat MCF7:5C cells with BP, BPA and 4OHT and compare the expression of the apoptosis related genes with the gene signature of the E2 treatment at 48 hrs. This particular time point was selected because the MCF7:5C cells undergo apoptotic changes after E2 treatment during this time period (Lewis et al., 2005) and also because after 48 hrs of E2 treatment the cells are committed to apoptosis, as 4OHT treatment cannot rescue these cells after this time point (56). Next, we analyzed the changes in the overall expression profiles of apoptotic genes by E2, 4OHT, BP, and BPA versus vehicle (Veh) treatment at 48 hrs (SFig.6A-D respectively) using the same apoptosis RT profiler. For any gene to be considered as differentially expressed we set the cut-off as 2.5 fold up- or down-regulation versus the vehicle treatment. Using this criterion we created a gene-list for up-regulated and down-regulated genes for each treatment group (Table 4).
SFig.6. Representation of 17-β estradiol (E2), 4-hydroxy tamoxifen, (4OHT), bisphenol, (BP) and bisphenol A, (BPA) regulated apoptotic genes in MCF7:5C cells after 48 hrs of treatment versus vehicle using volcano plots. A, B, C,D are the volcano plots of apoptotic genes regulated by E2, 4OHT, BP and BPA, respectively after 48 hrs treatment. Each circle in the plot represents one gene. Genes which are up-regulated at least 2.5 fold over vehicle treatment are denoted as red circles whereas the genes which are down-regulated at least 2.5 fold over vehicle are in green circles. The genes represented by black circles were not considered as differentially regulated. The circles above the blue horizontal line represent the genes which achieves the statistical significance of p value of 0.05. The p values are calculated based on a Student’s t-test of the replicate $2^\Delta (- \text{Delta Ct})$ values for each gene in the control group and treatment groups.
Table 4. Gene list of 17β estradiol, (E2), 4-hydroxy tamoxifen, (4OHT), bisphenol, (BP) and bisphenol A, (BPA) regulated apoptotic genes in MCF7:5C cells after 48 hrs of treatment versus vehicle. MCF7:5C cells were treated with vehicle, 1nM E2, 4OHT, BP and BPA for 48 hrs. Total RNA was isolated and reverse transcribed. Subsequently real-time PCR was performed using RT-profiler assay kits (see text for details) for apoptosis and the genes which were at least 2.5 fold up-regulated or down-regulated as compared to vehicle treatment were selected for creating this gene list. All the treatments were performed in triplicate, and the data presented is average fold regulation. *Gene S: Gene symbol; Fold Reg: Fold regulation.
We thereafter generated a heat map (Fig.14) in which we selected all the genes which were at least 2.5 fold up- or down-regulated by E₂ treatment and compared it with other ligand treatments. This heat map clearly demonstrates that the genes which are up-regulated at least 2.5 fold after 48 hrs of E₂ treatment are not upregulated in 4OHT or BP treatment. In contrast, the majority of the genes up-regulated by BPA treatment were shown to be the same genes up-regulated by the E₂ treatment. Many of these genes are up-regulated by BPA to the similar extent as E₂ and others show a distinct trend of over-expression as compared to vehicle (Fig. 14). Nevertheless, down-regulated genes follow a different pattern. The pattern of genes down-regulated by BP treatment resembles the pattern observed with E₂ and BPA treatment and not with the pattern of 4OHT treatment (Fig. 14 and Table 4). Approximately 53% and 61% of down-regulated genes are in common with E₂ treatment and with the treatment of BP and BPA respectively (Table 4).
Fig.14. Heat map of apoptotic genes which are at least 2.5 fold up- or down-regulated by 48 hrs of treatment of 17-β estradiol 10-9 M (E₂), versus vehicle and its relative comparison of their expression with 4-hydroxy tamoxifen, 1 μM (4OHT), bisphenol, 1 μM (BP) and bisphenol A, 1μM (BPA) treatment after 48 hrs in MCF7:5C cells. The maximum expressed level of any given gene is represented by red color and minimum levels are presented as green color. Control group and group 1, 2, 3, 4 are the re-presentation of the vehicle, E₂, 4OHT, BP and BPA treatments respectively. The gene expression levels in each treatment group are the average of three independent biological replicates.
IV.3 Discussion

The chemical structures of the ligands which bind to ERα are critical in determining the biological effects in the estrogen responsive cells and tissues. Minor changes in the ligand structures can alter the way these ligands interact with the ERα protein and transform the conformation of the liganded –ERα complex in the cells. Structure-function relationships have been studied extensively using various biological endpoints, such as modulation of prolactin gene expression in primary cell cultures of rat pituitary glands(97, 98, 117), or TGFα activation in stably transfected wt and mutant ERα in MDA:MB 231 cells (70). The current study dissects, compares and contrasts the mechanism of action of BP and BPA, two structurally similar ligands of ERα, which have opposing effects on apoptosis but not on the growth of estrogen responsive breast cancer cells. The results of this study established that unlike BPA and E2, BP was not functioning as an estrogen-agonist in inducing apoptosis in MCF7:5C cells while both compounds (BPA and BP) were estrogenic in inducing growth in MCF7 cells. This clearly indicated differential requirement of ERα mediated molecular action to achieve two distinct physiological responses in the breast cancer cells. Activation of estrogen responsive gene PS2 by these compounds in MCF7 cells suggested that higher concentrations of BPA was as effective as E2 but BP treatment failed to achieve E2-like stimulation, even with higher concentration. This phenomenon was observed because BP has a high ERα binding affinity and can maximally induce PS2 gene at lower concentration and raising the concentration did not enhance the induction because it failed to recruit sufficient co-activator (SRC3) at the PS2 gene promoter. This was most likely due to insufficient ERα recruitment at the promoter and inaccessibility of the co-activator interacting surface of BP liganded ERα. A recent study (103) however suggested that BP-liganded ERα cannot bind to a peptide containing the co-activator interacting domain.
This discrepancy can be attributed to the fact that our studies were performed in live cells chromatin as opposed to using an in vitro ELISA based system. This indicates that binding of liganded ERα and its interaction with other co-regulators can be modulated by other factors involved in transcriptional complex. On the other hand BPA at higher concentration engaged SRC3 to a similar level as E₂ treatment. The fact that higher concentration of BPA was required to recruit ERα and SRC3 to the similar levels as E₂ treatment is because it’s binding affinity with ERα is very low (relative binding affinity (RBA), 0.073) (113) and therefore higher concentrations of the ligand is required to drive the kinetics towards the activated state. In the case of BP, it has a strong binding affinity to the ERα (RBA, 96.0)(98) and therefore maximal activation is achieved at lower concentration and increasing concentration do not enhance the activation. Overall, these results indicate that binding mode of BPA and E₂ are similar whereas BP might bind differently to ERα. Indeed, our molecular docking studies determined that BPA binds to the ERα in two possible ways, both similar to agonistic mode of binding. Also docking scores calculated in this study predicted very low binding affinity of BPA to ERα, which is in excellent agreement with previous reports(24, 118, 119). In contrast, modeling studies suggested antagonistic mode of binding (as in 4OHT) for BP to the ERα. To confirm the molecular modeling we used a biological model system which can distinguish between planar and angular estrogen ligands (70, 112) by measuring the transcriptional activation of TGFα in MDA:MB 231 cells stably transfected with wt ERα (MC2 cells) or mtERα (D351G) (JM6 cells). Results (Fig 4B) show that BP treatment failed to activate TGFα transcription similar to 4OHT (70) in JM6 cells whereas BPA treatment was similar to E₂ action, albeit with lower potency. This consolidated our finding that the mode of action of BP is more like 4OHT rather than E₂. Importantly, the structure of BP is identical to 4OHT except for the basic di-methylamine-ethoxy
side chain. The absence of the side chain contributes towards the enhanced estrogenic properties of BP with AF-1 fully engaged in ER responses to stimulate growth, as H12 of the ERα protein liganded with BP may not be properly restrained. This contrasts with 4OHT or RAL, where the restricted structure of the coactivator-interacting interface for binding of SRC3 or other co-activators now has limited AF-1 and AF-2 activity for growth. Of note, 4OHT and BP liganded ERα was less efficiently recruited to the PS2 promoter ERE which may also contribute towards lesser recruitment of SCR3 for BP as recruitment of ERα precedes the co-activator binding (114). The fact that SRC3 is essential for E2- induced apoptosis in the MCF7:5C cells (96) as well as E2-mediated growth of MCF7 cells (95) coupled with the findings of this study, leads to the hypothesis that the estrogen-mediated growth of MCF7 cells is more sensitive and can be induced even if the conformation of the liganded-ERα complex allows only partial interaction of coactivators as in case of BP binding. In contrast, complete and robust interaction of co-activator with the liganded-ERα complex must be needed for rapid induction of apoptosis in MCF7:5C cells. Indeed, using an “apoptosis” pathway focused real-time PCR based profiler consisting of 370 genes, this study further illustrated that apoptosis related genes were similarly up-regulated by E2 and BPA treatments after 48 hrs of treatment whereas BP and 4OHT showed very few upregulated genes and the TPE based compounds did not have a similar profile of up-regulated genes during this time frame. By comparing the gene list (Table 3), which includes all the genes up- or down-regulated at least 2.5 fold by the treatments, it is evident that 66% of up-regulated genes are common between E2 and BPA treatment, whereas only 8% genes are commonly up-regulated by BP or 4OHT treatment. Interestingly, a different pattern was observed for the down-regulated genes as both BP and BPA treatment exhibited common down-regulated genes as E2 and distinctly different from 4OHT. This suggests that the conformational requirement of
liganded- ERα may be different for upregulation and down-regulation of genes. Furthermore, it indicates that the up-regulated apoptotic genes are responsible for triggering and executing apoptosis since up-regulated genes are differentially regulated by BP and BPA but not the down-regulated genes. These observations merits further investigations. By employing structurally related ligands and using MCF7:5C and parental MCF7 cells we have demonstrated that depending upon the biological response the same molecule can function as an E2-antagonist or – agonist respectively. Based on these data it is reasonable to speculate that genistein and related phytoestrogens may also induce apoptosis in MCF7:5C cells as their binding to ERα LBD is similar as E2 and DES (120) and function as type I estrogens (112). In conclusion, this study provides evidence that binding of ERα with different ligands that program conformational changes of the liganded-ERα, determines the transcriptional profile of the responsive genes by virtue of interaction with co-regulators.
CHAPTER V

Delayed triggering of estrogen induced apoptosis that contrasts with rapid paclitaxel induced breast cancer cell death.

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Endocrine therapy remains the standard of care in the treatment of estrogen receptor (ER) positive breast cancer(1). Tamoxifen inhibits estradiol (E2)-induced tumor growth; but continuous tamoxifen treatment of nude mice with transplantable ER positive tumors results in tumor growth with either E2 or tamoxifen (23, 24). After 5 years of re-transplantation and tamoxifen treatment, these serially transplanted tamoxifen stimulated tumors grow in response to tamoxifen but paradoxically rapidly regress with physiologic E2 treatment (31). Development of acquired resistance to long term (5 years) antihormonal therapy in breast cancer causes a reconfiguration of the tumor cells that now makes them vulnerable to physiologic E2-induced apoptosis. MCF7 breast cancer cells that are resistant to long term estrogen withdrawal undergo apoptosis in response to E2 (38, 39). Clinical trials (79, 80) have evaluated this concept and their results show that about thirty percent of patients with advanced breast cancer who have acquired resistant to antihormone therapy show an objective clinical response with estrogen therapy. The Women Health Initiative (WHI) trial (121), which compared conjugated equine estrogen (CEE) therapy with placebo in hysterectomised postmenopausal women, noted a paradoxical decrease in incidence of breast cancer compared to combination CEE and progestin (122, 123) and this observation was subsequently supported by results from the Million Women Study (124). In neither clinical study was a molecular mechanism offered to explain the apparent anomaly that CEE alone does not induce a profound significant increase in breast cancer risk. However reanalysis of the mature data from the WHI CEE alone study (81) now demonstrates a persistent and sustained decrease in the incidence and mortality of breast cancer in women who received E2 alone therapy. We recently reported that constituents of CEE cause apoptosis in long term E2-deprived MCF7 cells (83). Given that these laboratory observations translate to clinical
benefit for patients, it is appropriate to investigate the molecular events that precede the induction of apoptosis by E2.

Cancer chemotherapy induces rapid death of neoplastic cells(125, 126), but E2-induced apoptosis, in contrast, is a delayed event. Ariazi and colleagues (40), recently identified the total gene activation sequence that occurs over a 7 day period during E2 induced apoptosis. Endoplasmic reticulum stress is induced by E2, which activates unfolded protein response leading to upregulation of mitochondrial proapoptotic genes. Involvement of the extrinsic pathway in E2 induced apoptosis have been implicated but its exact role is not clearly defined (32, 37). However, nothing is known on the effect of cytotoxic chemotherapy in the MCF7:5C cells. Paclitaxel, a member of the drug family, the taxanes is a mitotic spindle inhibitor that prevents destabilization of microtubules (127, 128). Taxanes are used extensively as part of combination therapy in metastatic breast cancer (129, 130)and are the gold standard in the adjuvant therapy of early breast cancer where they decrease risk of cancer recurrence and mortality(131, 132).

The goal of this paper is to determine the critical trigger point for E2-induced apoptosis. We have explored the differential gene expression as a prelude to determining the early molecular events in E2 induced apoptosis in comparison to classic cytotoxic chemotherapy-induced apoptosis. Induction of mRNA levels of proapoptotic genes confirmed whether mitochondrial and tumor necrosis factor (TNF) apoptotic pathways were activated. We compared and contrasted the ability of E2 and paclitaxel to arrest cell cycle to advance the molecular understanding of the new biology of E2-induced apoptosis in therapy.
V.2. Results

V.2a. Cell growth and apoptotic effects of E₂ and paclitaxel on MCF7:5C cells

We sought to compare the antiproliferative activity between paclitaxel and E₂ in the MCF7:5C cell line and explore their potential to induce apoptosis. Paclitaxel induced rapid inhibition of growth in a concentration dependent manner with maximum inhibition at 0.1µM. Fifty percent growth inhibition was achieved by 24h (Fig.15A), which increased to almost 100% after 48h of treatment (Fig.15B). In contrast, E₂ achieved maximal growth inhibition at 0.1nM, and did not quantitatively prevent cell proliferation until after 72hrs (Fig.15C). Twenty five percent of growth inhibition occurred at 96h with E₂ treatment (Fig.15D) and this increased to 80% at the 120h time point (Fig.15E). The decrease in cell number observed with E₂ and paclitaxel was further investigated to determine whether the growth inhibition was due to apoptosis. An increased apoptotic response (Fig.16A) was detected by increasing the percentage of annexin V staining from control 3.92% to 21.49% by paclitaxel after 12h treatment, whereas an apoptotic effect was observed at 72h with E₂ (Fig.16B). An apoptotic response was not detected after 24h treatments with E₂ through annexin V staining (SFig.7A). Experiments were repeated three times and a summary of results are represented in SFig.7. Similar results were observed with a DNA binding dye, YO-PRO-1 (SFig8).
Fig.15. Effect of E2 and paclitaxel on the growth characteristics in the MCF7:5C cells. MCF5C cells were seeded in 24-well plate treated with the control vehicle (Veh) or E2 (♦) and paclitaxel (□) over a range of doses and cells were harvested after (A) 24h (B) 48h (C) 72h (D) 96h and (E) 120h. Data points shown are the average of 3 replicate +/- SD. [**p<0.02, ***p<0.0003, ****p<0.0001]
Fig.16. Differential apoptotic effects of E₂ and paclitaxel

MCF7:5C cells were treated with control or (A) paclitaxel (1µM) for 12h and 24h or (B) E₂ (1nM) for 72h and then stained with annexin V-FITC and propidium iodide and analysed by flow cytometry. Viable cells (left lower quadrant) are annexin V-FITC - and PI - , early apoptotic cells (right lower quadrant) are annexin V-FITC + and PI - , dead cells (left upper quadrant) are PI + and late apoptotic cells (right upper quadrant) are annexin V-FITC + and PI +. Increased staining for apoptosis is observed maximally in the right upper quadrant.
SFig.7. Annexin v analysis of apoptotic effects of E₂ and paclitaxel. MCF7:5C cells were treated with control or E₂ (1nM) for (A) 24h and (B) 72h or (C) paclitaxel (1µM) for 12h and 24h and then stained with annexin v-FITC and propidium iodide and analysed by flow cytometry. All the data shown were representative of at least three separate experiments with similar results.
SFig.8. Analysis of apoptotic effects of E$_2$ and paclitaxel. MCF7:5C cells were treated with control or (A) E$_2$(1nM) for 48h and 72h or (B) paclitaxel (1µM) for 12h and 24h and then stained with nucleic acid dye YO-PRO-1 and propidium iodide and analysed by flow cytometry. All the data shown were representative of at least three separate experiments with similar results.
V.2b. Determination of the critical trigger point of estradiol induced apoptosis

Although $E_2$ treatment induces apoptosis of MCF7:5C cells in a concentration dependent manner, the cells are unresponsive to the antiestrogen, 4OHT. Rather 4OHT blocks $E_2$ mediated apoptosis(76). To further investigate the delayed response to $E_2$ mediated apoptosis and determine the critical trigger point for $E_2$ induced apoptosis, we used 4OHT to block and rescue the cells from the apoptotic effect of $E_2$. In this way, we established when the cells are committed to cell death. MCF7:5C cells were treated with 1 nM of $E_2$ and subsequently 1$\mu$M of 4OHT was used to block the apoptotic effects of $E_2$ at the indicated time points over a range of 96h after the addition of $E_2$. Cells were then all collected for DNA assay on day 7. Apoptosis triggered by $E_2$ was competitively inhibited and rescued for up to 24h, and thereafter, it lost the ability to rescue cells committed to $E_2$ induced apoptosis (Fig.17). Between 24h- 36h, the cells are committed to apoptosis despite the antiestrogenic action of 4OHT. These data suggest that the critical trigger for the commitment of the cell to the induction of apoptosis by $E_2$ lies between 24h and 36h.
Fig. 17. Deciphering the trigger point for E₂-induced apoptosis

Cells were treated with vehicle (Veh) or E₂ (1nM) alone and 1µM 4OHT was added and used to block and reverse E₂ action at 6h, 12h, 24h, 36h, 48h, 60h, 72h, 84h, and 96h. The cells were harvested after 7 days of treatment. The extent of apoptosis was determined by measuring the DNA content of the remaining cells in each well. The experiment was done in triplicates and the data represent the mean of three independent experiments with 95% confidence intervals. The trigger point for E₂ mediated apoptosis was elucidated at the time when the apoptotic effects of E₂ could not be blocked by 4OHT.
V.2c. Differential gene expression of E2 mediated apoptosis at the critical trigger point

To identify genes associated with E2-induced apoptosis with a particular focus on the critical trigger time point, differential regulation of apoptotic gene expression in response to E2 was interrogated in the MCF7:5C cells. At 24h, as expected significant evidence of apoptotic gene induction is not apparent, rather proapoptotic genes such as BAD,BCL2L10 and Caspases 1, 9 and 10 are differentially downregulated by E2 (Table 5). TNF related genes, TNFRSF8 and TNFSF14 are induced by both E2 and 4OHT and they do not play a definitive role in the TNF mediated apoptosis but rather are involved in the T cell response. Interestingly, at 36h (Fig.18A), which represents the trigger point for apoptosis, E2 induces proinflammatory genes such as CEBPB, CEBPG, and DAPK1 and endoplasmic reticulum stress related genes; DDIT3 and ERN1. BCL2L11 (BIM), an important member of the mitochondrial pathway and an apoptosis activator is also upregulated by E2, suggesting an early involvement of the intrinsic pathway. Following 48h of E2 treatment (Fig.18B), the gene expression expands to involve the TNF related genes: FAS, TNFRSF21 and TNF and continued increased expression of endoplasmic reticulum stress and proinflammatory related genes. In addition p53 expression is increased at 48h. PMAIP 1 (also known as NOXA), a Bcl-2 homology (BH3) only family and a p53 regulated gene is also upregulated by E2. 4OHT acted as an antiestrogen and was able to block most of the effects of E2. The identified apoptosis related genes are listed in Table 5-7.
**Fig. 18. Heat map of E2-mediated apoptotic genes which are differentially expressed by 36h and 48 h of treatment.** Cells were parsed into groups of 3 replicates per treatment per time point, and then treated with either 0.1%ethanol (control group), 1nM E2 (group 1), 1 µM 4OHT (group 2), in the presence (group 3), or absence of E2 over a period of 48 h. Total RNA was extracted and reverse transcribed as described in materials and methods. Samples were loaded onto customized PCR array plates with primers for indicated apoptotic genes. Gene expression values were obtained and analyzed in comparison to the controls at (A) 36h and (B) 48h. The maximum expressed level of any given gene is represented by red color and minimum levels are presented as green color.
Table 5. Gene list of E2 (1nM) regulated apoptotic genes in MCF7:5C cells at 24h of treatment versus vehicle treatment in comparison to 4OHT. Apoptotic genes regulated by E2 and 4OHT after 24 h treatment were determined using RT-profiler assay kits for apoptosis. Data values include the genes which were at least 2.5 fold up-regulated (red) or down-regulated (blue) relative to the vehicle treatment. The genes which achieve the statistical significance of p value of 0.05 were selected for creating this gene list. *Gene S: Gene symbol; Fold reg: Fold regulation

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Table 6. Gene list of E2 (1nM) regulated apoptotic genes in MCF7:5C cells at 36h of treatment versus vehicle treatment in comparison to 4OHT. Apoptotic genes regulated by E2 and 4OHT after 36 h treatment were determined using RT-profiler assay kits for apoptosis. Data values include the genes which were at least 2.5 fold up-regulated (red) or down-regulated (blue) relative to the vehicle treatment. The genes which achieve the statistical significance of p value of 0.05 were selected for creating this gene list. *Gene S: Gene symbol; Fold reg: Fold regulation

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Table 7. Gene list of E2 (1nM) regulated apoptotic genes in MCF7:5C cells at 48h of treatment versus vehicle treatment in comparison to 4OHT. Apoptotic genes regulated by E2 and 4OHT after 48 h treatment were determined using RT-profiler assay kits for apoptosis. Data values include the genes which were at least 2.5 fold up-regulated (red) or down-regulated (blue) relative to the vehicle treatment. The genes which achieve the statistical significance of p value of 0.05 were selected for creating this gene list. *Gene S: Gene symbol; Fold reg: Fold regulation

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V.2d. Paclitaxel induces TNF family of apoptotic related genes in MCF7:5C cells.

We further investigated expressed genes activated by paclitaxel that may define a molecular mechanism. Based on the biological experiments shown above (Fig.15 and Fig. 16B), paclitaxel-induced apoptosis happened after 12h treatment and reached to a peak at 24h. We mainly focused on detecting gene regulation by paclitaxel at these two time points. Paclitaxel selectively activated the TNF family of apoptotic related genes. After an initial 12h of treatment (Fig.19A-B), paclitaxel stimulated TNFRSF10A (TNF receptor superfamily, member 10a) and TNFRSF10B (TNF receptor superfamily, member 10b) which are known to be activated by the ligand TNF-related apoptosis inducing ligand (TNFSF10/TRAIL), and causes death through the extramitochondrial pathway. TNFRSF19 (TNF receptor superfamily, member 19) induces apoptosis in a caspase-independent manner. In addition, TNF proapoptotic genes, including FAS, TNF and other TNF proinflammatory genes; LTA, LTB and TNFAIP3 are activated by 24h of treatment with paclitaxel (Fig. 19C-D). Paclitaxel further induces NOXA and CDKN1A (p21) which is known to inhibit the activity of cyclin-CDK2 or -CDK4 complexes at the G1 phase. Although these two p53 regulated genes were upregulated by paclitaxel, p53 induction was not observed at 24h. Unlike E2, which increases BIM and TNF mRNA levels (Fig.20A-B), paclitaxel was only able to induce TNF expression (Fig.20C-D). These results highlight the differences in apoptotic related genes induced by the two treatments.
Fig. 19. Determination of apoptotic genes induced by a cytotoxic chemotherapy in MCF7:5C cells. MCF7: 5C cells were treated with either 0.1% ethanol (control), or 1uM paclitaxel (group 1) for 12h, and 24h. Gene expression values were obtained and analyzed in comparison to the controls and volcano plots were generated at 12h of treatment (A) and the expressed genes listed (B). Similarly, gene expression levels are analyzed after 24h of paclitaxel treatment (C) and genes are listed in (D). The genes selected were at least 2.5 fold over-expressed or under-expressed as compared to vehicle at p value of 0.05. Genes upregulated are represented in red and downregulated genes are represented in green.
Fig. 20. E₂ activates both mitochondrial and extrinsic pathway of apoptosis, while paclitaxel activates only the extrinsic pathway. MCF7:5C cells were treated with Vehicle (Veh), 1 nM E₂, 1µM 4OHT or combination treatment of E₂ and 4OHT for 24, 36 and 48 h. Total RNA was reverse transcribed and assessed for (A) BIM and (B) TNF gene expression. Induction of (C) BIM and (D) TNF mRNA was determined in MCF7:5C cells treated either Veh or 1µM paclitaxel for 12h and 24h using RT-PCR. PCR data values are presented as fold difference versus vehicle treated cells ± SEM. [** p<0.02, *** p<0.0003, **** p<0.0001]
V.2e. Differential effect of paclitaxel in induction of G2 blockade in comparison to E2

Paclitaxel prevents progression of mitosis and activates the mitotic checkpoint, paving a path for apoptosis. To elucidate whether the apoptotic effects of paclitaxel in comparison to E2 were mediated through cell cycle arrest, we performed cell cycle analysis in MCF7:5C cells using flow cytometry. Our results reveal that paclitaxel treatment causes accumulation of cells in G2/M phase with a concomitant reduction in the number of cells in G1 phase and S phase (Fig.21) Cell cycle arrest in G2/M phase was about 3-fold higher compared with control. In contrast, a G1 or G2 blockade was not observed with E2 treatment. E2 dramatically enhanced S phase at 12h and rapidly increased to 7-fold by 48h. This is consistent with our recent publication (54) that E2 increased S phase after 72 hours treatment. Based on these observations, we hypothesize that the apoptotic effects of paclitaxel in MCF7:5C cells results from a perturbation in the cell cycle check points, whereas E2 induces cell proliferation finally resulting in apoptosis.
Fig. 21. Cell cycle analysis of the effects of E2 and paclitaxel in the MCF7:5C cells

Representative cell cycle profiles of MCF7:5C cells treated with either 0.1% ethanol (Veh), E2 (1nM) or paclitaxel (1µM) for 12h, 24h and 48 h. FL2-A represents the intensity of propidium iodide, and the y axis represents the cell number.
V.3. Discussion

The molecular sequence of events resulting in either E$_2$-induced apoptosis or paclitaxel- induced apoptosis is completely different. E$_2$-induced apoptosis appears to be unique. Paclitaxel rapidly induces apoptosis of MCF7:5C cells, whereas E$_2$ shows a delayed process for the induction of apoptosis. Using 4OHT to block and rescue E$_2$ induced events necessary for an apoptotic response, we observed that the trigger for apoptosis occurs after 24h and the cells become committed to apoptosis by and after 36h. There is activation by E$_2$ of endoplasmic reticulum stress related genes and proinflammatory genes at 36h. Activation of the mitochondrial pathway was indicated by increased expression of BCL2L11, BIM, which continued to be upregulated at 48h. Involvement of the extrinsic pathway was evidenced by induction of FAS, TNFRSF21 and TNF and TNFAIP3 at 48h. The TNF family genes are a group of cytokines that are involved in a number of processes including apoptosis(133, 134) and inflammation(135). The increased involvement of endoplasmic reticulum stress and inflammatory genes in E$_2$ induced apoptosis is not surprising because both pathways are known to intersect (136, 137). Multiple genes induced by E$_2$ are NF-$\kappa$B responsive which is a major regulator of inflammatory response(138, 139). Upregulation of the observed genes provide a potential mechanism for E$_2$ to target a variety of inflammatory and apoptotic genes.

The importance of BIM and Bax have previously been noted and verified by selective increased expression of both proteins by E$_2$ (39). Involvement of the extrinsic signaling pathway in E$_2$-induced apoptosis has been also observed. Osipo et al(32) showed that E$_2$ induced regression of tamoxifen stimulated breast cancer tumors, by activating the death receptor Fas and inhibiting the antiapoptotic/prosurvival factors NF-kB and HER2/neu. In addition, the growth of raloxifene resistant MCF7 cells *in vitro* and *in vivo* was inhibited by E$_2$ by increasing Fas
expression and reduced NF-kB activity (33). However unlike the present study, none of the previous studies investigated a time course of the intrinsic and extrinsic pathway in the MCF7:5C cells in E2-induced apoptosis. Similar to our PCR array results, RNA sequencing of E2 treated MCF7:5C cells revealed induction of multiple apoptotic related genes (54), therefore deletion of a single gene is unlikely to significantly affect E2 mediated apoptosis in the MCF7:5C cells. E2 induces apoptosis in osteoclasts within 24h (140) and is associated with upregulation of TGF-β and inhibition of E2 treated cells with anti-TGF-β antibody inhibited E2-induced apoptosis (141). Therefore, this study show a unique sequential activation of endoplasmic reticulum stress, inflammatory response genes as well as the intrinsic and extrinsic apoptotic related genes in E2 mediated apoptosis.

Paclitaxel, a cytotoxic chemotherapy extensively used in the treatment of breast cancer was used as a comparator to E2 to demonstrate differences in the expression of apoptosis related genes. Paclitaxel selectively induces the TNF proapoptotic genes, but BIM expression was not noted. On the other hand, paclitaxel kills the MCF-7 cells by displacement of BIM from the BIM/BCL2 complex (142). Knockdown of BIM with siRNA significantly impairs the ability of paclitaxel to cause apoptosis in MCF-7 cells (142, 143). In contrast, another study (144) showed that BIM was not required for paclitaxel mediated apoptosis in MCF-7 cells and these apparent discrepancies could be due to differences that exist from MCF-7 cell lines obtained from different sources. However, long term deprivation of E2 from the MCF7 cells may have induced changes in the microenvironment that may be responsible for the taxane to activate the TNF apoptosis related genes. Flow cytometry studies show that E2 causes both proliferation and apoptosis of the MCF7:5C cells indicating that before the trigger for apoptosis occurs, the cells grow in response to E2. Because cells continue to divide with elevated S phase of cell cycles, the
reduction of cell number by E2 do not become evident until after 4 days of treatment. In contrast, paclitaxel causes an immediate G2 blockade by 12h which may explain the rapid reduction of cell number.

In conclusion, the initial target site of E2 is ER. E2 induces endoplasmic reticulum stress and mitochondrial apoptotic genes and a later recruitment of the TNF family of apoptotic genes, whereas paclitaxel induces a G2/M blockade and rapidly induces TNF apoptotic related genes. The unique delayed aspect of E2 induced apoptosis in antihormone resistant breast cancer creates a new dimension in our opportunities to apply the knowledge for this targeted therapy of clinical significance (80, 81, 83). This natural process of E2-induced apoptosis may have significant applications in the further understanding of the cellular biology of cancer.
CHAPTER V1

Differences in the Rate of Oestrogen-induced Apoptosis in Breast Cancer by Estradiol and Triphenylethylene Bisphenol.

Obiorah I\textsuperscript{1} and Jordan VC\textsuperscript{1}

\textsuperscript{1}Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC 20057

VI.1. Introduction

Apoptosis triggered by physiological oestrogen levels in antihormone resistant oestrogen receptor (ER) positive breast cancer (30, 31, 37, 39), is a well-documented laboratory phenomenon, which has clinical significance. Low dose oestradiol causes 30% clinical benefit for the treatment of aromatase resistant breast cancer (80) and treatment of postmenopausal women in their 60’s with conjugated equine oestrogen causes a decrease in breast cancer incidence and mortality (81) in oestrogen deprived cancer in women who are 15 years postmenopausal. It is proposed that the key to triggering oestrogen-induced apoptosis is the selection of vulnerable ER positive tumour cell populations that evolve and eventually dominate the tumour during long oestrogen deprivation or antihormone therapy (78, 83, 145, 146). However unlike the immediate and catastrophic initiation of apoptosis by paclitaxel, there is a delay with commitment of the cell for 24h with E2 that completes the process by 96h (56). Oestrogen induced apoptosis is heralded by endoplasmic reticulum stress (ERS) and an unfolded protein response (UPR) (40, 54).

Oestrogen can be classified into planar (class 1) and angular (class II) ligands that create different shapes when complexed with the ER (70). A class 1 oestrogen such as E2 causes cell replication and apoptosis because the ligand is sealed by helix 12 in the ligand binding domain of the ER complex. By contrast, a class II angular oestrogen such as bisphenol causes cell replication but cannot cause oestrogen induced apoptosis in a short term 7 day in vitro assay (73, 147). In fact, BP blocks oestradiol-induced apoptosis in a manner similar to 4-hydroxytamoxifen (4OHT) (147). It appears that BP can adopt the conformation of the 4OHT-ER complex (103). However, these laboratory data are paradoxical as they do not conform to the known antitumour effects on class 1 and II oestrogens in the successful treatment of metastatic
breast cancer in postmenopausal women (15).

High dose oestrogen therapy was the first “chemical therapy” to be used successfully to treat any cancer. Haddow (15) demonstrated, after preliminary laboratory studies, that high doses of two structurally different oestrogens diethylstiboestrol (class I) and triphenylchlorethylene (class II), were both effective in producing a 30% response rate in post-menopausal women with metastatic breast cancer. Haddow (16) also noted that responses were more likely in breast cancer if the patient was more than 5 years post menopause. Today, it is recognized that oestrogen deprivation caused by menopause creates a selection pressure for breast tumour cells that results in the outgrowth of cellular populations more likely to die with oestrogens than grow (83).

We have addressed the paradox that an angular class II oestrogen, BP, can act as an inhibitor of oestrogen-induced apoptosis by adopting an “antiestrogenic conformation” for the BP:ER complex (103, 147), but related triphenylethylenes are effective antitumour agents in patients (15). We have found that the trigger for oestrogen-induced apoptosis is dependent not

**VI.2. Results**

**VI.2a. Differential expression of cell cycle genes induced by bisphenol and 17β oestradiol**

We have previously shown that BP, a triphenylethylene can induce the growth of MCF7 breast cancer cells as effectively as E₂ (73, 147). To identify cell cycle genes associated with BP induced cell growth, MCF7 cells were treated with 1µM BP for 6h, 12h and 24h and compared to 1nM E₂ and 1µM 4OHT as positive and negative regulators of cell replication respectively. The antiestrogen, 4OHT was used to block the stimulatory effects of BP and E₂. We used RT-PCR array kits that contain 4 x 96 well plates to profile the expression of 84 genes key to cell
cycle regulation. At 6h, E$_2$ induces several genes such as cyclin D1 (CCND1), CDK5R1, Herc5, CHEK2 and RBBP8 (Fig.22A). Bisphenol and 4OHT only induced HERC5. Interestingly CCND1 was downregulated by BP at this time point. There was increased expression of cell cycle related genes by E$_2$ at 12h (Fig.22B), which further increased by almost 2 fold at 24h (Fig.22C). Similarly, BP induced 60% and 50% of the cell cycle related genes that were up-regulated by E$_2$ at 12h and 24h respectively. The rest of the cell cycle related genes induced by BP show an obvious trend of overexpression when compared to the control. Similarly, all cell cycle genes downregulated by BP are equally decreased by E$_2$ treatment. List of genes induced by E$_2$ and BP are presented in Table 8. Furthermore, E$_2$ and BP decrease retinoblastoma protein (RB1) mRNA levels in a time dependent manner (SFig.9). Unlike the estrogens, 4OHT did not activate the cell cycle related genes but rather blocked the effects of E$_2$ and BP. These results demonstrate that BP induces similar cell cycle related genes as E$_2$, although not as effectively.
Fig. 22. Heat map of the time course pattern of E$_2$ and BP-regulated expression of cell cycle genes. MCF-7 breast cancer cells were treated with either control, E$_2$ (1nM), BP (1µM) or 4OHT (1µM) over a period of 24h and 4OHT was used to block the effects of E$_2$ and BP. Genes which are at least 2 fold up – or downregulated in comparison to the controls at (A) 6h, (B) 12h and (C) 24h are represented by the red and green color respectively.
Table 8. List of cell cycle regulated genes induced by E2 (1nM), BP (1µM) and 4OHT (1µM) combination treatments of 4OHT and E2 and 4OHT and BP in MCF-7 cells after 24 h of treatment versus control. Upregulated genes are in red and downregulated genes are in blue.

*Gene S: Gene symbol; Fold reg: Fold regulation.
SFig.9. **Inhibition of (retinoblastoma protein) RB1 by E2 and BP.** MCF-7 cells were treated with either Veh (control), E2 (1nM) or BP (1µM) for 4h, 8h, 12h and 24h. 4OHT (1µM) and combination of 4OHT with either E2 or BP were used as negative controls. Retinoblastoma protein (RB) mRNA levels were determined using RT-PCR.
VI.2b. Effect of bisphenol on apoptosis in MCF7:5C cells

The planar type 1 oestrogen, E\textsubscript{2} induces apoptosis in long term oestrogen deprived MCF7 (MCF7:5C) cells. On the other hand, the angular oestrogen BP does not initially induce apoptosis in MCF7:5C cells and blocks E\textsubscript{2} induced apoptosis in a similar manner as does 4OHT(147). To evaluate the long term effects of BP, we treated MCF7:5C cells with 1 µM BP, 1nM E\textsubscript{2} and vehicle control(0.1% ethanol). Growth of the cells was inhibited by E\textsubscript{2} after 3 days of treatment and the effect became maximal by 6 days of treatment (Fig.23A). On the other hand, BP increases the growth of the cells up to 6 days of treatment (Fig.23A) but causes 100% inhibition of growth by 9 days of treatment (Fig.23B). The inhibition of growth observed with BP was further investigated for apoptosis using flow cytometry. Following 6 days of treatment, BP caused an 7 fold increase in the percent of cells undergoing apoptosis (4.15% vs. 27.61%) compared to the control (Fig.23C) using Annexin V staining. A similar effect was observed using a DNA binding stain, YO-PRO-1 (SFig.10).
Fig. 23. Effect of BP in the growth and apoptosis of MCF7:5C breast cancer cells. (A) Cells were seeded in triplicates and treated with either control, E₂ (1nM) or BP (1µM) and the cells were harvested daily for 6 days. (B) Treatment with BP versus the control was extended for 13 days and the DNA content of the remaining cells in each well was quantified. The data represent the mean of three independent experiments. (C) MCF7:5C cells were treated with control or BP (1µM) for 6 days and then stained with annexin v-FITC and propidium iodide and analysed by flow cytometry. Viable cells (left lower quadrant) are annexin v-FITC − and PI −, early apoptotic cells (right lower quadrant) are annexin v-FITC + and PI −, dead cells (left upper
quadrant) are PI + and late apoptotic cells (right upper quadrant) are annexin v-FITC + and PI +. Increased late apoptotic effect is observed in the right upper quadrant.

SFig.10. Apoptotic effect of BP in MCF7:5C cells. MCF7:5C cells were treated with vehicle (control) or BP (1µM) for 5 days and then stained with nucleic acid dye YO-PRO-1 and propidium iodide and analysed by flow cytometry. Viable cells (left lower quadrant) are YO-PRO-1 − and PI −, early apoptotic cells (right lower quadrant) are YO-PRO-1 + and PI −, dead cells (left upper quadrant) are PI + and late apoptotic cells (right upper quadrant) are YO-PRO-1 + and PI +. Increased late apoptotic effect is observed in the right upper quadrant.
VI.2c. Determination of the point of commitment for BP induced apoptosis

Next, we investigated the delayed response of BP, MCF7:5C cells were treated with BP [1μM] and 4OHT [1μM] was used to block the antiproliferative and apoptotic effects of BP at daily intervals over a range of 9 days. Cells were harvested after 13 days of treatment and total DNA was quantified using a fluorescent DNA quantification kit. Apoptosis induced by BP was blocked by daily additions of 4OHT for up to 3 days and afterwards the cells became committed to apoptosis mediated by BP (Fig.24). Cells could not be rescued from BP induced apoptosis by 4OHT after 4 days of treatment suggesting that the cell commitment trigger for apoptosis has occurred. The experiment was repeated with ICI 182,780 as the estrogen antagonist and similar results were obtained (SFig.11). It is important to emphasize that each of the two “rescue” experiments adds antiestrogens 4OHT or ICI 182,780 at specific days after BP and measures cellular DA at 13 days. MCF7:5C cells are both committed to apoptosis after day 3 with either antiestrogen (Fig 3, Fig S4).
**Fig. 24. Determination of the trigger point for BP induced apoptosis.** MCF7:5C cells were treated in triplicates with BP (1μM) alone and 1μM 4OHT was added and used to block and reverse BP action daily over a period of 9 days. The cells were harvested after 14 days of treatment. The DNA content of the remaining cells was quantified using a fluorescent DNA quantification kit. The point of trigger for apoptosis induced by BP is determined by the time when the apoptotic effects of BP could not be blocked by 4OHT.
**SFig.11. Determination of the critical trigger of apoptosis.** MCF7:5C cells were treated in triplicates with BP (1µM) alone and ICI 182 780 (1µM) was added at the indicated time points and used to block and reverse BP action over a period of 9 days. The cells were harvested after 14 days of treatment. The DNA content of the remaining cells was quantified using a fluorescent DNA quantification kit.
VI.2d. Apoptosis related genes induced by bisphenol

To determine the early events preceding BP induced apoptosis, the induction of apoptosis related genes were investigated in MCF7:5C cells treated with BP [1µM], vehicle control (0.1% ethanol), 1µM 4OHT and BP in combination with 4OHT (in triplicates) for 3, 4, 5 days. We used 384 well RT-PCR profiler plates to monitor expression of 370 apoptosis related human genes (see Methods). Comparative analysis showed that significant evidence of apoptotic gene induction did not occur until after 3 days of treatment. At 4 days (Fig.25A) BP induces endoplasmic reticulum stress (ERS) related genes; DDIT3 and inflammatory stress (IS) response genes such as CEBPB, IFI6, IFI16 and DAPK1. At 5 days of treatment (Fig.25B) there is continued increase in the up-regulation of ERS and IS associated genes including LTA and caspase 4, an inflammatory caspase. Levels of LTA and caspase 4 mRNA were elucidated using RT-PCR (SFig.12). The apoptosis related genes detected using the PCR arrays are listed in Tables 9-10. Bim/BCL2L11 is important for E2 induced apoptosis. Its activation by E2 occurs by 36h of treatment(56) and E2 subsequently induces the TNF family of proapoptosis related genes. The induction of these genes by BP was investigated by extending the duration of treatment for 7, 8 and 9 days mRNA levels of BCL2L11 and TNF were quantified by RT-PCR. Upregulation of Bim/BCL2L11 (Fig.26A), TNF (Fig.26B), FAS (Fig.26C) and FADD (Fig.26D) was observed by 8 days of treatment with continued increase of all genes at 9 days of treatment with BP. These data indicate that there is a prolonged induction of ERS and IS associated genes by 4 days of treatment with subsequent up-regulation of mitochondrial and TNF related apoptosis genes.
Fig. 25. Determination of apoptotic genes differentially expressed by BP treatment in MCF7:5C cells. MCF7: 5C cells were treated with vehicle (control), 1 µM BP, 1 µM 4OHT, in the presence or absence of BP over a period of 5 days. Gene expression values were obtained and analyzed in comparison to the controls and heat maps were generated at (A) 96h and (B) 120h of treatment and the expressed genes listed. The selected genes were at least 2.5 fold over-expressed (red) or under-expressed (green) as compared to control at p value of 0.05.
SFig. 12. mRNA levels of caspase 4 and LTA. Cells were treated with either Vehicle (control), BP (1µM) and 4OHT (1µM) in the presence or absence of BP for 72h, 96h and 120h. Cells were harvested and caspase 4 and LTA mRNA levels were determined using RT-PCR.
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**Table 9.** List of apoptosis regulated genes induced by BP (1µM) and 4OHT (1µM) in the presence or absence of BP in MCF7: 5C cells after 96 h of treatment versus control. Upregulated genes are in red and downregulated genes are in blue. *Gene S: Gene symbol; Fold reg: Fold regulation.*
Table 10. List of apoptosis regulated genes induced by BP (1µM) and 4OHT (1µM) in the presence or absence of BP in MCF7: 5C cells after 120 h of treatment versus control. Upregulated genes are in red and downregulated genes are in blue. *Gene S: Gene symbol; Fold reg: Fold regulation.

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Fig. 26. Induction of apoptotic genes by BP. BP induces apoptotic genes after 7 days of treatment. MCF7:5C cells were treated with Vehicle (Veh), BP (1 µM), 4OHT, 1µM or combination treatment of BP and 4OHT for 7-9 days. Total RNA was isolated and reverse transcribed and (A) BIM and (B) TNF (C) FAS and (D) FADD mRNA levels was determined using RT-PCR. PCR data values are presented as fold difference versus vehicle treated cells ± SEM. [* P< 0.05, ** p<0.005, *** p<0.0001, ****p<0.0005]
VI.2e. Differential effect of bisphenol on cell cycle

Since the BP induced apoptosis is not apparent until the second week of treatment, we evaluated the effect of BP on the regulation of the cell cycle. MCF7:5C cells were treated with either vehicle control, 1nM E\textsubscript{2} or 1\mu M BP for 24h, 48h and 96h and performed cell cycle analysis using flow cytometry (Fig.27). As suspected, BP and E\textsubscript{2} cause a consistent increase in the S phase when compared to the control. Although, the trigger for apoptosis occurred for E\textsubscript{2} and BP at 36h and 96h respectively, no checkpoint blockade was noted after treatment with either compound, no checkpoint blockade was noted after treatment with either compound and contrasts dramatically with early cell cycle arrest at G2/M with paclitaxel(56).
Fig. 27. Diverse effects of BP and E2 on cell cycle progression. Distribution of the cells through the cell-cycle phases was analyzed by flow cytometry in cells treated with E2 (1nM), BP (1 µM), or control for 24 h, 48h and 96h. The percentage of the cells in each fraction is calculated using the ModFit software. The y axis represents the number of cells and FL2-A represents the intensity of propidium iodide.
VI.2f. Functional importance of caspase 4 in bisphenol induced apoptosis

Caspase 4, an inflammatory caspase, is upregulated in the MCF7:5C cells by 5 days of treatment with BP. To determine the role of caspase 4 in BP induced apoptosis, cells were treated with control or BP (1µM) and the effects of caspase 4 was blocked by caspase 4 inhibitor-z-LEVD-fmk (10µM). Growth inhibited by BP was reversed by z-LEVD-fmk (Fig.28A). Proliferation was determined after 12 days of exposure to BP and quantified by DNA mass per well. Apoptosis induced after 6 days of exposure to BP was completely reversed by z-LEVD-fmk (Fig.28B). Thus, the blockade of BP induced apoptosis by caspase 4 inhibitor-z-LEVD-fmk indicates that caspase 4 plays an important role for the induction of apoptosis by BP.
Fig.28. Caspase 4 is important for BP induced apoptosis. MCF7:5C cells were treated with control(0.1% ethanol) or BP (1µM) or caspase 4 (casp4) inhibitor with or without BP for either (A) 12 days and assessed for cell proliferation or (B) for 6 days and evaluated for apoptosis. Apoptosis and inhibition of growth of cells were blocked by caspase 4 inhibitor z-LEVD-fmk (10 µM).
VI.3. Discussion

The aim of our study is to elucidate the growth and induction of apoptosis by BP in fully oestrogenised and long term oestrogen deprived breast cancer cells. The ER in breast cancer cells can either initiate replication or trigger apoptosis based on the context of cell selection in estrogen replete or deprived environments (148). Originally, oestrogens including, E2 and TPE derivatives were discovered using a bioassay of the induction of vaginal cornification in ovariectomised mice. Replication and cornification of vaginal cells in the mouse was the early appropriate method of establishing the structure-function relationships of an oestrogenic TPE molecule (44). Initial structure-function studies in vitro established an ER mediated mechanism for E2 stimulate prolactin (an oestrogen responsive gene) synthesis in rat pituitary cells (149). However, BP and other TPE derivatives were found to act as partial agonists with antioestrogenic properties at the prolactin gene in vitro (97, 98). Structure-function relationship studies to modulate prolactin synthesis by extending the length of the “antioestrogenic side chain” created an antioestrogen that blocked oestrogen stimulated prolactin synthesis (98, 117).

These are the basic early facts of the pharmacological function of the oestrogen-ER complex that now allows us to interpret our current findings on the modulation of apoptosis.

Our results show that BP induces cell cycle regulated genes that are similar to those activated by E2 in MCF-7 cells. This correlates with the ability of BP to induce replication of MCF-7 cells in a comparable manner as E2 (73). On the other hand, 4OHT, which possesses a bulky alkylaminoethoxy side chain(72), failed to induce cell cycle regulated genes in a time dependent manner but rather blocks E2 and BP mediated activation of cell cycle genes, therefore confirming its role as an antioestrogen. Although BP possesses a bulky phenyl substituent, it does not have an alkylaminoethoxy side chain. Molecular modeling studies suggest that the
phenyl component of TPEs prevent the complete sealing of the ligand binding domain of the ER by helix 12(73, 147). The reduced number of gene changes noted with BP treatment compared with E2 (Fig.22) may be caused by differences in the structure of the ligand-ER complex, thus resulting in a reduction in the full oestrogenic potential of BP induced replication. Additionally, BP unlike E2 does not readily induce apoptosis in long term oestrogen deprived MCF-7 cells but rather appear to possess early antioestrogenic properties (147). Using cell proliferation assays, BP induces growth of MCF7:5C cells in the first week of treatment. In contrast, growth inhibition occurs after the third day of treatment with E2. Inhibition of growth in oestrogen deprived MCF7:5C cells with BP is seen after 8 days of treatment. Similarly, apoptotic effects of BP are observed following 6 days of BP treatment using flow cytometry studies(Fig.23, SFig.10). Previous studies have shown that MCF7:5C cells are resistant to the actions of 4OHT, which has the ability to reverse and block E2 mediated apoptosis(76). Using 4OHT or ICI 182,780 to block and rescue the cells from BP induced apoptosis suggests that the trigger for apoptosis occurs with BP after 4 days of treatment. There was no evidence of cell cycle arrest with either E2 or BP (Fig 6) prior to apoptosis. This contrasts dramatically with our previous publication of E2 and the rapid G2 blockade triggered by paclitaxel prior to apoptosis , no checkpoint blockade was noted after treatment with either compound and contrasts dramatically with early cell cycle arrest at G2/M with paclitaxel (56).

The apoptosis related genes clearly demonstrate that the majority of genes that are upregulated by BP at 4 days of treatment are ERS and IS response genes. DDIT3 also known as CHOP or GADD153 is a key ERS protein associated with cell death(58, 150) , whereas CEBPB, which is known to induce proinflammatory cytokines such as IL6(151), is activated by ERS and is important for nuclear transport of DDIT3(152, 153). There is a continued induction of similar
proapoptotic genes at 5 days of treatment including caspase 4, an inflammatory caspase that predominantly localizes to the endoplasmic reticulum and undergoes cleavage and induces effector caspases in response to ERS (154, 155). Upregulation of Bim, FAS, TNF and FADD mRNA are observed by 7 days of treatment with BP. Microarray analysis indicate ERS-mediated apoptosis as the top scoring pathway of apoptosis induced by E₂ in MCF7:5C cells (40). Oestradiol induces ERS and IS response genes by 36h of treatment and apoptotic genes such as Bim and TNF are activated by 48h of treatment (56). A similar trend is observed with BP, however there is a prolonged ERS and IS with subsequent induction of caspase 4 at 5 days of treatment and mitochondrial and extramitochondrial apoptotic genes at 7 days of treatment. After 48h treatment with BP, there is no induction of apoptotic genes (147) but an increase in growth (Fig.23A,27) and the cells can be rescued from apoptosis with antioestrogens (Fig.24).

The initial resistance to trigger apoptosis may also result from the antioestrogenic conformation BP creates with the ER. Angular TPEs such as BP have a reduced tendency to promote recruitment of coactivators containing LxxLL motif (103). We have previously shown that BP recruits the ER and SRC3 to the PS2 promoter ERE less efficiently when compared to planar estrogens (147, 156) thus indicating that complete sealing of helix 12 of the LBD and interaction of coactivators with the TPE-ER complex is necessary for the rapid activation of apoptosis observed with planar estrogens (76). Depletion of SRC3 in the MCF7:5C cells and MCF-7 cells leads loss of E₂ induced apoptosis (96) and growth (94, 95) respectively.

Since caspase 4 is specifically activated by ERS (155) and it was induced by 2 fold with E₂ within 24h (40) but by 2 fold by BP within 96h, a specific caspase 4 inhibitor (155) was used to block activation of caspase 4 in BP treated cells and this resulted in reversal of BP inhibited growth and apoptosis (Fig.28). We previously reported that E₂ induced apoptosis can be blocked
by a caspase 4 inhibitor (40). Together, these results suggest that BP activates IS and ERS related genes which interact with resultant induction of caspase 4 at 5 days of treatment and subsequent activation of mitochondrial and extramitochondrial related apoptotic genes in the second week of treatment. This delayed sequence for BP contrast with early activation by E2 (40, 56).

In summary, we have used cell based assays and gene profiling studies to demonstrate the biological response of the TPE, BP on both growth and apoptosis. TPEs were among the first chemical therapy used in the treatment of advanced breast cancer in postmenopausal women (15). These data support the apoptotic mechanism of TPEs in early clinical practice.
CHAPTER VII

Breast Cancer Cell Apoptosis with Phytoestrogens is dependent on an estrogen deprived state

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Cancer Prevention Research 2014 (revision submitted 04/24/2014)
VII.1. Introduction

Acquired resistance to antihormone therapy occurs despite the successful use of endocrine treatment to improve survival in breast cancer patients. Early laboratory models show that retransplantation of tamoxifen resistant tumors into ovariectomised athymic mice led to tumor growth in response to tamoxifen and estradiol (E$_2$) (23, 24). Continued retransplantation of the tamoxifen stimulated tumors in nude mice for up to 5 years resulted to a rapid regression of the tumors in response to E$_2$ (31). This correlates with the finding that E$_2$ induces apoptosis in long term estrogen deprived MCF-7 breast cancer cells (38, 39). The use of estrogens has been beneficial in the treatment of metastatic breast cancer in postmenopausal women with acquired resistance to endocrine therapy. A clinical study (79) found that high dose diethylstilbestrol induced an objective response in 30 percent of postmenopausal breast cancer patients who had previous exhaustive antihormone therapy. Ellis and colleagues (80) showed that postmenopausal women with aromatase inhibitors resistant metastatic breast cancer, had a 29% clinical benefit with low dose estrogen (6mg daily) but the same clinical benefit but more side effects with high dose estrogen (30mg daily). Additional clinical evidence for the antitumor action of low dose estrogen comes from the Women Health Initiative (WHI) trial which compared conjugated equine estrogen (CEE) therapy with placebo in hysterectomised postmenopausal women which show a persistent decrease in the incidence and mortality of breast cancer in women who received estrogen alone therapy (81, 82). Studies in vitro show that constituents of CEE cause apoptosis in long term estrogen deprived MCF7 cells (83). The clinical and laboratory studies suggest that the ability of estrogen therapy to treat or prevent tumors is most apparent in the postmenopausal state of a woman and how long they have been physiologically deprived of estrogen (83).
Phytoestrogens are plant-derived polyphenolic compounds that are structurally similar to E₂. Phytoestrogens consist of isoflavones (genistein, diadzein), coumestans (coumestrol), the lignans (enterolactone, enterodiol) and stilbenes (resveratrol). Isoflavones are principally found in soy-based products which are staple foods in many Asian countries and are becoming increasingly popular in Western countries. An inverse relationship found between soy consumption in Asian countries and decreased breast cancer risk has sparked a sustained interest in the use of phytoestrogens in breast cancer prevention. However, the clear beneficial effects of these estrogens remain controversial. Several meta-analyses (157-159) that assessed soy exposure and breast cancer risk revealed that studies conducted in Asian countries showed a significant trend of a reduced risk with increased soy intake and the lowest breast cancer risk was found in pre- and postmenopausal Asian women with high soy intake. On the other hand, no association was observed between soy consumption and breast cancer risk in low soy-consuming Western populations (157, 158), suggesting that consumption of soy products in amounts taken in the Asian population may have protective benefits. Evaluation of the breast cancer protective effects of isoflavones stratified by menopausal status is still undefined. Trock and colleagues (160) reported in their meta-analysis, a stronger association between soy exposure and breast cancer risk in premenopausal women. However, the analyses included studies with incomplete measurements, potential confounders and lack of a dose response that make the findings inconclusive. On the other hand, another study reported that adult or adolescent soy consumption was associated with reduced risk of premenopausal breast cancer (161) and no significant associations were reported for the risk of postmenopausal breast cancer. Furthermore, there is increased evidence that the chemo-protective effects of isoflavones are dependent on early exposure. High soy consumption during adolescence is associated with reduced risk of adult
breast cancer (162, 163). This concurs with the findings in animal model experiments, where prepubertal exposure to genistein causes mammary gland differentiation, thereby resulting in increased breast cancer prevention(164, 165). The effect of phytoestrogens in breast cancer cells have been extensively studied. At low pharmacologic concentrations, phytoestrogens stimulate the growth of estrogen receptor positive breast cancer cells (166-168). In contrast at high concentrations (>5µM), these plant derived estrogens inhibit the growth of the cancer cells (167, 169, 170). Ingestion of soy isoflavones in healthy premenopausal women resulted in increased breast tissue proliferation (171), epithelial hyperplasia (172) and a weak estrogenic response in inducing estrogen regulated markers (173). On the other hand in postmenopausal women, soy supplementation resulted in either a protective effect (174) or no effect (175-177) on breast cancer risk. Only one of the postmenopausal studies (177) consisted of healthy subjects, the rest included breast cancer patients. Fink et al (178) reported a decreased all-cause mortality in pre- and postmenopausal breast cancer patients who had a high intake of isoflavones, whereas a reduced breast cancer mortality was observed in postmenopausal women. However the DietCompLyf study (179) which investigated associations between phytoestrogens and breast cancer recurrence and survival found no significant associations between pre-diagnosis phytoestrogen intake and reduced breast cancer risk. Interestingly Shu and colleagues found that soy food consumption was significantly associated with decreased risk of death and recurrence in breast cancer patients (180).

In this study we have evaluated the apoptotic and potential chemopreventive effects of phytoestrogens using a unique cell model that simulates a postmenopausal cellular environment. Genistein, coumestrol and equol, a gastrointestinal metabolite of diadzein are used in comparison to E₂ and equilin a constituent of conjugated equine estrogen (CEE) in hormone replacement
therapy (HRT) to determine their proliferative and apoptotic potential using fully estrogentised and an estrogen deprived breast cancer cells respectively. Here, we test the hypothesis that the phytoestrogens have biologic effects similar to that of E2 and CEE in breast cancer prevention and this may have clinical implications for the strategic use of phytoestrogens as alternatives to HRT in postmenopausal populations.

VII.2. Result

VII.2a. Effect of phytoestrogens on breast cancer cells

Based on the controversy surrounding breast cancer risk and the use of phytoestrogens, we decided to determine the biological properties of the genistein, equol and coumestrol (SFig.13B) in comparison to E2 and equilin (SFig.13A) in two different models of breast cancer cell models. Estrogens have been shown to regulate the growth of ER positive MCF-7 breast cancer cells. First, we tested the ability of test compound to induce proliferation in MCF7:WS8 cells which are estrogen responsive breast cancer cells grown in fully estrogentised medium. MCF-7:WS8 cells were grown in estrogen free media for 3 days and treated with various concentrations of genistein, coumestrol and equol and their effects were compared to E2 and equilin (Fig. 29A). The phytoestrogens all stimulated cell growth in a concentration related manner with maximum stimulation occurring at 0.1µM, whereas E2 and equilin maximally induced cell growth at 10 pM and 0.1 nM respectively. The EC50 values of all estrogens are shown in Table 11. Growth inhibition was observed with the phytoestrogens at 10µM with genistein being the most effective. Next we investigated the growth properties of the genistein, equol and coumestrol in long term estrogen deprived MCF7:5C cells in comparison to E2 and equilin (Fig.29B). Genistein [IC50: 2.77 x 10⁻⁸], equol[IC50: 4.67 x 10⁻⁸] and coumestrol[IC50: 2.34 x 10⁻⁸]
drastically inhibited the growth of the MCF7:5C cells at higher concentrations compared to E₂.
Maximum growth inhibition was observed with all phytoestrogens at 0.1µM. E₂ [IC₅₀: 2.06 x 10⁻¹¹] achieved maximum growth inhibition at 0.1nM, while equilin [IC₅₀: 2.32 x 10⁻¹⁰] reached maximum growth inhibition at 1nM after 7 days of treatment.

SFig.13. Chemical structures of estrogens used in the experiments. (A) Steroidal estrogens
(B) phytoestrogens
Fig. 29. Growth characteristics of 17β-estradiol, equilin and phytoestrogens in breast cancer cells. (A) MCF7:WS8 cells were seeded in 24-well plate and treated with steroidal and phytoestrogens over a range of doses for seven days. Cell growth was assessed as DNA content in each well. (B) Inhibition of cell growth in MCF7:5C cells by genistein, equol and coumestrol was assessed in comparison to E₂ and equilin. Each data point is average +/- SD of three replicates.

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Table 11. EC50 values of the steroidal and phytoestrogens.
VII.2b. Phytoestrogens induce apoptosis in a long term estrogen deprived breast cancer cell line

Based on the fact that the decrease in cell growth observed with the steroidal estrogens is due to apoptosis (83), we investigated whether the antiproliferative effects of the phytoestrogens was also due to an increase in apoptosis. MCF7:5C cells were treated with $E_2$ (1nM), equilin (1nM), genistein(1µM), equol(1µM) and coumestrol(1µM) for 72h and stained with annexinV-FITC and PI fluorescence and cells were analyzed using the flow cytometry. In the control treated group, only 6.8% of cells stained for apoptosis, whereas $E_2$(24.56%), equilin(17.49%), genistein (14.79%), equol (14.89%) and coumestrol (17.83%) all show increased apoptotic staining compared to the control treated cells (Fig. 30A). A similar effect was noted using a DNA binding stain, YO-PRO-1 (SFig.14). $E_2$, equilin and all phytoestrogens induced apoptotic genes; $BCL2L11/BIM$, $TNF$, $FAS$ and $FADD$ (Fig. 30B-C) after 48h of treatment. Induction of these genes is consistent with the apoptotic status determined using the flow cytometry. Although evidence of apoptosis occurs with the phytoestrogens by 48h, a consistent increase in the S phase when compared to the control was observed with all estrogens(SFig.15). In contrast to other reports(169, 181) which indicate that genistein causes a G2/M arrest, no checkpoint blockade was noted after treatment with all compounds, indicating that the initial response of the cells to estrogens is growth, then apoptosis in MCF7:5C cells.
Fig.30. Induction of apoptosis by phytoestrogens and steroidal estrogens. (A) MCF7:5C cells were treated with 0.1% ethanol vehicle (control), or 1nM E2, 1nM equilin or phytoestrogens (1µM) for 72h and then stained with annexin v-FITC and propidium iodide and analysed by flow cytometry. Increased apoptotic effect is observed in the right upper and lower quadrant. E2, equilin and phytoestrogens increase (B) BIM, TNF (C) FAS and FADD mRNA levels. PCR data values are presented as fold difference versus vehicle treated cells ± SEM. [* P< 0.05]
SFig.14. Differential apoptotic effects of E₂ equilin and phytoestrogens. MCF7:5C cells were treated with control or E₂(1nM), equilin (1nM), phytoestrogens (1µM) for 72h and then stained with YO-PRO-1 and propidium iodide and analysed by flow cytometry.
SFig.15. Diverse effects of E₂, equilin and phytoestrogens on cell cycle progression.
Distribution of the cells through the cell-cycle phases was analyzed by flow cytometry in cells treated with E₂(1nM), equilin (1nM), genistein (1µM), equol (1µM), coumestrol (1µM) or control for 24 h, 48h and 72h. The percentage of the cells in each phase is calculated using the ModFit software. The y axis represents the number of cells and FL2-A represents the intensity of propidium iodide.
VII.2c. Phytoestrogens possess estrogenic properties mediated through the estrogen receptor in the MCF7:5C cells

We explored the ability of phytoestrogens to regulate estrogen response genes in comparison to E2 and equilin. Genistein, equol and coumestrol were all able to induce TFF1/PS2 and GREB1 (Fig. 31A). Phytoestrogens have been shown to induce apoptosis through an estrogen receptor (ER) independent mechanism(167, 182). To evaluate the involvement of ER in the effects of the phytoestrogens, we investigated their antiproliferative effects in the presence of 4-hydroxytamoxifen (4OHT) (Fig. 31B) and ICI 182 780 (SFig.16). The combination of various concentrations of 4OHT or ICI 182 780 with E2, equilin and each phytoestrogen blocked estrogen induced apoptosis suggesting that the phytoestrogens mediate apoptosis via the ER. We sought to examine the effects of genistein, equol and coumestrol on the ER. Following treatment of MCF7:5C cells with E2, equilin and the phytoestrogens for 24h, ERα levels were determined by western blotting. All phytoestrogens caused a decrease in the ERα protein levels in a comparable manner as E2 and equilin (Fig. 31C). Similarly the same effect was noted with all estrogens on the ERα mRNA levels (Fig. 31D). Interestingly, E2, equilin, genistein, equol and coumestrol, all have no effect on the ERβ protein and mRNA levels suggesting different regulatory effects the phytoestrogens may have on the ERα and ERβ.
Fig.31. Steroidal and phytoestrogens act as agonists via an estrogen receptor dependent mechanism. (A) MCF7:5C cells were treated with 0.1% ethanol vehicle (control), 1nM E2, 1nM equilin or phytoestrogens (1µM). Total RNA was isolated after 24h and reverse transcribed and PS2 and GREB1 mRNA levels was obtained using RT-PCR. (B) Various concentrations of 4-hydroxytamoxifen (4OHT) block steroidal estrogen- or phytoestrogen mediated growth inhibition (C) MCF-7:5C cells were treated with vehicle (control) and steroidal and phytoestrogens for 24 hours. ERα and ERβ protein was detected by Immunoblotting. (D) ERα and ERβ mRNA was quantified with real time PCR (RT-PCR). *, P < 0.05, compared with control.
SFig.16. Anti-estrogen, ICI 182 780 blocks phytoestrogen inhibited growth. MCF7:5C cells were treated with E₂(1nM), equilin (1nM), phytoestrogens (1µM) in presence of various concentrations of ICI 182 780. Total DNA was assessed in each well using a fluorescent DNA quantification kit.

VII.2d. ERα is important for steroidal and phytoestrogen induced apoptosis and growth inhibition

To determine whether ER α or β is required for the antiproliferative and apoptotic effects of the estrogens, MCF7:5C cells were transfected with either ERα or ERβ siRNA or nontarget siRNA(control) for 72h. Knockdown of ERα and ER β protein level was determined by western
blot (Fig. 32A, D). RNA-interference mediated inhibition of ER α abolished both steroidal and phytoestrogen induced apoptosis (Fig. 32B) and growth inhibition (Fig. 32C) compared with cells transfected with the control siRNA. Interestingly, loss of ERβ using siRNA did not prevent the ability of the steroidal or phytoestrogens to either induce apoptosis (Fig. 32E) or inhibit the growth (Fig. 32F) of the MCF7:5C cells. Taken together, this indicates that ERα is the initial site for the indicated estrogens to cause growth inhibition and apoptosis in the MCF7:5C cells. A relative ratio of ERα to ERβ in MCF7:5C cells is shown in (SFig. 17).

**SFig. 17. ERα and ERβ mRNA levels in MCF7:5C cells**

MCF7:5C cells were treated with 0.1% ethanol vehicle (control) and total RNA was isolated after 24h and reverse transcribed and ERα and ERβ mRNA levels was obtained using RT-PCR.
**Fig. 32. ERα is required for estrogen induced growth inhibition and apoptosis**

MCF7:5C cells were transfected with either non target RNA (consi) or siRNA of ERα for 72 h. (A) ERα was detected by immunoblotting. Then, cells were treated with either control (0.1% EtOH), 1nM steroidal estrogens or 1µM phytoestrogens for (B) 72h and apoptosis was determined using annexin V binding assay. (C) Growth inhibition in the transfected cells was assessed after 6 days of treatment with indicated compounds using DNA quantification assay. (D) Knockdown of ERβ did not prevent (E) estrogen induced apoptosis or (F) growth inhibition.
VII.2e. Phytoestrogens induce endoplasmic reticulum stress and inflammatory stress response genes

Microarray analysis indicates that endoplasmic reticulum stress (ERS) and inflammatory response genes are top scoring pathways associated with E₂ induced apoptosis(40). To investigate whether phytoestrogens induce ERS genes, we used RT-PCR to quantitate mRNA levels. After 48h of treatment, genistein, equol, coumestrol and equilin and E₂ all induce \textit{DDIT3}(also known as CHOP), a marker of ERS associated with cell death, and inositol requiring protein 1 alpha (\textit{IRE1\alpha}), an unfolded protein response sensor which is activated to relieve stress(Fig. 33A). Significant induction of \textit{IRE1\alpha} and phosphor-eukaryotic translation initiation factor-2α (p-eIF2α), another UPR sensor, protein levels occur by 24h (Fig. 33B). Next we determined whether genistein, coumestrol and equol induce proinflammatory response genes using RT-PCR. At 48h, E₂, equilin and all phytoestrogens activate caspase 4, an inflammatory caspase; \textit{CEBP\beta} which is known to bind to IL-1 response element in \textit{IL6} and a downstream target of ERS; \textit{IL6}, a proinflammatory cytokine; lymphotoxin beta (\textit{LTB}), an inducer of inflammation response, (Fig. 33C-D). This indicates that the phytoestrogens activate similar genes involved in the apoptotic pathway of E₂.
Fig.33. Endoplasmic reticulum stress and inflammatory stress response are involved in phytoestrogen induced apoptosis. (A) The indicated estrogens induce endoplasmic reticulum stress related genes, DDTT3 and IRE1α. (B) MCF-7:5C were treated with E2 (1nM), equilin (1nM) or phytoestrogen (1µM) for 24h. IRE1α and phosphorylated eIF2α were used as indicators of UPR activation and their protein expression were examined by immunoblotting. Total eIF2α and β-actin were determined for loading controls. Indicators of inflammatory stress response (C) caspase4, CEBP β, (D) IL6 and LTB were activated by E2, equilin and phytoestrogens. [* P< 0.05]
VII.2f. Inflammation is required for phytoestrogen mediated apoptosis

Next we investigated the importance of inflammatory response in phytoestrogen mediated apoptosis. Dexamethasone, a synthetic glucocorticoid with potent anti-inflammatory properties was used to inhibit inflammation in the MCF7:5C cells. Cells were treated with 1nM E₂ or equilin or 1µM phytoestrogens and various concentrations of dexamethasone were added to block the biological effects of the compounds. Although dexamethasone has an inhibitory effect in the MCF7:5C cells, it was able to reverse the steroidal estrogen or phytoestrogen inhibited growth(Fig. 34A). Similarly, flow cytometry studies revealed that 1µM dexamethasone reversed the apoptotic effects mediated by E₂, equilin, genistein, equol and coumestrol (Fig. 34B). To determine that inflammatory stress response was inhibited by dexamethasone, MCF7:5C cells were treated with the indicated estrogens for 48h and total RNA was extracted and reverse transcribed. Dexamethasone inhibited the ability of all estrogens to induce caspase 4, CEBP β, BIM and TNF (Fig. 34C-D). Together, this suggests that inflammation is important for both steroidal and phytoestrogen mediated apoptosis.
Fig. 34. Inflammation is important for phytoestrogen mediated apoptosis

(A) Cells were treated with the indicated estrogens in presence of increasing concentration of dexamethasone (dexa). (B) Dexamethasone completely reverses E2, equilin and all phytoestrogen induced apoptosis. Apoptosis was assessed using the flow cytometry. Dexamethasone blocked the induction of (C) CEBP β, caspase 4 (D) BIM, and TNF by E2, equilin and phytoestrogens.
VII.3. Discussion

Phytoestrogen consumption is associated with a decrease in the incidence of breast cancer in the Asian population probably due to early exposure to a high soy diet. This correlates to animal studies which suggest that it is due to mammary cell differentiation and a decrease in terminal end buds which are sites of early tumor proliferation(183, 184). Phytoestrogens increase cell growth of ER positive breast cancer cells but induce apoptosis at high concentrations in these cells. Although studies (157, 174) may support use of phytoestrogens in postmenopausal women, their full chemopreventive properties is yet to be clearly defined. E2 and CEE induce apoptosis in long term estrogen deprived breast cancer cells. Therefore we addressed the question of whether low concentrations of phytoestrogens will induce apoptosis in MCF7:5C cells which simulate a postmenopausal state that is dependent on the duration of estrogen deprivation following menopause. Genistein, equol and coumestrol all increase cell growth in MCF7:WS8, (which simulate the premenopausal or perimenopausal state) after 3 days of estrogen deprivation at physiologic concentrations. These cells have adapted to an estrogen rich environment and will grow with a natural resupply of estrogens provided with exogenous phytoestrogens treatment. This correlates with the results of Andrade and colleagues (185) who show that long-term consumption of low GEN doses (≤500 ppm) promotes MCF-7 tumor growth in vivo. However at low concentrations >1µM, all phytoestrogens inhibit cell growth. In contrast the phytoestrogens, although less potent than E2 and equilin, induce apoptosis in MCF-7 cells that have undergone long term estrogen deprivation. Therefore a potential use of phytoestrogens at physiologic concentrations will be in an estrogen deprived environment which is induced either by natural withdrawal of estrogens caused by menopause or by treatment with exhaustive anti-estrogen therapy for breast cancer with aromatase inhibitors or tamoxifen.
Studies (186, 187) suggest that phytoestrogens possess antiestrogenic properties which may be responsible for their chemopreventive effects. Here we show that the phytoestrogens do in fact induce estrogen responsive genes just like steroidal estrogens in the estrogen deprived MCF7-5C cells and that their growth inhibition and apoptosis are mediated through the ER. In contrast, it has been reported that genistein mediates apoptosis through an ER independent mechanism in the MCF-7 cells (182, 186) and the ability of phytoestrogens to induce apoptosis is observed maximally in the presence of E2. It is important to note however that apoptosis was mediated by the phytoestrogens only at high concentrations in these studies (182, 186). As another potential mechanism of apoptosis, phytoestrogens show increased binding affinity to ERβ(188) which is thought to be responsible for its growth inhibitory properties. In our study, loss of ERβ did not affect the anti-proliferative and apoptotic properties of the steroidal and phytoestrogens. However, we determined that knockdown of ERα prevents both steroidal and phytoestrogen mediated growth inhibition and apoptosis suggesting that ER α signaling is required for their biological actions.

Genistein, equol and coumestrol induce ERS and inflammatory stress response, intrinsic and extrinsic apoptosis related genes which correlates with results of differential gene expression in response to E2 interrogated using agilent based microarray analysis(40). Activated ERS genes indicate that E2 prevents protein folding leading to accumulation of unfolded proteins and widespread inhibition of protein translation and crosstalk with inflammatory response genes and subsequent induction of cell death. Inhibition of PERK/EIF2AK3, a key ERS sensor of UPR and inducer of pEIF2α (65) prevents E2 mediated apoptosis (54). PERK is also known to induce apoptosis by sustaining levels of DDIT3(189), another major ERS gene involved in apoptosis, which is known to dimerize with CEBPβ under stress conditions(152, 153). Ablation of CEBPβ
using siRNA decreases expression of *DDIT3* (152) suggesting a crosstalk between ERS and inflammatory stress response. Similarly, inhibition of caspase 4, an inflammatory response gene and a downstream target of ERS, using caspase 4 inhibitor-z-LEVD-fmk also blocks $E_2$ induced apoptosis. To show that inflammation is important in phytoestrogen induced apoptosis, dexamethasone was used to block inflammation globally, resulting in inhibition of all estrogen induced apoptosis and their ability to induce inflammatory response and apoptosis related genes. Therefore the clinical implication is that caution should be exercised in the use of steroidal anti-inflammatory agents in conjunction with these phytoestrogens, which could prevent the full chemopreventive benefits.

Successful use of estrogens to treat or prevent tumors is dependent on the timing of estrogen withdrawal (Fig.35). Estrogen therapy was the first chemical used in the treatment of advanced breast cancer in postmenopausal women and this therapy resulted in the regression of 30% of tumors in the first reported clinical trial (15). It was noted that “the beneficial responses were three times more frequent in women over the age of 60 years than in those under that age; that estrogens may, on the contrary, accelerate the course of mammary cancer in younger women, and that their therapeutic use should be restricted to cases 5 years beyond the menopause” (16). Stoll and colleagues (17) noted that objective remission rate from estrogen treatment in 407 patients with advanced breast cancer was higher in women more than 5 years postmenopausal (35%) when compared to women who were less than 5 years postmenopausal (9%). In more recent clinical studies, about thirty percent of patients with advanced breast cancer who have been exposed to exhaustive antihormone therapy show an objective clinical response with estrogen therapy (79, 80). CEEs reduced the incidence and mortality from breast cancer but this is probably because the majority of these women were over
65 years\(^{(81)}\). Furthermore, 10 years adjuvant tamoxifen therapy produced a further reduction in recurrence and mortality from breast cancer when compared to 5 years of tamoxifen therapy\(^{(4)}\) suggesting that it was the woman’s own estrogen that destroys the appropriately sensitive tamoxifen resistant micrometastasis once long term tamoxifen is stopped\(^{(30)}\).

In conclusion, it is important to note that in order to obtain the full breast cancer chemopreventive benefits of phytoestrogens, it is necessary to begin up to five years following menopause. Commencing soy consumption during perimenopause may cause growth of nascent ER positive breast tumors which may increase breast cancer risk, whereas phytoestrogen therapy 5 years after menopause will most likely induce apoptotic cell death and enhanced patient survival.
Fig. 35. The success of estrogen replacement therapy is dependent on menopausal status of a woman. A. Estrogen withdrawal in postmenopausal women causes ER positive dependent cells to die but some cells continue to grow independent of estrogen. B. Treatment of women immediately after menopause with CEE results in sustained growth of nascent ER positive tumors, whereas treatment 5 years after menopause causes apoptotic cell death. Source: Obiorah I and Jordan VC. Menopause 2013; 20:372-382.
Chapter VIII

General Conclusions and Future Directions

VIII.1 Clinical relevance

Before the clinical use of antiestrogen therapy, high dose estrogens were effective in the induction of tumor regression in metastatic breast cancer (15, 17). In more recent times, estrogen therapy show significant clinical benefit in postmenopausal women who have undergone extensive anti-hormone treatment(80). Development of tamoxifen stimulated tumors in athymic mice following a five year treatment with tamoxifen(31) suggest that the development of anti-hormone resistance over years of treatment reconfigures the survival mechanism of breast cancer so that estrogen is no longer a potent mitogen that stimulates cell proliferation but rather becomes a death signal. Preclinical data (39)clearly show that long term estrogen deprivation of ER positive MCF-7 breast cancers and subsequent treatment of the cells with $E_2$ causes apoptosis of these cells. Creation of an estrogen deprived environment either by withdrawal of estrogen treatment or by exhaustive anti-hormone therapy increases sensitivity of breast tumors to estrogen therapy which subsequently induces tumor regression. Similarly CEE alone reduces the incidence of breast cancer in hysterectomised postmenopausal women. This protective effect is not observed in women who received addition progesterone therapy, suggesting that the progestin may play a potential role in the increase in breast cancer seen in postmenopausal women who received combined hormonal therapy.
VIII.2. Conclusions

Due to the aforementioned clinical relevance, we tested and determined that a series of steroidal estrogens including the constituents of CEE could induce apoptosis in the long term estrogen deprived MCF-7 cells (Chapter III). However not all estrogens could readily induce apoptosis. The TPEs which are structurally similar to 4OHT showed an initial inhibition of the E2 induced apoptosis due to the antiestrogenic conformation they create with the ER. The TGFα assays and the molecular modelling data support this hypothesis that helix 12 is pushed back in the TPE-ER complex. The fact that the TPEs do not readily recruit coactivators further explains the delay in apoptosis observed with the TPEs since SRC3 is required for E2 induced apoptosis. The planar estrogens form compact estrogen-ER complex with and readily recruit SRC3 binding and therefore readily induce apoptosis in the MCF7:5C cells. We further showed that our molecular classification assay could be used to classify estrogens based on their shape by comparing bisphenol A and bisphenol which revealed that BPA a flat estrogen will induce apoptotic related genes at a comparable manner as E2, whereas BP was not able to do this in the first 72h (Chapter VI). Because the initial response of the MCF7:5C cells to E2 is growth, we determined the point of trigger for apoptosis occurs after 24h unlike that of a classic chemotherapy (paclitaxel), which causes a G2/M blockade and apoptosis by 12h (Chapter V). Performing a time course, we determined that E2 induces ERS and IS and initially activates the intrinsic pathway by 36h with a later recruitment of the extrinsic pathway, whereas paclitaxel induces apoptosis exclusively via the extrinsic pathway. In contrast, BP shows a prolonged ERS before it induces apoptosis related genes in the second week of treatment (Chapter VI). This is not surprising since the trigger for apoptosis occurs after 72h. Similar to E2, caspase 4 is required for BP induced apoptosis. Since phytoestrogens planar estrogens, we hypothesized that they will
induce apoptosis in the MCF7:5C cells. (Chapter VII) As expected they induced apoptosis and also showed that the ERα and not ERβ is required for apoptosis. We further showed that they induce similar apoptosis related genes as the steroidal estrogens and that an inflammatory response is required.

**VIII.3. Future Directions**

An inflammatory response is required for estrogen induced apoptosis, it is important to further explore if there is a crosstalk between ERS and IS. DDIT3 is a key ERS protein associated with cell death(58, 150), whereas CEBPB, which is known to induce proinflammatory cytokines such as IL6(151), is activated by ERS and is important for nuclear transport of DDIT3(152, 153). It will be important using ChIP assays to show that this interaction is required for E2 induced apoptosis. Overexpression and siRNA transfection assays will further give evidence for the need for this interaction. Studies (67, 68) showed that loss of Bim expression resulted in protection from ERS induced apoptosis and that increased Bim levels noted with ERS induction was dependent on transcriptional activation of DDIT3(69). Therefore it will be important to connect the ERS pathway to Bim induction by using siRNA transfection assays to knockdown Bim and note the effect on DDIT3 expression levels. The effect of overexpression of DDIT3 will be performed to determine any effect on the bcl-2 protein.

We showed that inflammatory stress response (chapter VII) is important for estrogen induced apoptosis. Therefore further investigation is warranted to see if there is a crosstalk between IS and ERS. CEBPB, which is known to induce proinflammatory cytokines such as IL6(151), is activated by ERS and is important for nuclear transport of DDIT3(152, 153). DDIT3 has been shown to dimerize with CEBPβ under stress conditions(152, 153). Ablation of CEBPβ using siRNA decreases expression of DDIT3 (152). It will be important to use ChIP assays and
protein immunoprecipitation experiments to show interaction between DDIT3 and CEBP β. Secondly the effect of loss of CEBP β, using siRNA transfection, on the expression of DDIT3 and induction of apoptosis can be evaluated to determine their importance. These experiments will connect the stress pathways involving inflammation and ERS whose interaction will then activate apoptosis via the intrinsic mitochondrial pathway.

VIII.4. Therapeutic use of estrogen in clinical practice

Since steroidal and phytoestrogens induce apoptosis in our long term estrogen deprived cells we explain that the laboratory studies (Chapters III and VII)show that estrogens in the CEE and phytoestrogens were able to cause proliferation of MCF7 cells after growing these cells in an estrogen free medium for 3 days. “This cell population is adapted to an environment rich in estrogen, so naturally all the cells grow with a “resupply” of natural steroidal estrogens. However, these same estrogens induce apoptosis to a similar extent as E2 in MCF-7 cells that have been deprived of estrogen treatment for many years. The ability of estrogen therapy to treat or prevent tumors is related to the menopausal status of a woman and how long they have been physiologically deprived of estrogen. In the Stoll data(17) (Table 1), the rate of remission of advanced breast cancer was significantly less in women who were less than 5 years postmenopausal(9%), and there was a 35% remission rate in women who were more than 5 years postmenopausal. It is important to stress that majority of the women in the WHI CEE trial were above 60 years and the mean age at screening was 63.6 years. Here, the overall result was a reduction in breast cancer and mortality. There is a need for an “estrogen holiday” before starting estrogen therapy. Induction of menopause in a woman gradually deprives the cells of estrogen. However immediate treatment with estrogens may cause growth of nascent ER positive breast tumors which may increase breast cancer risk (Fig. 35). The cells vulnerable to death with
estrogens in CEE, have been selected because estrogen deprivation at menopause causes estrogen dependent nascent breast cancers to die, but all do not die. Remaining cells that survive learn to grow without estrogen. These cells will continue to grow to produce breast cancer unless exogenous estrogens induce apoptotic death. Therefore 5 years of CEE treatment or phytoestrogens immediately after menopause will cause sustained continuing growth of ER positive tumor cells. Because nascent ER+ tumor cells have been estrogen deprived in women who are 5 to 10 years postmenopausal, 5 years of CEE or phytoestrogen therapy induces massive apoptotic cell death and subsequent tumor cell death and an enhanced patient survival”(83).
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