Selective Estrogen Mimics for the Treatment of Tamoxifen-Resistant, PKCα-Overexpressing Breast Cancer

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THESIS

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

4-OHT	4-hydroxytamoxifen
AI	Aromatase inhibitor
AR	Androgen receptor
AP-1	Activator protein-1
ATCC	American Type Culture Collection
BTC	2-(4-hydroxyphenyl)-benzo[b]thiophen-6-ol
BZA	Bazedoxifene
CAF	Cyclophosphamide, doxorubicin, and 5-FU
CMF	Cyclophosphamide, methotrexate, and 5-FU
CREB	cAMP response element binding protein
CSC	Cancer stem-like cell
DAG	Diacylglycerol
DAPI	4', 6-Diamidino-2-phenylindole
DBD	DNA binding domain
DCIS	Ductal carcinoma in situ
DES	Diethylstilbestrol
DISC	death-induced signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
E2	17β-Estradiol
EGFR	Epidermal growth factor receptor

LIST OF ABBREVIATIONS (continued)

ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
ERE	Estrogen response element
FADD	Fas-associated death domain
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
GPCR	G protein-coupled receptor
GPER	G protein-coupled estrogen receptor 1
HER2	Human epidermal growth factor receptor 2
IF	Immunofluorescence
JAK	Janus kinase
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
Neo	Neomycin
PARP	Poly ADP-ribose polymerase
PAT	palmitoylacyltransferase
PELP-1	Proline-, glutamic acid- and leucine-rich protein
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PR	Progesterone receptor

LIST OF ABBREVIATIONS (continued)

PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
SEM	Selective estrogen mimic
SERM	Selective estrogen receptor modulator
siRNA	Silencing ribonucleic acid
SP1	Specificity protein 1
STAT	Signal transducer and activator of transcription
TAC	Docetaxel, doxorubicin, and cyclophosphamide
TNF	Tumor necrosis factor
TPA	12-*O*-Tetradodecanoylphorbol-13-acetate
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor

SUMMARY

A women's lifetime risk of developing breast cancer is 1 in 8, making it the leading female malignancy in the United States. Up to 75% of breast cancers express estrogen receptor α (ERα) and are therefore candidates to receive endocrine therapies aimed to block estrogen signaling, such as selective estrogen modulators (SERMs) or aromatase inhibitors (AIs). The SERM tamoxifen (TAM) is the most widely prescribed endocrine therapy for the treatment and prevention of breast cancer. The development of resistance to TAM, either *de novo* or acquired, limits its clinical effectiveness, leading to disease progression. Currently there is a lack of effective therapeutic options for women whose breast cancers no longer respond to conventional endocrine therapy approaches. 17β-estradiol (E2) has clinical efficacy in this setting, but due to unfavorable side effects it is no longer used for treatment. Further, there are currently no biomarkers to predict a positive response to an estrogenic treatment. The purpose of my study was to identify novel selective estrogen mimics (SEMs) that could achieve the positive therapeutic effects of E2 treatment in TAM-resistant breast cancers, while minimizing the side effects as well as to further explore the use of PKCα as a potential biomarker in predicting a positive therapeutic response to E2.

The Tonetti lab developed and previously described a preclinical TAM-resistant model where PKCα is stably overexpressed in the T47D:A18 breast cancer cell line (1). When T47D:A18/PKCα cells are grown in mice as xenograft tumors, E2 administration inhibits tumor growth and induces complete tumor regression in established tumors (2). In the work described herein, I demonstrate that all tested cell lines inhibited by E2, either *in*

vitro or *in vivo*, overexpress PKCα. Further I demonstrate that decreased cell viability by E2 may require PKCα, together suggesting that PKCα expression may predict a positive response to therapy with an estrogenic agent.

The mixed antagonist/agonist activity of TAM is also associated with side effects such as increased incidence of endometrial cancers, which led to the development of second generation SERMs. Raloxifene (RAL) is a benzothiophene SERM with a favorable antiestrogenic profile in the uterus and has proven safe over 15 years of clinical use in postmenopausal osteoporosis and breast cancer chemoprevention. In collaboration with Dr. Gregory Thatcher's lab we used the benzothiophene core structure of RAL as a starting point for rational drug design with the goal of developing novel selective estrogen mimics (SEMs), which could achieve the positive therapeutic effects of E2 treatment in TAM-resistant breast cancers, while minimizing the side effects. *In vitro* screening identified two SEMs, BTC and TTC-352, which displayed estrogenic activity in breast cancer cell lines. BTC and TTC-352 treatment resulted in significant tumor regression in two xenograft models of TAM-resistant, PKCα-overexpressing breast cancer. Interestingly E2 (3) and SEM induced T47D:A18/PKCα tumor regression was accompanied by translocation of ERα to extranuclear sites, possibly suggesting a direction to study the mechanism through which these SEMs initiate tumor regression. SEM treatment, however, did not result in growth of parental, TAM-sensitive xenograft tumors. Endometrial thickening, caused by both E2 and TAM, is directly associated with gynecological carcinogenesis and uterine cancer. Importantly, SEM treatment did

not increase uterine weight in mice suggesting negligible hormonal stimulation in gynecological tissues. Both BTC and TTC-352 resulted in regression of two TAM-resistant breast cancer models, while displaying enhanced safety compared to E2 and TAM.

These studies suggest PKCα may be a potential biomarker for estrogenic treatment therapy. These data also suggest SEMs may be superior to E2 in the treatment of endocrine-resistant breast cancer. Further development of SEMs targeted to TAM-resistant, PKCα-overexpressing breast cancer is warranted and may someday have an impact on clinical outcomes of breast cancer patients.

CHAPTER 1

INTRODUCTION

1.1 Breast Cancer

Breast cancer is the most common malignancy in women (4) and the development of metastatic disease is the most common cause of breast cancer-related mortality (5). In 2011, the American Cancer Society estimates that 230,480 women were diagnosed with invasive breast cancer in the U.S. Approximately 39,520 died from the disease in 2011 making it the 2nd leading cause of cancer related death in women. Advances in the detection, diagnosis and treatment of breast cancer have led to a 50% reduction in mortality rates since 1970 (6, 7).

Risk factors for breast cancer include age, early menarche, use of hormone-replacement therapy, late pregnancy, late menopause and early menarche. Pregnancy before age 30, breastfeeding, late menarche and early menopause are protective factors that reduce a women's chance of contracting breast cancer. A women's lifetime risk of developing breast cancer increases with the time she is exposed to estrogen (8). Only about 15% of breast cancers are thought to be heritable (9). Mutations in the BRCA1 or BRCA2 genes account for genetically driven breast cancers. These genes encode for proteins, which regulate deoxyribose nucleic acid (DNA) repair. Women with mutations in BRCA1 or BRCA2 have an 80% risk of developing breast cancer and a 55% risk of developing ovarian cancer (10).

1.2 Breast Cancer Subtypes

Breast cancer is a heterogeneous disease originating from cells within the breast tissue. This phenomenon has long been recognized clinically as evidenced by the

immunohistological classifications that dictate disease treatment (i.e. ERα, PR, HER2). More recently technological advances have allowed researchers to carry out expression analysis of thousands of genes simultaneously to classify tumors on a molecular level. Microarray studies first identified five molecular subtypes which breast tumors could be divided into based on gene expression profiles. These five subtypes have been suggested to originate from different precursors cells and include: Luminal A, Luminal B, HER2-enriched, basal-like, and normal-like (11-13). Classifications continue to emerge based on microarray studies such as molecular apocrine (14) and claudin-low (15).

Luminal A subtype represents 50-60% of total breast cancers, making it the most common molecular subtype. This group is primarily characterized by genes activated by transcription of the ER and low levels of genes related to proliferation (11, 12). The relapse rate for patients with this subtype is 27.8% which is significantly lower than other subtypes (16). The immunohistochemical profile of the luminal A subtype is characterized by expression of ERα, PR, Bcl-2 and cytokeratin CK8/18. These breast cancers also lack expression of HER2, have a low proliferative index and histological grade and compared to the other subtypes express the highest levels of GATA3. Recurrence primarily occurs within the bone. This subgroup is mainly treated with third generation aromatase inhibitors, SERMs such as tamoxifen (TAM) or SERDs such as fulvestrant (17).

The Luminal B subtype represents between 10-20% of all breast cancers. These breast cancers are more aggressive, have a higher proliferative index and histological grade as well as a worse prognosis compared to luminal A. Although luminal B breast cancers still express ERα they express higher levels of proliferative genes such as KI67

and Cyclin B1. The luminal B subtype often also expresses HER2 and EGFR (18). Prognosis for Luminal B cancers is worse than Luminal A despite treatment with TAM or aromatase inhibitors (19). Treatment of this subtype is challenging and numerous clinical trials are currently underway to improve therapeutic outcome for these patients.

Currently, ER-positive node-negative breast cancer patients are routinely treated with both adjuvant hormone therapy as well as adjuvant chemotherapy in order to reduce disease recurrence. To prevent unnecessary use of chemotherapy in patients several diagnostic systems have been developed based on gene-expression profiles of breast tumors. Commercially available systems such as Mammaprint^{TM} (20) and Oncotype DX^{TM} (21) use different platforms and are superior to conventional histological grade classifications in predicting a patient's response to therapy.

Fifteen to twenty percent of breast cancers fall into the HER2 positive molecular subtype. These cancers overexpress genes related to proliferation, most notably high expression of HER2 and genes associated with the HER2 pathway. These tumors are highly proliferative and the majority are of high histological grade. Anti-HER2 based therapies have significantly improved survival of patients both in the initial and metastatic stages of the disease (22, 23). In neoadjuvant studies the HER2 subtype also has higher chemosensitivity compared to the luminal subtypes (24). Clinical trials are currently underway which combine therapies that inhibit the HER2 pathway at different locations. HER2 positive breast cancers are typically treated with trastuzumab, lapatinib, pertuzumab or a combination of agents with taxanes, which will be discussed further in the following section.

The basal-like subtype makes up 10-20% of all breast carcinomas. P-cadherin, caveolin-1, nestin, CD44, EGFR and cytokeratins CK5 and CK17 are commonly expressed in this subtype, which are also genes commonly expressed in normal myoepithelial cells. They are often characterized by high histological grade and being of large tumor size at diagnosis (25). Metastatic relapse tends to be aggressive predominantly located in the lung, central nervous system and lymph nodes (26). Although these tumors respond to chemotherapy, they have a worse prognosis compared to luminal cancers (12). Tumors with BRCA1 mutations also fall into this subtype (27). Most relevant to this tumor type however is the absence of ER, PR and HER2. Clinically the terms basal-like and triple negative are often used interchangeably although these terms are not equivalent as there has been up to 30% discordance between the groups (28). Using IHC a core group of 5 markers has been used to accurately characterize basal like tumors and include ER, PR, HER2, EGFR and CK5/6 (29).

Normal-like breast cancers are rare making up between 5-10% of breast cancers diagnosed. These cancers usually do not respond well to neoadjuvant chemotherapy and present with an intermediate prognosis between luminal and basal type breast cancers. They can be classified as triple negative as they do not express ER, PR or HER2, but are not classified as basal due to lack of epidermal growth factor receptor (EGFR) and CK5 expression (11). Normal-like breast tumors express genes associated with adipose tissue and usually cluster with fibroadenoma and normal breast samples (30).

The claudin-low subtype was first identified as a new intrinsic subtype in 2007 (31). Tight junction and cellular adhesion genes such as claudin -3, -4, -7, occludin and E-cadherin are expressed at low levels in these cancers. This subtype clusters near the basal-like subtype suggesting expression similarities between the two groups. Unlike basal-like cancers, claudin-low tumors express a unique set of 40 genes related to immune response (15). Claudin-low tumors express low levels of genes related to proliferation yet have poor prognosis. Genes associated with mesenchymal differentiation and epithelial-mesenchymal transition are overexpressed in this subgroup, which are also features associated with acquisition of the Cancer Stem Cell (CSC) phenotype (32). These tumors are generally classified as triple negative but about 20% express hormone receptors (33).

Molecular subtype classification of breast cancer has proven to be an important tool in understanding the pathways and genes that contribute to the development of the disease. Different molecular subtypes associate with different prognoses and vary in their response to therapy. Breast cancer molecular subtypes do not currently dictate therapeutic selection. However as previously mentioned, gene arrays such as Mammaprint™ and Oncotype DX™ are used to assess the likelihood of disease recurrence in early stage ER-positive breast cancer patients and determine if the addition of chemotherapy to the therapeutic regimen will be beneficial to the patient.

TABLE 1. ER, PR and HER2 status of molecular breast cancer subtypes.

	ER	PR	HER2
Luminal A	**+**	**+/-**	**-**
Luminal B	**+**	**+/-**	**+**
HER2+	**-**	**-**	**+**
Basal-like	**-**	**-**	**-**
Normal-like	**-**	**-**	**-**

1.3 Breast Cancer Treatment

Women diagnosed with breast cancer are treated by a combination of the following approaches: surgery, radiation therapy, chemotherapy, biologic therapy, and/or endocrine therapy. Selection of therapy and prognosis are dictated by a number of clinical and pathological factors including: the age and menopausal status of the patient, stage of the disease, histologic grade of the tumor, expression of ER, PR and HER2 and the proliferative index of the tumor.

Surgery and radiation therapy are considered local therapies, which treat at the site of the tumor. Most women diagnosed with breast cancer will undergo surgery (lumpectomy or mastectomy) followed by adjuvant therapy such as radiation, chemotherapy, hormone or targeted therapy. In some cases chemotherapy or hormone therapy is used before surgery, termed neoadjuvant therapy.

Chemotherapy refers to the use of cytotoxic antineoplastic drugs. Chemotherapeutics generally target all rapidly dividing cells leading to adverse systemic side effects. For advanced stage breast cancer, chemotherapy reduces the risk of recurrence. Chemotherapeutic regimen depends on tumor size and grade as well as lymph node involvement. For breast cancer common adjuvant regimens may include CAF (Cyclophosphamide, doxorubicin, and 5-FU), TAC (Docetaxel, doxorubicin, and cyclophosphamide), CMF (cyclophosphamide, methotrexate, and 5-FU) or TCH (taxotere, carboplatin, and Herceptin)(34).

As previously mentioned, approximately 20% of breast cancers are driven by HER2 signaling. Biologic, antibody-based therapies such as trastuzumab can effectively inhibit signal transduction by HER2 and are used to treat HER2-positive

breast cancers (35). Trastuzamab was first introduced into the metastatic disease setting (36) followed by combined use with chemotherapy in the adjuvant setting (22) reducing relapse in 50% of patients and improving survival in approximately 33% of cases. Resistance to trastuzamab eventually occurs in all metastatic breast cancer patients. Clinical trials have determined that dual targeting of the HER2 receptor by the combination of trastuzamab and lapatinib with paclitaxel improves pathological complete response (37). Lapatinib is a duel tyrosine kinase inhibitor that disrupts HER2 and EGFR pathways (38). Pertuzumab is a monoclonal antibody that blocks homodimerization and heterodimerization of the HER2 receptor (39). It has been approved for use in breast cancer patients with metastatic HER2 positive cancer in combination with trastuzumab and docetaxel.

1.3.1 Endocrine Therapies

ERα expression is an important prognostic marker in breast cancer and provides an index of response to endocrine therapies (40). Up to 75% of breast cancers are classified as ERα positive and are therefore candidates to receive various forms of endocrine therapy. The current strategy to treat hormone dependent breast cancers is to block estrogen signaling in tumor cells either by preventing estrogen from binding to the estrogen receptor using SERMs, preventing estrogen synthesis using aromatase inhibitors, or by degrading the ER using a pure antiestrogen such as fulvestrant. The antitumor effects of estrogen ablation were first discovered over 100 years ago with the use of oophorectomy to treat premenopausal breast cancer patients (41). Estrogen ablation or inhibition of estrogen function later became the target of pharmacological therapies for the treatment of ER positive breast cancer.

Estrogen production by the ovaries ceases at menopause. Circulating androgens are the main source of estrogens in postmenpausal women primarily through the conversion of androstenedione into estrone (42, 43). Adrenalectomy and hypophysectomy were shown in the 1950s to have antitumor effects in postmenopausal women (44), due to the fact that the adrenal gland provides androgens for the peripheral aromatization of androgens to estrogens. The unsuccessful antiepileptic aminoglutethimide successfully suppressed estrogen synthesis revealing antitumor activity in breast cancer patients (45). Aminoglutethimide was shown to act as an aromatase inhibitor (46) albeit with toxic side effects. Angela Brodie's lab identified 4-hydroxyandrostenedione (later named formestane) (47) through the investigation of androstenedione derivatives as substrate-binding blockers of the aromatase enzyme (48). Formastane (Lentaron) is classified as a 2^{nd} –generation steroidal aromatase inhibitor which binds irreversibly to the substrate binding site of the aromatase enzyme leading to protein-drug degradation, also referred to as suicide inhibitors. Another drug belonging to this class of steroidal aromatase inhibitors is exemestane (Aromasin). Non-steroidal aromatase inhibitors, such as anastrozole (Arimidex) and letrozole (Femara), bind to the p450 site of the aromatase complex.

The ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial was a randomized controlled clinical trial comparing the efficacy of anastrozole, TAM or both for five years, followed by a five-year follow-up (49). Results from the study suggested that the preferred treatment for post-menopausal women is aromatase inhibitors due to lower incidence of side effects, a prolonged disease-free survival and reduced rate of distant metastases.

In contrast to postmenopausal women, whose estrogen is produced in the peripheral tissues of the body, estrogen in premenopausal women is produced primarily in the ovaries. Five years TAM therapy is the current standard for pre-menopausal ER-positive breast cancer patients. However, it was recently reported that 10 years of TAM reduced the risk of late recurrence and increased overall survival compared to 5-years of TAM therapy (50). TAM, the most widely prescribed selective estrogen receptor modulator (SERM), has been used clinically for over 30 years. TAM can be used to treat early and advanced stage breast cancer and can also used for chemoprevention in high-risk women (51). It reduces disease recurrence by 47% and overall mortality by 26% (52). TAM acts as an ER antagonist in the breast and as a partial agonist in the liver, bone, and uterus therefore leading to increased risk of both endometrial cancer and thromboembolic events (53). Efforts have been made to develop novel SERMs potentially more effective than TAM. These include triphenylethylene derivatives such as toremifene and idoxifene, and benzothiophene derivatives such as raloxifene (RAL) and arzoxifene (54). However, these SERMs failed to have increased efficacy in the treatment of advanced disease.

TAM was also the first drug approved by the FDA as a chemopreventative agent. The Breast Cancer Prevention Trial (BCPT) demonstrated that TAM prevented breast cancer in women at high risk of developing the disease. Women taking TAM had 45% fewer invasive breast cancers compared to placebo after a 7 year follow-up (55). Results from the MORE (Multiple Outcomes of RAL Evaluation) trial indicated that the second-generation SERM RAL reduced the incidence of breast cancer in postmenopausal patients with osteoporosis (56).

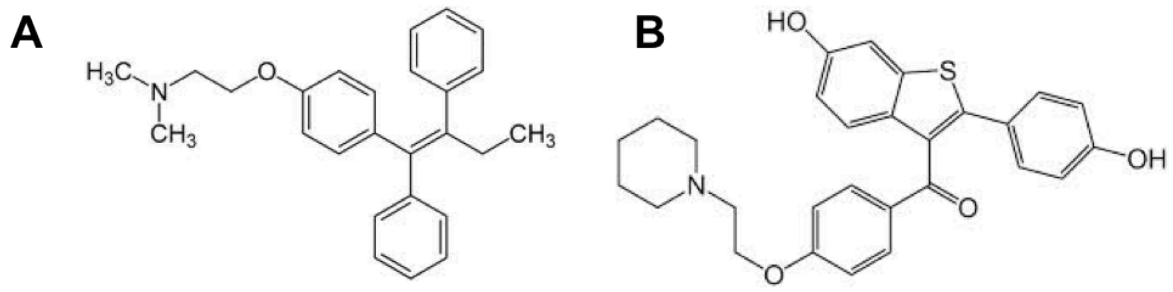


Figure 1. Chemical structures of tamoxifen and raloxifene. A) tamoxifen and **B)** raloxifene.

The Study of Tamoxifen and Raloxifene (STAR) trial was conducted to determine how the drugs compared in reducing the rate of breast cancer in high-risk postmenopausal women. The major findings of the trial were that women taking RAL had 36% fewer uterine cancers and 29% fewer blood clots than women taking TAM and that RAL was as effective as TAM in reducing the risk of developing invasive breast cancer (53). RAL "may be offered to reduce the risk of ER-positive invasive breast cancer in postmenopausal women" who are at high risk of developing the disease (57).

Bazedoxifene (BZA) is being developed for the treatment and prevention of osteoporosis in postmenopausal women and in combination with conjugated equine estrogens for the treatment of postmenopausal symptoms (58-60). It is currently in the late phases of review by the US FDA and was already approved by the European Union in 2009. BZA binds to both ERα and ERβ and has a 10-fold lower affinity to ERα then E2 (58). It was also shown that BZA does not stimulate the endometrium or result in breast cancer cell proliferation (60). Recently, BZA was demonstrated to effectively inhibit the growth of both TAM-sensitive and TAM-resistant breast cancer xenografts (61), suggesting it may have potential as a therapeutic option for patients with advanced disease.

1.3.2 Endocrine Therapy Resistance

Endocrine therapy is a pivotal treatment for ER positive breast cancer. Although endocrine therapy is an effective treatment against breast cancer, 25% of patients with early breast cancer and all patients harboring metastatic tumors will eventually relapse (62). A variety of cellular changes have been implicated in compensatory survival mechanisms leading to endocrine resistance. Many of the pathways deregulated in resistance to endocrine therapies have been studied in the preclinical setting with an emphasis on TAM resistance.

The tumor microenvironment has been implicated to play a role in endocrine resistance. The use of sophisticated *in vitro* and *in vivo* experimental models have implicated components of the tumor microenvironment such as various stromal cells, elements of the extracellular matrix, soluble factors as well as conditions within the tumor microenvironment such as hypoxia or pH to play a role in resistance to endocrine therapies (63, 64). Most pathways leading to endocrine resistance stem from genetic or epigenetic changes in the tumor cells themselves.

The expression of the ER itself and coregulators, which alter the function of the ER, may contribute to endocrine resistance. Although rare, the loss of ER expression can lead to an endocrine insensitive phenotype (65, 66). Expression of ER splice variants (ERα36) (67) as well as estrogen-related receptors have also been associated with endocrine resistance. ER corepressors or coactivators can directly alter the balance between the agonist or antagonist activity of SERMs and the ligand-independent activity of the ER. Overexpression of AIB1, an ER coactivator, is associated with TAM resistance in the laboratory and in the clinic (68). Downregulation

of the ER corepressor NCoR was found in experimental tumors refractory to TAM (69). TAM resistance has been associated with increased levels and activity of transcription factors AP-1 (70-72) and NFκB (73). Overexpression of positive regulators of the cell cycle such as cyclin E1 and D1 can block the antiproliferative effects of endocrine therapy (74, 75). Inactivation of RB (retinoblastoma) tumor suppressor or reduced activity, stability or expression of p21 or p27 has been associated with a poor response to endocrine therapy (76, 77).

Until very recently the number of ERα mutations found in clinical breast cancer specimens have been relatively low considering mutations in a drug target is a common mechanism of drug resistance (78). The Y537N ERα mutant was discovered in a metastatic breast tumor and may play potential roles in regulating ligand binding and transactivation of ERα (79). The 303 position of ERα has also been shown to be mutated from Arginine to Lysine allowing ERα to be more highly phosphorylated by Akt signaling (80) and PKA (81). Overexpression of the K303R ERα mutant conferred decreased TAM-sensitivity (82), resistance to anastrozole (80) and hypersensitivity to estrogen (83). The presence of this mutation has been associated with poor clinical outcomes (84) although the frequency of K303R mutation is still an area of contention (85, 86). Recently, Robinson and colleagues, as part of a clinical sequencing program, identified 6 of 11 advanced breast cancer patients whose tumors harbored mutations in the ligand-binding domain (LBD) of ERα (87). All of these patients had previously received endocrine therapy, suggesting that activating mutations in ERα are a possible mechanism of acquired endocrine resistance. Toy and colleagues also recently identified LBD mutations in 14 of 80 ER-positive metastatic breast tumors (88).

Mutations affecting Tyr537 and Asp538 promoted expression of ER regulated genes in the absence of E2. Mutations in these amino acids also rendered MCF-7 cells resistant to hormone deprivation *in vivo*. Although these mutations resulted in decreased sensitivity to fulvestrant and TAM *in vitro*, higher doses of the drugs were able to inhibit ER signaling suggesting that more potent or specific antagonists may be beneficial to patients harboring these ER mutations.

Alternative proliferative or survival stimuli can be provided to tumors by growth factors and other cellular kinase pathways when the ER pathway is effectively inhibited using various forms of endocrine therapy. Increased signaling from alternative pathways can also circumvent the inhibitory effects of endocrine therapy via modulation of the ER or crosstalk with the ER. Pathways such as HER2/neu (89), EGFR (90), IGF-1R (91), Src (92), VEGF (93), and FGF (94) have been implicated in endocrine resistance. Increased signaling through various cascades including Akt (95), PTEN (96), PKCα (1, 97) and MAPK (98) have also been implicated in endocrine resistance. Signaling through the androgen receptor (AR) has been shown to bypass ER inhibition leading to endocrine resistance (99). Clinical strategies have recognized EGFR and HER2 as contributing to endocrine resistance and have focused on co-targeting these pathways along with ER to improve patient outcomes (100, 101).

It has also been suggested that breast cancer stem-like cells (CSCs) within tumors may also contribute to endocrine resistance. Accumulating evidence supports the concept that epithelial and other solid tumors contain a cellular developmental hierarchy containing CSCs and more differentiated progenitor cells. The frequency of CSCs in breast tumors is disputed but may depend largely on the molecular subtype,

tumor grade and stage (102, 103). Al Hajj and colleagues were the first to identify a population of cells that could initiate breast tumors when as few as 200 were implanted into immune-deficient NOD/SCID mice. These $CD44^{+}/CD24^{lo}/ESA^{+}/lineage^{-}$ cells developed into tumors containing phenotypically diverse non-tumorigenic cells (104). A common feature of CSCs is that they are resistant to both chemotherapy as well as radiation therapy (105, 106). The normal mouse mammary stem cell population consists of less then 0.01% ER positive cells (107). One mechanism of endocrine resistance may lie in the possibility that an ER negative, endocrine treatment-resistant CSC population exists that is able to produce a more differentiated ER positive treatment sensitive cell population. These ER negative/low progenitor-like cells may be able to seed metastases and cause relapse despite endocrine therapy being effective on the bulk tumor population. Weinberg's group has linked the mesenchymal phenotype to the stem cell population in normal tissue and in CSCs (108). Normal breast epithelial stem cells are dependent on EGFR and other growth factor signaling pathways, and as previously mentioned these pathways are upregulated in endocrine breast cancers. The upregulation of EGFR and other related pathways may reflect an increase in the proportion of breast CSCs. Therapies that effectively target the breast CSC population in combination with endocrine agents may have the potential to overcome resistance in the clinical setting.

1.3.3 Estradiol as a Treatment for Breast Cancer

In the 1940s, high dose E2 was used to treat metastatic breast cancer and was the first chemical therapy to treat any cancer successfully (109). Before the introduction of TAM, breast cancer patients received high-dose E2 or diethystilbesterol

(DES) treatment. Although similar response rates were observed with these two treatments (110, 111), TAM treatment became the mainstay due to a lower incidence of side effects. A long-term follow up study, in fact, indicated a survival advantage for patients treated with the synthetic estrogen DES compared to patients treated with TAM (112).

Recently, the use of E2 or an E2-like compound has re-emerged as a possible treatment strategy for patients exhibiting endocrine therapy resistant breast cancers (113-116). Clinical trials have demonstrated the efficacy of E2 in this setting (117, 118). The basis for the clinical use of estrogens is supported by a number of preclinical laboratory models (2, 119-126). In addition, these paradoxical inhibitory effects of E2 have been observed in prostate cancer cells (127), breast cancer cells transfected with ER (128), osteoclasts (129), neuronal cells (130) and thymocytes (131). The MCF-7:5C model was derived from MCF-7 cells treated in estrogen deprived conditions long-term to mimic the effects of long–term aromatase inhibitor treatment (132). Treatment of MCF-7:5C cells with physiological concentrations of E2 leads to growth inhibition and apoptosis *in vitro* (125). Santen and colleagues observed the upregulation of ERα, MAPK, PI3K and mTOR (mammalian target of rapamycin) growth factor pathways when long-term estrogen deprived (LTED) MCF-7 cells became hypersensitive to E2 (133). Although the exact mechanism of E2-induced apoptosis is unknown, evidence suggests the involvement of both the extrinsic and intrinsic apoptotic pathways.

The extrinsic apoptotic pathway delivers an apoptotic signal from the extracellular environment to initiate an intacellular signaling cascade which begins when ligands interact with surface receptors such as Fas, tumor necrosis factor (TNF) or death

receptors 3-6 (DR3-6) (134). Caspases 8 and 10 are recruited leading to activation of effector caspases 3 and 7 resulting in cell death. Using LTED MCF-7 cells Song and colleagues were the first to demonstrate that E2 sensitive LTED cells had increased expression of Fas compared to wild-type MCF-7 cells and that when these cells were treated with E2, FasL expression increased (124). Fas expression was also induced when Osipo and colleagues treated TAM-resistant tumors with E2 resulting in tumor regression (120). Our lab has also shown that PKCα-overexpressing TAM-resistant T47D:A18/PKCα tumors upregulate Fas/FasL proteins when regressing in the presence of E2 (135).

The intrinsic apoptotic pathway is dependent on the loss of mitochondrial membrane integrity. Mitochondrial membrane permeablization is controlled by pro-apoptotic and anti-apoptotic Bcl-2 family members which regulate apoptosis by controlling cytochrome c release. PI3K/Akt, Ras/MAPK and JAK (Janus Kinase)/STAT (signal transducer and activator of transcription) pathways regulate phosphorylation of Bcl-2 family member regulating mitochondrial homeostasis (136). In MCF-7:5C cells, E2-induced apoptosis is dependent on expression of pro-apoptotic proteins Bax and Bim. In addition, overexpression of anti-apoptotic Bcl-XL blocked E2-induced apoptosis. E2 treatment led to cytochrome c release resulting in activation of caspases 7 and 9 and cleavage of poly(ADP-ribose)polymerase (PARP) (137).

The PI3K/Akt pathway has also been implicated in E2-induced growth inhibition. The pathway was found to regulate the phosphorylation/inactivation of pro-apoptotic factors controlling cytochrome c release as well as the activation of anti-apoptotic genes (138, 139). Our lab demonstrated that E2 treatment resulted in downregulation of

phospho-Akt in T47D:A18/PKCα xenografts (135). Basal phospho-Akt is increased in MCF-7:5C cells and is reduced upon E2 treatment (140) as well.

Recently, V. Craig Jordan's group has demonstrated an important role for c-Src kinase in E2-induced growth inhibition. MCF-7:5C cells treated with E2 had increased levels of phosphorylated c-Src which activated the unfolded protein response pathway (UPR) leading to phosphorylation of eukaryotic translation initiation factor-2α (eIF2α). Reactive oxygen species (ROS) production was also increased in response to E2 treatment. Inhibition of c-Src decreased ROS production, inhibited eIF2α phosphorylation and prevented E2-induced apoptosis (141).

1.4 Estrogen Receptor

1.4.1 Estrogen Receptor structure

Estrogen receptors, ERα and ERβ, belong to the nuclear steroid receptor superfamily and are capable of activating transcription of estrogen-regulated genes. Various species of ERα and ERβ have been discovered (142-145). ERα and ERβ are located on different chromosomes and are products of individual genes (146, 147). Both ER subtypes contain 6 functional domains (142). The A/B region contains the transactivation domain (AF1), which is responsible for ligand independent function and also contains a coregulatory domain that binds coactivators and corepressors. The C-region contains the DNA-binding domain (DBD), which allows binding to the promoter of estrogen responsive genes. The D region contains several functional regions including the nuclear localization signal, the hinge region and part of the ligand-independent activating domain. The E and F carboxy-terminal region contains the ligand-binding domain containing ligand-dependent transactivation function (AF2). This region is also

responsible for nuclear localization, receptor dimerization and binding of co-regulatory and chaperone proteins (142). Various isoforms of ERα exist including ER46 and ER36. ERα 46 is devoid of the N-terminus A/B domain, but otherwise is identical to full length ERα (148). ERα36 has no intrinsic transcriptional activity consistent with the fact that it lacks both transcriptional activation domains, AF-1 and AF-2, found in full length ERα66 (149, 150). ERα36 is mainly expressed at the plasma membrane and is expressed in ERα66-negative breast cancer cell lines (151) and patient specimens (152, 153). Expression of ERα36 is also associated with TAM resistance in ERα positive breast cancers (152). Various spice variants of ERβ have also been described due to alternative splicing of the last coding exon.

ERα and ERβ are able to regulate common and different genes (154). The same genes can also be differentially regulated by the ERs. The divergence in gene expression can be accounted for by the varied affinity for different ligands. The AF1

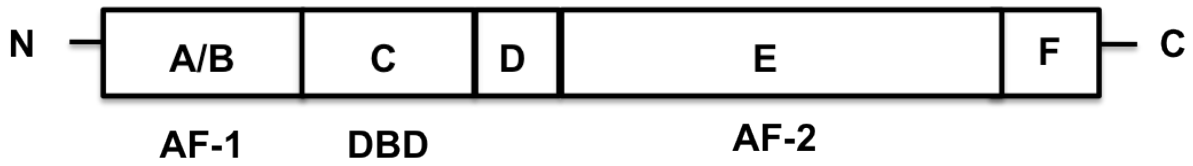


Figure 2. Domain structure of ERα. The A/B region contains the AF-1 transactivation domain. The C-region contains the DNA-binding domain (DBD). The D-region contains the nuclear localization signal. The E and F regions contain the ligand-binding domain.

domain is poorly conserved between ERα and ERβ, which is responsible for the recruitment of coactivators and corepressors. Conformational changes induced by agonist recruit coactivators to ERα but coactivators and corepressors to ERβ. TAM recruits corepressors on to ERα (155, 156). Activity of ERs can also be affected by heterodimerization and transcriptional response may be dependent on the cellular ratio

of ERα and ERβ. ERα and ERβ have been shown to exert opposite effects on cell proliferation and angiogenesis (157).

1.4.2 Estrogen Signaling

Estrogen binds to the ER leading to receptor dimerization and binding to estrogen response elements (EREs) on the promoter of target genes (158). Ligand binding induces a conformational change in the receptor leading to coactivator recruitment (159). ERs can regulate gene expression through a number of mechanisms that deviate from this classical model of gene transcription. Target genes activated by ERα are generally involved in survival of breast cancer cells and include cyclin D1, Bcl-2, insulin-like growth factor receptor (IGF-1R), ERα itself, progesterone receptor (PR) and vascular endothelial growth factor (VEGF) (160-162).

Not all genes regulated by ERs contain an ERE-like sequence. About one-third of genes regulated by ERs do not require direct binding of ERs to DNA (163). Gene expression can be modulated by protein-protein interactions where ERs tether to other classes of transcription factors in the nucleus. Activator protein-1 (AP-1) is an example of a transcription factor that can form a complex with ER leading to ERE-independent genomic functions. Other transcription factors such as Sp-1 and nuclear factor kappa B (NF-kB) also have the ability to exert ERE-independent genomic function when in complex with ER. Other members of the nuclear receptor superfamily also use this mechanism of transcription and it is referred to as transcriptional cross-talk (164).

In the absence of ligand ER activity can be modulated through phosphorylation. Extracellular signals such as growth factors and cytokines can activate pathways leading to the phosphorylation of the AF-1 domain of ERs in the absence of ligand

(165). The ligand-independent action of ER may also play a role in endocrine resistance.

1.4.3 Extranuclear ER

It is widely accepted that estrogens are capable of exerting non-genomic or extranuclear functions (166). These extranuclear actions are rapid and cannot be accounted for by RNA or protein synthesis. Steroid hormones in general have been known to exert non-genomic actions which frequently activate kinase cascades (166). The biological effects of non-genomic signaling are often difficult to study since most cellular processes involve both genomic and non-genomic signaling. Extranuclear ER signaling modulates migration, invasion and proliferation by controlling intranuclear ER signaling. Proline-, glutamic acid-, and leucine-rich protein-1 (PELP-1) functions as a regulator of both intracellular and extranuclear ER signaling. PELP-1 can act as a scaffold bringing membrane localized ER in physical association with other signaling molecules such as Src (167). PELP-1 also acts as a coactivator for steroid receptors (168).

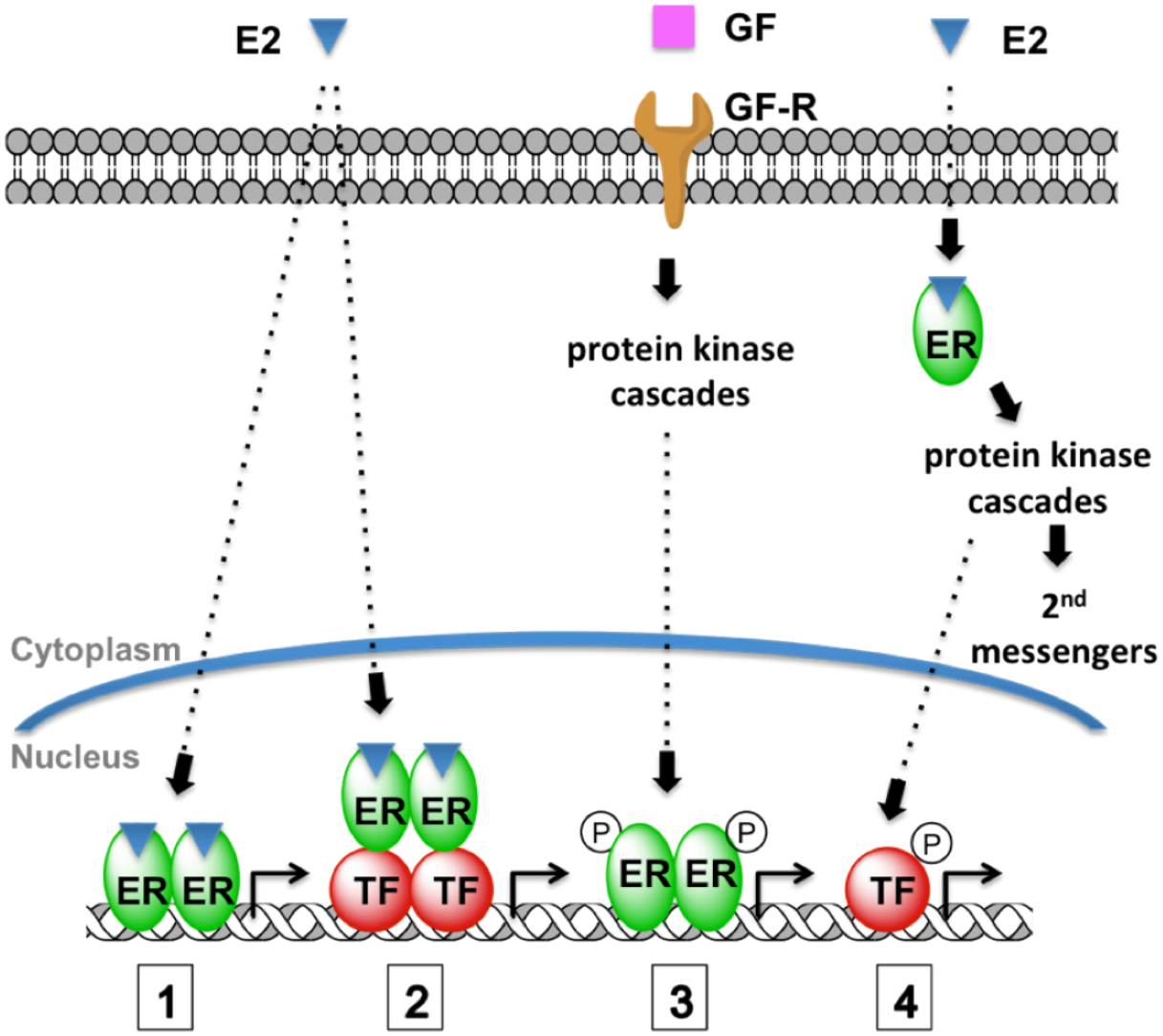


Figure 3. Schematic representing ER signaling mechanisms. **1)** The classical pathway includes ligand activation followed by direct binding to estrogen response elements (EREs) on the DNA. **2)** The tethered pathway involves interaction with other transcription factors (TF) following ligand activation of ER. These DNA interactions do not directly involve ER or EREs. **3)** Ligand-independent pathways occur through activation of kinase cascades typically by growth factor (GF) signaling which in turn activate ERs through phosphorylation. **4)** The extranuclear ER pathway leads to rapid cellular effects due to activation of protein kinase cascades increasing second messengers such as eNOS and calcium. This pathway also leads to activation of protein kinase cascades which can phosphorylate transcription factors leading to transcriptional activation.

Recent work has strongly supported that extranuclear or membrane ERα appears to be identical to classical nuclear ERα in several cell lines. For example, nuclear and membrane ERs were identified in CHO cells transfected with ERα and ERβ suggesting both nuclear and membrane receptors were derived from the same transcript (169). Using mass spectrometry, Pedram and colleagues found ER in the membrane which was capable of binding E2 to be identical to nuclear ER (170). The membrane ER pool is estimated to be only 3-10% of the nuclear receptors (170, 171). After analyzing 3200 clinical specimens Welsh and colleagues concluded that cytoplasmic ERα was only present in ~1.5% of cases (172). Pathologists designate ER positivity based on nuclear localization of the receptor. One inherent challenge with clinical detection of cytoplasmic ERs may lie in the immunodetection techniques. Cytoplasmic pools may be 10 times lower then nuclear pools, making cytoplasmic staining seem non-specific (173). More advanced techniques may be required to reliably determine the number of human breast cancers displaying cytoplasmic ERα.

Non-genomic actions of E2 include mobilization of intracellular calcium (174), stimulation of adenylate cyclase activity and cAMP production (175), as well as activation of MAPK (176) and PI3K (177) signaling pathways. The non-genomic actions of E2 at the plasma membrane are exerted by the classical ERs, ERα and ERβ (169), but other ER isoforms have also been suggested to exert non-genomic effects such as ERα46 (148) and ER-X (178). ER-X was discovered in the mouse neocortex and uterus in 2002. This 63 kDa protein can be detected with ERα antibodies against the E and C domains of ERα however the structure of the receptor is not known.

The localization of ERα to the plasma membrane requires palmitoylation at a highly conserved sequence in the E domain of the receptor (179). DHHC-7 and DHHC-21 are the palmitoylacyltransferase (PAT) proteins responsible for the palmitoylation of ERα (180), leading to a productive interaction with caveolin-1, an association required for membrane localization of ERα (181). DHHC-7 and DHHC-21 are also responsible for palmitoylation of the progesterone and androgen receptor (180). Hsp27 promotes palmitoylation of ERα presumably through alteration in the structure of ERα allowing access to the palmitoylation site and is required for ER trafficking to the plasma membrane (182). ERs do not contain a plasma membrane localization sequence and must associate with scaffold proteins at the membrane. ERs at the plasma membrane associate with caveolin-1 (183), G-proteins (184), ras (176), Src kinase (185), the p85α subunit of PI3K (186), Shc (187) and MNAR (188). ERα can interact directly with HER2 (189) and can also activate EGFR leading to downstream activation of MAPK and Akt (190). Activation of kinase cascades by non-genomic E2 signaling can lead to genomic signaling through activation of various transcription factors. E2 treatment can lead to the phosphorylation of Elk-1 (191), C/EBPβ and CREB (cAMP response element binding protein)(192).

The membrane only estrogen receptor (MOER) alpha mouse was developed to investigate the potential roles of membrane ERα in the normal development of the reproductive tract and mammary gland (193). The MOER mouse expresses a functional E domain of ERα at the plasma membrane, but no nuclear of cytoplasmic ERα. Extracellular signal- regulated kinase (ERK) and PI3K were activated in liver cells by E2 from MOER and wild type mice but not in cells from ERα knockout mice.

Furthermore the female reproductive organs of MOER mice were extremely atrophic and the mammary gland of MOER mice did not develop properly. The authors concluded that nuclear ERα is required for normal development of adult female mice and could not be rescued by membrane ERα domain expression alone. Signaling from membrane ERα does not compensate for nuclear ERα expression.

Another model has recently been developed by Adlanmerinia and colleagues in an attempt to differentiate the effects of membrane versus nuclear ERα (194). This group generated a mouse with a point mutation at the palmitoylation site of ERα, allowing for the loss of membrane-specific effects of ERα. Similar to the MOER mouse, female mice with this point mutation were infertile. E2 action in the uterus, however, was similar to wild-type mice. Rapid dilation of vasculature and acceleration of endothelial repair by E2 were abrogated in ERα palmitoylation impaired mice. These membrane initiated vascular effects of E2 *in vivo* were also suggested by a study done by John Katzenellenbogen's group using an estrogen-dendrimer conjugate (EDC), a compound which selectively activates non-nuclear ER (195). MCF-7 xenograft growth was not stimulated by EDC. In addition, unlike the effects of E2, the uteri of mice treated with EDC were not enlarged. Suggesting, that non-nuclear ER does not play a role in breast cancer and uterine growth stimulation elicited by E2.

In 2000, it was reported that the rapid effects of E2 on the activation of ERK was dependent on the expression of an orphan G protein-coupled receptor (GPCR), GPR30 or G protein-coupled estrogen receptor 1 (GPER) (196). Like other GPCRs, GPER is a 7-transmembrane spanning protein unrelated to nuclear receptors. E2 was found to bind GPER in breast cancer cells lines as well as in GPER-transfected COS7 and

HEK293 cells (197, 198). Using fluorochromes linked to E2 Revankar and colleagues estimated that GPER was primarily localized to the endoplasmic reticulum and the Golgi apparatus in breast cancer cells. The fluorochromes were only able to stain permeabilized membrane suggesting that GPER was not located at the plasma membrane (198). Otto et al., confirmed endoplasmic reticulum localization of GPER in MDA-MB-231 cells. GPER is widely expressed throughout murine and rat tissues, specifically it was found in the mammary gland, uterus, ovary, testis, pituitary gland, heart, lung, brain and renal pelvis (199, 200). The protein is also more highly expressed in neoplastic tissues compared to normal tissue (198, 200), although no relationship was found between GPER expression level and histological type, grade, or overall survival in ovarian cancer(201). GPER activation by treatment with the GPER specific agonist G1, inhibited MCF-7 hormone-dependent cell growth (202). In MCF-7 cells exposed long-term to TAM, inhibition of GPER reversed the stimulatory effects of TAM on cell growth suggesting a role for GPER in the development of TAM resistance (203). In breast cancer patients treated with TAM, GPER expression negatively correlated with relapse free survival (204).

1.5 Protein Kinase C

A major mechanism through which external stimuli are transformed into cellular events is through intracellular protein phosphorylation. Stabilization of certain conformational states of proteins can be achieved through phosphorylation leading to altered biological activity. Protein Kinase C (PKC) is a family of serine/threonine protein kinases and has been implicated in a variety of cellular processes including proliferation, cell cycle, gene expression, differentiation, cell migration and apoptosis

(205). Subcellular localization of both enzyme and substrate are key in the regulation of PKC function.

All members of the PKC family share a common structure: a N-terminal regulatory domain and a C-terminal Catalytic domain linked by a flexible hinge region. The PKCs are divided into 3 subfamilies based on structural and regulatory characteristics. The classical or conventional PKCs consist of regulatory domains C1 and C2 which confer binding to diacylglycerol (DAG), phorbol esters, phosphatidylserine (PS) and Ca2+. PKCα, PKC-βI, PKC-βII, and PKC-γ isozymes are classified as classical PKCs and require DAG, PS and Ca2+ for activation. Novel PKCs contain a C1 domain and a novel C2 domain requiring PS and DAG but not Ca2+ for activation. Members of the novel PKCs include PKCδ, PKCε, PKCη, PKC θ and PKC μ. In contrast the atypical PKCs contain a C1 domain that does not bind DAG or Ca2+ but only requires PS for activation. Atypical PKCs include PKCζ and PKCι/λ. The C3 and C4 regions of PKCs contain the ATP and substrate binding domains. Plasma membrane recruitment is a key step in activation of most PKCs, although some isoforms have been reported to go to the nucleus. Substrates are phosphorylated when PKCs are in their active, membrane-bound, open form. The N-terminus of PKCs contains a pseudosubstrate sequence, which resembles a substrate phosphorylation motif. The enzyme is autoinhibited and maintained in an inactive state by the pseudosubstrate motif sterically blocking the catalytic domain. Binding of cofactors such as DAG and Ca2+ confer a conformational change to the enzyme exposing its kinase domain and allowing the enzyme to exert catalytic function.

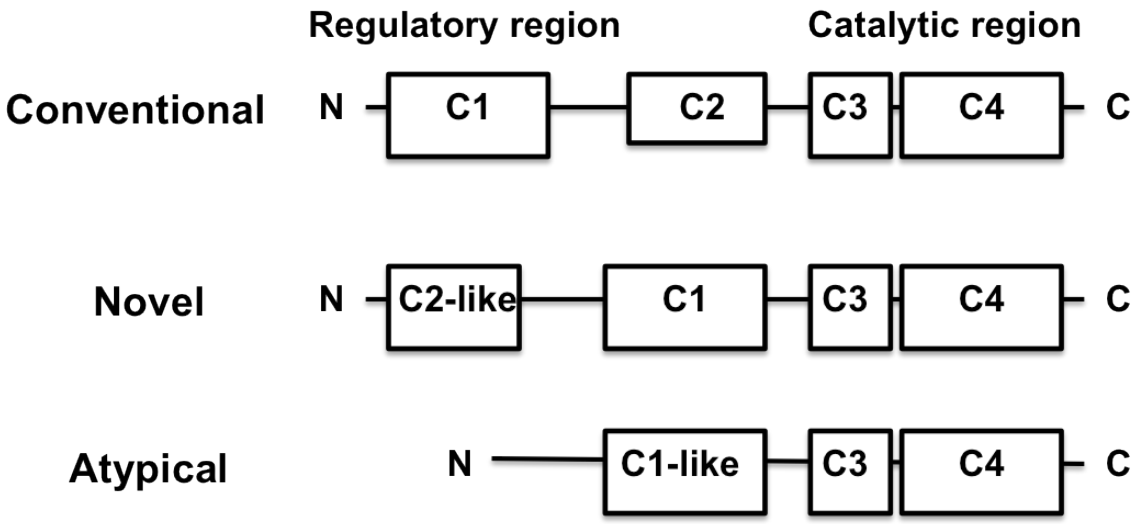


Figure 4. Primary structure of the PKC family. Conventional PKCs consist of regulatory domains C1 and C2 which confer binding to diacylglycerol (DAG), phorbol esters, phosphatidylserine (PS) and Ca2+. Novel PKCs contain a C1 domain and a novel C2 domain requiring PS and DAG but not Ca2+ for activation. Atypical PKCs contain a C1 domain that does not bind DAG or Ca2+ but only requires PS for activation. The C3 and C4 regions of PKCs contain the ATP and substrate binding domains.

Modulation of PKCs localization and expression occur during mammary gland differentiation and involution. Different PKC isoforms are involved in mitogenic and/or apoptosis during these processes (206). MEK/ERK (207) and PI3K/Akt (208) are two major downstream pathways activated by PKCs. Through the phosphorylation of target proteins such as BAD the PI3K pathway is capable of inhibiting apoptotic response (209). The deregulation of expression and/or activation of PKCs can contribute to enhanced proliferation or survival processes. The PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has long been known as a tumor promoter. TPA induces activation of PKC by binding to the DAG-binding site (210). PKC activity is also increased in malignant compared to normal breast tissue (211). ER-negative

status in breast cancer is associated with poor clinical outcome and a negative correlation between ERα status and PKC has been described (212-215).

1.5.1 Protein Kinase C alpha in Breast Cancer

Upon stimulation PKCα translocates mainly to the plasma membrane but also to specialized compartments such as the nucleus or focal adhesions in certain cell types. In fibroblasts specific stimuli have been shown to lead to nuclear translocation of PKCα although the kinase does not contain a nuclear localization sequence (216). PKCα activation is dependent on threonine 497, threonine 638 and serine 657 phosphorylation (217). Subcellular localization of PKCα is a marker of PKCα activation as well as phosphorylation of serine 657 (218). Dephosphorylation by phosphatases results in kinase inhibition of PKCα (219). PKCα is ubiquitously expressed within tissues. Cell type specific substrates, modulators and anchoring proteins regulate the dynamic cell specific effects of PKCα.

An important role has been suggested for the upregulation of PKCα in breast cancer (97, 212, 213, 220-222) and abnormal expression has been found in human tumors and transformed cell lines. In various tumor cell lines its half-life has been estimated to between 7-24 hours (223). PKCα has been mapped to chromosome 17q22-23.2. Chromosome 17q is highly susceptible to rearrangements in breast cancer. Other genes such as HER2 and BRCA1, which are associated with breast tumorigenesis, are also located on chromosome 17q (224). There are very few cases in which point mutations in PKCs have been linked to a transformed phenotype. PKCα point mutations have been found in human pituitary adenomas, thyroid cancers and follicular neoplasms (225, 226).

PKCα expression in breast cancer cell lines has been associated with increased proliferative potential. PKCα expressing cell lines MCF-7, MDA-MB-231 and MDA-MB-468 demonstrated enhanced proliferation compared to non-PKCα expressing T47D breast cancer cells (227). Work from the Tonetti lab has demonstrated that PKCα plays a role in the hormone-independent and TAM-resistant phenotype. Hormone independent breast cancer cell lines T47D:C42 and MCF-7:5C had increased PKCα expression compared to their hormone-dependent counterparts, T47D:A18 and MCF-7:WS8. The ectopic overexpression of PKCα in T47D breast cancer cells (T47D:A18/PKCα) led to hormone-independent and TAM-resistant phenotype. Additionally basal AP-1 activity was elevated in all PKCα overexpressing hormone-independent clones (1).

"PKC*α* overexpression may be predictive of TAM treatment failure, since expression is high in the primary biopsy and does not increase in the second biopsy following TAM exposure" (221). PKCα has also been demonstrated to regulate the multidrug resistance phenotype (228-230). When MCF-7 cells were transfected with the *mdr-1* gene, doxorubicin resistance emerged and elevated PKCα levels were detected. PKCα knockdown in these cells inhibited P-glycoprotein allowing doxorubicin to be retained within the cells (230).

PKCα positivity is associated with poor patient survival and breast cancer aggressiveness (227). Laboratory research has further confirmed the association between PKCα and a more aggressive phenotype. PKCα overexpression enhanced the invasive potential of MCF-7 cells (97) and non-tumorigenic MCF10A cells (231). Unpublished work from the Tonetti lab has suggested that overexpression of PKCα in

T47D breast cancer cells (T47D:A18/PKCα) leads to loss of E-cadherin, increased *in vitro* invasive and migratory potential. The selective PKCα inhibitor aV5-3 inhibited lung metastasis development without affecting primary tumor growth in a murine breast cancer model. This inhibitor reduced cell migration and inhibited matrix metalloproteinase-9 activity, which was accompanied by a reduction in NF-κB activity (232). Although preliminary, these studies suggest that inhibition of PKCα activity may potentially reduce lung metastases in patients with breast cancer.

The PKCα antisense oligonucleotide aprinocarsen (LY900003, ISIS 3521) has been the only PKCα inhibitor given therapeutically to breast cancer patients. Patients with metastatic breast cancer who had failed one chemotherapeutic regimen received the agent as a continuous infusion (233). No information was available in PKCα levels suggesting that the patient population was chosen indiscriminately and affects may have been seen in patients with cancer driven by PKCα. In addition to PKC inhibitors, non-specific PKC activators such as bryostatin-1 have been used in numerous clinical trials for melanoma, colon cancer, as well as non-small cell lung cancer but showed little to no benefit for trial participants (234-236).

In an effort to identify signaling networks utilized by CSCs, Tam et al. (237), used gene arrays to identify PKCα as a kinase significantly increased following induction of EMT, a program known to increase CSC frequency (108). FRA1, a member of the FOS family, transcriptionally drives CSC function downstream of PKCα. Pharmacological inhibition of PKCα was able to selectively inhibit the CSC population (237). As previously mentioned, CSC are resistant to conventional therapeutic regimens (106).

This study suggests that inhibition of PKCα may be a viable option to improve therapeutic outcomes for certain types of breast cancer.

2. HYPOTHESIS

Overexpression of PKCα in primary breast tumors may predict resistance to endocrine therapies (221). Patients whose tumors overexpress PKCα are in need of alternative therapeutic options. Tonetti and colleagues have previously shown that overexpression of PKCα in T47D breast cancer cells conferred both a TAM-resistant as well as E2-inhibited phenotype *in vivo* (1, 2).

Based on current data, we hypothesize that PKCα may be a potential biomarker for the use of an estrogenic treatment for breast cancer. Furthermore, I hypothesize that compounds that selectively have estrogenic action in the breast may be a viable option for TAM-resistant breast cancer patients. In this thesis I aim to further define the potential use of PKCα as a biomarker for estrogenic therapy as well as identify alternative options for TAM-resistant breast cancer.

3. MATERIALS AND METHODS

3.1 Reagents

For in vitro experiments DMSO, ethanol, E2, 4-hydroxytamoxifen (4-OHT) and RAL were obtained from Sigma-Aldrich (St. Louis, MO USA). For in vivo experiments E2 and TAM were obtained from Sigma. RAL (Evista®, Eli Lilly and Company, Indianapolis, IN USA) was purchased from the University of Illinois at Chicago Hospital Pharmacy. Cell culture reagents were obtained from Life Technologies (Carlsbad, CA USA). Tissue cultureware was purchased from Becton-Dickinson (Franklin Lakes, NJ USA). The following antibodies were used: rabbit monoclonal ERα (for tissue and cells, SP1, Lab Vision, Thermo Scientific, Kalamazoo, MI USA), rabbit polyclonal ERα (for colonies, HC20, Santa Cruz Biotechnology, Santa Cruz, CA USA), β-actin (mouse monoclonal, 1:1000, Sigma-Aldrich) and PKCα (rabbit polyclonal, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies included: anti-rabbit Alexa Fluor 488 (Life Technologies, Carlsbad, CA USA), anti-mouse Cy3 (Jackson Immunoresearch Laboratories, West Grove, PA USA) and HRP-cojungated anti-rabbit and anti-mouse (GE Healthcare UK Limited, Buckinghamshire, UK).

3.2 Cell culture conditions

Stable transfectant cell lines T47D:A18/neo and T47D:A18/PKCα (1) were maintained in RPMI1640 (phenol red) supplemented with 10% fetal bovine serum (FBS) containing G418 (500 μg/ml). MCF-7:5C cells were maintained in phenol red-free RPMI 1640 supplemented with 10% 3X dextran-coated charcoal treated FBS (E2-depleted media) as previously described (125). LTED MCF-7 cells were maintained in phenol red-free Improved Minimal Essential Medium (IMEM) as previously described (124).

The T47D:A18-TAM1 cell line was created by maintaining T47D:A18 breast cancer cells long-term (6-12 months) in 1uM of 4-hydroxytamoxifen (4-OHT) in E2-depleted RPMI 1640. Single cell clones were derived using the limiting dilution method. Prior to treatment cell lines were cultured in E2-depleted medium for 3 days. Cell lines were routinely tested for Mycoplasm contamination (MycoAlertTM Mycoplasm Detection Kit, Lonza Ltd., Rockland, ME, USA).

3.3 DNA growth assay

Cells were plated at a density of 15,000 cells/well in 24-well plates. Treatment media (vehicle, DMSO [0.1%], E2 [10-9M], 4-OHT [10-7M], RAL [10-7M], BTC [10-9M, 10-8M, 10-7M], TTC-352 [10-9M, 10-8M, 10-7M] was added the following day (Day 1) and changed every three days. Growth was determined by incubating cells with Hoechst 33342 cell permeable dye (Life Technologies, Carlsbad, CA USA) for 1 h at 37°C and reading fluorescence at excitation 355 nm/emission 460 nm on a Perkin Elmer Victor3 V (Waltham, MA USA) plate reader.

3.4 siRNA mediated knockdown of PKCα

400,000 cells were seeded onto a 6-well plate 24hrs prior to transfection. Cells were transfected with a final concentration of 50 nM siRNA (silencing ribonucleic acid) using DharmaFECT 1 transfection reagent according to the manufacture's instructions (Dharmacon, Lafayette, CO). Nontargeting siRNA and PKCα targeting siRNA pools with the following sequences were used.

TABLE 2. siRNA Sequences

Gene	ON-TARGET plus SMARTpool siRNA sequences (Dharmacon)
Non-targeting control	UGGUUUACAUGUCGACUAA UGGUUUACAUGUUGUGUGA UGGUUUACAUGUUUUCUGA UGGUUUACAUGUUUUCCUA
PRKCA	UAAGGAACCACAAGCAGUA UUAUAGGGAUCUGAAGUUA GAAGGGUUCUCGUAUGUCA UCACUGCUCUAUGGACUUA

Media was changed 24 hrs post transfection and cells were plated for proliferation assay at 48hrs post transfection.

3.5 Trypan blue exclusion viability assay

Following 5 days of treatment, cells were trypsinized and resuspended in medium. Cell suspension was then mixed 1:1 with 0.4% trypan blue solution. Cells were then counted using a hemocytometer. The percent of viable cells was calculated by dividing the number of viable cells by the total number of cells in each treatment group.

3.6 Synthesis and oral bioavailability of benzothiophene SEMs

The synthesis of BTC and TTC-352 was preformed as described by Dr. Gregory Thatcher's laboratory (238).

3.7 Transient transfection and luciferase assays

Cells were transiently transfected by electroporation with 5 μg ERE-tk-Luc plasmid containing the luciferase reporter gene controlled by a triplet vitellogenin consensus ERE (239) and 1 μg pCMVβ-galactosidase (β-gal) expressing plasmid. After 24 hours the cells were treated and incubated overnight at 37°C. Cells were lysed and luciferase activity and β-gal signals were read by a Monolight 3010 luminometer (Becton Dickinson, Franklin Lakes, NJ USA).

3.8 Matrigel colony formation assay

Treatments (ethanol [0.1%], E2 [10-9M], 4-OHT [10-7M] or RAL [10-7M]) were added to liquefied phenol-red free Matrigel matrix (BD Biosciences, Franklin Lakes, NJ USA) and used to coat 6-well plates and solidified at 37°C for 30 min. Cells (5000) were seeded in E2-depleted media containing treatments on top of pre-gelled Matrigel and incubated at 37°C with 5% CO2. Treatment media were changed every three days and colonies were counted on Day 20. Colonies were stained with 0.25% crystal violet (Sigma-Aldrich, St. Louis, MO USA) solution for 30 min and then destained with 0.9% saline for 20 min at room temperature. Colony number was determined by counting five 1.0 cm2 areas.

3.9 Tumor growth *in vivo*

T47D:A18/neo, T47D:A18/PKCα, T47D:A18-TAM1, T47D:A18-RAL5 and T47D:A18-RAL9 tumors were established as previously described (2). E2 was administered via silastic capsules (1.0 cm) implanted subcutaneously between the scapulae, producing a mean serum E2 level of 379.5 pg/mL (240). RAL, BTC and TTC-352 were administered p.o. at a dose of 1.5 mg/animal daily for 2 weeks as previously described for other SERMs (2). RAL was administered p.o. at a dose 1.5 mg/animal daily for 2 weeks. Tumor cross-sectional area was determined weekly using Vernier calipers and calculated using the formula: length / 2 × width / 2 × π. Mean tumor area was plotted against time in weeks to monitor tumor growth. “The mice were sacrificed by CO2 inhalation and cervical dislocation [and] tumors [and uteri] were excised” (135). The Animal Care and Use Committee of the University of Illinois at Chicago approved all of the procedures involving animals.

3.10 Tumor immunofluorescent (IF) confocal microscopy and co-localization analysis

Tumors sections (4 μm) were prepared from paraffin blocks for IF staining by deparaffinization and rehydration. Antigen retrieval was performed by incubating slides in Tris-EDTA (pH = 9.0) buffer at 90◦C and allowed to cool at room temperature for 45 min. Slides were blocked with antibody diluent (DAKO, Carpinteria, CA USA) for 20 min followed by primary antibody at 1:100 in antibody diluent for 1 h at room temperature. Slides were incubated with fluorescence-conjugated secondary antibodies at 1:100 in antibody diluent for 45 min at room temperature followed by 4', 6-diamidino-2-phenylindole (DAPI) [1 μg/mL], DAKO, Carpinteria, CA USA) for 15 min and mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA USA). Confocal microscopy was performed with a Zeiss LSM 510 microscope (Carl Zeiss, Incorporated, North America, Thornwood, NY USA).

3.11 Western Blot

Whole cell extracts of cultured cells were prepared in lysis buffer (200 mM Tris, 1% Triton X-100, 5mM EDTA) with protease and phosphatase inhibitor cocktails (1:50, both from Sigma-Aldrich) after scraping from the culture plates. Protein concentration was measured using the Bradford method (Bio-Rad, Hercules, 26 CA). Proteins were separated under denaturing conditions, blotted onto nitrocellulose membrane (Bio-Rad) using a wet transfer system (Bio-Rad). Images of blots were acquired on a Bio-Rad ChemiDoc System following incubation with SuperSignal West Dura luminol solution (Thermo Fisher Scientific). Protein bands were quantified using densitometry measured in Adobe Photoshop CS4 (San Jose, CA) and normalized to β-actin.

3.12 Cell IF microscopy

Cells were seeded in phenol red-containing media onto Lab-Tek II 4-well chamber slides (Millipore, Billerica, MA) at a density of 3 × 104 cells/well. The following day cells were placed in E2-depleted media for 3 days then given treatment media (DMSO [0.1%], E2 [10-9M], 4-OHT [10-7M] or RAL [10-7M]). For IF, cells were fixed in 100% methanol overnight at -20° C and stained as described above for tissue sections. Cells were imaged using Zeiss Axiovision Observer D1 microscope (Carl Zeiss, LLC, Thornwood, NY USA).

3.13 Colony IF microscopy

Colonies were formed by seeding cells in Matrigel as described above and treated with DMSO (0.1%), E2 (10-9M), 4-OHT (10-7M) or RAL (10-7M). Colonies were extracted from the Matrigel by adding ice-cold PBS-EDTA to the rinsed and aspirated wells. Gel was lifted from the bottom of the well with a cell scraper and plates were shaken gently on ice. Colonies were then transferred to a conical tube and shaken on ice for an additional 30 min until Matrigel was completely dissolved, collected by centrifugation at 115g for 2 min and pipetted onto a slide. Slides were then fixed in ice cold methanol and stored at -80° C until staining (as described above). Confocal microscopy was performed with a Zeiss LSM 510 microscope.

3.14 Statistical analysis

The specific statistical test applied to the data is described in the figure legends. When comparisons were performed between two groups, two-way unpaired Student's t-test was used to determine statistical significance between groups. One-way Analysis of Variance (ANOVA) followed by Tukey's post-hock test was used when comparing

between multiple groups. All of the statistics on the data were performed using GraphPad Prism 5.02 Software (La Jolla, CA USA).

4. RESULTS

4.1 T47D:A18 breast cancer cells exposed long-term to antiestrogens display increased PKCα expression, TAM-resistance and an E2-inhibited phenotype

Previous studies from our laboratory and others indicate that PKCα overexpression correlates with a TAM-resistant phenotype in breast cancer cell lines and tumor biopsies from patients (1, 213, 221). The ectopic overexpression of PKCα in T47D:A18 breast cancer cells (T47D:A18/PKCα) led to TAM-resistance and a unique phenotype in which these cells are inhibited by E2 only in 3D Matrigel or *in vivo* conditions but not in 2D *in vitro* culture. This led us to hypothesize that T47D:A18 breast cancer cells that acquire resistance to anti-estrogens through long-term exposure to 4-hydroxytamoxifen (4-OHT) or raloxifene (RAL) may also gain an E2-inhibited phenotype *in vivo*.

To address this hypothesis I exposed T47D:A18 breast cancer cells to 1μM 4-OHT or RAL for 1 year. Single cell clones were derived using the limiting dilution method and analyzed by to the DNA growth assay. All clones tested demonstrated resistance to both 4-OHT (100 nM) and RAL (100 nM) and displayed hormone-independent growth *in vitro* (data not shown). To determine the effects of TAM and E2 *in vivo* clone 1 (T47D:A18-TAM1) was chosen for further study. Similar to the previously described T47D:A18/PKCα model, T47D:A18-TAM1 xenografts regressed when exposed to E2 *in vivo* but were unaffected by this ligand *in vitro* (Figure 5A and B). T47D:A18-TAM1 xenografts grew hormone-independently and were stimulated by TAM *in vivo* (Figure 5B). Furthermore, T47D:A18-TAM1 colony formation was also

inhibited by E2 in 3D Matrigel (data not shown). Based on *in vitro* growth clone 5 (T47D:A18-RAL5) and clone 9 (T47D:A18-RAL9) were also chosen for further characterization *in vivo*. Although both T47D:A18-RAL5 and T47D:A18-RAL9 cells were resistant to RAL *in vitro* (Figure 5C and E), only T47D:A18-RAL9 xenografts grew in the presence of RAL (1.5mg/day) *in vivo* (Figure 5F). Both T47D:A18-RAL5 and T47D:A18-RAL9 xenografts were TAM-resistant, hormone-independent and regressed when treated with E2 *in vivo* (Figure 5D and F). These studies suggest that long-term treatment with antiestrogens leads to an E2-inhibited phenotype *in vivo*, accompanied by upregulation of PKCα.

4.2 PKCα-overexpression in breast cancer cells correlates with sensitivity to E2

PKCα-overexpression in clinical specimens predicted resistance to TAM (221). The ectopic overexpression of PKCα in T47D cells (T47D:A18/PKCα) led to a TAM-resistant, E2-inhibited phenotype *in vivo* (2) suggesting that PKCα may also predict a positive response to estrogenic therapeutic intervention.

Following the acquisition of a TAM-resistant, E2-inhibited phenotype *in vivo* T47D:A18-TAM1, T47D:A18-RAL5 and T47D:A18-RAL9 cells displayed increased PKCα expression compared to the TAM-sensitive parental T47D:A18 cell line (Figure 5G). MCF-7:5C cells are TAM- and RAL-resistant and inhibited by E2 both *in vitro* and *in vivo* (125). Santen and colleagues developed LTED cells by long-term estrogen deprivation and display sensitivity to E2 both *in vitro* (124). MCF-7:5C and LTED cells also displayed increased expression of PKCα compared to parental MCF-7:WS8 cells

(Figure 5G). E2-inhibited cell lines T47D:A18/PKCα, T47D:A18-TAM1, T47D:A18-RAL5, T47D:A18-RAL9, MCF-7:5C and LTED display overexpression of PKCα compared to parental E2-stimulated T47D:A18/neo, T47D:A18, MCF-7:WS8 cell lines respectively (Figure 5G) suggesting that PKCα may play a role in E2-induced growth inhibition. The knockdown of PKCα in T47D:A18/PKCα cells led to a partial reversal of the E2-inhibited phenotype (241). Together this suggests that PKCα may either play a role in E2-induced growth inhibition or possibly be a marker for E2-induced growth inhibition. This led us to ask whether PKCα is required for E2-induced growth inhibition in cells that endogenously over-express PKCα.

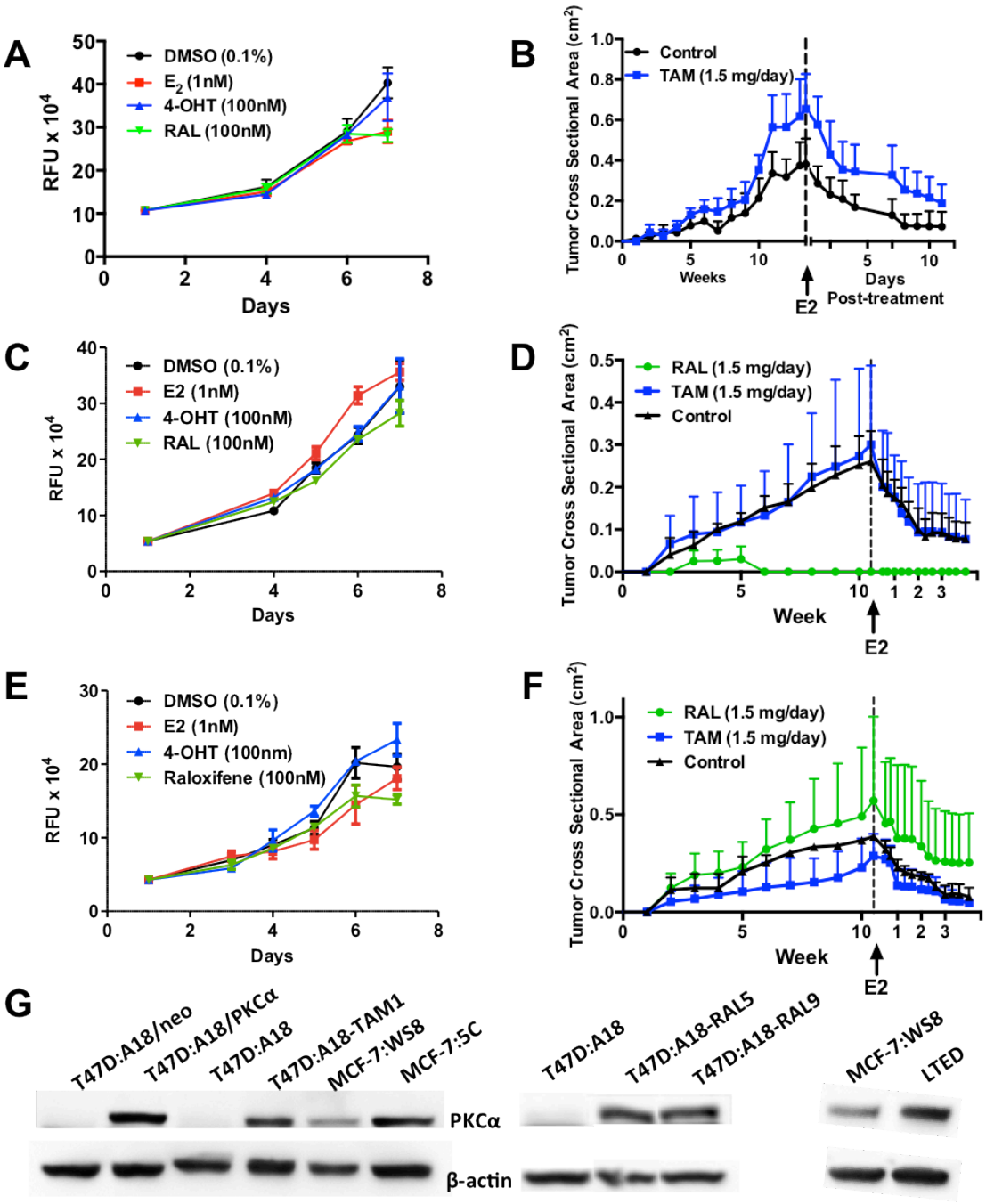


Figure 5. Effect of long-term exposure to antiestrogens on T47D:A18 breast cancer cells. T47D:A18-TAM1 cell growth *in vitro* **(A)** and *in vivo* **(B)**. T47D:A18-RAL5 cell growth *in vitro* **(C)** and *in vivo* **(D)**. T47D:A18-RAL9 cell growth *in vitro* **(E)** and *in vivo* **(F). G)** Western blot analysis of PKCα expression in E2-stimulated (T47D:A18/neo, T47D:A18, MCF-7:WS8) and E2-inhibited (T47D:A18/PKCα, T47D:A18-TAM1, MCF-7:5C, T47D:A18-RAL5, T47D:A18-RAL9 and LTED) cell lines.

4.3 PKCα may modulate cell viability in the presence of E2

The ectopic overexpression of PKCα in T47D cells (T47D:A18/PKCα) led to a TAM-resistant, E2-inhibited phenotype *in vivo* (2) suggesting that PKCα may also predict a positive response to estrogenic therapeutic intervention. PKCα overexpression also correlates with an E2-inhibited phenotype in multiple cell lines (Figure 5G). To determine if PKCα modulates cell viability in the presence of E2, I inhibited PKCα using siRNA in LTED and MCF-7:5C cells that endogenously over-express PKCα. Five days following E2 (1nM) or vehicle (0.1% DMSO) treatment cell viability was assessed by the trypan blue exclusion method. PKCα knockdown significantly reversed the effects of E2 on LTED cell viability (Figure 6A). Although PKCα knockdown altered the effect of E2 on MCF-7:5C cell viability following 5 days of treatment, E2 was still capable of reducing cell viability in the presence of PKCα siRNA in MCF-7:5C cells (Figure 6B). I speculate that a stronger knockdown of PKCα in MCF-7:5C cells may have an enhanced effect on the reversal of cell viability in the presence of E2. Interestingly, both MCF-7:5C and LTED cells were derived using similar cell culture conditions, but were derived from different parental MCF-7 cell stocks in independent laboratories, likely accounting for the variability in reliance on PKCα expression in producing decreased cell viability in the presence of E2.

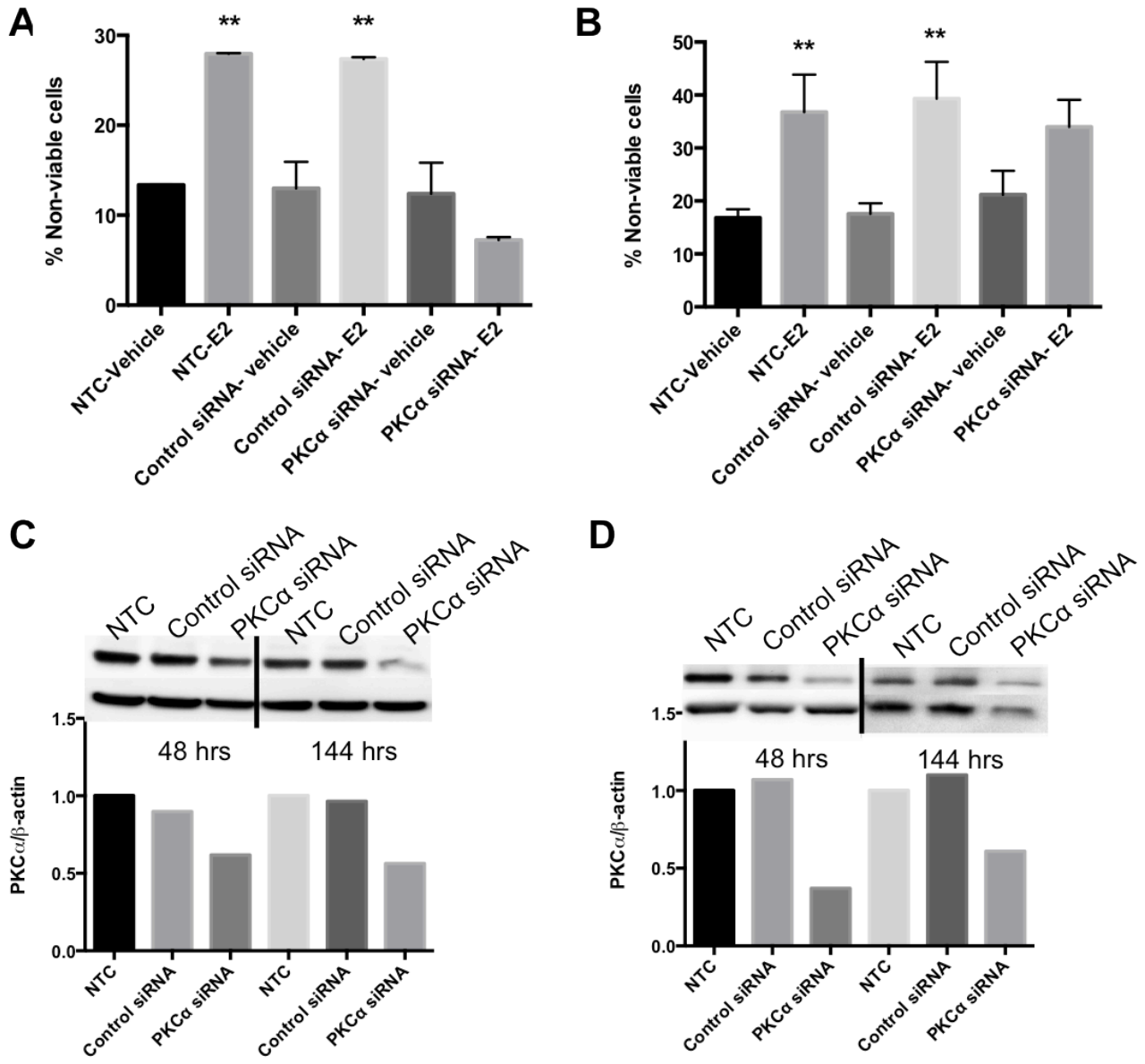


Figure 6. PKCα modulates cell viability in the presence of E2.
Cell viability following PKCα knockdown in E2 treated **(A)** LTED and **(B)** MCF-7:5C cells on day 5. One-way ANOVA way used for statistical analysis. N=3. **, $P < 0.01$ compared to NTC. NTC: non-treated control cells. Western blot analysis of PKCα expression following PKCα knockdown in **(C)** LTED and **(D)** MCF-7:5C cells at 48hrs transfection (day 1 of treatment) and 144hrs post transfection (day 5 of treatment).

4.4 T47D:A18/PKCα cells are resistant to RAL *in vitro* and are partially inhibited by RAL *in vivo*

Many SERMs have been developed in an effort to overcome the uterotrophic side effects associated with TAM treatment as well as to combat TAM-resistance. Cross-resistance often develops to drugs within the same class or when drugs have a similar mechanism of action. RAL is a second-generation benzothiophene SERM developed to overcome the mixed antagonist/agonist activity of TAM, which is associated with side effects such as increased incidence of endometrial cancers. RAL has a favorable antiestrogenic profile in the uterus (242) and has proven safe over 15 years of clinical use in postmenopausal osteoporosis. Although cross-resistance occurs with drugs in the same class, TAM and RAL differ structurally. In addition, T47D:A18/PKCα cells display a unique phenotype in which E2 has no effect on 2D growth *in vitro* but inhibits growth in 3D Matrigel or *in vivo*. However, E2 is also associated with significant side effects prompting us to seek potential clinical alternatives. I therefor sought to further characterize the effect of RAL on T47D:A18/PKCα cells both *in vitro* and *in vivo.*

To determine the effects of RAL on T47D:A18/PKCα cell growth *in vitro*, I treated cells with 100nM RAL and I measured DNA content as an index of proliferation at various time points. RAL had no effect on the proliferation of T47D:A18/PKCα cells *in vitro* (Figure 7A) as compared to vehicle treated controls.

As previously mentioned, E2 is unable to effect the growth of T47D:A18/PKCα cells in 2D *in vitro* conditions but is capable of inhibiting colony formation in 3D Matrigel. I therefore wanted to determine if like E2 RAL was capable of inhibiting colony formation

in 3D Matrigel. I found that even at the highest concentration tested (1uM) RAL was unable to significantly inhibit T47D:A18/PKCα colony formation in Matrigel (Figure 7B)(3).

Although RAL had no effect on T47D:A18/PKCα 3D Matrigel colony formation, due to the variability of T47D:A18/PKCα response to E2 *in vitro* vs. *in vivo* and the clinical applicability of RAL, it was important to determine if RAL had an effect on T47D:A18/PKCα xenograft regression *in vivo*. T47D:A18/PKCα cells were bilaterally injected into the mammary fat pads of 15 athymic nude mice. Following tumor establishment I randomized mice into 3 treatment groups: control, E2 (1cm capsule) or RAL (1.5mg/day). Mice receiving RAL exhibited tumor regression following 4 weeks of treatment, however the effect of RAL was modest compared to that of E2. Overall these results indicate that *(1)* RAL is capable of partially inhibiting the growth of T47D:A18/PKCα TAM-resistant tumors and *(2)* RAL exerts contradictory *in vitro* and *in vivo* growth effects on T47D:A18/PKCα cells in a manner similar to E2. The distinction between E2 and RAL activity is that E2 but not RAL inhibits colony formation in 3D culture (3).

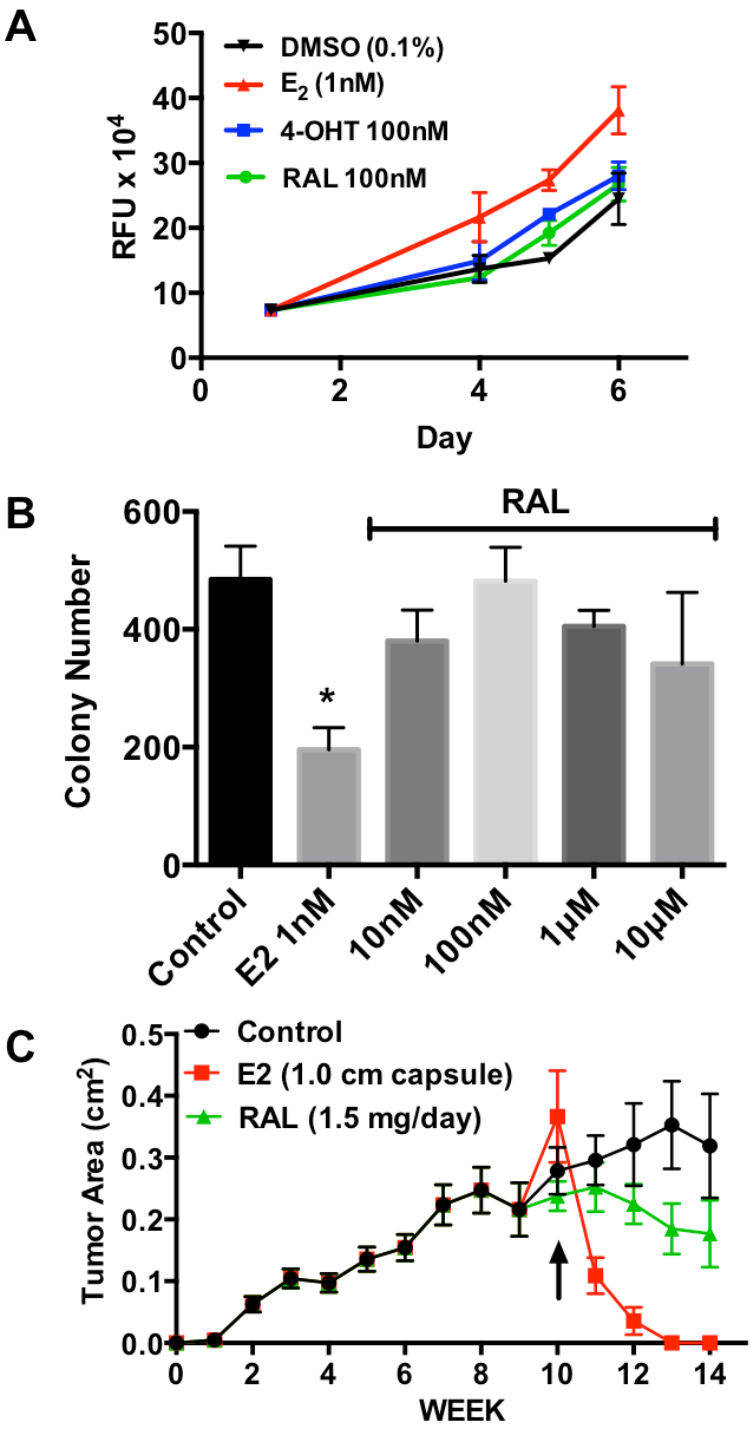


Figure 7. Effect of RAL on T47D:A18/PKCα cell growth. A) Effect of RAL on T47D:A18/PKCα cells growth *in vitro*. Graph is representative of 3 independent experiments. **B)** Effect of RAL on T47D:A18/PKCα colony formation. *, $P < 0.05$ versus DMSO. **C)** *In vivo* effect of RAL on T47D:A18/PKCα xenograft regression. Arrow indicates treatment initiation.

4.5 Benzothiophene selective estrogen mimics display an estrogenic profile and are capable of inhibiting PKCα-overexpressing, TAM-resistant breast cancer cell lines *in vitro*

Unlike TAM, RAL does not stimulate uterine tissue. The specific conformations of the ER-ligand complexes determine gene transcription and ultimately influence the tissue-selective outcomes of these ligands. Since RAL displays an enhanced safety profile due to decreased uterotrophic effects compared to both TAM and E2 and was partially able to inhibit T47D:A18/PKCα growth *in vivo*, Dr. Gregory Thatcher's group used the benzothiophene core (BTC) structure of RAL to rationally design a series of selective estrogen receptor mimics (SEMs). Ideally SEMs would combine the efficacy of an estrogenic compound for treating TAM-resistant breast cancer with the enhanced safety of RAL.

To determine whether SEMs act as estrogen agonists in hormone-dependent, TAM-sensitive T47D:A18/neo cells, stimulating proliferation in 2D culture, cells were treated and DNA content was measured as an index of proliferation. T47D:A18/neo cells proliferated in the presence of BTC and TTC-352 (Figure 8 A-D). Based on chemical structure and estrogenic action in T47D:A18/neo cells, BTC and TTC-352 were selected for further characterization in TAM-resistant, PKCα-overexpressing T47D:A18/PKCα, T47D:A18-TAM1 and MCF-7:5C cell lines.

T47D:A18/PKCα cells proliferated in the presence of BTC and TTC-352 at all concentrations tested (1 nM, 10 nM, 100 nM) and maximal efficacy was similar to that of E2 (1 nM) observed *in vitro* (Figure 8E and F). Interestingly, only the higher concentrations of BTC (100 nM) and TTC-352 (100 nM) showed effects on the

proliferation of T47D:A18/neo cells comparable to E2 (1 nM) treatment (Figure 8 C and D). Analogous to the effect on T47D:A18/PKCα cells, BTC and TTC-352 showed similar efficacy to E2 on T47D:A18-TAM1 cell growth *in vitro* (Figure 8 G and H).

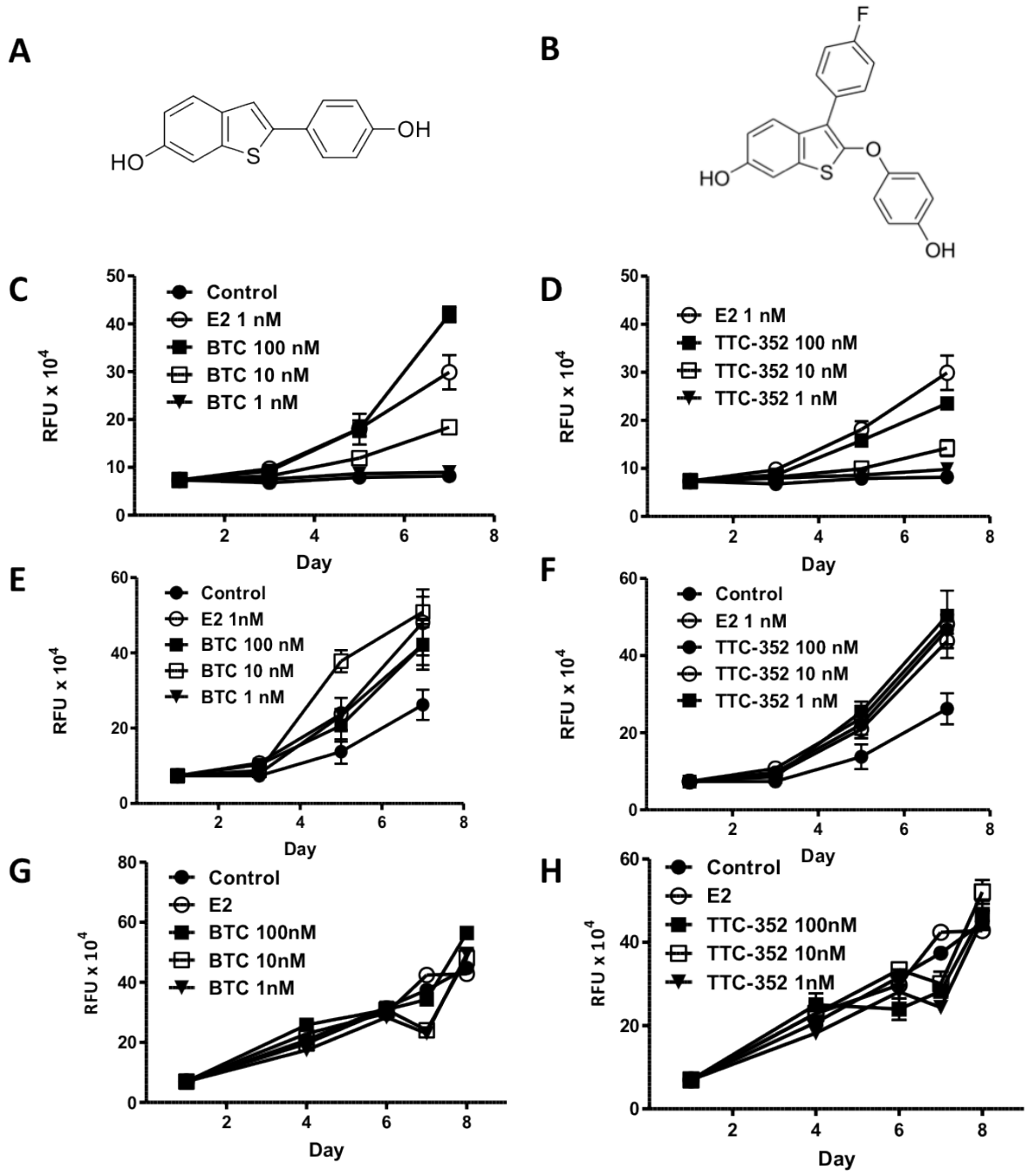


Figure 8. Effect of BTC and TTC-352 on proliferation of T47D:A18/neo and T47D:A18/PKCα cells *in vitro*. A) Structure of BTC. B) Structure of TTC-352. Effect of BTC and TTC-352 treatment on the growth T47D:A18/ PKCα cells (C and D respectively), T47D:A18/neo cells (E and F respectively) and T47D:A18-TAM1 cells (G and H respectively). DNA assays were performed as described in Materials and Methods. Graphs show mean ± SEM and are representative of three independent experiments.

TAM-resistant MCF-7:5C cells display a unique phenotype in which these cells are inhibited by E2 not only *in vivo* but also *in vitro* (125). We therefore sought to determine if BTC and TTC-352 have the ability to inhibit growth of MCF-7:5C cells *in vitro*. Cells were treated on day 1, media was changed every 2-3 days and cells were counted on day 9. Both BTC and TTC-352 significantly inhibited growth of MCF-7:5C cells compared to vehicle treated cells (Figure 9A). As expected based on the effects of BTC and TTC-352 in T47D:A18/neo cells, hormone-dependent MCF-7:WS8 cells proliferated in the presence of both BTC and TTC-352 (Figure 9B).

E2 inhibits T47D:A18/PKCα colony formation in Matrigel (135), in part recapitulating the E2 inhibitory effect on tumor establishment (2). To determine if BTC and TTC-352 similar to E2, can inhibit the growth of T47D:A18/PKCα and T47D:A18-Tam1 colonies in 3D culture, colony formation in Matrigel was examined. As expected based on the proliferative effects in 2D culture, BTC and TTC-352 treatment resulted in increased T47D:A18/neo colony formation (Figure 10A) and significantly inhibited T47D:A18/PKCα and T47D:A18-TAM1 colony formation in 3D Matrigel (Figure 10B and C, respectively). These results suggest that BTC and TTC-352 are estrogenic *in vitro* and will likely inhibit growth of T47D:A18/PKCα and T47D:A18-TAM1 xenografts.

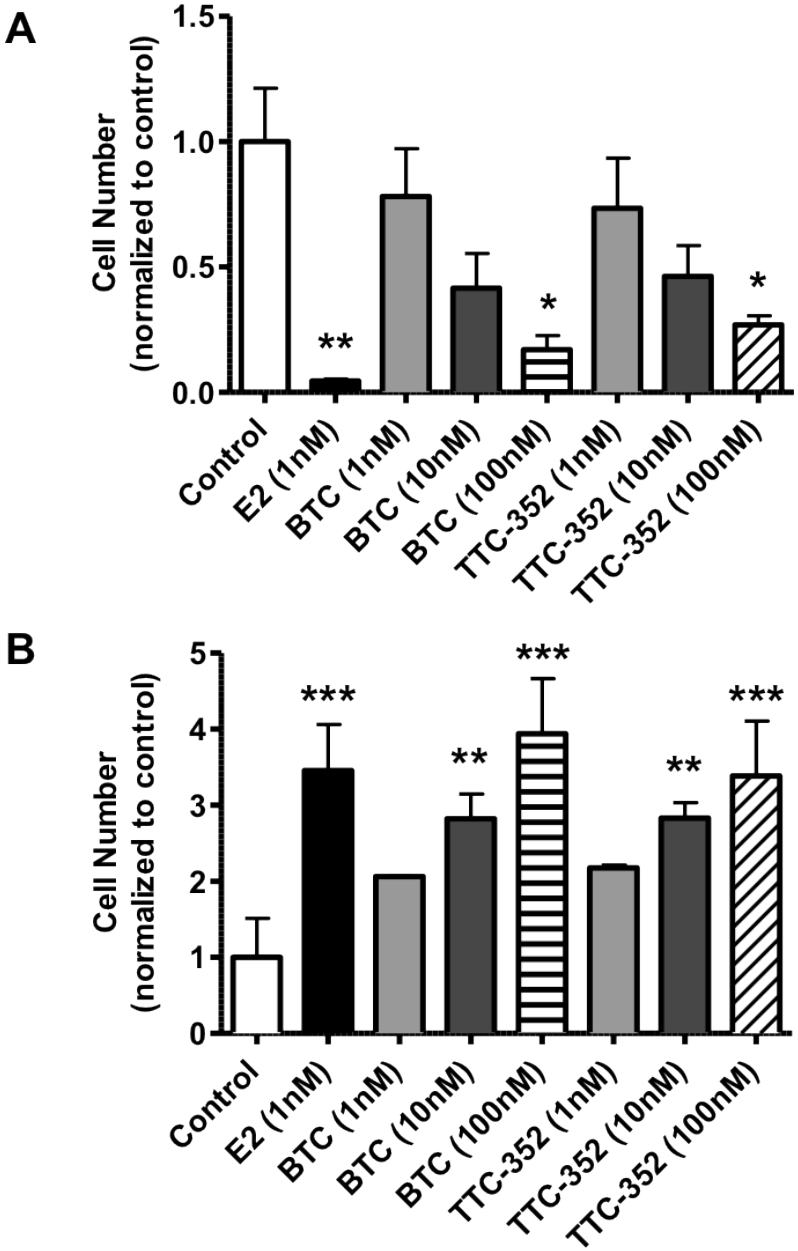


Figure 9: Effect of BTC and TTC-352 on growth of MCF-7:5C and MCF-7:WS8 cells (A) MCF-7:5C proliferation assay demonstrating growth inhibition induced by E2, BTC and TTC-352. (B) MCF-7:WS8 proliferation assay demonstrating growth stimulation induced by E2, BTC and TTC-352. 200,000 cells were plated on day 0 , treated on day 1 and counted on day 9. Media was changed every 3 days. Graphs are representative of 3 independent experiments. One-way ANOVA way used for statistical analysis. *, $P \leq 0.05$ versus DMSO. **, $P \leq 0.01$ versus DMSO. ***, $P \leq 0.001$ versus DMSO. Graph shows mean ± SEM of 3 independent experiments.

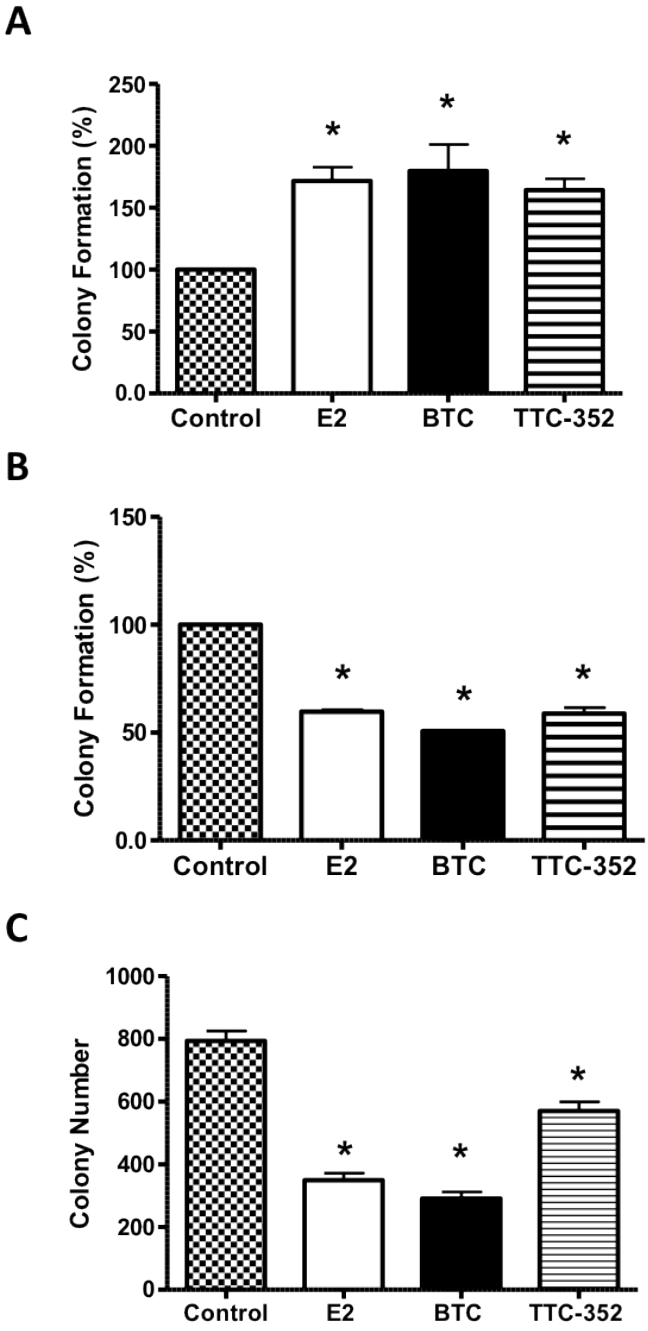


Figure 10. BTC and TTC-352 inhibit T47D:A18/PKCα and T47D:A18-TAM1 colony formation in 3D Matrigel™. Colonies were established as described in Materials and Methods and treated for 10 days (Control [0.1% DMSO], E2 1 nM, BTC 100 nM, TTC-352 100 nM). One-way ANOVA was used for statistical analysis. *, $P < 0.05$ versus DMSO. Graph shows mean ± SEM of 3 independent experiments. A) T47D:A18/neo. B) T47D:A18/PKCα. C) T47D:A18-TAM1.

4.6 Induction of ERα transcriptional activity by BTC and TTC-352 in breast cancer cells

To confirm that the *in vitro* effects of BTC and TTC-352 were due to estrogenic activity of the compounds, transcriptional activation of ERα was examined using an estrogen response element (ERE)-luciferase reporter construct (239). Cells were treated with vehicle control (0.1% DMSO), E2 (1 nM), BTC (1 nM, 10 nM, 100 nM), or TTC-352 (1 nM, 10 nM, 100 nM) 24 hours prior to determining ERE-luciferase and β-gal activity. In T47D:A18/neo cells, BTC and TTC-352 treatment resulted in an increase in ERα transcriptional activity at the highest concentration of 100 nM (Figure 11A). T47D:A18/PKCα cells were more sensitive than T47D:A18/neo cells to BTC ERE-luciferase induction at 100 nM and 10 nM (Figure 11B). Figure 11D demonstrates baseline T47D:A18/PKCα ERE-luciferase activity in increased compared to T47D:A18/neo as previously described (214). E2 (1 nM) and BTC (100 nM) also significantly induced ERE-luciferase activity in T47D:A18-TAM1 cells following 24 hrs of treatment (Figure 11C). As expected based on 2D and 3D *in vitro* assays, these data suggest that BTC and TTC-352 act as ER agonists in T47D:A18/neo, T47D:A18/PKCα and T47D:A18-Tam1 cell lines *in vitro*. Based on *in vitro* data these SEMs are likely to inhibit the growth of T47D:A18/PKCα and T47D:A18-TAM1 cells in xenografts.

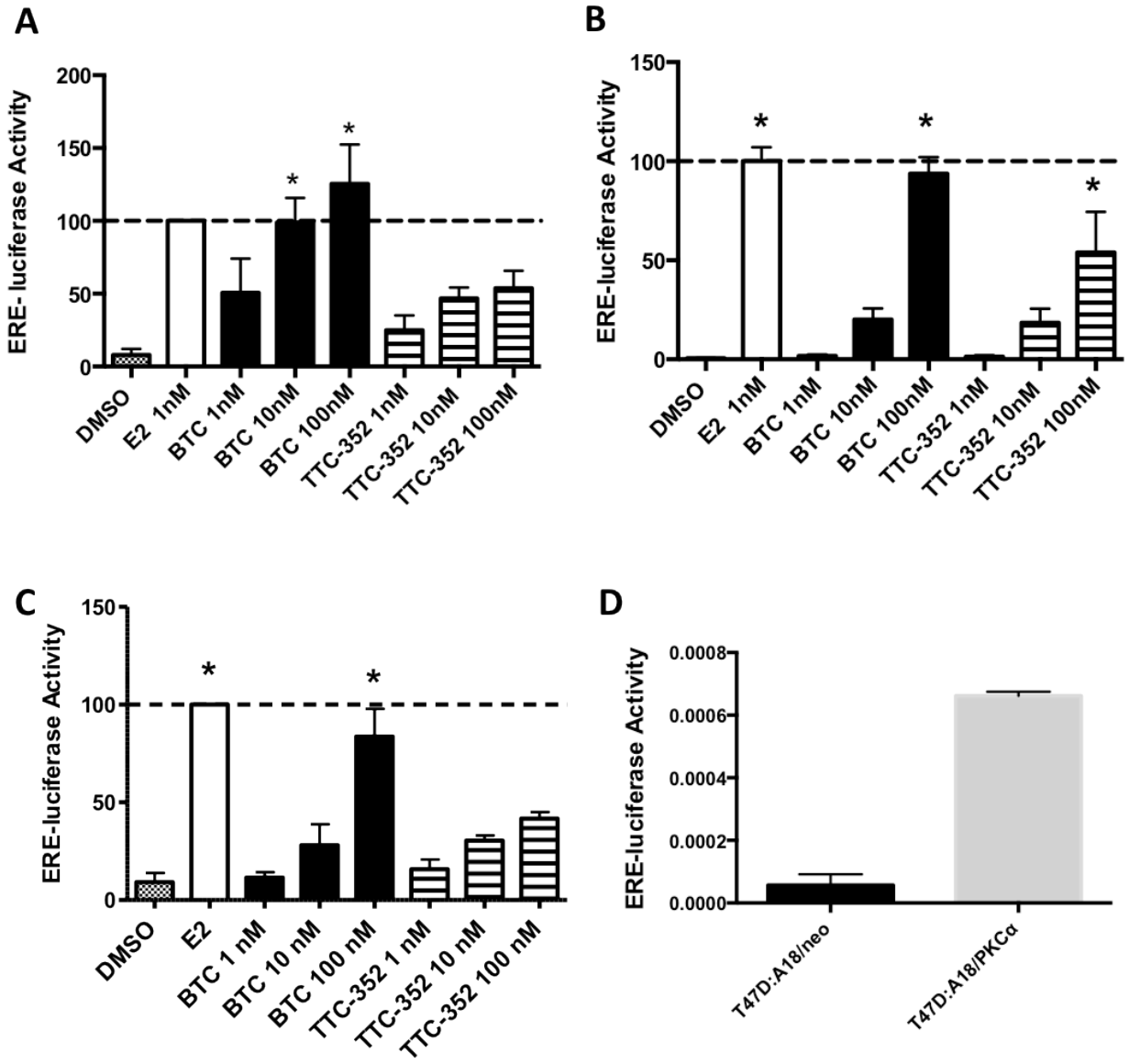


Figure 11. BTC and TTC-352 induce ERα transcriptional activity in T47D:A18/neo, T47D:A18/PKCα and T47D:A18-TAM1 cell lines. A) T47D:A18/neo, **B)** T47D:A18/PKCα and **C)** T47D:A18-TAM1 cell lines. Data is expressed normalized to E2 (100%). One-way ANOVA was used for statistical analysis. *, $P < 0.05$ versus DMSO. Graph shows mean ± SEM of 3 independent experiments. **D)** Unnormalized ERE-luciferase values for T47D:A18/neo and T47D:A18/PKCα cells treated with vehicle, demonstrating increased ERE-luciferase activity in T47D:A18/PKCα cells at baseline.

4.7 Inhibitory effect of BTC and TTC-352 treatment on hormone-independent, TAM-resistant T47D:A18/PKCα and T47D:A18-TAM1 xenografts

We have recently reported that RAL treatment results in significant regression of TAM-resistant T47D:A18/PKCα tumors (3). However, RAL did not produce an effect as robust as E2 on tumor regression and was unable to inhibit Matrigel colony formation (3). In contrast to RAL, BTC and TTC-352 are estrogenic in 2D culture and like E2 have the ability to inhibit T47D:A18/PKCα and T47D:A18-TAM1 colony formation in Matrigel (Figures 10B and C). To determine if these compounds could initiate T47D:A18/PKCα tumor regression, T47D:A18/PKCα cells were injected into 40 athymic mice and were left untreated for seven weeks (mean tumor size was ~0.5 cm^2, 100%). At seven weeks, the mice were randomized to either continue on the untreated control arm (9 mice), received implants of an E2 capsule (9 mice), oral RAL 1.5 mg/day (9 mice), oral BTC 1.5 mg/day (9 mice), or oral TTC-352 1.5 mg/day (4 mice). Following two weeks, all treatments significantly reduced tumor volume compared to non-treated controls ($P<$ 0.05). BTC treated T47D:A18/PKCα tumors regressed by ~88% to a size of ~0.07 cm^2 (Figure 12A) at two weeks. Mice treated with TTC-352 also exhibited a decrease in tumor volume at two weeks regressing by ~70% with a mean tumor volume of ~0.18 cm^2 (Figure 12A). The effect of BTC and TTC-352 was only surpassed by E2 treatment which resulted in ~98% regression at 2 weeks. Both BTC and TTC-352 resulted in a decrease in T47D:A18/PKCα tumor volume that surpassed regression exhibited by RAL (~50%). Furthermore, unlike RAL, regression induced by BTC and TTC-352 was sustained for at least four weeks post-treatment (Figure 12A).

Since TAM-resistant T47D:A18-TAM1 cells were growth inhibited by E2, BTC or TTC-352 in 3D Matrigel (Figure 10C), we hypothesized that similar to T47D:A18/PKCα cells, T47D:A18-TAM1 cells may be growth inhibited *in vivo* by these agents. To test this hypothesis T47D:A18-TAM1 xenograft tumors were established in 11 athymic nude mice. Following tumor establishment, mice were randomized into four treatment groups: untreated control arm (2 mice), E2 capsule (3 mice), oral BTC 1.5 mg/day (3 mice), or oral TTC-352 1.5 mg/day (3 mice). Following 2 weeks of daily treatment BTC and TTC-352 resulted in significant regression of T47D:A18-TAM1 tumors (Figure 12B).

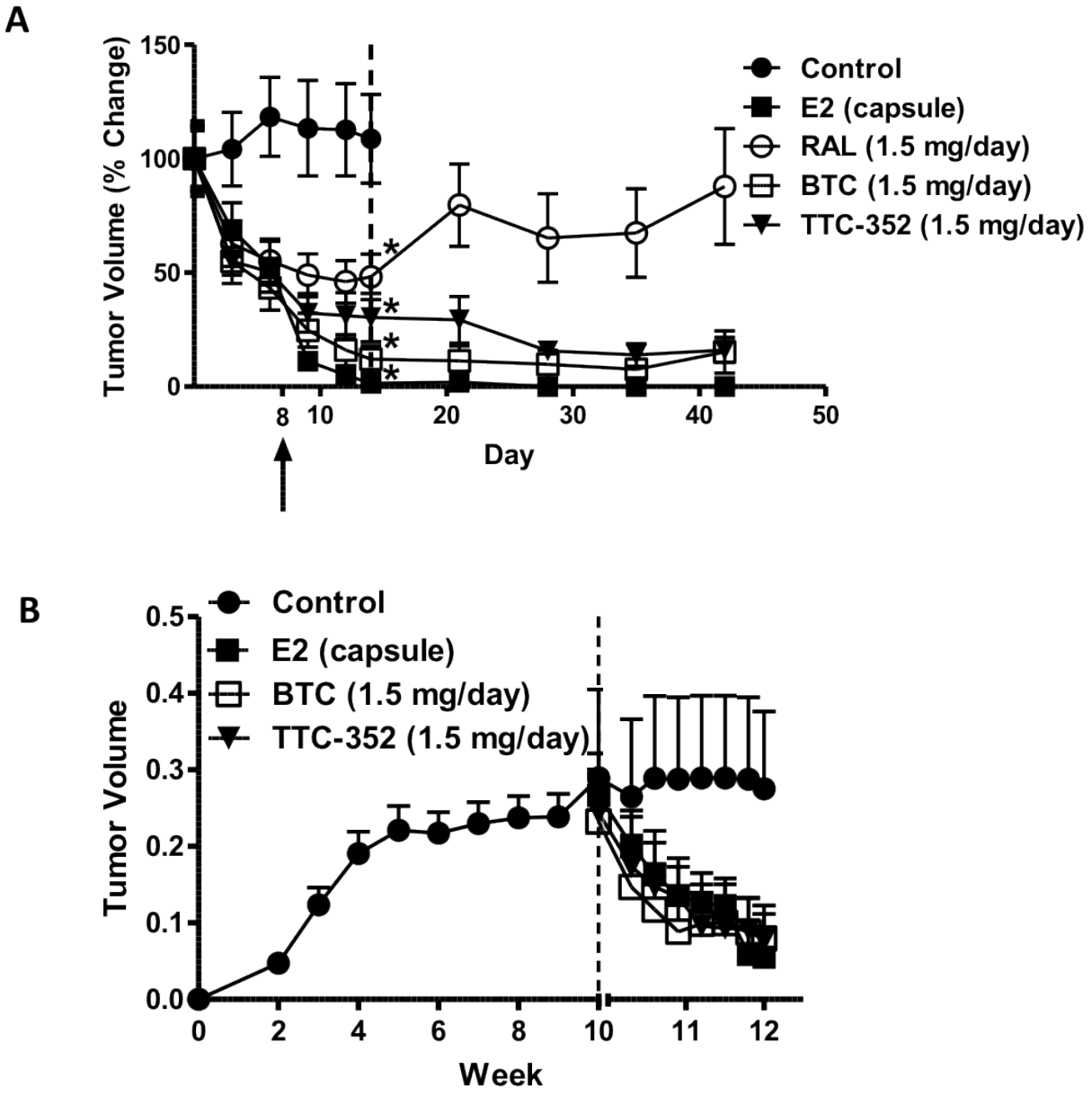


Figure 12. BTC and TTC-352 inhibit T47D:A18/PKCα and T47D:A18-TAM1 xenograft tumors. A) BTC and TTC-352 treatment result in regression of T47D:A18/PKCα tumors. Graph shows percentage of tumor regression (100% ~0.5 cm^2). Dotted line indicates when treatment was ended. Arrow designates where tumors used in Figure 14 were obtained. One-way ANOVA was used for statistical analysis. *, $P < 0.05$ versus control. Graph shows mean ± SEM. B) T47D:A18-TAM1 tumors regress when treated with BTC and TTC-352. Dotted lines represents start of treatment.

4.8 BTC and TTC-352 have no significant effect on hormone-dependent T47D:A18/neo xenografts

The ER positive hormone-dependent T47D:A18/neo control breast cancer cell line requires E2 for growth *in vitro* and *in vivo* (1, 2). Since BTC and TTC-352 treatment result in growth of T47D:A18/neo cells in 2D culture (Figure 8C and D) and in 3D Matrigel (Figure 10A), we next sought to determine if BTC and TTC-352 could sustain the growth of T47D:A18/neo tumors *in vivo*. T47D:A18/neo cells were bilaterally injected into the mammary fat pads of 20 athymic mice and divided into six treatment groups (3 non-treated control, 3 E2 capsule, 3 oral TAM 1.5 mg/day, 3 oral RAL 1.5 mg/day, 4 oral BTC 1.5 mg/day, or 4 oral TTC-352 1.5 mg/day). Following seven weeks of treatment, mice treated with E2, as expected, harbored T47D:A18/neo tumors that reached an average size of ~0.35 cm^2 (100%), tumors treated with BTC and TTC-352, grew to an average size of ~0.04 cm^2 and ~0.1 cm^2, respectively (Figure 12A). Although at higher concentrations, BTC and TTC-352 (Figure 8C and D) stimulated the growth of T47D:A18/neo cells *in vitro*, neither compound was able to significantly stimulate the growth of T47D:A18/neo xenograft tumors *in vivo*. Interestingly, the dose capable of causing robust regression of T47D:A18/PKCα and T47D:A18-TAM1 tumors had no effect on the growth of T47D:A18/neo tumors *in vivo*. Additionally, no significant weight loss was observed over the seven-week treatment period (Figure 13B).

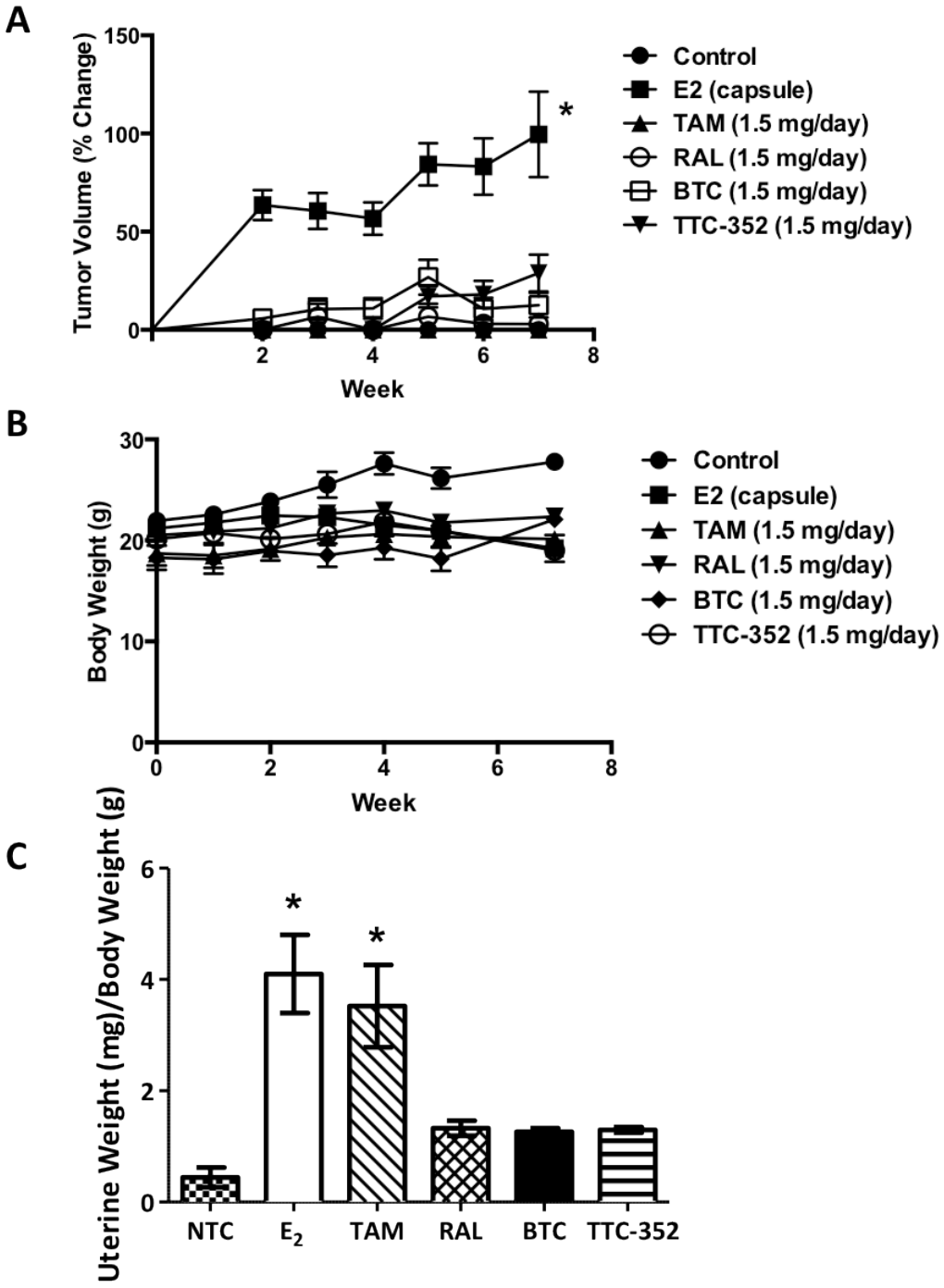


Figure 13. BTC and TTC-352 have no effect on T47D:A18/neo tumor growth, body weight or uterine weights of ovariectomized mice. A) BTC and TTC-352 do not result in growth of T47D:A18/neo tumors. B) Body weights of treated mice from (A). C) Uterine weights from mice in (A). Weights are reported as uterine weight (mg)/body weight (g). One-way ANOVA was used for statistical analysis. *, $P < 0.05$ versus control. Graphs show mean ± SEM.

4.9 BTC and TTC-352 have no effect on uterine weights of athymic mice

E2 has a proliferative effect on the endometrium resulting in an increase in uterine weight. TAM has an estrogenic effect on endometrial growth, which leads to an increased risk of developing endometrial cancer (243). In ovariectomized rats at a minimally effective dose, RAL did not increase uterine weight in contrast to E2 and TAM, and at doses up to 10 mg/kg/day did not increase luminal epithelial cell thickness (244-247). Mindful of the estrogen agonist actions of BTC and TTC-352 in Ishikawa cells, we sought to compare the effects of BTC and TTC-352 on uterine weight with those of RAL, TAM and E2. Following 7 weeks of treatment the uteri from ovariectomized mice in Figure 13A were excised and weights determined. Interestingly, there was no significant increase in the uterine weights of mice treated with BTC or TTC-352 (Figure 13C). The significant proliferative actions associated with both TAM and E2 were absent from BTC and TTC-352, suggesting that these SEMs would deliver an improved safety profile compared to TAM and E2.

4.10 Extranuclear ER translocation correlates with E2- and SEM-induced T47D:A18/PKCα tumor regression

The Tonetti lab has previously reported that ERα and the extracellular matrix (ECM) are required for T47D:A18/PKCα tumor regression and that plasma membrane-associated ERα is likely to mediate the inhibitory effects of E2 (135). To test our hypothesis that extranuclear ERα participates in E2-induced T47D:A18/PKCα tumor regression, we asked whether ERα localization differs in E2, RAL, BTC and TTC-352-

induced T47D:A18/PKCα regressing tumors compared with TAM-stimulated T47D:A18/PKCα tumors or E2-stimulated T47D:A18/neo tumors.

IF confocal microscopy of T47D:A18/neo E2-stimulated tumors and T47D:A18/neo TAM- and RAL-regressing tumors illustrates that ERα is mainly localized in the nucleus (Figure 14) (3). The T47D:A18/neo no treatment (NT) group is not available for comparison since T47D:A18/neo cells required E2 for tumor growth. Similarly, ERα is located within the nucleus in T47D:A18/PKCα NT and TAM treatment groups. However, ERα is almost completely localized to extranuclear sites in E2, RAL, BTC and TTC-352 induced regressing T47D:A18/PKCα tumors. Interestingly, following withdrawal of RAL (RAL W/D) tumors resume growth and ERα re-localizes to the nucleus. TAM and RAL which oppositely regulate T47D:A18/PKCα tumor growth, induces differential ERα subcellular localization (3). Furthermore, T47D:A18/PKCα tumor regression induced by either E2, RAL, BTC or TTC-352 is associated with extranuclear ERα.

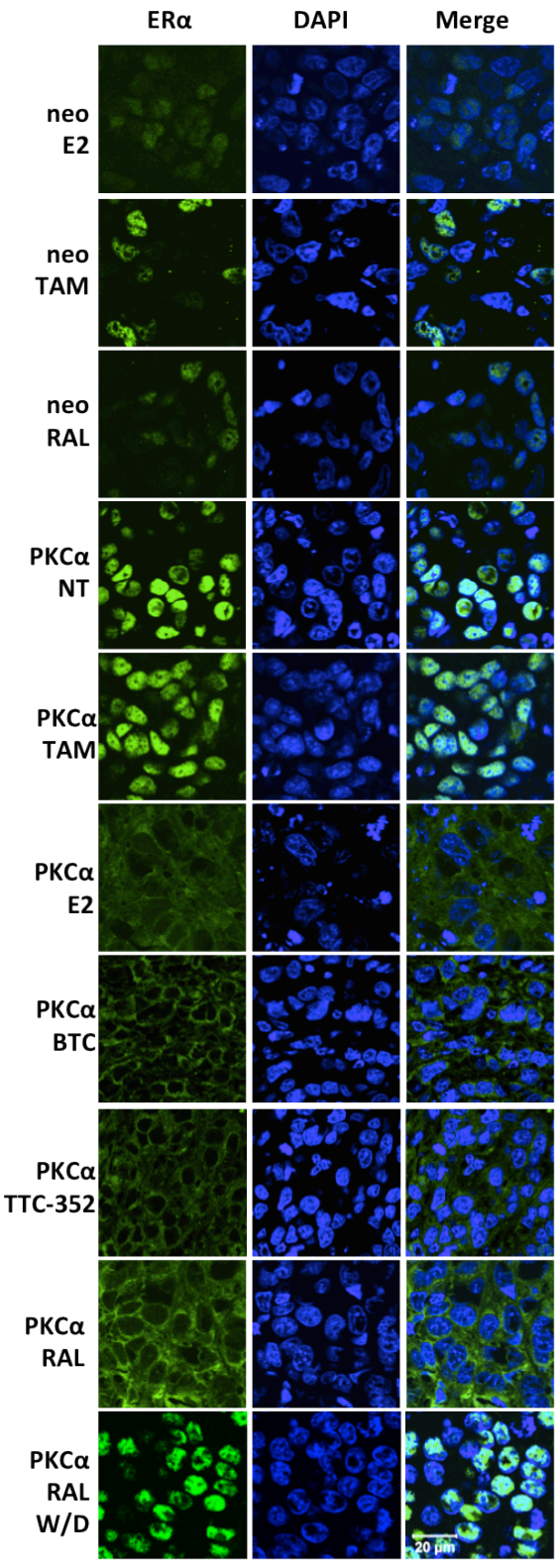


Figure 14. ERα localizes to extranuclear sites in E2, RAL, BTC and TTC-352 induced T47D:A18/PKCα regressing tumors. Tissue sections were immunostained as described in materials and methods. Images are representative photographs of immunostained tumor sections. Sections were costained for ERα (green) and nuclei (blue). Scale bar = 20 µm. PKCα, T47D:A18/PKCα; neo, T47D:A18/neo; W/D, withdrawal (Ref 3).

4.11 E2 induces ERα translocation to extranuclear sites in T47D:A18/PKCα colonies grown in 3D Matrigel

I next wanted to determine whether extranuclear ERα correlates with inhibition of colony formation in 3D Matrigel. Inhibition of colony formation by E2 in 3D culture is analogous to the *in vivo* phenotype whereby E2 prevents tumor establishment (2). However, unlike the *in vivo* phenotype, E2 is incapable of initiating regression of an established T47D:A18/PKCα colony in Matrigel. To determine whether extranuclear ERα is a response to E2 and RAL treatment in 3D culture or whether ERα translocation occurs only during regression in tumors, we compared ERα subcellular localization in T47D:A18/neo and T47D:A18/PKCα cells grown in 3D culture. To address this, T47D:A18/neo and T47D:A18/PKCα cells were plated in Matrigel. Colonies were allowed to establish for 10 days when treatments were initiated and continued for either 24h or 10 days with E2, 4-OHT or RAL. In contrast to E2-induced tumor regression seen *in vivo*, treating established colonies did not cause a decrease in colony number or size. Following 24 h treatment of established T47D:A18/neo colonies, there was no ERα expression in the vehicle and E2 treatment groups and sparse staining in the 4-OHT and RAL groups. Examination of T47D:A18/PKCα colonies under the same conditions, shows strong ERα nuclear staining in the vehicle, 4-OHT and RAL treated groups. However, in the 24 h E2 treatment group, some colonies showed nuclear staining while other colonies showed membrane and/or cytoplasmic staining (data not shown). To determine if treating established colonies for a longer period would lead to the complete translocation of ERα from the nucleus to the cytoplasm, we extended treatment for 10 days with media changes every three days before IF staining. Under

these conditions, ERα is localized to the nucleus in all groups of T47D:A18/neo colonies as well as T47D:A18/PKCα vehicle control, 4-OHT and RAL groups (Figure 15)(3). However, under conditions that mimic tumor regression, T47D:A18/PKCα colonies exhibit complete ERα translocation out of the nucleus in response to E2 after 10 days and this effect is seen as early as 24 h. While E2 administration to established colonies in Matrigel induces ERα translocation to extranuclear sites, ERα translocation alone is not sufficient to induce regression likely due to the requirement of additional factors found in the tumor microenvironment, but not in Matrigel (3).

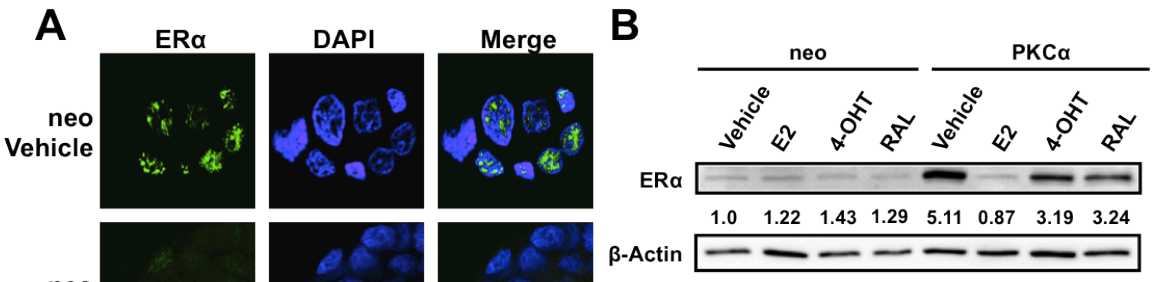


Figure 15. E2 induces relocalization of ERα in established T47D:A18/ PKCα colonies after 10 days. A. T47D:A18/neo colonies (neo) and T47D:A18/PKCα colonies (PKCα) colonies were immunostained for ERα (green) and nuclei (blue). Colonies were grown for 10 days then treated for 10 days with vehicle (EtOH, 0.1%), E2 (10^{-9} M), 4-OHT (10^{-7} M) or RAL (10^{-7}M). Scale bar = 20 μm. **B**. Expression of ERα in whole cell colony lysates. Values represent β-actin-normalized ERα expression relative to T47D:A18/neo E2-treated colonies (Ref 3).

4.12 Pharmacologic inhibition of PKCα does not reverse E2-induced extranuclear ERα translocation in T47D:A18/PKCα colonies

The finding that ERα is localized to the nucleus during RAL and TAM-induced T47D:A18/neo tumor regression suggests that it is not simply regression that triggers ERα to exit from the nucleus, but localization may be influenced by PKCα overexpression. To determine if PKCα activity is required for E2-induced ERα extranuclear translocation in T47D:A18/PKCα colonies, colonies were grown for 10 days followed by 10 days of treatment (Vehicle, 1 nM E2, 1μM Gö6976, or 1nM E2 + 1μM Gö6976). Gö6976 is a PKCα and PKCβ1 selective inhibitor. As expected E2 treatment resulted in extranuclear translocation of ERα in Matrigel colonies, but the addition of Gö6976 did not effect translocation of ERα by E2 (Figure 16). These results suggest that PKCα activity may not be necessary for E2 induced translocation of ERα, but PKCα expression itself may still potentially play a role in ERα translocation.

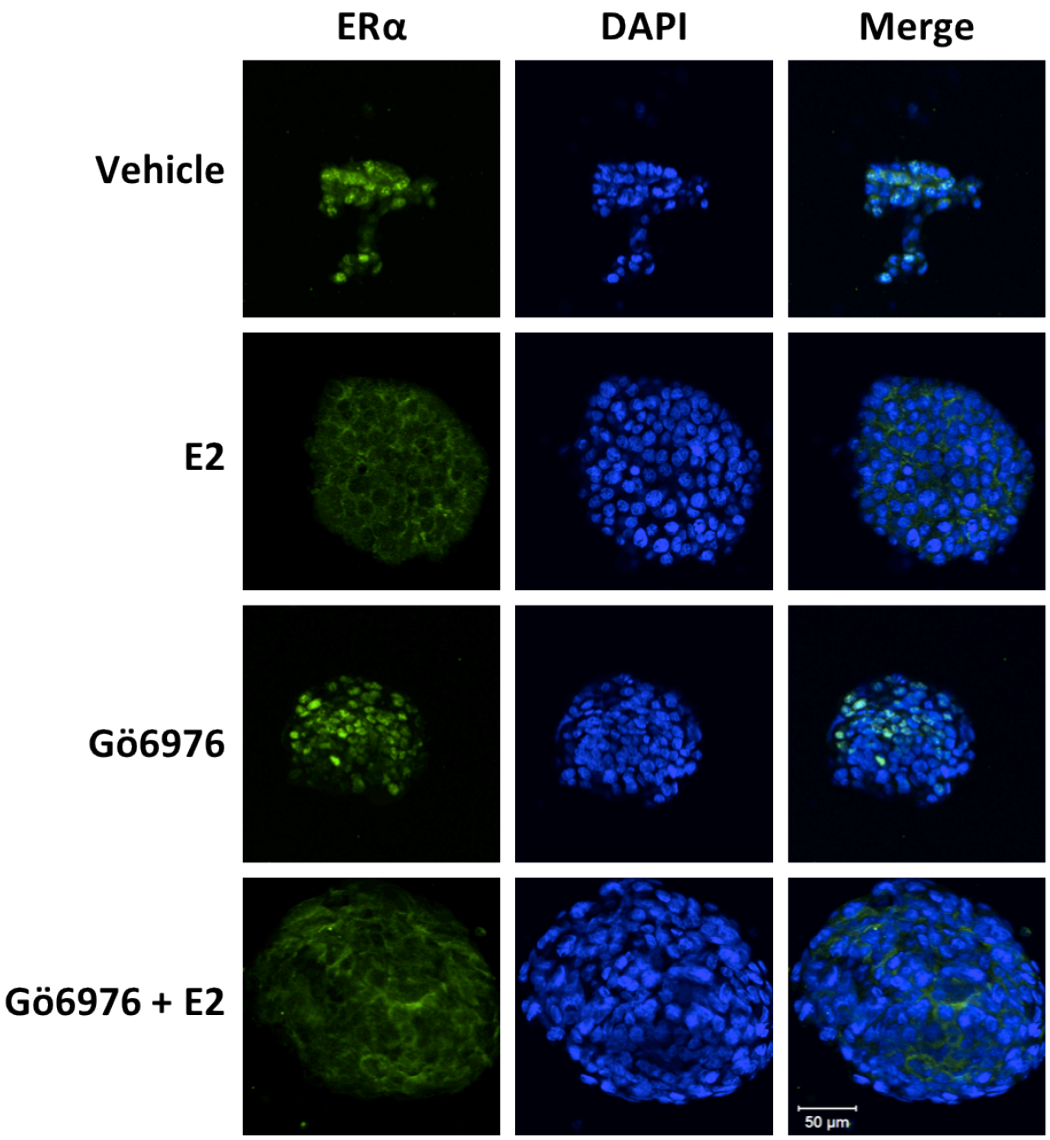


Figure 16. PKCα inhibition does not reverse E2-induced ERα extranuclear localization in T47D:A18/PKCα colonies grown in Matrigel. T47D:A18/PKCα colonies were immunostained for ERα (green) and nuclei (blue). Colonies were grown for 10 days then treated for 10 days with vehicle (EtOH, 0.1%), E2 (10^{-9} M), Gö6976 (10^{-6} M) or Gö6976 + E2. Scale bar = 50 µm.

4.13 ERα extranuclear translocation is not observed in all models of E2-induced tumor regression

I next sought to determine if the translocation of ERα to extranuclear sites is a universal phenomenon observed in other models of E2-induced tumor regression, specifically T47D:A18-TAM1 and MCF-7:5C models. Both T47D:A18-TAM1 and MCF-7:5C overexpress PKCα and regress when treated with E2 *in vivo*. Following 2 weeks of treatment with E2, BTC or TTC-352 T47D:A18-TAM1 xenografts significantly regressed (Figure 12B) and tumor tissue collected. At this time point IF microscopy revealed nuclear ERα localization in all groups including the non-treated control (Figure 17). Although probable, it is not clear if tumors would continue to regress as the study was ended at this time point due to limited animal numbers. It is also unclear if extranuclear translocation of ERα is observed at earlier time points during tumor regression. A larger study is currently underway to determine if ERα translocation to extranuclear sites occurs at an earlier time point in regressing T47D:A18-TAM1 xenografts.

Next, MCF-7:5C xenografts were established in 20 athymic nude mice. Following tumor establishment mice were randomized to either receive E2 capsule implantation (10 mice) or continue to be left untreated (10 mice) (Figure 18B). Following 50% regression by E2 mice were sacrificed and tumors were again subjected to IF microscopy to identify ERα subcellular localization. ERα was observed in the nucleus of all non-treated control tumors. The majority of E2 treated tumors (4/5) displayed nuclear ERα localization, however 1 of 5 tumors display extranuclear ERα localization (Figure 18C). I observed no apparent difference in the size at tumor

excision or in the morphology of the cells within the tumor possibly suggesting heterogeneity between xenografts established from the same cell line. MCF-7:5C cells are also inhibited by E2 *in vitro* allowing for interrogation of ERα localization following E2 treatment *in vitro*. At 24 and 48 hrs following E2 treatment MCF-7:5C displayed nuclear ERα localization (Figure 18D). Although the tumor microenvironment differs greatly from *in vitro* conditions, at the very least these data suggest that ERα translocation to extranuclear sites is not a requirement for E2-induced growth inhibition in MCF-7:5C cells.

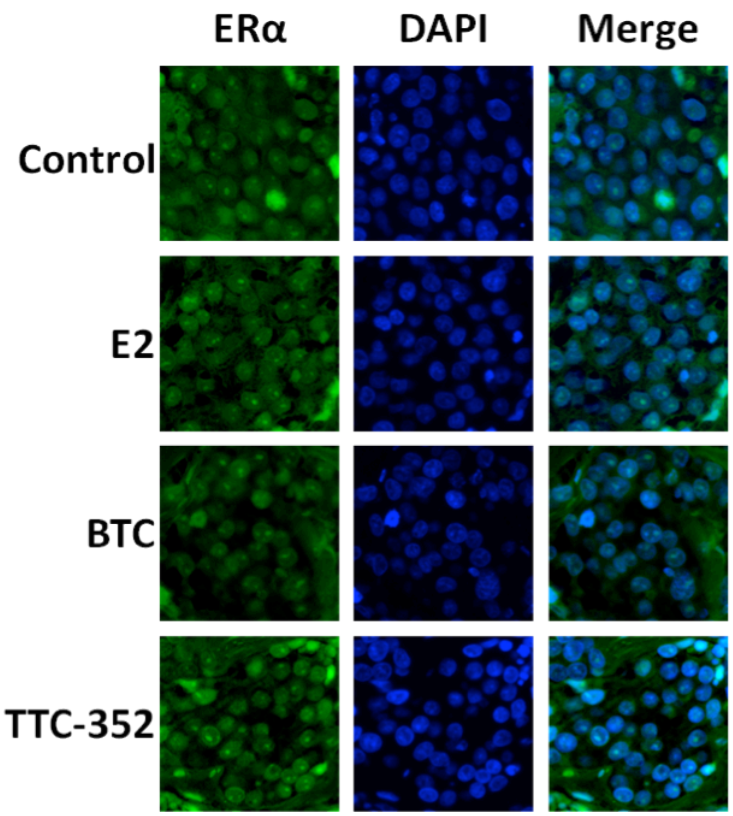


Figure 17. ERα extranuclear localization is not observed in T47D:A18-TAM1 regressing tumors. T47D:A18-TAM1 tumors from (Figure 12B) were immunostained for ERα (green) and nuclei (blue).

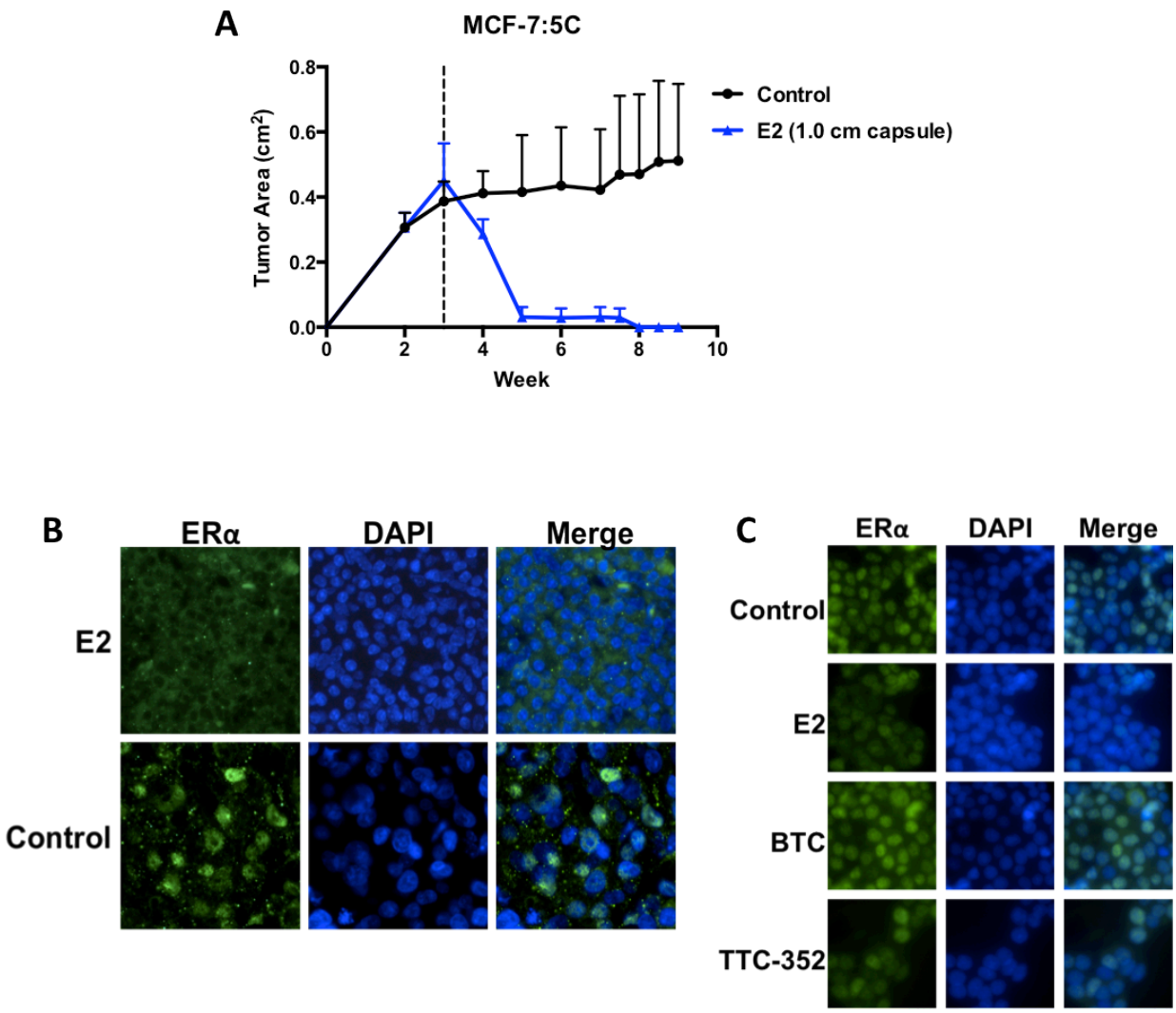


Figure 18. ERα extranuclear localization is not observed in all MCF-7:5C regressing tumors. A) MCF-7:5C xenografts were established then treated with E2. Tumors **(B)** and cells **(C)** were immunostained for ERα (green) and nuclei (blue).

5. Discussion

Seventy-five percent of breast cancers express the estrogen receptor. Endocrine therapy refers to treatments that target estrogen receptor signaling, either by antagonizing ER function with SERMs such as TAM or by estrogen deprivation with aromatase inhibitors. *De novo* or acquired resistance to endocrine therapies is a major obstacle encountered in the clinical setting. Although recent efforts have been made to combat resistance, there is currently a lack of effective therapeutic options for women who no longer respond to conventional endocrine therapy approaches.

Recent clinical trials have demonstrated the efficacy of E2 as a potential treatment option following exhaustive use of TAM or aromatase inhibitors. Before the advent of TAM in the 1970s, E2 was successfully used as a treatment for breast cancer. TAM and E2 were compared head to head in a clinical trial published in 1981 by Ingle and colleagues which concluded that both treatments were comparable after a 4-year follow up but TAM was significantly better tolerated by patients (111). Since then agents which block estrogen signaling have been the treatment of choice for patients with ER positive breast cancer. A long-term follow-up study conducted 20-years later by Peethambaram and colleagues observed that 16% of patients from the TAM treated group and 35% of patients from the DES treated group were alive at 5 years (112). Another interesting finding from this study was that 30% of patients who were switched from the TAM arm responded to DES therapy. These findings suggest that mechanistically E2 therapy may differ from TAM therapy and revisiting the use of E2 may be a viable therapeutic option.

Optimal management of breast cancer requires a way to predict response or resistance to endocrine therapy, whether estrogenic or antiestrogenic, prior to treatment. In the previous chapter, I further explored the use of PKCα as a potential biomarker to predict a positive therapeutic response to E2 treatment. The exogenous overexpression of PKCα in hormone-dependent T47D breast cancer cells led to a TAM-resistant, E2-inhibited phenotype *in vivo* suggesting a crucial role for this protein in the shift from an E2-stimulated to an E2-inhibited phenotype. Here I demonstrate that following long-term exposure to antiestrogens, T47D breast cancer cells acquire an E2-inhibited phenotype *in vivo* (Figure 5). PKCα expression was detected in a panel of E2-inhibited cell lines compared to their E2-stimulated counterparts (Figure 5G). All E2-inhibited cell lines displayed increased PKCα expression compared to E2-stimulated breast cancer cell lines.

To further explore the role of PKCα in the E2-inhibited phenotype and determine if PKCα is required for E2-induced growth inhibition, PKCα was knocked down in the two models which are inhibited by E2 *in vitro*, MCF-7:5C and LTED MCF-7 cells. Interestingly these cell lines displayed opposing effects to E2 following PKCα knockdown. PKCα knockdown in MCF-7:5C cells had little effect on the decrease in cell viability elicited by E2 (Figure 6B). However, PKCα was required for E2 to result in decreased viability of LTED MCF-7 cells (Figure 6A). It would be interesting to determine if enhanced or stable knockdown of PKCα in MCF-7:5C cells would alter the viability of the cells in the presence of E2. Both MCF-7:5C and LTED cell lines were derived from MCF-7 cells cultured long-term in estrogen free conditions ie: phenol-red free medium containing dextran-coated charcoal stripped FBS. The discrepancies

observed between these two cell lines highlights the variability between MCF-7 sublines. Clonal variants of MCF-7 cells are abundant in the literature and cells vary from lab to lab possibly due to selective pressures from various culture conditions or from the intrinsic capacity of stem-like cells within the population to generate heterogeneity. Inhibition of PKCα in T47D:A18-TAM1 cells which endogenously overexpress PKCα would be a useful alternative to determine if PKCα is required for E2-induced inhibition. However, this would require a stable TET-inducible knockdown of PKCα due to the requirement of the *in vivo* microenvironment for E2 to elicit growth inhibition. Very few models of E2-induced growth inhibition currently exist. T47D:A18-TAM1 cells are an additional model of breast cancer pathobiology to explore the role of PKCα in E2-induced growth inhibition.

The major drawback in determining whether PKCα can predict a positive response to an estrogenic therapy is the lack of tumor tissue from patients who have been treated with E2 or DES. Multigene tests are currently used to determine whether early stage breast cancer patients will benefit from the addition of chemotherapy to their hormonal therapy regimen. Although PKCα expression itself may possibly predict a positive response to an estrogenic therapy, it is likely that a multigene set would make a more accurate prediction. Biopsies from patients who respond to E2 (responders) can be compared to biopsies from patients who do not respond to E2 treatment (non-responders) to determine if there are differences in PKCα expression between the two groups. Biopsies could also be subjected to gene arrays to determine which genes are differentially regulated in responders versus non-responders in order to identify a gene

microarray pattern or set of genes associated with response to estrogenic therapeutic intervention.

In collaboration with Drs. Lucy Chen and Elizabeth Wiley, Dr. Tonetti currently has plans to conduct a small phase II clinical trial to determine if PKCα can predict a positive response to E2 in metastatic breast cancer patients. Biopsies of patients will be taken before treatment is initiated and PKCα levels detected using immunohistochemistry techniques. Although not required by MCF-7:5C cells, PKCα was required for E2 to elicit a decrease in cell viability in LTED cells. PKCα was also required for E2-induced tumor regression in the T47D:A18/PKCα model (241). PKCα was therefore necessary for E2 to elicit its effects in 2 of the 3 models probed. Furthermore, all known models of E2 inhibited breast cancer overexpress PKCα. Based on current data, one would hypothesize that the clinical trial will reveal that patients whose breast cancer overexpresses PKCα will positively respond to E2 treatment.

PKCα has recently been established as a central signaling node for breast CSCs (237). An increase in the CSC population has been suggested as a possible mechanism of endocrine resistance. PKCα is overexpressed in endocrine-resistant breast cancer (1, 213) and may be responsible for the increase in CSCs within this population. The effect of E2 on the CSC population in endocrine-resistant breast cancer has not been studied. In particular the effect of E2 on endocrine-resistant, E2-inhibited breast cancer cells is not known. Normal mammary stem cells do not express ERα and in order to respond to systemic hormonal signaling, must rely on paracrine signaling from neighboring cells (248). There have been conflicting reports in the literature on the effects of E2 on the CSC population in breast cancer cell lines, with

evidence suggesting that E2 both increases and decreases the CSC population (249, 250). Our lab as demonstrated that E2 treated T47D:A18/PKCα xenografts regress when treated with E2 and no resumption of tumor growth is seen upon discontinuation of E2 treatment for up to 31 weeks (3). Since the E2 capsules maintain constant serum E2 levels for only 8–10 weeks, we are confident that the E2 capsule is depleted by week 20 and have confirmed no detectable serum E2 by mass spectrometry at 31 weeks. The fact that these xenografts never resume growth suggests the possible inhibition of the CSC population by E2. It would be interesting to further define the effects of E2 on the CSC population in E2-inhibited breast cancer cell lines.

As previously mentioned the side effects associated with E2 treatment led to the discontinuation of its clinical use. An important aspect of E2 and DES's limited clinical use may stem from public misconceptions of the drugs; the most pertinent being the Women's Health Initiative (WHI). The WHI was a set of clinical trials conducted in healthy post-menopausal women designed to test the effects of post-menopausal hormone-therapy, diet modification and vitamin D and calcium supplementation on breast and colorectal cancer, heart disease and bone fractures. Women receiving estrogen plus progestin had an increased risk of thromboembolic complications, stroke, coronary heart disease and breast cancer resulting in early termination of the study (251). Thromboembolic complications were also increased in the estrogen only group. By the mid-1990s 40% of women in the United States were prescribed hormone therapy (252). From 2 years prior to the initial publication to 5 months after the publication hormone therapy prescriptions decreased by 46% in the United States (253). Recently the results of a 13-year follow-up, which ended in 2010, were published and offer a

more complete picture of the effects of hormone replacement therapy. If women started hormone therapy within 10 years of their last menstrual cycle there was less risk associated with the use of hormone replacement therapy. Women treated with estrogen only showed less adverse health outcomes than women taking estrogen plus progestin. Most importantly, a reduction in breast cancer risk was reported for hysterectomized women treated with estrogen only (254). Additionally, women diagnosed with breast cancer who took estrogen alone had a 63% reduction in deaths from the disease compared to the placebo group (255). These results differ drastically from the initial results published 10 years prior and suggest that there may be a benefit to estrogen treatment therapy for the management of postmenopausal symptoms with less side effects then initially suggested.

Public misconceptions regarding the use DES therapy may also be related to its use in pregnant women from 1938 to 1971. At the time it was believed that miscarriages and premature deliveries were due to low levels of estrogen in the women's body (256). It was later found that infants exposed to DES in utero were more likely to have complications with their reproductive systems including cervical dysplasia, infertility and problems during pregnancy (257). Women who took DES while pregnant may also be at an increased risk of developing breast cancer (258). Although extreme side effects associated with the use of DES in this context are noted, these side effects are not pertinent to the use of DES as a treatment for advanced breast cancer.

In the previous chapter, I described the identification of two novel benzothiophene compounds that display estrogenic activity in breast cancer cell lines (Figure 8). The benzothiophene SERM RAL was used as a starting point for rationale

drug design due to the fact that *(1)* RAL treatment caused regression of T47D:A18/PKCα xenografts and *(2)* because RAL, unlike TAM or E2, does not cause proliferation of the endometrium and does not increase a patient's risk of developing endometrial cancer. Table 3 summarizes the estrogenic activity of E2, TAM, RAL and SEMs in the breast and uterine tissue. The intention was to identify compounds that display estrogenic effects in breast tissue but not in the uterus. The clinical community may be more willing to revisit the use of an estrogenic therapy for the treatment of breast cancer if the estrogenic effects were more selective.

TABLE 3. Estrogenic activity of compounds in breast and uterine tissue.

	E2	TAM	RAL	SEMs
Breast	Estrogenic	Anti-estrogenic	Anti-estrogenic	Estrogenic
Uterus	Estrogenic	Estrogenic	Anti-estrogenic	Anti-estrogenic

By screening a small library of benzothiophene analogs synthesized by Dr. Greg Thatcher's lab I identified two compounds, BTC and TTC-352, which demonstrated estrogenic activity *in vitro*. Both BTC and TTC-352 inhibited TAM-resistant T47D:A18/PKCα and T47D:A18-TAM1 colony formation in 3D Matrigel (Figure 10B and 10C) as well as inhibited growth of TAM-resistant MCF-7:5C cells *in vitro* (Figure 9A). T47D:A18/PKCα and T47D:A18-TAM1 xenograft tumors significantly regressed when treated with BTC or TTC-352 *in vivo* (Figure 12A and 12B, respectively). Although E2 induced growth of hormone-dependent, TAM-sensitive, parental T47D:A18/neo tumors *in vivo,* neither BTC nor TTC-352 were able to support T47D:A18/neo tumor growth (Figure 13A). Furthermore, in contrast to E2, neither BTC nor TTC-352 treatment

resulted in an increase in the uterine weight of mice (Figure 13C) indicating that BTC and TTC-352, unlike E2 and TAM, may have enhanced tissue specificity.

Interestingly structurally related compounds, variously showing classical ERα antagonist activity (RAL), or classical agonist activity (BTC, TTC-352) elicit the same tumor regressing actions in T47D:A18/PKCα xenografts, although the failure of RAL-induced regression to persist after drug withdrawal is noted. That the estrogen agonists, BTC and TTC-352, did not stimulate growth of estrogen-sensitive T47D:A18/neo xenografts or uterine tissues is most simply rationalized by the relatively low potency of these agonists, possibly indicating involvement of a pathway that is not simply classical ERα mediated in T47D:A18/PKCα and T47D:A18-TAM1 xenografts and may include involvement of ERβ, GPER, ERX or another yet undiscovered estrogen binding molecule. Alternatively, it is possible that the oral dose of 1.5 mg/day achieved a plasma concentration within a therapeutic window capable of causing regression of T47D:A18/PKCα and T47D:A18-TAM1 xenografts but unable to stimulate uterine growth in mice or hormone-dependent T47D:A18/neo xenograft growth. Nonetheless, if the therapeutic window is large enough, the off target effects of SEMs would be negligible. Studies are currently underway to determine if lower doses can effectively cause regression of TAM-resistant xenografts.

Different SERMs, such as TAM and RAL, exhibit various ER agonist and antagonist activity on different genes depending on the cell type that these agents are acting on. This can most simply be explained by different ligand induced conformational changes in ER (259), followed by distinct interactions with coregulators based on specific conformational changes, leading to differential transcription of ER regulated

genes (260). Here I demonstrate that SEMs are estrogenic in breast cancer cells both *in vitro* (Figure 11) and *in vivo* (Figure 12), but do not display estrogenic action in the uterus of mice *in vivo* (Figure 13C). Differential coregulator expression in breast vs uterine cells may explain the selectivity observed with SEMs *in vivo* (261). Although the mechanism of the estrogenic selectivity of SEMs is not completely understood, further understanding of what mediates tissue selectivity of these compounds may improve SEM design to meet clinical needs.

Mutations in the LBD of the ER have recently been shown to play a role in resistance to endocrine therapies (87, 88). The presence of the ER mutation was shown to favor the agonist conformation of the receptor in both studies. However, Robinson and colleagues show that anti-estrogen sensitivity is not effected by LBD mutations in the ER (87) while Toy and colleagues show reduced efficacy of ER antagonists (88). Toy et al. conclude that more potent ER antagonists may overcome clinical resistance to hormonal therapy and as for SEMs the same may be true. More potent ER agonists such as BTC may be favored compared to weaker ER agonists such as TTC-352 when LBD mutations are present in the ER. It would be of interest to determine the effects of ER LBD mutations on the efficacy of SEMs as these mutations are present in the target population of endocrine resistant breast cancer patients.

In the previous chapter I have shown by immunofluorescent confocal microscopy that ERα translocates from the nucleus to the extranuclear space upon E2, RAL (3), BTC and TTC-352-induced tumor regression in our T47D:A18/PKCα preclinical TAM-resistant model (Figure 14). Extranuclear ERα was previously reported to play a role in endocrine-resistant breast cancers specifically by interacting with growth factor

receptors to activate proliferative and pro-survival signals (262, 263). However I demonstrate here that ERα translocation is associated with tumor regression in T47D:A18/PKCα tumors (3). To our knowledge this is the first study to report an association of extranuclear ERα with tumor regression, as opposed to the activation of growth factor receptor signaling. These studies underline the unique role of extranuclear ERα in E2 and SEM induced tumor regression.

ERα translocation to extranuclear sites does occur in Matrigel in response to E2 (Figure 15) (3). Matrigel results reveal that the translocation of ERα may be an early event as ERα was seen in the membrane and cytoplasm in some colonies at 24 h further illustrating a rapid response to E2 treatment. Colony regression is not initiated by E2 perhaps because a component in the tumor microenvironment is also required to initiate the regression, but ERα still translocates to extranuclear sites. Proliferating Matrigel colonies exhibit extranuclear ERα suggesting that ERα translocation to extranuclear sites may not be the mechanism of T47D:A18/PKCα tumor regression, but an event that occurs concurrently with tumor regression (3).

Extranuclear ERα translocation was observed in T47D:A18/PKCα colonies and xenografts in response to E2 treatment but not in T47D:A18/neo colonies or xenografts suggesting a potential role for PKCα in extranuclear translocation of ERα. Pharmacological inhibition of PKCα did not reverse the effects of E2 on ERα translocation in T47D:A18/PKCα colonies indicating that PKCα activity may not be necessary for ERα translocation (Figure 16). Current work in our lab is focused on developing PKCα kinase-dead mutants to further address whether the kinase activity of PKCα is necessary for ERα translocation as PKCα inhibitors may have off target effects.

I am not, however, out ruling the potential physical association of PKCα with various signaling molecules in the cytoplasm that may account for ERα translocation. Addressing this would require genetic knockdown of PKCα in the T47D:A18/PKCα cell line. Longo *et al*. has shown that a PKCα-src kinase-ERα interaction is critical in the modulation of estrogen responsiveness and the differentiation process in osteoblasts (264). However, we were unable to detect a physical interaction between PKCα and ERα, Her2 or src in our tumor model (3).

We have observed that translocation of ERα from the nucleus to cytoplasm is a common feature of treatments that cause regression of T47D:A18/PKCα tumors, but not those that are ineffective, i.e. TAM (3). The similarity with the diarylthiohydantoin antiandrogens (e.g. Enzalutamide, ARN509, RD162) that cause a similar translocation of the androgen receptor (AR) in prostate cancer cells is of interest, in particular because this feature is seen as a clinical advantage over older antiandrogens (265, 266). Enzalutamide is currently approved for treatment of castration-resistant prostate cancer. The mechanism by which these antiandrogens cause translocation have not been defined, and as for benzothiophene SEMs could include stabilization of cytoplasmic or destabilization of nuclear receptor complexes.

The breast tumor microenvironment clearly plays a role in tumor regression elicited by E2. The Tonetti lab has previously demonstrated the requirement of the ECM in E2-induced inhibition of T47D:A18/PKCα cells (135). The ECM is also required for E2-induced inhibition of T47D:A18-TAM1 as these cells are inhibited in 3D matrigel (Figure 10C) and *in vivo* by E2 (Figure 5B), but not inhibited by E2 in 2D *in vitro* conditions (Figure 5A). Different immune and stromal cells types make up the breast

tumor microenvironment such as fibroblasts and adipocytes (267). Matrigel itself does not consist of immune or stromal cells of any type. The Matrigel matrix consists primarily of collagen, laminin and growth factors that resemble the complex extracellular microenvironment. T47D:A18/PKCα and T47D:A18-TAM1 colony formation is inhibited by E2 in the presence of Matrigel suggesting that interactions with stromal cells such as fibroblasts or immune cells are likely not required for E2-induced growth inhibition. Growth factor reduced Matrigel also did not affect the ability of E2 in inhibit T47D:A18/PKCα colony formation (135). Taken together, this suggests that the physical interaction of tumor cells with ECM proteins is the main driver of E2-induced inhibition, although I would not rule out the potential influence of stromal and immune cells on tumor regression.

With respect to the tumor microenvironment, PKCα expression may also influence E2-induced inhibition through enhanced integrin signaling. PKCα has been shown to directly interact with β1 integrin (268). In the presence of E2, enhanced PKCα/integrin signaling may lead to a down stream pro-apoptotic response.

PKCα may regulate E2-induced apoptosis through modulation of the Akt pathway. The Tonetti lab has previously demonstrated that T47D:A18/PKCα E2-induced regression is accompanied by down-regulation of the Akt pathway (135). It has also been demonstrated that E2 can directly bind and activate PKCα (269). It is possible that E2 directly activates PKCα, leading to downregulation/inhibition of the Akt prosurvival pathway resulting in apoptosis (Figure 19). In fact, selective activation of PKCα in prostate cancer cells led to apoptosis due to inactivation of the Akt pathway (270).

PKCα may also play a role in E2-induced growth inhibition by modulating ERα extranuclear translocation. I have demonstrated here that ERα translocation is associated with tumor regression only in PKCα overexpressing tumors in response to E2, RAL (3), BTC and TTC-352 (Figure 14). ERα localization to the plasma membrane requires palmitoylation at a highly conserved sequence in the E domain of the receptor (179). DHHC-7 and DHHC-21 are the palmitoylacyltransferase (PAT) proteins responsible for the palmitoylation of ERα (180), leading to a productive interaction with caveolin-1, an association required for membrane localization of ERα (181). It is interesting to note that ERα/caveolin-1 complex formation correlates with durable tumor regression produced with E2, but not with transient tumor regression as observed with RAL, nor with proliferating T47D:A18/PKCα tumors (3). The PAT DHHC-7 contains several potential PKC phosphorylation sites. Taken together these results suggest that perhaps PKCα is capable of modifying the interaction of ERα and caveolin-1 in the presence of E2, potentially through increased PAT activity at the membrane to then effect tumor regression (Figure 19). It is also important to note that although ERα translocation to extranuclear sites does occur in Matrigel in response to E2 (Figure 15), colony regression is not initiated perhaps because a component in the tumor microenvironment is also required to initiate the regression signal (3). Experiments are currently underway in the Tonetti lab to further define the mechanism through which PKCα modulates ERα translocation and subsequent tumor regression.

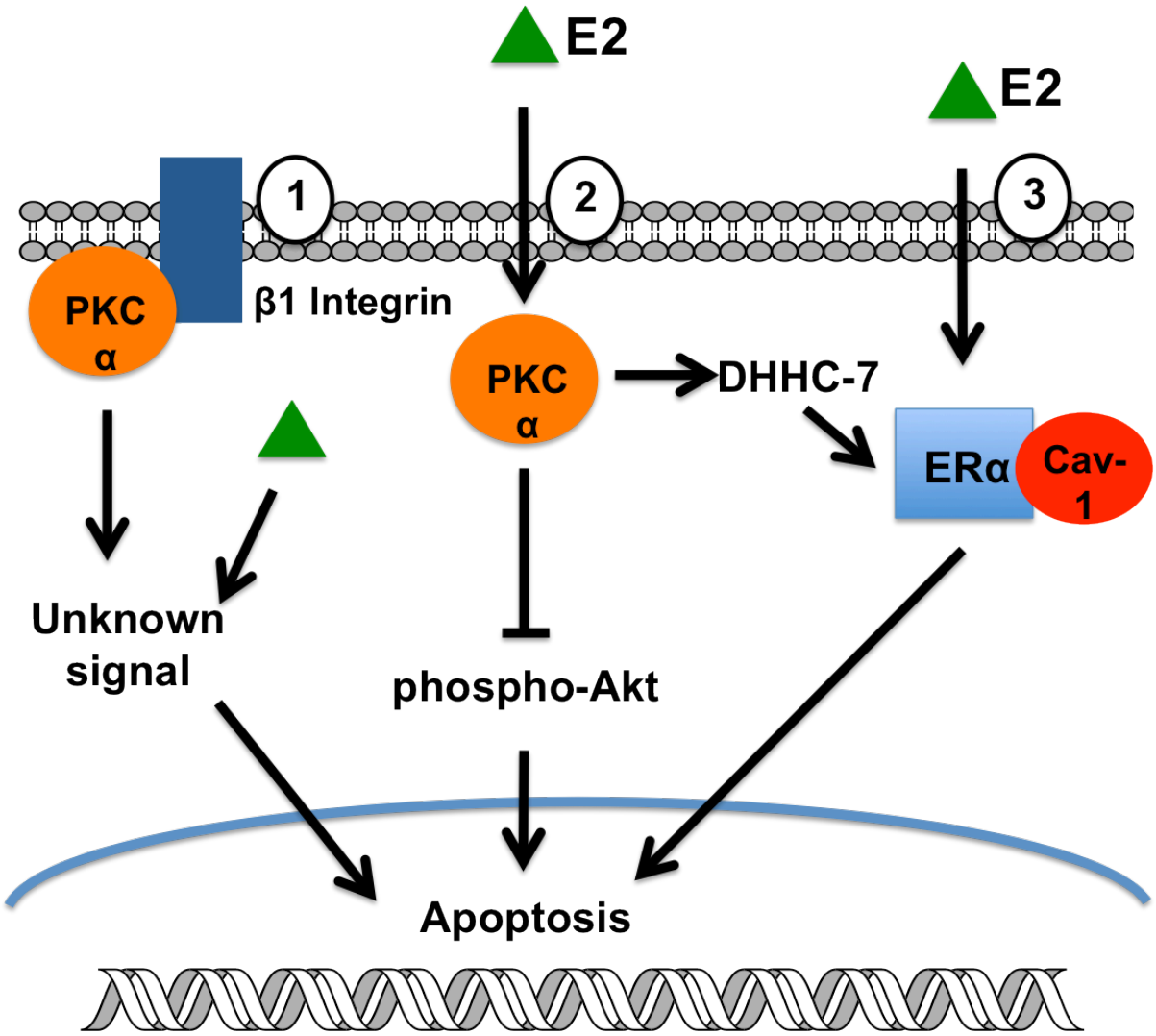


Figure 19. Potential mechanisms through which PKCα may regulate E2-induced apoptosis in PKCα-overexpressing breast cancer. **1)** PKCα can directly interact with β1 integrin and in the presence of E2, enhanced PKCα/integrin signaling may initiate a yet unknown signal leading to a down stream pro-apoptotic response. **2)** E2 can directly activate PKCα, leading to downregulation/inhibition of the Akt prosurvival pathway resulting in apoptosis. **3)** Increased PKCα signaling due to direct binding of E2 can activate the PAT DHHC-7, resulting in an increased extranuclear ERα due to ERα-Cav-1 interaction. Extranuclear ERα is associated with tumor regression/ apoptosis in PKCα-overexpressing breast cancer.

In order to determine if extranuclear translocation of ERα is a phenomenon observed in other models of E2-induced tumor regression T47D:A18-TAM1 and MCF-7:5C xenografts were analyzed for ERα localization. T47D:A18-TAM1 regressing xenografts displayed nuclear ERα localization (Figure 17). These tumors were excised following 75% regression and it is possible that kinetics may play a factor in extranuclear ERα translocation. Current studies are underway to determine ERα localization at various time points during tumor regression. Interestingly only 1 of 5 MCF-7:5C E2 treated xenografts displayed extranuclear ERα (Figure 18), while the majority displayed nuclear ERα localization. MCF-7:5C cells treated with E2 *in vitro* also displayed nuclear ERα localization suggesting that extranuclear ERα translocation may not be a requirement for E2-induced inhibition, at least in the MCF-7:5C model.

Extranuclear ERα is not currently measured clinically. The inability to identify extranuclear ER pools may lie in the fact that extranuclear steroid receptors may be 10-fold lower then nuclear ER. In addition, although pathologists may observe extranuclear ER staining, it is not reported because breast cancers are designated as ER positive or ER negative based on nuclear staining. Welsh and colleagues sought to detect non-nuclear ERα in nearly 3200 clinical breast cancer specimens and found the average incidence to be only 1.5% (172). More sensitive techniques may be required to detect the very small ERα pools located outside of the nucleus. In addition the tissue used in the Welsh study was obtained from a variety of institutions and the methods of tissue handling in many cases was not known. The upcoming clinical trial conducted by Tonetti and colleagues will allow for rigorous detection of ERα localization in patient samples. It will also allow the investigators to determine ERα localization in human

breast specimens following E2 treatment, which was certainly not the case for the tissue used in the Welsh study.

The results of the present study support the use of a second-line SEM with selective estrogenic effects on the breast for use in patients that no longer respond to conventional endocrine therapy and whose tumors overexpress PKCα. I have described two novel benzothiophene SEMs that cause tumor regression in the TAM-resistant T47D:A18/PKCα xenografts, T47D:A18-TAM1 xenografts and MCF-7:5C cell *in vitro*, while having minimal effect on growth of parental hormone-dependent T47D:A18/neo tumors. Importantly treatment with BTC and TTC-352 had minimal effects on proliferation within the uteri of mice *in vivo* suggesting that the estrogenic effects of these agents are specific to the breast. Both BTC and TTC-352 are potential alternatives to E2 treatment and represent chemical probes and lead compounds for further optimization towards new treatment options in the management of endocrine resistant breast cancer.

6. CITED LITERATURE

1. Tonetti DA, Chisamore MJ, Grdina W, Schurz H, Jordan VC. Stable transfection of protein kinase C alpha cDNA in hormone-dependent breast cancer cell lines. Br J Cancer. 2000;83:782-91.

2. Chisamore MJ, Ahmed Y, Bentrem DJ, Jordan VC, Tonetti DA. Novel antitumor effect of estradiol in athymic ice injected with a T47D breast cancer cell line overexpressing protein kinase C alpha. Clinical Cancer Research. 2001;7:3156-65.

3. Perez White B, Molloy M, Zhao H, Zhang Y, Tonetti D. Extranuclear ERalpha is associated with regression of T47D PKCalpha-overexpressing, tamoxifen-resistant breast cancer. Molecular Cancer. 2013;12:34.

4. Horner MJ RL, Krapcho M, Neyman N, Aminou R, Howlader N, Altekruse SF, Feuer EJ, Huang L, Mariotto A, Miller BA, Lewis DR, Eisner MP, Stinchcomb DG, Edwards BK (eds). . SEER cancer statistics review. National Cancer Institute Bethesda, MD. 1975-2006.

5. Ganz PA. Breast cancer, menopause, and long-term survivorship: critical issues for the 21st century. The American Journal of Medicine. 2005;118:136-41.

6. Bosetti C, Malvezzi M, Chatenoud L, Negri E, Levi F, La Vecchia C. Trends in cancer mortality in the Americas, 1970-2000. Annals of Oncology. 2005;16:489-511.

7. Levi F, Lucchini F, Negri E, Boyle P, La Vecchia C. Cancer mortality in Europe, 1995-1999, and an overview of trends since 1960. International Journal of Cancer. 2004;110:155-69.

8. Ma H, Bernstein L, Pike MC, Ursin G. Reproductive factors and breast cancer risk according to joint estrogen and progesterone receptor status: a meta-analysis of epidemiological studies. Breast Cancer Res. 2006;8:R43.

9. Hemminki K, Forsti A, Bermejo JL. The 'common disease-common variant' hypothesis and familial risks. PLoS One. 2008;3:e2504.

10. Claus EB, Petruzella S, Matloff E, Carter D. Prevalence of BRCA1 and BRCA2 mutations in women diagnosed with ductal carcinoma in situ. JAMA: the journal of the American Medical Association. 2005;293:964-9.

11. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406:747-52.

12. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proceedings of the National Academy of Sciences. 2001;98:10869-74.

13. Hu Z, Fan C, Oh D, Marron J, He X, Qaqish B, et al. The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics. 2006;7:96.

14. Farmer P, Bonnefoi H, Becette V, Tubiana-Hulin M, Fumoleau P, Larsimont D, et al. Identification of molecular apocrine breast tumours by microarray analysis. Breast Cancer Research. 2005;7:P2.11.

15. Prat A, Parker J, Karginova O, Fan C, Livasy C, Herschkowitz J, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Research. 2010;12:R68.

16. Kennecke H, Yerushalmi R, Woods R, Cheang MCU, Voduc D, Speers CH, et al. Metastatic behavior of breast cancer subtypes. Journal of Clinical Oncology. 2010;28:3271-7.

17. Guarneri V, Conte P. Metastatic breast cancer: therapeutic options according to molecular subtypes and prior adjuvant therapy. The Oncologist. 2009;14:645-56.

18. Debled M, MacGrogan G, de Mascarel I, Brouste V, Bonnefoi H, Mauriac L. Expression profiling in breast carcinoma: new insights on old prognostic factors? Journal of Clinical Oncology. 2007;25:4316-7.

19. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. New England Journal of Medicine. 2004;351:2817-26.

20. van't Veer LJ, Dai H, Van De Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002;415:530-6.

21. Paik S. Development and clinical utility of a 21-gene recurrence score prognostic assay in patients with early breast cancer treated with tamoxifen. The Oncologist. 2007;12:631-5.

22. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. New England Journal of Medicine. 2005;353:1659-72.

23. Gianni L, Dafni U, Gelber RD, Azambuja E, Muehlbauer S, Goldhirsch A, et al. Treatment with trastuzumab for 1 year after adjuvant chemotherapy in patients with HER2-positive early breast cancer: a 4-year follow-up of a randomised controlled trial. The Lancet Oncology. 2011;12:236-44.

24. Parker JS, Mullins M, Cheang MCU, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. Journal of Clinical Oncology. 2009;27:1160-7.

25. Bosch A, Eroles P, Zaragoza R, Vina JR, Lluch A. Triple-negative breast cancer: Molecular features, pathogenesis, treatment and current lines of research. Cancer treatment reviews. 2010;36:206-15.

26. Smid M, Wang Y, Zhang Y, Sieuwerts AM, Yu J, Klijn JGM, et al. Subtypes of breast cancer show preferential site of relapse. Cancer Research. 2008;68:3108-14.

27. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proceedings of the National Academy of Sciences. 2003;100:8418-23.

28. Kreike B, van Kouwenhove M, Horlings H, Weigelt B, Peterse H, Bartelink H, et al. Gene expression profiling and histopathological characterization of triple-negative/basal-like breast carcinomas. Breast Cancer Research. 2007;9:R65.

29. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clinical Cancer Research. 2004;10:5367-74.

30. Peppercorn J, Perou CM, Carey LA. Molecular subtypes in breast cancer evaluation and management: divide and conquer. Cancer investigation. 2008;26:1-10.

31. Herschkowitz J, Simin K, Weigman V, Mikaelian I, Usary J, Hu Z, et al. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biology. 2007;8:R76.

32. Hennessy BT, Gonzalez-Angulo A-M, Stemke-Hale K, Gilcrease MZ, Krishnamurthy S, Lee J-S, et al. Characterization of a naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics. Cancer Research. 2009;69:4116-24.

33. Prat A, Perou CM. Deconstructing the molecular portraits of breast cancer. Molecular oncology. 2011;5:5-23.

34. Roche H, Vahdat LT. Treatment of metastatic breast cancer: second line and beyond. Annals of Oncology. 2011;22:1000-10.

35. Rexer BN AC. Intrinsic and acquired resistance to HER2-targeted therapies in HER2 gene-amplified breast cancer: mechanisms and clinical implications. . Crit Rev Oncog. 2012;17:1-16.

36. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. New England Journal of Medicine. 2001;344:783-92.

37. Blackwell KL, Burstein HJ, Storniolo AM, Rugo H, Sledge G, Koehler M, et al. Randomized study of lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer. Journal of Clinical Oncology. 2010;28:1124-30.

38. Burris HA. Dual kinase inhibition in the treatment of breast cancer: initial experience with the EGFR/ErbB-2 inhibitor lapatinib. The Oncologist. 2004;9:10-5.

39. de Bono JS, Bellmunt J, Attard G, Droz JP, Miller K, Flechon A, et al. Open-Label Phase II Study Evaluating the Efficacy and Safety of Two Doses of Pertuzumab in Castrate Chemotherapy-Naive Patients With Hormone-Refractory Prostate Cancer. Journal of Clinical Oncology. 2007;25:257-62.

40. Hortobagyi GN. Treatment of breast cancer. New England Journal of Medicine. 1998;339:974-84.

41. Beatson G. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases The Lancet. 1896;148:162-5.

42. Grodin JM, Siiteri PK, Macdonald PC. Source of estrogen production in postmenopausal women. Journal of Clinical Endocrinology & Metabolism. 1973;36:207-14.

43. Macdonald PC, Rombaut RP, Siiteri PK. Plasma precursors of estrogen. I. Extent of conversion of plasma androstenedione to estrone in normal males and nonpregnant normal, castrate and adrenalectomized females. Journal of Clinical Endocrinology & Metabolism. 1967;27:1103-11.

44. Pyrah LN, Smiddy FG. Mammary cancer treated by bilateral adrenalectomy The Lancet. 1954;263:1041-7.

45. Lipton A, Harvey HA, Santen RJ, Boucher A, White D, Bernath A, et al. Randomized trial of aminoglutethimide versus tamoxifen in metastatic breast cancer. Cancer Research. 1982;42:3434s-6s.

46. Samojlik E, Veldhuis JD, Wells SA, Santen RJ. Preservation of androgen secretion during estrogen suppression with aminoglutethimide in the treatment of metastatic breast carcinoma. The Journal of Clinical Investigation. 1980;65:602-12.

47. Coombes RC, Dowsett M, Goss P, Gazet JC, Brodie A. 4-Hydroxyandrostenedione in the treatment of postmenopausal patients with advanced breast cancer The Lancet. 1984;324:1237-9.

48. Brodie AMH, Marsh D, Brodie HJ. Aromatase inhibitors. Regression of hormone-dependent, mammary tumors in the rat with 4-acetoxy-4-androstene-3,17-dione. Journal of Steroid Biochemistry. 1979;10:423-9.

49. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet. 2005;365:60-2.

50. Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. The Lancet. 2012.

51. Clemons M, Danson S, Howell A. Tamoxifen (Nolvadex): a review: Antitumour treatment. Cancer Treatment Reviews. 2002;28:165-80.

52. Abe O, Abe R, Enomoto Kt, Kikuchi K, Koyama H, Nomura Y, et al. Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet. 1998;351:1451-67.

53. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes. JAMA: the journal of the American Medical Association. 2006;295:2727-41.

54. Johnston SRD. Endocrine manipulation in advanced breast cancer: recent advances with SERM therapies. Clinical Cancer Research. 2001;7:4376s-87s.

55. Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst. 2005;97:1652-62.

56. Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA, Purdie DW, et al. Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial. Breast Cancer Res Treat. 2001;65:125-34.

57. Visvanathan K, Hurley P, Bantug E, Brown P, Col NF, Cuzick J, et al. Use of pharmacologic interventions for breast cancer risk reduction: American Society of Clinical Oncology clinical practice guideline. Journal of Clinical Oncology. 2013;31:2942-62.

58. Miller CP, Collini MD, Tran BD, Harris HA, Kharode YP, Marzolf JT, et al. Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. Journal of medicinal chemistry. 2001;44:1654-7.

59. Gruber C, Gruber D. Bazedoxifene (Wyeth). Current opinion in investigational drugs (London, England: 2000). 2004;5:1086-93.

60. Komm BS, Kharode YP, Bodine PV, Harris HA, Miller CP, Lyttle CR. Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. Endocrinology. 2005;146:3999-4008.

61. Wardell SE, Nelson ER, Chao CA, McDonnell DP. Bazedoxifene exhibits antiestrogenic activity in animal models of tamoxifen-resistant breast cancer:

implications for treatment of advanced disease. Clinical Cancer Research. 2013;19:2420-31.

62. Howell A, Wardley AM. Overview of the impact of conventional systemic therapies on breast cancer. Endocrine-Related Cancer. 2005;12:S9-S16.

63. Pontiggia O, Rodriguez V, Fabris V, Raffo D, Bumaschny V, Fiszman G, et al. Establishment of an in vitro estrogen-dependent mouse mammary tumor model: a new tool to understand estrogen responsiveness and development of tamoxifen resistance in the context of stromal-epithelial interactions. Breast Cancer Res Treat. 2009;116:247-55.

64. Generali D, Berruti A, Brizzi MP, Campo L, Bonardi S, Wigfield S, et al. Hypoxia-inducible factor-1 alpha-expression predicts a poor response to primary chemoendocrine therapy and disease-free survival in primary human breast cancer. Clinical Cancer Research. 2006;12:4562-8.

65. Encarnacion CA, Ciocca DR, McGuire WL, Clark GM, Fuqua SAW, Osborne CK. Measurement of steroid-hormone receptor in breast-cancer patients on tamoxifen. Breast Cancer Res Treat. 1993;26:237-46.

66. Gutierrez MC, Detre S, Johnston S, Mohsin SK, Shou JN, Allred DC, et al. Molecular changes in tamoxifen-resistant breast cancer: Relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. Journal of Clinical Oncology. 2005;23:2469-76.

67. Shi L, Dong B, Li ZW, Lu YW, Tao OY, Li JF, et al. Expression of ER-alpha 36, a novel variant of estrogen receptor alpha, and resistance to tamoxifen treatment in breast cancer. Journal of Clinical Oncology. 2009;27:3423-9.

68. Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SAW, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst. 2003;95:353-61.

69. Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen TM, Schiff R, et al. Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. Proc Natl Acad Sci U S A. 1998;95:2920-5.

70. Schiff R, Reddy P, Ahotupa M, Coronado-Heinsohn E, Grim M, Hilsenbeck SG, et al. Oxidative stress and AP-1 activity in tamoxifen-resistant breast tumors in vivo. J Natl Cancer Inst. 2000;92:1926-34.

71. Dumont J, Bitonti A, Wallace C, Baumann R, Cashman E, Cross-Doersen D. Progression of MCF-7 breast cancer cells to antiestrogen-resistant phenotype is accompanied by elevated levels of AP-1 DNA-binding activity. Cell Growth Differ. 1996;7:351-9.

72. Johnston SRD, Lu B, Scott GK, Kushner PJ, Smith IE, Dowsett M, et al. Increased activator protein-1 DNA binding and c-Jun NH2-terminal kinase activity in human breast tumors with acquired tamoxifen resistance. Clinical Cancer Research. 1999;5:251-6.

73. Zhou YM, Yau C, Gray JW, Chew K, Dairkee SH, Moore DH, et al. Enhanced NF kappa B and AP-I transcriptional activity associated with antiestrogen resistant breast cancer. BMC Cancer. 2007;7.

74. Span PN, Tjan-Heijnen VCG, Manders P, Beex L, Sweep CGJ. Cyclin-E is a strong predictor of endocrine therapy failure in human breast cancer. Oncogene. 2003;22:4898-904.

75. Musgrove EA, Sutherland RL. Biological determinants of endocrine resistance in breast cancer. Nat Rev Cancer. 2009;9:631-43.

76. Chu IM, Hengst L, Slingerland JM. The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. Nat Rev Cancer. 2008;8:253-67.

77. Perez-Tenorio G, Berglund F, Merca AE, Nordenskjold B, Rutqvist LE, Skoog L, et al. Cytoplasmic p21(WAF1/CIP1) correlates with Akt activation and poor response to tamoxifen in breast cancer. Int J Oncol. 2006;28:1031-42.

78. Roodi N, Bailey LR, Kao W-Y, Verrier CS, Yee CJ, Dupont WD, et al. Estrogen receptor gene analysis in estrogen receptor-positive and receptor-negative primary breast cancer. J Natl Cancer Inst. 1995;87:446-51.

79. Zhang Q-X, Borg A, Wolf DM, Oesterreich S, Fuqua SA. An estrogen receptor mutant with strong hormone-independent activity from a metastatic breast cancer. Cancer Research. 1997;57:1244-9.

80. Barone I, Iacopetta D, Covington KR, Cui Y, Tsimelzon A, Beyer A, et al. Phosphorylation of the mutant K303R estrogen receptor alpha at serine 305 affects aromatase inhibitor sensitivity. Oncogene. 2010;29:2404-14.

81. Cui Y, Zhang M, Pestell R, Curran EM, Welshons WV, Fuqua SAW. Phosphorylation of estrogen receptor alpha blocks its acetylation and regulates estrogen sensitivity. Cancer Research. 2004;64:9199-208.

82. Giordano C, Cui Y, Barone I, Ando S, Mancini MA, Berno V, et al. Growth factor-induced resistance to tamoxifen is associated with a mutation of estrogen receptor alpha and its phosphorylation at serine 305. Breast Cancer Res Treat. 2010;119:71-85.

83. Fuqua SA, Wiltschke C, Zhang QX, Borg A, Castles CG, Friedrichs WE, et al. A hypersensitive estrogen receptor alpha mutation in premalignant breast lesions. Cancer Research. 2000;60:4026-9.

84. Herynk MH, Parra I, Cui Y, Beyer A, Wu M-F, Hilsenbeck SG, et al. Association between the estrogen receptor alpha A908G mutation and outcomes in invasive breast cancer. Clinical Cancer Research. 2007;13:3235-43.

85. Conway K, Parrish E, Edmiston S, Tolbert D, Tse C-K, Geradts J, et al. The estrogen receptor-alpha A908G (K303R) mutation occurs at a low frequency in invasive breast tumors: results from a population-based study. Breast Cancer Research. 2005;7:R871 - R80.

86. Tokunaga E, Kimura Y, Maehara Y. No hypersensitive estrogen receptor-alpha mutation (K303R) in Japanese breast carcinomas. Breast Cancer Res Treat. 2004;84:289-92.

87. Robinson DR, Wu Y-M, Vats P, Su F, Lonigro RJ, Cao X, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nature genetics. 2013;45:1446-51.

88. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. Nature genetics. 2013.

89. Arpino G, Wiechmann L, Osborne CK, Schiff R. Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: Molecular mechanism and clinical implications for endocrine therapy resistance. Endocr Rev. 2008;29:217-33.

90. Arpino G, Green SJ, Allred DC, Lew D, Martino S, Osborne CK, et al. HER-2 amplification, HER-1 expression, and tamoxifen response in estrogen receptor-positive metastatic breast cancer. Clinical Cancer Research. 2004;10:5670-6.

91. Kahlert S, Nuedling S, van Eickels M, Vetter H, Meyer R, Grohe C. Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. J Biol Chem. 2000:M910345199.

92. Morgan L, Gee J, Pumford S, Farrow L, Finlay P, Robertson J, et al. Elevated Src kinase activity attenuates tamoxifen response in vitro and is associated with poor prognosis clinically. Cancer Biol Ther. 2009;8:1550-8.

93. Qu Z, Van Ginkel S, Roy AM, Westbrook L, Nasrin M, Maxuitenko Y, et al. Vascular endothelial growth factor reduces tamoxifen efficacy and promotes metastatic colonization and desmoplasia in breast tumors. Cancer Research. 2008;68:6232-40.

94. McLeskey SW, Kurebayashi J, Honig SF, Zwiebel J, Lippman ME, Dickson RB, et al. Fibroblast growth factor 4 transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. Cancer Research. 1993;53:2168-77.

95. Jordan NJ, Gee JMW, Barrow D, Wakeling AE, Nicholson RI. Increased constitutive activity of PKB/Akt in tamoxifen resistant breast cancer MCF-7 cells. Breast Cancer Res Treat. 2004;87:167-80.

96. Shoman N, Klassen S, McFadden A, Bickis MG, Torlakovic E, Chibbar R. Reduced PTEN expression predicts relapse in patients with breast carcinoma treated by tamoxifen. Mod Pathol. 2004;18:250-9.

97. Ways DK, Kukoly CA, deVente J, Hooker JL, Bryant WO, Posekany KJ, et al. MCF-7 breast cancer cells transfected with protein kinase C-alpha exhibit altered expression of other protein kinase C isoforms and display a more aggressive neoplastic phenotype. The Journal of Clinical Investigation. 1995;95:1906-15.

98. Gee JMW, Robertson JFR, Ellis IO, Nicholson RI. Phosphorylation of ERK1/2 mitogen-activated protein kinase is associated with poor response to anti-hormonal therapy and decreased patient survival in clinical breast cancer. International Journal of Cancer. 2001;95:247-54.

99. De Amicis F, Thirugnansampanthan J, Cui YK, Selever J, Beyer A, Parra I, et al. Androgen receptor overexpression induces tamoxifen resistance in human breast cancer cells. Breast Cancer Res Treat. 2010;121:1-11.

100. Johnston SRD. New strategies in estrogen receptor-positive breast cancer. Clinical Cancer Research. 2010;16:1979-87.

101. Johnston SRD. Clinical efforts to combine endocrine agents with targeted therapies against epidermal growth factor receptor/human epidermal growth factor receptor 2 and mammalian target of rapamycin in breast cancer. Clinical Cancer Research. 2006;12:1061S-8S.

102. Park SY, Lee HE, Li H, Shipitsin M, Gelman R, Polyak K. Heterogeneity for stem cell-related markers according to tumor subtype and histologic stage in breast cancer. Clinical Cancer Research. 2010;16:876-87.

103. Pece S, Tosoni D, Confalonieri S, Mazzarol G, Vecchi M, Ronzoni S, et al. Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell. 2010;140:62-73.

104. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proceedings of the National Academy of Sciences. 2003;100:3983-8.

105. Zhang M, Atkinson RL, Rosen JM. Selective targeting of radiation-resistant tumor-initiating cells. Proceedings of the National Academy of Sciences. 2010;107:3522-7.

106. Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A, et al. Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proceedings of the National Academy of Sciences. 2009;106:13820-5.

107. Asselin-Labat M-L, Shackleton M, Stingl J, Vaillant F, Forrest NC, Eaves CJ, et al. Steroid hormone receptor status of mouse mammary stem cells. J Natl Cancer Inst. 2006;98:1011-4.

108. Mani SA, Guo W, Liao M-J, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133:704-15.

109. Haddow A, Watkinson JM, Paterson E, Koller P. Influence of synthetic oestrogens on advanced malignant disease. British medical journal. 1944;2:393.

110. Kennedy BJ. Massive estrogen administration in premenopausal women with metastatic breast cancer. Cancer. 1962;15:641-8.

111. Ingle JN, Ahmann DL, Green SJ, Edmonson JH, Bisel HF, Kvols LK, et al. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. New England Journal of Medicine. 1981;304:16-21.

112. Peethambaram PP, Ingle JN, Suman VJ, Hartmann LC, Loprinzi CL. Randomized trial of diethylstilbestrol vs. tamoxifen in postmenopausal women with metastatic breast cancer. An updated analysis. Breast Cancer Res Treat. 1999;54:117-22.

113. Ingle J. Estrogen as therapy for breast cancer. Breast Cancer Res. 2002;4:133 - 6.

114. Song RXD, Santen RJ. Apoptotic action of estrogen. Apoptosis. 2003;8:55-60.

115. Ingle JN. Sequencing of endocrine therapy in postmenopausal women with advanced breast cancer. Clinical Cancer Research. 2004;10:362s-7s.

116. Jordan VC, Obiorah I, Fan P, Kim HR, Ariazi E, Cunliffe H, et al. The St. Gallen Prize Lecture 2011: Evolution of long-term adjuvant anti-hormone therapy: consequences and opportunities. The Breast. 2011;20, Supplement 3:S1-S11.

117. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer. JAMA: The Journal of the American Medical Association. 2009;302:774-80.

118. Lønning PE, Taylor PD, Anker G, Iddon J, Wie L, Jørgensen L-M, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat. 2001;67:111-6.

119. Osipo C, Gajdos C, Cheng D, Jordan VC. Reversal of tamoxifen resistant breast cancer by low dose estrogen therapy. The Journal of Steroid Biochemistry and Molecular Biology. 2005;93:249-56.

120. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst. 2003;95:1597-608.

121. Yao K, Lee E-S, Bentrem DJ, England G, Schafer JIM, ORegan RM, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clinical Cancer Research. 2000;6:2028-36.

122. Liu H, Lee E-S, Gajdos C, Pearce ST, Chen B, Osipo C, et al. Apoptotic action of 17β-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst. 2003;95:1586-97.

123. Santen RJ, Song RX, Zhang Z, Yue W, Kumar R. Adaptive hypersensitivity to estrogen. Clinical Cancer Research. 2004;10:337s-45s.

124. Song RX-D, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst. 2001;93:1714-23.

125. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. The Journal of Steroid Biochemistry and Molecular Biology. 2005;94:131-41.

126. Shim W-S, Conaway M, Masamura S, Yue W, Wang J-P, Kumar R, et al. Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. Endocrinology. 2000;141:396-405.

127. Robertson CN, Roberson KM, Padilla GM, O'Brien ET, Cook JM, Kim C-S, et al. Induction of apoptosis by diethylstilbestrol in hormone-insensitive prostate cancer cells. J Natl Cancer Inst. 1996;88:908-17.

128. Jiang S-Y, Jordan VC. Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. J Natl Cancer Inst. 1992;84:580-91.

129. Saintier D, Khanine V, Uzan B, Ea H, de Vernejoul M, Cohen-Solal M. Estradiol inhibits adhesion and promotes apoptosis in murine osteoclasts in vitro. The Journal of steroid biochemistry and molecular biology. 2006;99:165-73.

130. Nilsen J, Mor G, Naftolin F. Estrogen-regulated developmental neuronal apoptosis is determined by estrogen receptor subtype and the Fas/Fas ligand system. Journal of neurobiology. 2000;43:64-78.

131. Okasha SA, Ryu S, Do Y, McKallip RJ, Nagarkatti M, Nagarkatti PS. Evidence for estradiol-induced apoptosis and dysregulated T cell maturation in the thymus. Toxicology. 2001;163:49-62.

132. Jiang S-y, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Molecular and Cellular Endocrinology. 1992;90:77-86.

133. Santen RJ, Song RX, Masamura S, Yue W, Fan P, Sogon T, et al. Adaptation to estradiol deprivation causes up-regulation of growth factor pathways and hypersensitivity to estradiol in breast cancer cells. Innovative Endocrinology of Cancer: Springer; 2008. p. 19-34.

134. Peter ME, Krammer P. The CD95 (APO-1/Fas) DISC and beyond. Cell Death & Differentiation. 2003;10:26-35.

135. Zhang Y, Zhao H, Asztalos S, Chisamore M, Sitabkhan Y, Tonetti DA. Estradiol-induced regression in T47D:A18/PKCα tumors requires the estrogen receptor and interaction with the extracellular matrix. Molecular Cancer Research. 2009;7:498-510.

136. Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nature Reviews Molecular Cell Biology. 2010;11:621-32.

137. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst. 2005;97:1746-59.

138. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997;91:231-41.

139. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science. 1997;278:687-9.

140. Lewis-Wambi JS, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit. Breast Cancer Res. 2009;11:206.

141. Fan P, Griffith OL, Agboke F, Anur P, Zou X, McDaniel RE, et al. c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. Cancer Research. 2013.

142. Herynk MH, Fuqua SAW. Estrogen receptor mutations in human disease. Endocr Rev. 2004;25:869-98.

143. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proceedings of the National Academy of Sciences. 1996;93:5925-30.

144. Walter P, Green S, Greene G, Krust A, Bornert JM, Jeltsch JM, et al. Cloning of the human estrogen receptor cDNA. Proceedings of the National Academy of Sciences. 1985;82:7889-93.

145. Toft D, Gorski J. A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proceedings of the National Academy of Sciences. 1966;55:1574-81.

146. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, et al. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. Journal of Clinical Endocrinology & Metabolism. 1997;82:4258-65.

147. Gosden J, Middleton P, Rout D. Localization of the human oestrogen receptor gene to chromosome 6q24,Üí q27 by in situ hybridization. Cytogenetic and Genome Research. 1986;43:218-20.

148. Li L, Haynes MP, Bender JR. Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells. Proceedings of the National Academy of Sciences. 2003;100:4807-12.

149. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF. Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. Biochemical and Biophysical Research Communications. 2005;336:1023-7.

150. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF. A variant of estrogen receptor-alpha, hER-alpha36: Transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling. Proceedings of the National Academy of Sciences. 2006;103:9063-8.

151. Lee LMJ, Cao J, Deng H, Chen P, Gatalica Z, Wang Z-Y. ER-alpha36, a novel variant of ER-alpha, is expressed in ER-positive and -negative human breast carcinomas. Anticancer Research. 2008;28:479-83.

152. Shi L, Dong B, Li Z, Lu Y, Ouyang T, Li J, et al. Expression of ER-alpha36, a novel variant of estrogen receptor alpha, and resistance to tamoxifen treatment in breast cancer. Journal of Clinical Oncology. 2009;27:3423-9.

153. Vranic S, Gatalica Z, Deng H, Frkovic-Grazio S, Lee LMJ, Gurjeva O, et al. ER-alpha36, a novel isoform of ER-alpha66, is commonly over-expressed in apocrine and adenoid cystic carcinomas of the breast. Journal of Clinical Pathology. 2011;64:54-7.

154. Fox EM, Davis RJ, Shupnik MA. ER beta in breast cancer- onlooker, passive player, or active protector? Steroids. 2008;73:1039-51.

155. Smith CL, Nawaz Z, O,ÄôMalley BW. Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Molecular Endocrinology. 1997;11:657-66.

156. Webb P, Valentine C, Nguyen P, Price R, Marimuthu A, West B, et al. ERbeta binds N-CoR in the presence of estrogens via an LXXLL-like motif in the N-CoR c-terminus. Nuclear Receptor. 2003;1:4.

157. Fox EM, Davis RJ, Shupnik MA. ERbeta in breast cancer- onlooker, passive player, or active protector? Steroids. 2008;73:1039-51.

158. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, et al. Mechanisms of estrogen action. Physiological Reviews. 2001;81:1535-65.

159. Rosenfeld MG, Glass CK. Coregulator codes of transcriptional regulation by nuclear receptors. Journal of Biological Chemistry. 2001;276:36865-8.

160. Klinge CM. Estrogen receptor interaction with estrogen response elements. Nucleic Acids Research. 2001;29:2905-19.

161. Sanchez R, Nguyen D, Rocha W, White JH, Mader S. Diversity in the mechanisms of gene regulation by estrogen receptors. BioEssays. 2002;24:244-54.

162. Frasor J, Danes JM, Komm B, Chang KCN, Lyttle CR, Katzenellenbogen BS. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology. 2003;144:4562-74.

163. O'Lone R, Frith MC, Karlsson EK, Hansen U. Genomic targets of nuclear estrogen receptors. Molecular Endocrinology. 2004;18:1859-75.

164. Gottlicher M, Heck S, Herrlich P. Transcriptional cross-talk, the second mode of steroid hormone receptor action. J Mol Med. 1998;76:480-9.

165. Metzger D, Ali S, Bornert J-M, Chambon P. Characterization of the amino-terminal transcriptional activation function of the human estrogen receptor in animal and yeast cells. Journal of Biological Chemistry. 1995;270:9535-42.

166. Bjornstrom L, Sjoberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Molecular Endocrinology. 2005;19:833-42.

167. Haas D, White SN, Lutz LB, Rasar M, Hammes SR. The modulator of nongenomic actions of the estrogen receptor (MNAR) regulates transcription-independent androgen receptor-mediated signaling: evidence that MNAR participates in G protein-regulated meiosis in Xenopus laevis oocytes. Molecular Endocrinology. 2005;19:2035-46.

168. Nair SS, Mishra SK, Yang Z, Balasenthil S, Kumar R, Vadlamudi RK. Potential role of a novel transcriptional coactivator PELP1 in histone H1 displacement in cancer cells. Cancer Research. 2004;64:6416-23.

169. Razandi M, Pedram A, Greene GL, Levin ER. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in chinese hamster ovary cells. Molecular Endocrinology. 1999;13:307-19.

170. Pedram A, Razandi M, Levin ER. Nature of functional estrogen receptors at the plasma membrane. Molecular Endocrinology. 2006;20:1996-2009.

171. Levin ER. Membrane oestrogen receptor alpha signalling to cell functions. The Journal of Physiology. 2009;587:5019-23.

172. Welsh AW, Lannin DR, Young GS, Sherman ME, Figueroa JD, Henry NL, et al. Cytoplasmic estrogen receptor in breast cancer. Clinical Cancer Research. 2012;18:118-26.

173. Levin ER. Elusive extranuclear estrogen receptors in breast cancer. Clinical Cancer Research. 2012;18:6-8.

174. Improta-Brears T, Whorton AR, Codazzi F, York JD, Meyer T, McDonnell DP. Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. Proceedings of the National Academy of Sciences. 1999;96:4686-91.

175. Aronica SM, Kraus WL, Katzenellenbogen BS. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proceedings of the National Academy of Sciences. 1994;91:8517-21.

176. Migliaccio A, Di Domenico M, Castoria G, De Falco A, Bontempo P, Nola E, et al. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. The EMBO journal. 1996;15:1292.

177. Castoria G, Migliaccio A, Bilancio A, Di Domenico M, de Falco A, Lombardi M, et al. PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. EMBO J. 2001;20:6050-9.

178. Toran-Allerand CD, Guan X, MacLusky NJ, Horvath TL, Diano S, Singh M, et al. ER-X: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. The Journal of Neuroscience. 2002;22:8391-401.

179. Pedram A, Razandi M, Sainson RCA, Kim JK, Hughes CC, Levin ER. A conserved mechanism for steroid receptor translocation to the plasma membrane. Journal of Biological Chemistry. 2007;282:22278-88.

180. Pedram A, Razandi M, Deschenes RJ, Levin ER. DHHC-7 and -21 are palmitoylacyltransferases for sex steroid receptors. Molecular Biology of the Cell. 2012;23:188-99.

181. Razandi M, Alton G, Pedram A, Ghonshani S, Webb P, Levin ER. Identification of a structural determinant necessary for the localization and function of estrogen receptor alpha at the plasma membrane. Molecular and Cellular Biology. 2003;23:1633-46.

182. Razandi M, Pedram A, Levin ER. Heat shock protein 27 Is required for sex steroid receptor trafficking to and functioning at the plasma membrane. Molecular and Cellular Biology. 2010;30:3249-61.

183. Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER. ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions. Molecular Endocrinology. 2002;16:100-15.

184. Kumar P, Wu Q, Chambliss KL, Yuhanna IS, Mumby SM, Mineo C, et al. Direct interactions with G alpha i and G alpha gamma mediate nongenomic signaling by estrogen receptor alpha. Molecular Endocrinology. 2007;21:1370-80.

185. Wong C-W, McNally C, Nickbarg E, Komm BS, Cheskis BJ. Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. Proceedings of the National Academy of Sciences. 2002;99:14783-8.

186. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature. 2000;407:538-41.

187. Song RX-D, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R, et al. Linkage of rapid estrogen action to MAPK activation by ERalpha-Shc association and Shc pathway activation. Molecular Endocrinology. 2002;16:116-27.

188. Barletta F, Wong C-W, McNally C, Komm BS, Katzenellenbogen B, Cheskis BJ. Characterization of the interactions of estrogen receptor and MNAR in the activation of cSrc. Molecular Endocrinology. 2004;18:1096-108.

189. Chung Y-L, Sheu M-L, Yang S-C, Lin C-H, Yen S-H. Resistance to tamoxifen-induced apoptosis is associated with direct interaction between Her2/neu and cell membrane estrogen receptor in breast cancer. International Journal of Cancer. 2002;97:306-12.

190. Razandi M, Pedram A, Park ST, Levin ER. Proximal events in signaling by plasma membrane estrogen receptors. Journal of Biological Chemistry. 2003;278:2701-12.

191. Duan R, Xie W, Burghardt RC, Safe S. Estrogen receptor-mediated activation of the serum response element in MCF-7 cells through MAPK-dependent phosphorylation of Elk-1. Journal of Biological Chemistry. 2001;276:11590-8.

192. Kousteni S, Han L, Chen J-R, Almeida M, Plotkin LI, Bellido T, et al. Kinase-mediated regulation of common transcription factors accounts for the bone-protective effects of sex steroids. The Journal of Clinical Investigation. 2003;111:1651-64.

193. Pedram A, Razandi M, Kim JK, O'Mahony F, Lee EY, Luderer U, et al. Developmental phenotype of a membrane only estrogen receptor alpha (MOER) mouse. Journal of Biological Chemistry. 2009;284:3488-95.

194. Adlanmerini M, Solinhac R, Abot A, Fabre A, Raymond-Letron I, Guihot A-L, et al. Mutation of the palmitoylation site of estrogen receptor alpha in vivo reveals tissue-specific roles for membrane versus nuclear actions. Proceedings of the National Academy of Sciences. 2013:201322057.

195. Chambliss KL, Wu Q, Oltmann S, Konaniah ES, Umetani M, Korach KS, et al. Non-nuclear estrogen receptor alpha signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice. The Journal of Clinical Investigation. 2010;120:2319.

196. Filardo EJ, Quinn JA, Bland KI, Frackelton AR. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Molecular Endocrinology. 2000;14:1649-60.

197. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology. 2005;146:624-32.

198. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science. 2005;307:1625-30.

199. Hazell GG, Yao ST, Roper JA, Prossnitz ER, O'Carroll A-M, Lolait SJ. Localisation of GPR30, a novel G protein-coupled oestrogen receptor, suggests multiple functions in rodent brain and peripheral tissues. Journal of Endocrinology. 2009;202:223-36.

200. Chevalier N, Vega A, Bouskine A, Siddeek B, Michiels J-F, Chevallier D, et al. GPR30, the non-classical membrane G protein related estrogen receptor, is overexpressed in human seminoma and promotes seminoma cell proliferation. PLoS One. 2012;7:e34672.

201. Kolkova Z, Casslen V, Henic E, Ahmadi S, Ehinger A, Jirstrom K, et al. The G protein-coupled estrogen receptor 1 (GPER/GPR30) does not predict survival in patients with ovarian cancer. J Ovarian Res. 2012;5.

202. Ariazi EA, Brailoiu E, Yerrum S, Shupp HA, Slifker MJ, Cunliffe HE, et al. The g protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. Cancer Research. 2010;70:1184-94.

203. Ignatov A, Ignatov T, Roessner A, Costa SD, Kalinski T. Role of GPR30 in the mechanisms of tamoxifen resistance in breast cancer MCF-7 cells. Breast Cancer Res Treat. 2010;123:87-96.

204. Ignatov A, Ignatov T, WeiBenborn C, Eggemann H, Bischoff J, Semczuk A, et al. G-protein-coupled estrogen receptor GPR30 and tamoxifen resistance in breast cancer. Breast Cancer Res Treat. 2011;128:457-66.

205. Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel PA, et al. Protein kinase C isozymes and the regulation of diverse cell responses. American Journal of Physiology - Lung Cellular and Molecular Physiology. 2000;279:L429-L38.

206. Masso-Welch PA, Verstovsek G, Ip MM. Alterations in the expression and localization of protein kinase C isoforms during mammary gland differentiation. Eur J Cell Biol. 1999;78:497-510.

207. Marshall CJ. Cell signalling - Raf gets it together. Nature. 1996;383:127-8.

208. Balendran A, Hare GR, Kieloch A, Williams MR, Alessi DR. Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms. FEBS Lett. 2000;484:217-23.

209. Fang X, Yu S, Eder A, Mao M, Bast Jr RC, Boyd D, et al. Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway. Oncogene. 1999;18:6635-40.

210. Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. The FASEB Journal. 1995;9:484-96.

211. Gordge P, Hulme M, Clegg R, Miller W. Elevation of protein kinase A and protein kinase C activities in malignant as compared with normal human breast tissue. European Journal of Cancer. 1996;32:2120-6.

212. Borner C, Wyss R, Regazzi R, Eppenberger U, Fabbro D. Immunological quantitation of phospholipid/CA2+-dependent protein kinase of human mammary carcinoma cells: Inverse relationship to estrogen receptors. International Journal of Cancer. 1987;40:344-8.

213. Assender JW, Gee JMW, Lewis I, Ellis IO, Robertson JFR, Nicholson RI. Protein kinase C isoform expression as a predictor of disease outcome on endocrine therapy in breast cancer. Journal of Clinical Pathology. 2007;60:1216-21.

214. Tonetti DA, Gao W, Escarzaga D, Walters K, Szafran A, Coon JS. PKC alpha and ER alpha are associated with triple-negative breast cancers in african american and caucasian patients. International journal of breast cancer. 2012;2012.

215. Fournier DB, Chisamore M, Lurain JR, Rademaker AW, Jordan VC, Tonetti DA. Protein kinase C alpha expression is inversely related to ER status in endometrial carcinoma: possible role in AP-1-mediated proliferation of ER-negative endometrial cancer. Gynecologic oncology. 2001;81:366-72.

216. Schmalz D, Hucho F, Buchner K. Nuclear import of protein kinase C occurs by a mechanism distinct from the mechanism used by proteins with a classical nuclear localization signal. Journal of Cell Science. 1998;111:1823-30.

217. Nakashima S. Protein kinase c alpha (PKCalpha): regulation and biological function. Journal of Biochemistry. 2002;132:669-75.

218. Bornancin F, Parker PJ. Phosphorylation of protein kinase C- alpha on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state. Journal of Biological Chemistry. 1997;272:3544-9.

219. Parekh DB, Ziegler W, Parker PJ. Multiple pathways control protein kinase C phosphorylation. The EMBO journal. 2000;19:496.

220. O'Brian CA, Vogel VG, Eva Singletary S, Ward NE. Elevated protein kinase C expression in human breast tumor biopsies relative to normal breast tissue. Cancer Research. 1989;49:3215-7.

221. Tonetti DA, Morrow M, Kidwai N, Gupta A, Badve S. Elevated protein kinase C alpha expression may be predictive of tamoxifen treatment failure. Br J Cancer. 2003;88:1400-2.

222. Frankel L, Lykkesfeldt A, Hansen J, Stenvang J. Protein Kinase C α is a marker for antiestrogen resistance and is involved in the growth of tamoxifen resistant human breast cancer cells. Breast Cancer Res Treat. 2007;104:165-79.

223. Dean NM, McKay R. Inhibition of protein kinase C-alpha expression in mice after systemic administration of phosphorothioate antisense oligodeoxynucleotides. Proceedings of the National Academy of Sciences. 1994;91:11762-6.

224. Latham C, Zhang A, Nalbanti A, Maner S, Zickert P, Blegen H, et al. Frequent co-amplification of two different regions on 17q in aneuploid breast carcinomas. Cancer genetics and cytogenetics. 2001;127:16-23.

225. Prevostel C, Alvaro V, Deboisvilliers F, Martin A, Jaffiol C, Joubert D. The natural proetin kinase C alpha mutant is present in human thyroid neoplasms Oncogene. 1995;11:669-74.

226. Knauf JA, Ward LS, Nikiforov YE, Nikiforova M, Puxeddu E, Medvedovic M, et al. Isozyme-specific abnormalities of PKC in thyroid cancer: Evidence for post-transcriptional changes in PKC epsilon. Journal of Clinical Endocrinology & Metabolism. 2002;87:2150-9.

227. Lonne G, Cornmark L, Zahirovic I, Landberg G, Jirstrom K, Larsson C. PKCalpha expression is a marker for breast cancer aggressiveness. Molecular Cancer. 2010;9:76.

228. Gupta KP, Ward NE, Gravitt KR, Bergman PJ, O'Brian CA. Partial reversal of multidrug resistance in human breast cancer cells by an N-myristoylated protein kinase C- pseudosubstrate peptide. Journal of Biological Chemistry. 1996;271:2102-11.

229. Gill PK, Gescher A, Gant TW. Regulation of MDR1 promoter activity in human breast carcinoma cells by protein kinase C isozymes α and θ. European Journal of Biochemistry. 2001;268:4151-7.

230. Ahmad S, Glazer RI. Expression of the antisense cDNA for protein kinase C alpha attenuates resistance in doxorubicin-resistant MCF-7 breast carcinoma cells. Molecular Pharmacology. 1993;43:858-62.

231. Abeyweera TP, Chen X, Rotenberg SA. Phosphorylation of alpha6-tubulin by protein kinase C alpha Activates motility of human breast cells. Journal of Biological Chemistry. 2009;284:17648-56.

232. Kim J, Thorne SH, Sun L, Huang B, Mochly-Rosen D. Sustained inhibition of PKC alpha reduces intravasation and lung seeding during mammary tumor metastasis in an in vivo mouse model. Oncogene. 2011;30:323-33.

233. Lahn M, Kohler G, Sundell K, Su C, Li S, Paterson BM, et al. Protein kinase C alpha expression in breast and ovarian cancer. Oncology. 2004;67:1-10.

234. Propper D, Macaulay V, O'Byrne K, Braybrooke J, Wilner S, Ganesan T, et al. A phase II study of bryostatin 1 in metastatic malignant melanoma. British Journal of Cancer. 1998;78:1337-41.

235. Zonder JA, Shields AF, Zalupski M, Chaplen R, Heilbrun LK, Arlauskas P, et al. A phase II trial of bryostatin 1 in the treatment of metastatic colorectal cancer. Clinical Cancer Research. 2001;7:38-42.

236. Winegarden JD, Mauer AM, Gajewski TF, Hoffman PC, Krauss S, Rudin CM, et al. A phase II study of bryostatin-1 and paclitaxel in patients with advanced non-small cell lung cancer. Lung Cancer. 2003;39:191-6.

237. Tam WL, Lu H, Buikhuisen J, Soh BS, Lim E, Reinhardt F, et al. Protein kinase C alpha is a central signaling node and therapeutic target for breast cancer stem cells. Cancer Cell. 2013;24:347-64.

238. Abdelhamid R, Luo J, Vandevrede L, Kundu I, Michalsen B, Litosh VA, et al. Benzothiophene selective estrogen receptor modulators provide neuroprotection by a novel GPR30-dependent mechanism. ACS chemical neuroscience. 2011;2:256-68.

239. Catherino WH, Jordan VC. Increasing the number of tandem estrogen response elements increases the estrogenic activity of a tamoxifen analogue. Cancer Letters. 1995;92:39-47.

240. O'Regan RM, Cisneros A, England GM, Chatterton R, Dragan YP. Effects of the antiestrogens tamoxifen, toremifene, and ICI 182,780 on endometrial cancer growth. J Natl Cancer Inst. 1998;90:1552-8.

241. Lin X, Yu Y, Zhao H, Zhang Y, Manela J, Tonetti DA. Overexpression of PKCα is required to impart estradiol inhibition and tamoxifen-resistance in a T47D human breast cancer tumor model. Carcinogenesis. 2006;27:1538-46.

242. Cummings Sr ESKKA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: Results from the more randomized trial. JAMA: The Journal of the American Medical Association. 1999;281:2189-97.

243. Fisher B, Costantino JP, Redmond CK, Fisher ER, Wickerham DL, Cronin WM, et al. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. J Natl Cancer Inst. 1994;86:527-37.

244. Grese TA, Sluka JP, Bryant HU, Cullinan GJ, Glasebrook AL, Jones CD, et al. Molecular determinants of tissue selectivity in estrogen receptor modulators. Proc Natl Acad Sci U S A. 1997;94:14105-10.

245. Grese TA, Cho S, Finley DR, Godfrey AG, Jones CD, Lugar CW, 3rd, et al. Structure-activity relationships of selective estrogen receptor modulators: modifications to the 2-arylbenzothiophene core of raloxifene. J Med Chem. 1997;40:146-67.

246. Black LJ, Sato M, Rowley ER, Magee DE, Bekele A, Williams DC, et al. Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest. 1994;93:63-9.

247. Fuchs-Young R, Glasebrook AL, Short LL, Draper MW, Rippy MK, Cole HW, et al. Raloxifene is a tissue-selective agonist/antagonist that functions through the estrogen receptor. Ann N Y Acad Sci. 1995;761:355-60.

248. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat M-L, et al. Generation of a functional mammary gland from a single stem cell. Nature. 2006;439:84-8.

249. Harrison H, Simoes B, Rogerson L, Howell S, Landberg G, Clarke R. Oestrogen increases the activity of oestrogen receptor negative breast cancer stem cells through paracrine EGFR and Notch signalling. Breast Cancer Research. 2013;15:R21.

250. Simoes BM, Piva M, Iriondo O, Comaills V, Lopez-Ruiz JA, Zabalza I, et al. Effects of estrogen on the proportion of stem cells in the breast. Breast Cancer Res Treat. 2011;129:23-35.

251. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick M, et al. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. Jama. 2002;288:321-33.

252. Grady D, Rubin SM, Petitti DB, Fox CS, Black D, Ettinger B, et al. Hormone therapy to prevent disease and prolong life in postmenopausal women. Annals of internal medicine. 1992;117:1016-37.

253. Buist DS, Newton KM, Miglioretti DL, Beverly K, Connelly MT, Andrade S, et al. Hormone therapy prescribing patterns in the United States. Obstetrics & Gynecology. 2004;104:1042-50.

254. Manson JE, Chlebowski RT, Stefanick ML, et al. Menopausal hormone therapy and health outcomes during the intervention and extended poststopping phases of the women's health initiative randomized trials. Jama. 2013;310:1353-68.

255. Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in

postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. The Lancet Oncology. 2012;13:476-86.

256. Smith OW. Diethylstilbestrol in the prevention and treatment of complications of pregnancy. Obstetrical & Gynecological Survey. 1949;4:190-1.

257. Hoover RN, Hyer M, Pfeiffer RM, Adam E, Bond B, Cheville AL, et al. Adverse health outcomes in women exposed in utero to diethylstilbestrol. New England Journal of Medicine. 2011;365:1304-14.

258. Titus-Ernstoff L, Hatch E, Hoover R, Palmer J, Greenberg E, Ricker W, et al. Long-term cancer risk in women given diethylstilbestrol (DES) during pregnancy. British Journal of Cancer. 2001;84:126.

259. Paige LA, Christensen DJ, Gr√∏n H, Norris JD, Gottlin EB, Padilla KM, et al. Estrogen receptor (ER) modulators each induce distinct conformational changes in ER alpha and ER beta. Proceedings of the National Academy of Sciences. 1999;96:3999-4004.

260. McKenna NJ, Lanz RB, O'Malley BW. Nuclear Receptor Coregulators: Cellular and Molecular Biology 1. Endocr Rev. 1999;20:321-44.

261. Dutertre M, Smith CL. Molecular Mechanisms of Selective Estrogen Receptor Modulator (SERM) Action. Journal of Pharmacology and Experimental Therapeutics. 2000;295:431-7.

262. Nicholson RI, Johnston SR. Endocrine therapy- current benefits and limitations. Breast Cancer Res Treat. 2005;93:3-10.

263. Schiff R, Massarweh SA, Shou J, Bharwani L, Arpino G, Rimawi M, et al. Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. Cancer chemotherapy and pharmacology. 2005;56:10-20.

264. Longo M, Brama M, Marino M, Bernardini S, Korach KS, Wetsel WC, et al. Interaction of estrogen receptor alpha with protein kinase C alpha and c-Src in osteoblasts during differentiation. Bone. 2004;34:100-11.

265. Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, et al. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science. 2009;324:787-90.

266. Clegg NJ, Wongvipat J, Joseph JD, Tran C, Ouk S, Dilhas A, et al. ARN-509: A novel antiandrogen for prostate cancer treatment. Cancer Research. 2012;72:1494-503.

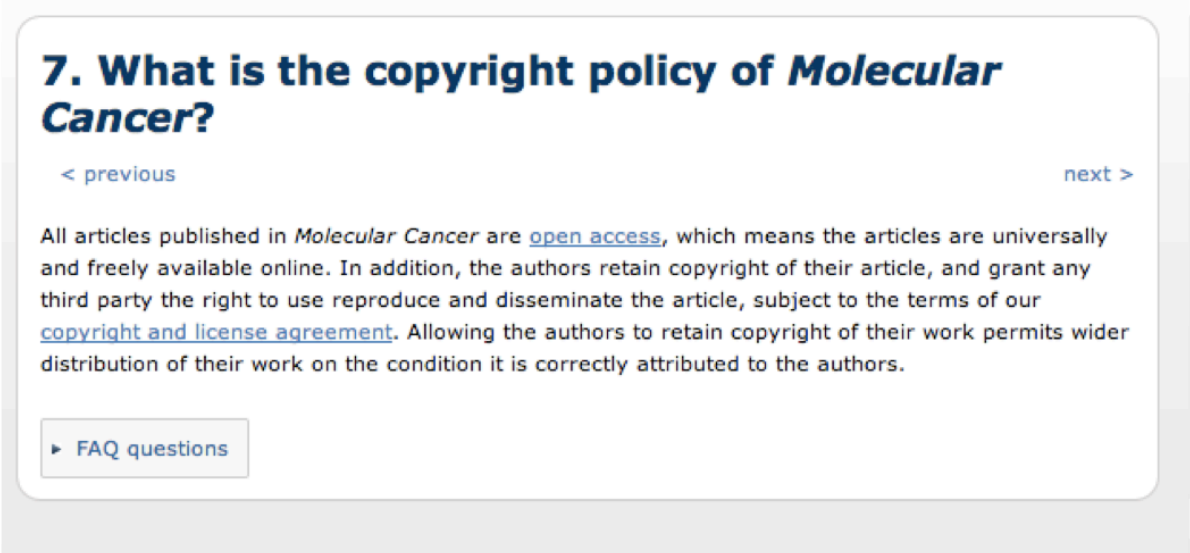
267. Place A, Jin Huh S, Polyak K. The microenvironment in breast cancer progression: biology and implications for treatment. Breast Cancer Research. 2011;13:227.

268. Parsons M, Keppler MD, Kline A, Messent A, Humphries MJ, Gilchrist R, et al. Site-directed perturbation of protein kinase C-integrin interaction blocks carcinoma cell chemotaxis. Molecular and Cellular Biology. 2002;22:5897-911.

269. Alzamora R, Harvey BJ. Direct binding and activation of protein kinase C isoforms by steroid hormones. Steroids. 2008;73:885-8.

270. Tanaka Y, Gavrielides MV, Mitsuuchi Y, Fujii T, Kazanietz MG. Protein Kinase C Promotes Apoptosis in LNCaP Prostate Cancer Cells through Activation of p38 MAPK and Inhibition of the Akt Survival Pathway. Journal of Biological Chemistry. 2003;278:33753-62.

7. APPENDIX I- Copyright permissions



7. What is the copyright policy of *Molecular Cancer*?

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APPENDIX II- Animal Care Committee Approval Letters

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

October 31, 2013

Debra A. Tonetti
Biopharmaceutical Sciences
M/C 865

Dear Dr. Tonetti:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **9/17/2013**. *The protocol was not initiated until final clarifications were reviewed and approved on* **10/19/2013**. *The protocol is approved for a period of* ***3*** years with annual continuation.

Title of Application: Hormonal and Targeted Therapies for Breast Cancer

ACC Number: 13-154

Initial Approval Period: 10/19/2013 to 9/17/2014

Current Funding: ***Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol. UIC is the only performance site currently approved for this protocol.***

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW's "*What Investigators Need to Know about the Use of Animals*" (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/*ss*
cc: BRL, ACC File, Huiping Zhao, Mary Ellen Molloy

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (OACIB) (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

10/19/2012

Debra A. Tonetti
Biopharmaceutical Sciences
M/C 865

Dear Dr. Tonetti:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and **renewed on 10/19/2012.**

Title of Application: **Hormonal and Targeted Therapies for Breast Cancer**
ACC NO: **10-172**
Original Protocol Approval: **11/1/2010 (3 year approval with annual continuation required).**
Current Approval Period: **10/19/2012 to 10/19/2013**

Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Grant Title**			**Portion of Grant Matched**
NIH	*PKCalpha as a Marker of Logical Therapeutic Approaches to Breast Cancer*			*Matched*
Grant Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Grant PI**
RO1 CA122914 (yrs 1-5) A1 version	*Funded*	*2006-07410*	*UIC*	*Debra Tonetti*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/*kg*
cc: BRL, ACC File

Phone (312) 996-1972 • Fax (312) 996-9088

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (OACIB) (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

10/19/2011

Debra A. Tonetti
Biopharmaceutical Sciences
M/C 865

Dear Dr. Tonetti:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and **renewed on 10/19/2011.**

Title of Application: **Hormonal and Targeted Therapies for Breast Cancer**
ACC NO: **10-172**
Original Protocol Approval: **11/1/2010 (3 year approval with annual continuation required).**
Current Approval Period: **10/19/2011 to 10/19/2012**

Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Grant Title**			**Portion of Grant Matched**
NIH	*PKCalpha as a Marker of Logical Therapeutic Approaches to Breast Cancer*			*Matched*
Grant Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Grant PI**
RO1 CA122914 (yrs 1-5) A1 version	*Funded*	*2006-07410*	*UIC*	*Debra Tonetti*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Richard D. Minshall

Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/*kg*
cc: BRL, ACC File

Phone (312) 996-1972 • Fax (312) 996-9088

8. VITA

Education:

2008 -	**Ph.D.**, Biopharmaceutical Sciences (Advisor: Prof. Debra A. Tonetti) **University of Illinois at Chicago**, College of Pharmacy, Chicago, Illinois USA
2004 - 2008	**B.S.**, Biological Sciences **University of Illinois at Chicago**, College of Liberal Arts and Sciences, Chicago, Illinois USA

Honors and Awards:

2013	**2nd Place Graduate Student Poster Competition** Great Lakes Chapter of ASPET 2013 Annual Meeting
2013	**Innovation Award** Project Title: Selective Estrogen Mimics for the Treatment of Tamoxifen-Resistant Breast Cancer. College of Pharmacy Research Day 2013, Office of Technology Management, UIC, Chicago, IL
2012	**Deiss Award for Graduate Student Research** Project Title: Novel Benzothiophene SERMs for the Treatment of Tamoxifen-Resistant Breast Cancer. Graduate College, UIC, Chicago, IL
2011	**Graduate College Student Presenter Award** Graduate College, UIC, Chicago, IL
2011	**Graduate Student Council Travel Award** Graduate College, UIC, Chicago, IL
2009-2010	**Pharmacological Sciences Training Program-Ruth L. Kirschstein-National Research Service Award T32** NIH-NIGMS (National Institute of General Medical Sciences)
2009	**Women in Science and Engineering (WISE) Travel Award** UIC, Chicago, IL
2009	**Graduate College Student Presenter Award** Graduate College, UIC, Chicago, IL
2009	**Graduate Student Council Travel Award** Graduate College, UIC, Chicago, IL
2008	**Departmental Distinction in Biological Sciences** College of Liberal Arts and Sciences, UIC, Chicago, IL

Professional and Extracurricular Activities:

Oct. 2012 -	**Member**, The American Society for Pharmacology and Experimental Therapeutics (ASPET)
Sept. 2010 -	**Member,** American Association for Cancer Research (AACR)
Sept. 2010 -	**Member,** European Association for Cancer Research (EACR)
Aug. 2009 -	**Member,** American Association for the Advancement of Science (AAAS)
Aug. 2009 -	**Member,** Controlled Release Society Student Chapter (CRS-IL), UIC, Chicago, IL
Sept. 2007 -	**Member,** American Association of Pharmaceutical Scientists (AAPS) Student Chapter, UIC, Chicago, IL

Academic Experience:

Aug. 2008 - IL	**Graduate Student**, Department of Biopharmaceutical Sciences, UIC, Chicago,
Aug. 2011 - IL	**Research Assistant**, Department of Biopharmaceutical Sciences, UIC, Chicago,
Aug. 2008 - May 2009	**Teaching Assistant**, Department of Biopharmaceutical Sciences, UIC, Chicago, IL

Students Supervised:

Graduate Students

May 2013 -	**Jennifer Le**, PharmD Student, UIC, Chicago, IL
Jan. 2013 - April 2013	**Sabrina Sanchez**, PharmD Student, UIC, Chicago, IL
Jan. 2011 - May 2011	**Lamiaa El-Shennawy,** Fullbright Scholar, Biopharmaceutical Sciences, UIC, Chicago, IL

Undergraduate Students

Nov. 2011- May 2012	**Helen Sweiss,** Biological Sciences (Honors College), UIC, Chicago, IL
Dec. 2011	**Sean Forte**, Molecular and Cellular Biology, University of Illinois, Urbana,IL

Publications:

Research Papers

Kim HY, Sohn J, Wijewickrama GT, Edirisinghe P, Gherezghiher T, Hemachandra M, Lu PY, Chandrasena RE, Molloy ME, Tonetti DA, Thatcher GR. Click synthesis of estradiol-cyclodextrin conjugates as cell compartment selective estrogens. Bioorg Med Chem. 2010 Jan 15;18(2):809-21.

Perez White B*, Molloy ME*, Zhao H, Zhang Y, Tonetti D. Extranuclear ERalpha is associated with regression of T47D PKCalpha-overexpressing, tamoxifen-resistant breast cancer. Molecular Cancer. 2013;12:34. * Authors contributed equally

Conference Proceedings

1. Molloy ME, White BP, Michalsen BT, Zhao H, Thatcher GRJ, Tonetti DA. Selective estrogen mimics for the treatment of tamoxifen-resistant breast cancer. Great Lakes Chapter of ASPET 2013 Annual Meeting **2013**, Chicago, IL**.**

2. Molloy ME, White BP, Michalsen BT, Zhao H, Thatcher GRJ, Tonetti DA. Selective estrogen mimics for the treatment of tamoxifen-resistant breast cancer. College of Pharmacy Research Day **2013**, Chicago, IL**.**

3. Molloy ME, Tonetti DA. Overexpression of PKCα in T47D breast cancer cells alters the $CD44^{high}/CD24^{low}$ population. UIC Cancer Center Research Forum **2012**, Chicago, IL**.**

4. Molloy ME, Tonetti DA. Overexpression of PKCα in T47D breast cancer cells alters the $CD44^{high}/CD24^{low}$ population. College of Pharmacy Research Day **2012**, Chicago, IL**.**

5. White BP, Zhao H, Kundu M, Molloy ME, Tonetti DA. Protein kinase C α overexpression is associated with loss of membrane-associated E-cadherin and β-catenin in T47D xenograft breast tumors. American Association for Cancer Research (AACR) National Meeting **2012**, Chicago, IL**.**

6. Molloy ME, Tonetti DA. Overexpression of PKCα in T47D breast cancer cells alters the $CD44^{high}/CD24^{low}$ population. AACR- Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications **2011**, San Francisco, CA**.**

7. Molloy ME, Tonetti DA. Potential role for Src kinase inhibitors in PKCα-overexpressing breast cancer. College of Pharmacy Research Day **2011**, Chicago, IL**.**

8. Molloy ME, Tonetti DA. Potential role for Src kinase inhibitors in PKCα-overexpressing breast cancer. American Association for Cancer Research (AACR) National Meeting **2011**, Orlando, FL**.**

9. Molloy ME, Thatcher GRJ, Bolton JL, Tonetti DA. 2-(4-hydroxyphenyl)-benzo[b]thiophen-6-ol, an estrogen-like compound, induces apoptosis in T47D/PKCα breast cancer cells. ASPET **2010**, Chicago, IL.

10. Molloy ME, Thatcher GRJ, Bolton JL, Tonetti DA. 2-(4-hydroxyphenyl)-benzo[b]thiophen-6-ol, an estrogen-like compound, induces apoptosis in T47D/PKCα breast cancer cells. Pharmaceutical Graduate Student Research Meeting **2009**, West Lafayette, IN.

11. Molloy ME, Thatcher GRJ, Bolton JL, Tonetti DA. 2-(4-hydroxyphenyl)-benzo[b]thiophen-6-ol, an estrogen-like compound, induces apoptosis in

T47D/PKCα breast cancer cells. San Antonio Breast Cancer Symposium **2009**, San Antonio, TX.