IDENTIFYING NEW ACTIONS OF ESTROGEN RECEPTOR TARGETABLE WITH SMALL MOLECULES

BY

NEAL ANDRUSKA

DISSERTATION

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Doctoral Committee

Professor David Shapiro, Chair
Professor Paul Hergenrother
Associate Professor Lin-Feng Chen
Assistant Professor Eric Bolton
ABSTRACT

Estrogens, acting via estrogen receptor α (ERα), stimulate the proliferation and metastatic potential of breast cancers, and likely some ovarian, endometrial, and cervical cancers. Endocrine therapy targeting these cancers often leads to development of resistance. Ovarian cancers do not respond to endocrine therapy. The presence of ERα in many resistant tumors suggested the existence of additional modes of ERα action that could be targeted with small molecule biomodulators.

To identify new small molecule inhibitors that target the estrogen-ERα axis in cancer cells resistant to current therapies, we developed and implemented an unbiased pathway-directed screen of ~150,000 small molecules. Using screening and functional validation, I identified BHPI, a potent non-competitive small molecule ERα inhibitor. At 100 nM, BHPI completely inhibited estrogen-induced proliferation in ERα containing breast, ovarian and endometrial cancer cells with no effect at 10,000 nM in counterpart ERα negative cells. BHPI effectively targets cancer cells that no longer depend on estrogens for growth, kills drug-resistant breast and ovarian cancer cells, and restores paclitaxel sensitivity to multi-drug resistant cancer cells. In a mouse xenograft, BHPI at 15 mg/kg daily for 10 days induced rapid and substantial regression of 48/52 large tumors and was not toxic.

BHPI's effectiveness in a broad range of ERα-containing breast, ovarian and endometrial cancer cells is due to its ability to target the endoplasmic reticulum stress sensor pathway, the unfolded protein response (UPR). In ERα positive cancer cells, BHPI opens the endoplasmic reticulum IP3R calcium channel, triggering efflux of
calcium into the cytosol, strongly activating all three arms of the UPR. BHPI activates the PERK arm of the UPR, which leads to phosphorylation of eukaryotic initiation factor 2α (eIF2α), and potent inhibition of protein synthesis. To restore endoplasmic reticulum calcium, calcium-ATPase pumps are activated, but the calcium rapidly leaks back out through the open IP₃R channel, creating an ATP-depleting futile cycle. ATP depletion activates the energy sensor, AMPK. The influx of calcium into the cytosol leads to phosphorylation and inactivation of eukaryotic elongation factor 2 (eEF2), inhibiting the elongation step of protein synthesis. Inhibiting protein synthesis at a second site prevents synthesis of chaperones and other proteins that normally resolve UPR stress. BHPI kills ERα positive cancer cells by triggering a lethal cascade encompassing sustained activation of the UPR, persistent inhibition of protein synthesis, and depletion of ATP stores.

BHPI selectively targets the UPR in ERα positive cancer cells by distorting a previously unknown ability of estrogen-ERα to stimulate a weak and transient UPR activation that protects cancer cells from subsequent stress. This anticipatory activation of the UPR by estrogen-ERα is essential for estrogen-ERα-induced gene expression and cell proliferation, and is a new paradigm by which estrogens promote therapy-resistance and tumor progression. Bioinformatic analysis of data from ≈1,000 ERα positive breast tumors shows that estrogen-ERα and UPR activity become elevated during tumor development and that a UPR gene signature is a powerful new prognostic biomarker predictive of resistance to tamoxifen therapy, time to relapse, and overall survival.
We employed a novel approach to identifying new pathways and cancer biomarkers in which unbiased high throughput screening is used to “interrogate the cell”. For a well-studied protein, such as ERα, it was unclear whether this approach could identify new pathways and biomarkers and promising new drug candidates. These studies demonstrate the potential of small molecules identified through targeted cell-based screening to reveal and validate new pathways of action, therapeutic drug targets, and small molecule therapeutic candidates; even in a system as intensively studied as ERα positive breast cancer.
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LIST OF ABBREVIATIONS

4-OHT, 4-hydroxy-tamxofien
AF-1, activation function 1
AF-2 activation function 2
AMPK, AMP kinase
ATF6α, activating transcription factor 6 α
BHPI, 3,3-bis(4-hydroxyphenyl)-7-methyl-1,3, dihydro-2H-indol-2-one
CHOP, CCAAT/enhancer-binding protein homologous protein
DNA, deoxyribonucleic acid
DBD, DNA binding domain
E2, 17β-Estradiol
eEF2, eukaryotic elongation factor 2
EGF, epidermal growth factor
EGFR, epidermal growth factor receptor
eIF2α, eukaryotic initiation factor 2α
EnR, endoplasmic reticulum
ER, estrogen receptor
ERα, estrogen receptor α
ERβ, estrogen receptor β
ERE, estrogen response element
ERK, extracellular receptor kinase
GREB1, growth regulation by estrogen in breast cancer 1
ICI, ICI 182,780/Fulvestrant/Faslodex
IGF-1, insulin-like growth factor-1
IRE1α, inositol-requiring enzyme 1α
ISR, integrated stress response
LBD, ligand binding domain
PERK, protein kinase R (PKR)-like endoplasmic reticulum kinase
PI3K, phosphoinositide-3-kinase
SDF-1, stromal cell-derived factor 1
SERM, selective estrogen receptor modulator
SRC, steroid receptor coactivator
RAL, raloxifene
TFF1, trefoil factor 1/ps2
THG, thapsigargin
TUN, tunicamycin
UPR, unfolded protein response
CHAPTER 1
INTRODUCTION

Estrogen Receptors

Estrogens play a fundamental role in the development of the female reproductive system, and regulate the growth and function of tissues such as the breast, uterus, and ovaries. The most potent estrogen, 17β-estradiol (E₂), is present in the circulation of females from the onset of puberty to menopause, and can promote abnormal cell growth and eventually lead to the development of cancer. The effects of estrogens are largely mediated through binding to estrogen receptor (ER), which belongs to the superfamily of steroid nuclear receptors.

There are two major subtypes of estrogen receptor, ERα and ERβ. ERα is found on chromosome 6q and encodes a 595 amino acid protein, while ERβ is found on chromosome 14q and encodes a 530 amino acid protein. ER isoforms have distinct tissue expression patterns. ERα is expressed widely throughout the body with significant expression in the uterus, ovary (theca cells), breast, bone, and regions of the brain. ERβ is also expressed widely throughout the body, but is expressed in particularly high amounts in the prostate, ovary (granulosa), colon, lung, bone marrow, and testis, and in relatively low amounts in the uterus (1).

Estrogen receptors are composed of six functional regions or domains. The N-terminal (A/B) domain contains a ligand-independent and functionally minor activating function (AF)-1 domain (2, 3). The DNA binding domain (DBD) or C region, consists of 66-amino acid motif that forms two zinc fingers that interact with DNA. The hinge domain (D), contains the nuclear localization signal and interacts with heat shock
proteins. The E region, or ligand binding domain (LBD), interacts with E₂, and overlaps with the ligand-dependent, major transcriptional activation function (AF)-2 domain. The C-terminal region, or F domain, inhibits dimerization of the receptor until it is bound by ligand.

The Genomic Actions of Estrogen Receptor α (ERα)

The best characterized action of estrogens, acting through ERα, is the regulation of nuclear gene transcription. In the nucleus, unliganded monomeric ERα is complexed with heat shock proteins (HSPs). When estrogens diffuse into the cell and bind to the LBD of the ERα-HSP complex, ERα dissociates from HSPs. The LBD of ERα then undergoes a critical conformation change in which helix 12 covers the ligand binding pocket of ERα. ERα homodimerizes with another ERα molecule and binds to specific DNA response elements, or estrogen response elements (EREs). Once bound to EREs, the AF-2 domain of ERα, which contains an LXXLL-like motif, mediates the recruitment and docking of an array of coactivators including the p160 superfamily (SRC-1, SRC-2/GRIP1/TIF2, and SRC-3/AIB1) (4-6). The C-terminal domain of p160 coactivators then recruits additional coactivators including the p300/CBP histone acetyltransferase, CARM1 methyltransferase, and ubiquitin ligases, Ubc and Ubl. These additional coactivators acetylate, methylate, or ubiquitinate specific residues in the multi-protein complex that leads to chromatin remodeling and recruitment of RNA polymerase (RNAP) to the promoter of ERα-regulated genes, resulting in the transcriptional activation of ERα-target genes.
While ERα commonly interacts directly with estrogen-response elements (EREs) (7-9), ERα can also act through a tethering mechanism to indirectly regulate gene transcription. E₂-ERα can bind to ERE half sites and be brought to DNA through interactions with activator protein 1 (AP1), Sp1, and NFκB transcription factors bound at SP1 and AP-1 sites (10-15). Through these mechanisms estrogen receptor modulates transcription of hundreds of genes, leading to the numerous physiological responses of estrogens.

**Non-Genomic Pathways of Estrogen Receptor Action**

While estrogens promote a slow transcriptional response, they also trigger rapid, non-genomic signaling cascades that contribute to estrogen-stimulated proliferation, survival, and migration. These events occur within seconds to minutes and can be activated by estrogen receptor lacking a nuclear localization signal or by ER targeted to the plasma membrane, suggesting that these non-genomic effects of estrogen are mediated through additional receptors for estrogens that are not localized to the nucleus. A fraction of the ERα and ERβ in many cells localizes to the plasma membrane and to cytoplasmic organelles including the mitochondria and endoplasmic reticulum (16, 17). While there is general agreement that some sort of estrogen-binding receptor is present on the plasma membrane, there remains considerable disagreement as to the nature of the receptor. The most popular candidates are some forms of the nuclear receptors ERα and ERβ that allow membrane localization, and GRP30 or some other type of membrane receptor.
Rapid estrogen signaling from the plasma membrane was first discovered many decades ago (18, 19), and it is suggested that 5-10% of cellular ERα is localized to the plasma membrane in most cells. Numerous reports suggest that ERα can associate with the plasma membrane through posttranslational modifications (20). ERα is trafficked to the plasma membrane through interactions with the protein, caveolin-1 (21), which assists transport of ERα to caveolae rafts in the membrane. Studies have shown that cysteine 447 in the E domain of ERα functions as part of a larger palmitoylation motif, which allows ERα to interact with caveolin-1 (20, 22). Membrane-localized ERα has been shown to associate with and activate several G proteins, such as Gα and Gβγ proteins (23, 24), resulting in the generation of cAMP and calcium, and the activation of numerous kinases (Src, PI3K, ERK, and AKT). While the precise functions of plasma membrane localized ERα remains elusive, these effects of plasma membrane localized ERα may modulate the transcriptional effects of nuclear ERα (25).

Numerous studies have also investigated whether the non-genomic effects of E2 are mediated through the orphan GPCR, known as GPR30. GPR30 is overexpressed in ERα+ breast cancer cell compared to ERα- cells (26). In addition, GPR30 is expressed in a number of tissues with the highest levels of expression in breast, placenta, and heart tissue (26). It is also expressed in the endometrium, ovary, and vascular endothelium. Nanomolar amounts of E2, tamoxifen, and fulvestrant all bind to GPR30 (27). However, ERα ligands like DES, have minimal binding affinity for GPR30. While GPR30 may contribute to the nongenomic effects of estrogens, considerable controversy exists concerning the localization and functional role of the receptor in
estrogen signaling. While non-genomic signaling by ERα likely helps to facilitate the genomic actions of ERα, it alone cannot stimulate growth (28), suggesting the estrogen-dependent proliferation in the breast requires nuclear genomic signaling. Furthermore, four different GPR30 knockout mice have been created, and each has shown minimal phenotypic alterations with no disruption of normal development of mammary or reproductive tissue (29). In contrast, ERα knockout mice produce profound effects on development of the uterus, mammary gland, and ovary (30).

Several truncated ERα and ERβ proteins have also been reported to play a role in the non-genomic actions of estrogens (31), and another putative estrogen receptor has been reported in the central nervous system. In the hypothalamus, estradiol is believed to bind to a GPCR, which is unrelated to GPR30 (32). Other studies have shown that a diphenylacrylamide ligand, known as STX, displayed no binding affinity for ERα or ERβ, but mimicked the effects of E2 in animal studies and may play a role in energy homeostasis (33). Thus, ongoing work continues to identify additional estrogen receptors, which may play important roles in promoting the actions of estrogens.

The most notable nongenomic effects of estrogen-ERα include rapid activation of phosphatidylinositol-3-kinase leading to the activation of Akt (34-36), activation of p42/p44 mitogen-activated protein kinases (MAPKs) (37), and increases in intracellular calcium through activation of PKA and PLCγ (38-40). Activation of these pathways and others helps to support the genomic actions of ERα, and the integration of these molecular events is required to obtain the complete cellular response to estrogens.
Endocrine Therapy in Breast Cancer Treatment

Sustained exposure to estrogens is a well-established cause of breast cancer (41, 42), underpinning the development of targeted therapies that inhibit the actions of estrogens (43-45). At diagnosis, more than two-thirds of breast cancers are classified as ER\(\alpha\) positive (46), and interference with estrogen action has remained a mainstay in breast cancer treatment for many decades (43, 45, 47). Endocrine therapies fall into two broad categories: (i) tamoxifen, fulvestrant/Faslodex/ICI 182,780 and other antiestrogens that compete with estrogens for binding to ER\(\alpha\) and (ii) aromatase inhibitors that inhibit the production of estrogen.

The functional significance of ER\(\alpha\) in breast cancer has made it the foremost target for the development of anti-hormonal therapies aimed at preventing and treating breast cancer. Antiestrogens fall into two broad categories. The first class is represented by selective estrogen receptor modulators (SERMs) and includes tamoxifen, raloxifene, and toremifene. Theses SERMs inhibit AF-2 activation and thereby prevent ER\(\alpha\) activity, but do not prevent activation of the AF-1 domain of ER\(\alpha\). Since ER\(\alpha\) activity in the breast epithelium is largely due to AF-2 activation, tamoxifen and other SERMs function as antagonists in breast cells. In other tissues, such as the uterus, AF-1 activity is more significant and some SERMs function as partial agonist. Thus, SERMS function as mixed agonists/antagonists. Binding of SERMs, such as 4-OHT and raloxifene, induces a distinct conformational change in ER\(\alpha\) that prevents coactivator recruitment and enhances recruitment of corepressor molecules. At the molecular level, the side-chains of antiestrogens protrude from the ligand-binding pocket of ER\(\alpha\), which prevents helix-12 from sealing the ligand-binding-pocket, which
prevents recruitment of coactivators to ERα (48, 49). In large-scale randomized clinical trials, initiating tamoxifen therapy for 5-years immediately following surgery in patients diagnosed with early-stage ERα+ breast cancer reduced breast cancer mortality by 28% (50).

The second class of antiestrogens are the ‘pure’ antagonists, which includes ICI 182,780 (Fulvestrant/Faslodex). Pure antagonists prevent activation of the AF-1 and AF-2 domains of ERα. At the molecular level, the long hydrophobic side-chain of ICI 182,780 (ICI) prevents the binding of helix-12 to the surface of the ligand-binding domain, which prevents ERα transcriptional activation. In addition, ICI 182,780 (ICI) promotes the rapid destruction of ERα in breast cancer cells in cell culture, the mouse uterus, and breast tumors in vivo (51-53). While ICI has shown considerable efficacy in cell culture at concentrations between 100-1000 nM, the average concentration of ICI in patients after 6 months of dosing was only 10 nM (54), demonstrating that it is difficult to achieve comparable circulating plasma concentrations of ICI in patients. Even though fulvestrant has not proven to be better than SERMs, ICI is not cross-resistant with tamoxifen in several athymic models of tamoxifen-stimulated breast and endometrial cancer (55-57), and therefore represents an additional treatment option for women with breast cancer whose disease fails to respond to other therapies.

As the second arm of endocrine therapy, aromatase inhibitors block the synthesis of estrogens, and therefore abolish an endocrine response in ERα+ breast cancers. Since most cases of breast cancer occur in post-menopausal women, ovarian synthesis of estrogens has ceased. However, studies in post-menopausal women have shown that estrogen concentrations within breast tumors can be 20-fold higher than the
blood plasma concentration of estrogen, suggesting that non-ovarian sources of E₂ play an important role in driving ERα⁺ breast cancers. Local estrogen synthesis is dependent on the p450 enzyme, aromatase, which converts androgens (testosterone and androstenedione) into estrogens (estradiol and estrone). Thus, blocking estrogen synthesis using aromatase inhibitors (AIs) represents an effective way of indirectly targeting ERα in breast cancer. There are two classes of agents used to prevent the CYP19 aromatase enzyme from synthesizing estrogen: competitive inhibitors (letrozole or anastrozole) and suicide inhibitors (exemestane). Both experimental and clinical data now have confirmed that aromatase inhibitors achieve greater response rates compared to tamoxifen (58-60), which may be due to the fact that tamoxifen functions as a partial agonist and therefore may limit tamoxifen's clinical efficacy.

**Resistance to Current Endocrine Therapies**

While current endocrine therapies targeting estrogen represent a significant advance in breast cancer therapy, many women develop resistance to these therapies. Selection and outgrowth of breast cancers resistant to endocrine therapy is common, and most deaths due to breast cancer are in patients with ERα⁺ tumors (61). In ERα⁺ breast cancer, one-third of women treated with tamoxifen for 5 years will develop recurrent disease within 15 years. The development of resistance to tamoxifen and to aromatase inhibitors remains a key problem in breast cancer treatment (62). A number of mechanisms are involved in the development of resistance to endocrine therapies.

Numerous studies have suggested that altered expression of ERα plays an important role in resistance to tamoxifen therapy. Loss of ERα expression remains the
primary mechanism of de novo or intrinsic resistance to endocrine therapies. While some studies have implicated the loss of ERα expression as a mechanism of acquired resistance, this only occurs in 15-20% of resistant breast cancers, and represents a small subset of therapy resistant tumors. In contrast, ERα overexpression occurs in the majority of breast cancers in postmenopausal women, and gross overexpression of ERα has been associated with increased recurrence and reduced response to tamoxifen therapy.

Given the complexity of ERα transcriptional activation, endocrine resistance is often linked to changes in the levels and activity of several proteins involved in forming transcriptional complexes with ERα. This includes other transcription factors, such as activator protein 1 (AP1) and NF-κB, which interact with ERα and are often associated with endocrine resistance. It also includes expression of ERα co-activator molecules, particularly nuclear receptor co-activator 3 (nCOA3/AIB1/SRC3), which can lead to constitutive ERα-mediated transcription, and can confer resistance in xenograft models and is associated with reduced responsiveness to tamoxifen therapy. The coactivator pElp1 is also associated with tamoxifen resistance. pElp1 localizes to the cytosol and functions as a scaffolding protein, which allows ERα to interact with and activate SRC, which in turn leads to activation of MAPKs.

Accumulating data suggest that growth-factor-receptor signaling pathways might be overexpressed in tumors resistant to tamoxifen, suggesting that over time, breast cancer cells use alternative intracellular signaling pathways to enhance and activate ERα signaling. While ERα activity has classically been viewed as requiring ligand binding, bidirectional cross-talk between ERα and growth factor receptor signaling
pathways can stimulate post-translational modifications of ERα and stimulate ERα activation in a ligand-independent fashion (63-65). Increased expression of members of the epidermal growth factor receptor (EGFR) family, such as EGFR and ERBB2 (HER2/neu), and the insulin-like growth factor receptor (IGFR) family, are both linked to tamoxifen-resistance (63, 64, 66). Activation of the MAPK/ERK pathway by upstream growth factors (ERBB2, EGFR), the AKT/PI3K pathway by IGF growth factors, and activation of the p38 MAPK pathway by stress and/or cytokines can all lead to phosphorylation of key residues of the AF-1 domain of ERα and ligand-independent activation of ERα (59).

While estrogen-deprivation therapy might be more effective than tamoxifen in delaying resistance, eventually tumors become resistance to aromatase inhibitors. One important mechanism allowing tumors to escape suppression by aromatase inhibitors is the ERα hypersensitivity mechanism, which increases the sensitivity of ERα to lower circulating levels of E2. In wild-type MCF-7 cells, the cells respond maximally to doses of E2 in the range of $10^{-11}$ M. However, in MCF-7 cells deprived of estrogens for extended periods (LTED cells), the cells adapt and respond maximally to E2 concentrations of $10^{-13}$ (67-70). In addition, there is considerable evidence that signaling cross-talk between growth-factor-signaling pathways and ERα readily occurs at the time of tumor relapse. This can lead to hyperactivation of ERα and increased sensitivity of ERα to lower levels of estrogens, via cross-talk through the MAPK and the IGF/AKT signaling pathways. Increased expression of ERBB2/ERBB3, MAPK, and IGFR might therefore activate residual and increased levels of ERα, similar to cells that have become resistant to
tamoxifen (71-74). Thus, ERα signaling remains integral to breast cancer therapy, even in the context of aromatase inhibitors.

**Estrogen Action and Endocrine Therapy in Gynecological Malignancies**

Most epithelial ovarian cancer (EOC) presents at an advanced stage (Stage 3 or 4). Although 30-70% of these tumors are ERα positive (75), most tumors display de novo resistance to endocrine therapy (76-78). Tumors are treated with combination chemotherapy using taxanes and platinum, but after several cycles of treatment, tumors typically recur (79). Therapeutic options for these resistant tumors are poor and approximately two-thirds of ovarian cancer patients die within 5 years (80). However, the presence of ERα in most therapy-resistant gynecologic cancers suggests that there may exist additional modes of ERα action, which can be targeted with small molecules.

**Overview of Thesis**

Here I present a novel approach to breast cancer therapy and to identification of new modes of ERα action. We used high throughput screening to identify a novel ERα biomodulator, BHPI; demonstrated that BHPI is a promising new anticancer drug, effective in ERα containing tumor cells resistant to current therapies; identified anticipatory activation of the endoplasmic reticulum stress sensor, the unfolded protein response, as the surprising pathway of action of BHPI; showed that BHPI is distorting a normal action of ERα; and showed that the weak anticipatory activation of the UPR is an important initial signal required for subsequent estrogen-ERα mediated cell proliferation and gene expression. I also show that elevated expression of a UPR gene
index is a powerful new prognostic marker in ERα positive breast cancer, tightly correlated with reduced time to tumor recurrence, tamoxifen resistance and poor survival.

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CHAPTER 2

EVALUATION OF A LUCIFERASE-BASED REPORTER ASSAY FOR INHIBITORS OF ESTROGEN RECEPTOR α ACTION AS A SCREEN FOR INHIBITORS OF ESTROGEN-ERα-INDUCED PROLIFERATION OF BREAST CANCER CELLS

Abstract

Since estrogens (E₂), acting through estrogen receptor α (ERα), stimulate proliferation of breast cancer cells, ERα is an attractive drug target. Because screening small molecule inhibitors of cell proliferation is challenging in 384-well format, inhibition of luciferase-based reporters is often used as a surrogate end-point. To identify novel small molecules inhibitors of E₂-ERα-stimulated cell proliferation, we established a cell-based screen for inhibitors of 17β-estradiol (E₂)-ERα induction of an estrogen response element ((ERE)₃-Luciferase) reporter. 75 “hits” were selected to evaluate the effectiveness of the luciferase assay in predicting inhibitors of E₂-ERα induced proliferation of breast cancer cells. Only ~10% of the hits from the luciferase screen inhibited estrogen-induced proliferation of ERα positive MCF-7 and T47D cells, but not control ERα negative MDA-MB-231 cells. While a few compounds inhibited E₂-ERα-stimulated proliferation in only one of the ERα positive cell lines, most compounds either

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were toxic and inhibited growth of all the cell lines, or exhibited little or no ability to inhibit \(E_2\)-ER\(\alpha\)-stimulated cell proliferation. Representative compounds were evaluated in more detail and a lead ER\(\alpha\) inhibitor was identified. HTS using luciferase reporter assays represent a viable, but imperfect, way to identify small molecule inhibitors of cell proliferation.

**Introduction**

High throughput screens based on luciferase reporter assays are widely used to evaluate the effects of compounds on a pathway or target of interest (1). Reporter assays are often used as surrogate markers for a process such as cell proliferation that is more difficult to establish for full 384 or 1536 well HTS. Although there have been several studies of off-target effects due to direct interaction of a compound with the luciferase protein (2, 3), much less is known about the ability of a simple luciferase reporter assay to predict small molecule effects on a complex process, such as cell proliferation. Using inhibitors of estrogen receptor \(\alpha\) (ER\(\alpha\)) as a model, we evaluated how effectively a primary screen based on inhibition of a luciferase reporter predicted the ability of small molecules to selectively inhibit estrogen-ER\(\alpha\)-dependent growth of human breast cancer cells.

The complex of estrogens, such as 17\(\beta\)-estradiol (\(E_2\)) and ER\(\alpha\) plays a critical role in the growth and metastases of breast cancer. The important role of estrogens in breast cancer is illustrated by the widespread therapeutic use of aromatase inhibitors that block estrogen production, and the selective estrogen receptor modulators
tamoxifen and faslodex/fulvestrant/ICI 182,780 that work by competing with estrogens for binding to ERα (4). The progressive development of tumors resistant to tamoxifen and other ERα antagonists (5), and to aromatase inhibitors (6), underscores the continued importance of ERα as a therapeutic target.

It is widely accepted that the ability of E2-ERα to regulate nuclear gene expression plays a key role in the ability of estrogens to stimulate proliferation of ERα positive breast cancer cells (7, 8). The E2-ERα complex regulates gene expression by direct binding to DNA sequences termed estrogen response elements (EREs) and closely related sequences and by tethering to DNA through other proteins bound at AP1 and SP1 sites. The E2-ERα complex can also act in the cytosol to rapidly activate several membrane-associated protein kinase-based signaling pathways. However, rapid activation of the pro-growth ERK1/2 signaling pathway by E2-ERα complex is not sufficient for E2-dependent growth of breast cancer cells (9).

Recent reports have described assays using growth as a biological end-point (10). Although effective in 96-well plates, our assays for E2-ERα-stimulated proliferation of breast cancer cells did not exhibit sufficient reproducibility and precision for use in 384-well HTS. We therefore developed a screen using endogenous ERα in T47D, human breast cancer cells, stably transfected to express a luciferase reporter containing 3 consensus EREs ((ERE)3-luc) (11). The readout is the ability of small molecules to inhibit the E2-ERα induction of the (ERE)3-luc reporter. Although E2-ERα regulated gene expression is essential for E2-ERα-dependent cell proliferation, we found that there was an imperfect relationship between the inhibitory potency of compounds in the luciferase
reporter assay and their potency as selective inhibitors of estrogen-dependent cell proliferation. This led us to characterize in some detail the properties of 75 verified hits from the reporter assay. ~10% of the verified hits functioned as selective inhibitors of E$_2$-ER$_{\alpha}$-stimulated proliferation of breast cancers cells. We identified several causes for the failure of most of the hits to function as selective inhibitors. While imperfect predictors of selective inhibitory potency in cell proliferation assays, luciferase reporter assays nevertheless represent a useful primary screen.

**Materials and Methods**

**Cell Culture**

Human breast cancer cell lines were maintained in the following culture media. MCF-7: phenol red-free minimal essential medium (MEM), supplemented with 5% FBS; T47D: MEM, supplemented with 10% FBS; T47D-KBluc: RPMI-1640, supplemented with 10% FBS; (12) MDA-MB-231: phenol red-free MEM, supplemented with 10% FBS. Prior to experiments, ER$_{\alpha}$(+) cell lines were maintained for at least 3 days in phenol red-free charcoal-dextran (CD) treated serum. T47D cells: 4 days in MEM containing 10% CD-FBS; MCF-7 cells: 4 days in MEM + 5% CD-FBS; T47D-KBluc cells: 3 days in RPMI1640 + 10% CD-FBS.

**Automated HTS and Manual 384-Well Plate Luciferase Assays**
HTS luciferase-based assays were carried out by adding test compounds to plates using a Matrix PlateMate Plus instrument (ThermoScientific), equipped with a 384-well pin-transfer apparatus. To reach the desired final concentration for screening with limited volume options available using the pin transfer apparatus, 0.1 µl of each 10 mM compound stock in DMSO was transferred into 70 µl of serum-free RPMI-1640. 60 µl of medium containing each test compound was then withdrawn from the plates using a 384-well tip cartridge, leaving 10 µl of each compound at 14.28 µM. Cells were harvested at a density of 1-million cells/mL in RPMI-1640, supplemented with 10% CD-FBS. A 1:500 dilution of 17β-Estradiol (E2) in ethanol (EtOH) and a vehicle-ethanol control, was added to the (+)E2 and (-)E2 cell stocks, respectively. Cell were plated at a density of 10,000 cells/well by pipetting 10 µl of cells into each well using a Matrix Wellmate dispenser. The final concentration of test compounds was 7.14 µM. The screening medium contained 0.1% (v/v) EtOH, 0.07% (v/v) DMSO, and 10 nM E2. Plates were centrifuged for 2 minutes at 500 rpm, and incubated for 24 hours (37°C/5% CO2).

“Hits” from the primary HTS-screen were reconfirmed by diluting 10 mM compound stocks in DMSO to 20 µM in serum-free RPMI-1640. Cells were harvested at a density of 1-million cells/mL in RPMI-1640, supplemented with 10% CD-FBS. A 1:500 dilution of 17β-Estradiol (E2) in ethanol (EtOH) and a vehicle-ethanol control, was added to the (+)E2 and (-)E2 cell stocks, respectively. Cells were plated at a density of 10,000 cells/well by pipetting 10 µl of cells into each well. The final concentration of test compounds was 10 µM and the medium contained 0.1% (v/v) EtOH, 0.1% (v/v) DMSO,
and 10 nM E2. Plates were centrifuged for 2 minutes at 500 rpm, and incubated for 24 hours (37\(^\circ\)C/5% CO\(_2\)).

All plates were frozen at -20 \(^\circ\)C overnight following the 24-hour incubation, and thawed to room temperature. 10 \(\mu\)l of BrightGlow reagent (Promega, WI) was added to each well, and allowed to incubate for 15 minutes on the bench before measuring luminescence.

### 96-Well Luciferase Assays

5-6 days before the experiment T47D-KBluc cells were subcultured and plated at high density (about 30-40% confluence) in RPMI1640 + 10% FBS. 2 days later, the medium was changed to RPMI +10% CD-FBS. After 3 or 4 days with a medium change on day 2 the cells were harvested, counted and 50,000 cells in 100 \(\mu\)l of medium were added to each well of a 96-well white-wall-clear bottom plate (BD Biosciences, NJ) in RPMI + 10% CD-CS. The medium was replaced the next day with medium containing the test compounds with or without hormone. After 24 hours, the medium was aspirated off, and 30 \(\mu\)l of Bright Glow reagent (Promega, WI) was added. To help lyse the cells, the plate was placed on a shaker for 5 min. To remove any bubbles in the wells, the plate was subject to centrifugation at 2500 RPM for 2 min.

### MTS Growth Assays

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Cells were harvested and plated in 96-well plates at a density of 1,000 cells/well. MCF-7 cells were plated into MEM, supplemented with 10% CD-Calf serum; T47D cells were plated into MEM, supplemented with 10% CD-FBS; and MDA-MB-231 cells were plated into MEM, supplemented with 10% FBS. The medium was replaced with treatment medium the following day, and plates were incubated at 37°C in 5% CO2 for 3 days. 20 μl of CellTiter 96 Aqueous One Solution Reagent (Promega, WI) was added to each well and the cells were incubated at 37°C in 5% CO2 for 1 hour. A490 was then measured to assess cell viability. For each cell line, cell number was calculated from a standard curve of the number of cells plated versus A490 (12).

**Western Blotting**

Cells were trypsinized, resuspended in MEM supplemented with 10% CD-Calf serum and plated into 6-well plates at a density of 300,000 cells/well. The medium was replaced with treatment medium the following day, and the cells were treated for 24 hours. The cells were washed in ice-cold PBS and whole-cell extracts were prepared in lysis buffer containing: 1x radioimmunoprecipitation assay buffer, 1 mM EGTA, 30 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 tablet of protease inhibitor cocktail (Roche, IN). Cells were collected, and debris was pelleted by centrifugation at 15,000 x g for 10 min at 4°C. The supernatants were collected, and stored at -20°C. 20 μg of total protein was loaded onto 10% (v/v) SDS-PAGE gels, separated, and transferred to nitrocellulose. Membranes were incubated with monoclonal ERα [6F11] antibody (Biocare Medical, CA) or control monoclonal α-Tubulin antibody (Sigma, MO).
Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and chemiluminescent immunodetection with an ECL Detection Kit (GE Healthcare, NJ), and were visualized using a PhosphorImager.

**qRT-PCR**

pS2 mRNA levels were analyzed by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). RNAs were extracted with TRIzol reagent, purified with the RNAeasy mini-kit (QIAGEN, CA), and 0.5 μg of RNA was reverse transcribed using a DyNAmo cDNA synthesis kit (Finnzymes, Finland). 50 ng of the cDNA product was added to 1 μl of forward and reverse primer mix (10 μM). Primers used in qRT-PCR were: pS2, forward (5’-ACCGGACACCTCAGACACG) and reverse (5’-CTGTGTTGTGAGCCGAGGC); 36B4, forward (5’-GTGTTGACAATGGCAGCAT) and reverse (5’-GACACCCTCCAGGAAGCGA). The fold change in expression of each gene was calculated using the ∆∆Ct method with 36B4 as the internal control.

**Data Analysis**

Compounds that inhibited the (ERE)₃-luciferase reporter in the primary HTS screen by more than 50% were designated as “Hits”. Inhibition of E₂-ERα-mediated gene expression was calculated as follows:

\[
\text{Luciferase Inhibition (\%)} = \left(1 - \frac{\text{Compound}_{(+)}E₂ - \text{Negative Control}_{(+)}E₂}{\text{Positive Control}_{(+)}E₂ - \text{Negative Control}_{(-)}E₂}\right) \times 100
\]

Eq. (1)
Compounds were screened in parallel for their ability to inhibit a prostate specific antigen (PSA)-luciferase reporter induced by dihydrotestosterone AR in stably transfected HeLa cells (12). Inhibition of DHT-AR-mediated gene expression was calculated as follows:

\[
\text{Luciferase Inhibition} \, \% = \left( 1 - \frac{\text{Compound} \, _{(+)\text{DHT}} - \text{Negative Control} \, _{(+)\text{DHT}}}{\text{Positive Control} \, _{(+)\text{DHT}} - \text{Negative Control} \, _{(+)\text{DHT}}} \right) \times 100 \quad \text{Eq. (2)}
\]

Reconfirmed “Hits” were evaluated for their ability to inhibit E\(_2\)-ER\(\alpha\) stimulated proliferation of ER\(\alpha\) positive breast cancer cells using a cut-off of 50% to classify compounds as inhibitors of E\(_2\)-ER\(\alpha\)-stimulated cell proliferation. Percent inhibition of E\(_2\)-ER\(\alpha\)-stimulated proliferation of MCF-7 cell and T47D cells growth was calculated as follows:

\[
\text{Growth Inhibition} \, \% = \left( 1 - \frac{\text{Compound} \, _{(+)\text{E2}} - \text{Negative Control} \, _{(+)\text{E2}}}{\text{Positive Control} \, _{(+)\text{E2}} - \text{Negative Control} \, _{(+)\text{E2}}} \right) \times 100 \quad \text{Eq. (3)}
\]

Compounds were also evaluated for their ability to inhibit ER\(\alpha\) negative MDA-MB-231. Compounds were classified as “non-toxic”, if they inhibited growth of the control ER\(\alpha\) negative MDA-MB-231 cell by <30%, or if the cell growth inhibition was more than two-fold greater in the ER\(\alpha\) positive cell lines compared to the MDA-MB-231 cells. (Inhibition of E\(_2\)-dependent growth cannot exceed 100% for this calculation. Thus all compounds inhibiting MDA-MB-231 cell growth by more than 50% were classified as “toxic”). Percent inhibition of MDA-MB-231 cell growth was calculated as follows.

\[
\text{Growth Inhibition} \, \% = \left( 1 - \frac{\text{Compound}}{\text{Control}} \right) \times 100 \quad \text{Eq. (4)}
\]
Z’ values were calculated, as previously described (13).

Results

A Cell-based Screen for Inhibitors of $E_2$-ER$\alpha$ Induction of an (ERE)$_3$-Luciferase Reporter Gene

Regulation of nuclear gene expression is central to the ability of estrogens bound to ER$\alpha$ to induce proliferation of breast cancer cells. The widely used breast cancer therapeutic tamoxifen acts by competing with estrogens for binding to ER$\alpha$ and interfering with recruitment of coactivators critical for ER$\alpha$-mediated gene expression. To identify novel small molecules that directly or indirectly inhibit $E_2$-ER$\alpha$-mediated gene expression, a cell-based primary screen was developed using ER$\alpha$ positive T47D human breast cancer cells stably transfected to express a luciferase reporter whose expression is driven by 3 copies of the consensus estrogen response element (ERE)$_3$-luciferase (11). Dose-response studies show that $E_2$ robustly and reproducibly induces expression of the luciferase reporter (Fig. 1A). Some cell-based luciferase reporter screens have not been robust screens, as indicated by a low Z’-factor (14). In HTS, our assay was robust with a mean Z’-factor of 0.55 (Fig. 1B).

In some screens, a constitutively active luciferase reporter can provide a useful indicator of the specificity and toxicity of potential small molecule inhibitors. However, small molecule inhibitors of $E_2$-ER$\alpha$ induced gene expression should also inhibit the proliferation of ER$\alpha$ positive human breast cancer cells, and might thereby decrease the activity of a constitutively active Renilla luciferase internal standard. To test this, we
compared the effect of several well-established, specific and non-toxic, inhibitors of ERα with a mildly toxic compound identified in our follow-on assays. The well known therapeutics tamoxifen, raloxifene and faslodex and the toxic compound all produced similar substantial declines in expression of the constitutively active luciferase reporter gene (Fig. 2). Since the constitutively active luciferase could not distinguish toxic compounds from bona-fide ER inhibitors, we used tiered assays to filter out toxic compounds.

Small molecule “hits” were first screened for inhibition of dihydrotestosterone (DHT)-androgen receptor (AR) induction of a prostate specific antigen-luciferase (ARE-luciferase) reporter in stably transfected HeLa cells (12). This provided a way to initially flag compounds as toxic, which was later reconfirmed in subsequent toxicity assays. A second reporter also functioned as a crude method for assessing the nuclear receptor specificity of small molecules, given that ERα and AR share a high degree of structure homology and conservation in upstream signaling pathways. It also provides a way to detect inhibitors of luciferase enzyme activity. Alternatively, inhibitors of luciferase enzyme activity could have been detected by growing the T47D-kBLuc cells in the presence of estrogen alone, lysing the cells, and then adding the small molecule being tested and the luciferase reagent.

Compounds were considered hits in the primary screen if, at a concentration of 7.1 μM, they reduced luciferase units by at least 50%. All compounds reaching the 50% cut-off had reached statistical significance, as defined by ±3 SD from the negative reference. In order to evaluate the effective size of inhibition, strictly standardized mean difference was used as a secondary metric in lead selection.
Evaluation of 75 Verified inhibitors of E₂-ERα Induction of the (ERE)₃-Luciferase Reporter Gene

To evaluate the effectiveness of our luciferase-based assays in identifying useful lead inhibitors that selectively inhibit E₂-ERα induced proliferation of ERα positive human breast cancer cells, we characterized a randomly selected set of 75 hits from a subset of approximately 16,000 compounds. Data from the primary HTS screen for each compound screened at 7.1 μM is shown for inhibition of (ERE)₃-luciferase and ARE-luciferase in Figure 3, and in terms of SSMD in Figure 4. Compounds were further characterized in terms of their effects on cell growth (Fig. 5A and 5B) after reconfirming the compounds as inhibitors of (ERE)₃-luciferase activity at 10 μM (Fig. 5C, T47D-KBluc; Table 1). Each of the 75 compounds was evaluated at 10 μM for its ability to inhibit E₂-dependent proliferation of ERα positive MCF-7 and T47D, human breast cancer cells and for non-specific toxicity as evidenced by inhibition of the proliferation of control ERα negative MDA-MB-231 cells (Fig 5C, T47D and MCF-7). This is a rigorous control as small molecules readily inhibit proliferation of MDA-MB-231 cells (12). Based on their effects on E₂-ERα-dependent and E₂-ERα-independent cell proliferation, the compounds clustered into 4 distinct categories. (i) “Lead” compounds that inhibited proliferation of both ERα positive cell lines with minimal effects on proliferation of the ERα negative MDA-MB-231 cells (Fig. 5C, 1-8); (ii) “Cell Selective” compounds that inhibited proliferation of only one of the two ERα positive cell lines with minimal effects on proliferation of the ERα negative MDA-MB-231 cells (Fig. 5C, 9-17); (iii) “No Growth
Inhibition” compounds that exhibited little or no ability to inhibit E₂-ERα-stimulated proliferation (Fig. 5C, 18-38); and (iv) “Toxic” compounds that inhibited proliferation of the ERα negative MDA-MB-231 cells (Fig. 5C, 39-75). The toxic compounds could be further subdivided into “Toxic to All” compounds that were toxic in all three cell lines (Fig. 5C, 39-68) and “Toxic to 231” compounds that were primarily toxic in the MDA-MB-231 cells (Fig. 5C, 69-75).

**Detailed Characterization of Selected Compounds**

We examined the properties of four structurally unrelated representative compounds in more detail (Fig. 6A). For the four compounds that were selected, we performed dose-response studies of inhibition of E₂-ERα-mediated expression of the (ERE)₃-luciferase reporter (Fig. 6B) and of E₂-ERα-stimulated proliferation (Fig. 7).

At all concentrations tested, Compound 45, strongly inhibited proliferation of both the ERα positive MCF-7 and T47D cells and the ERα negative MDA-MB-231 cells (Fig. 7B). Its overall toxicity is responsible for the ability of Compound 45 to inhibit expression of the (ERE)₃-luciferase reporter (Fig. 6B). The shorter time cells were exposed to compounds in the reporter assay, 1 day as opposed to 3 days, likely accounts for the more limited inhibition seen in the reporter assay. Since Compound 45 was toxic to all the cells across a broad range of concentrations (Fig. 7B), it was not analyzed further.

Compound 4 was identified as a potential selective inhibitor of ERα action (Fig. 5C). Compound 4 elicited a dose-dependent inhibition of E₂-ERα-induced (ERE)₃-luciferase with an IC₅₀ of ~4.0 μM (Fig. 6B). From 2.5-10 μM, Compound 4 selectively inhibited
proliferation of the ERα positive cells compared to the ERα negative MDA-MB-231 cells (Fig. 7A). The IC₅₀ values for inhibition of the proliferation of the ERα positive cells were 3.7 and 1.7 μM for the MCF-7 and T47D cells, respectively and 12.8 μM for the ERα negative MDA-MB-231 cells (Fig. 7A). We also evaluated two small molecules that selectively inhibited one of the two ERα positive cell lines. Compound 14 was a moderately effective inhibitor of (ERE)₃-luciferase with an IC₅₀ of ~11.1 μM (Fig. 6B). While Compound 14 had little effect on cell proliferation at 1-5 μM, at concentrations above 10 μM it robustly inhibited proliferation of the T47D cells with little effect on the MCF-7 cells (Fig. 7C). Compound 11 inhibited (ERE)₃-luciferase with an IC₅₀ of ~3.4 μM (Fig. 6B). Compound 11 stimulated growth of the T47D cells, but strongly inhibited growth of the MCF-7 cells and exhibited moderate dose-independent inhibition of the MDA-MB-231 cells (Fig. 7D). Thus, this unusual small molecule has opposite effects on the proliferation of the two ERα positive cell lines.

**Effect of Selected Compounds on the Level of ERα and on Estrogen induction of pS2 mRNA**

Several ERα inhibitors act in part by decreasing the level of ERα (12, 15). We therefore examined whether the lead inhibitor, Compound 4 and the two cell type selective inhibitors, Compounds 11 and 14, influence levels of ERα in T47D and MCF-7 cells. ICI 182,780/fulvestrant/faslodex and TPSF are competitive and non-competitive ERα inhibitors known to function in part by reducing ERα levels (12, 15, 16). As expected, ICI 182,780 and TPSF dramatically reduced ERα levels in the MCF-7 and
T47D cells (Fig. 8A and 8B). The lead ER{alpha} inhibitor, Compound 4, strongly reduced ER{alpha} levels in both cell lines. In contrast, the two cell line selective inhibitors either had no effect (Compound 14) or elicited a small decline in ER{alpha} levels (Compound 11) (Fig. 8A and 8B).

We carried out our initial assays for inhibition of E{sub 2}-ER{alpha}-mediated gene expression using a stably transfected (ERE){sub 3}-luciferase reporter. While this stably transfected reporter gene will exhibit a more nearly native chromatin structure than a transiently transfected reporter gene, it is still likely different from the chromatin structure of a true endogenous gene. We therefore examined the ability of the three small molecules to inhibit E{sub 2}-ER{alpha}-induction of the widely studied endogenous pS2 gene in the same cell line in which we performed the (ERE){sub 3}-luciferase assays (17, 18). We used quantitative RT-PCR to measure pS2 mRNA levels (Fig. 8C). E{sub 2} induced pS2 mRNA 5.8 fold. The induction of pS2 mRNA was largely blocked by the control ER{alpha} inhibitors ICI 182,780 and TPSF and by the lead inhibitor reported here, Compound 4. There was good agreement between the ability of 5 {mu}M of Compound 4 to inhibit E{sub 2}-ER{alpha}-induction of the (ERE){sub 3}-luciferase reporter gene and the endogenous pS2 mRNA (~76% inhibition of (ERE){sub 3}-luciferase (Fig. 6B) and ~67% inhibition of pS2 mRNA (Fig. 8C)). In contrast, the weak inhibitor of (ERE){sub 3}-luciferase, Compound 14 did not inhibit E{sub 2} induction of pS2 mRNA and 10 {mu}M Compound 11 inhibited induction of pS2 mRNA by ~35% (Fig. 8C). While the lead compound, which inhibited proliferation in both ER{alpha} positive cell lines, robustly inhibited E{sub 2} induction of the endogenous pS2 mRNA, the 2
cell selective inhibitors were ineffective and are unlikely to act by directly targeting ERα action.

Discussion

In evaluating a single reporter luciferase-based assay as a surrogate assay for estrogen-induced proliferation of cancer cells, two potential sources of off-target effects stand out. Small molecules that inhibit because they exhibit direct interaction with luciferase and small molecules that are broadly toxic. Both types of small molecule can reduce luciferase activity and be scored as hits in the primary screen (2, 3).

Approximately 37% (28 of 75, Fig. 5) of the hits identified in the luciferase assay did not reach the cutoff for inhibition of E2-ERα-stimulated proliferation in both, MCF-7 and T47D, human breast cancer cells. This included the 21 compounds in the “No Growth Inhibition” category (Fig. 5, 18-37) and the 7 compounds in the “Toxic” category that only inhibited cell growth in MDA-MB-231 cells (Fig. 5, 69-75). Nearly all of the latter compounds (6 of 7) inhibited cell growth modestly (>25%) in both ERα positive breast cancer cell lines. The more pronounced effects on cell growth in the ERα negative MDA-MB-231 cells can be attributed to the higher sensitivity of this cell line to damage (12). The “No Growth” inhibitors, could be further sub-classified as (i) compounds that modestly inhibited cell proliferation, but did not reach the 50% cutoff (Fig. 5, 18-28) or (ii) compounds that exhibited little or no ability to inhibit E2-ERα-stimulated proliferation of the breast cancer cells (Fig. 5, 29-38). Partial growth inhibition may reflect different dose-response curves for inhibition of gene expression and cell
proliferation. Consistent with this idea, the concentration of the non-competitive ERα inhibitor TPSF required to inhibit cell proliferation is ~3 fold higher than is required to inhibit the luciferase reporter (12). Compounds that showed no ability to inhibit E₂-ERα-stimulated proliferation of the breast cancer cells might be acting as direct inhibitors of luciferase enzyme activity. However, 8 of the 9 compounds that failed to inhibit growth (Fig. 5, 29-38), also failed to significantly inhibit the DHT-AR induced PSA-luciferase reporter (Fig. 3, 29-38). This suggests that these compounds are not functioning as luciferase inhibitors.

Approximately 49% (37 of 75, Fig. 5) of the compounds were toxic, as a result of their ability to inhibit cell growth in ERα negative MDA-MB-231 cells. Approximately 81% of the toxic compounds displayed global toxicity (Fig 5, 39-68), while 19% selectively inhibited MDA-MB-231 cells (Fig. 5, 69-75). The large number of toxic compounds is readily attributed to the high sensitivity of MDA-MB-231 cells to damage, the rigorous 4-day treatment interval used to assess for growth effects, and the more stringent growth inhibition cut-offs used to classify compounds as “toxic” (30% cut-off compared to 50% cut-off for ERα positive cell lines). Our data suggests that use of an alternative reporter system in parallel with the primary screen can provide useful predictive value in identifying potentially toxic compounds. Approximately 70% of the compounds considered toxic inhibited the ARE-luciferase reporter by >30% (Fig. 3, 39–75). These compounds all displayed SSMD scores <-2 (Fig. 4, 39–75), demonstrating that these reductions in ARE-luciferase were statistically significant at the 95% confidence level. Conversely, only 11% of “nontoxic” compounds inhibited the DHT-AR-induced ARE-luciferase reporter by >30% (Fig. 3, 1–38). Yet, 3 of these nontoxic compounds
represent “lead” compounds (Fig. 3, 5, and 8). This suggests that data from a secondary reporter system should aid in evaluating toxicity and should be used in concert with additional toxicity assays, rather than serve as a rigid cutoff for evaluating candidate leads. The identification of leads that inhibit both reporters may reflect modulation of receptor activity via direct interactions with small molecules at structurally homologous sites or through indirect interaction via targeting-conserved upstream signaling pathways. Importantly, the ARE-reporter provides a preliminary measure of nuclear receptor specificity for small molecule “leads” and provides a starting point for tailoring subsequent assays toward subsets of “leads.”

Perhaps most surprising was our finding that some small molecule hits from the ERE-luc reporter assay inhibited proliferation in either T47D cells or MCF-7 cells, but not in both cell lines. MCF-7 and T47D cells are the most widely used lines of ERα positive breast cancer cells. These cell lines were independently derived from different tumors. Since the hits were identified using a primary screen for inhibitors of ERE-luc expression in stably transfected T47D cells, and T47D cells contain less ERα than MCF-7 cells (19), we anticipated we might identify small molecules that were more effective in T47D cells than MCF-7 cells. Instead we identified some small molecules that were toxic in MDA-MB-231 cells but only damaged one of the two cell lines and a few small molecules that preferentially inhibited proliferation of each of the cell lines without damaging MDA-MB-231 cells. Compound 14 exhibited a dose-dependent inhibition of the proliferation of T47D cells with little effect on proliferation of MCF-7 cells. At 10 μM, Compound 14 inhibited proliferation of the T47D cells by ~81% with no effect on proliferation of the MCF-7 cells or MDA-MB-231 cells (Fig. 7C). In contrast,
Compound 11 killed all the MCF-7 cells and actually stimulated estrogen-dependent growth of the T47D cells from 2.5-20 µM (Fig. 7D). The surprising properties of Compound 11 demonstrate that effects on cell proliferation can be entirely dissociated from effects on reporter gene expression. It is unlikely that Compounds 11 and 14 directly target ERα action as they had little or no ability to inhibit estrogen induction of pS2 mRNA in the same T47D line used for the luciferase assays (Fig. 8C). These data illustrate the importance of early evaluation of primary hits using more than one cell line.

About 10% of the primary hits from the luciferase-screen represented leads that inhibited estrogen-dependent proliferation of both the MCF-7 and T47D cells with minimal effects on proliferation of the ERα negative MDA-MB-231 cells (Fig. 5, 1-8). From 1-5 µM, Compound 4 elicited progressive inhibition of the proliferation of MCF-7 and T47D cells with little effect on proliferation of MDA-MB-231 cells (Fig. 7A). Since Compound 4 shows some toxicity in MDA-MB-231 cells at 10 µM, we used 5 µM to test its effect on estrogen induction of pS2 mRNA. Consistent with its effect on the ERE-luc reporter and on E2-ERα-stimulated cell proliferation, Compound 4 effectively inhibited induction of pS2 mRNA and is a lead compound for further development.

The application of cell-based assays in HTS remains inherently challenging. The large number of biological targets and high degree of crosstalk between integrated signaling pathways predisposes cell-based assays to higher “Hit” frequencies, lower confirmation rates, and higher numbers of toxic compounds in follow-up assays (20, 21). Approximately 10% of the verified hits from the luciferase-based-screen ultimately met our end-point for selective inhibition of estrogen-dependent proliferation of ERα.
positive cell lines. While this might seem to argue for a cell-proliferation based screen, a primary screen based on inhibition of cell proliferation would have exposed many of the same issues. Since single cell proliferation assays do not usually distinguish between toxic compounds and those targeting the pathway of interest, many of the hits from a proliferation-based screen would have emerged as toxic when tested in a control cell line. Our finding that some compounds exhibit cell line specific inhibition of estrogen-dependent proliferation means that compounds identified in a single cell line may still fail in other cell lines because they do not target ER$\alpha$. In our hands, assays for estrogen-dependent cell proliferation assays in 384 well plates exhibited less precision and reproducibility than luciferase-based assays and require several days compared to one day for luciferase assays. While luciferase-based assays offer the advantage of improved efficiency in HTS, assay interference remains an important obstacle to generating high quality leads. Although the role of assay interference in our screen is largely unknown, the coupling of multiple cell proliferation assays to the primary screen provided an effective filter for rapidly eliminating compounds that lack bioactivity. Alternatively, this empirically driven approach of asking cells to tell us what small molecules are capable of inhibiting E$_2$-ER$\alpha$-stimulated proliferation, provides a way to select for bioactive compounds, independent of their ability to elicit assay interference in the primary screen. Recent studies have shown that several important small molecules function as dual inhibitors of ATP-dependent kinases and luciferases, including resveratrol and the MEK1/2 inhibitor, PD090859 (22-24). Since the role of E$_2$-ER$\alpha$ in gene expression is essential to stimulating breast cancer cell proliferation, and a number of pathways can modulate ER$\alpha$ transcriptional activity through post-translational
modifications (25), our screen would not preclude identification of such bioactive compounds. Small molecules generally target several sites in cells, and through signal integration and unintended crosstalk, can elicit toxicity that is readily observed in downstream readouts like E2-ERα-mediated gene expression. Carrying out multiple cell proliferation assays, using cells with and without the target, provides a very effective toxicity filter. This is reflected by the large numbers of toxic compounds we identified. With appropriate follow-on verification assays carried out in multiple cell lines, a luciferase-based primary screen represents a useful surrogate assay for a more complex process, such as hormone-stimulated cell proliferation.
Figure 2.1. The ERE-luciferase reporter assay. (A) Dose response study of E$_2$-ER$\alpha$ induction of ERE-luciferase. The data represents the average $\pm$ S.E.M. of quadruplicate assays carried out in 96 well plates. (B) Assessment of screen robustness using $Z'$-factor. $Z'$-factor for each plate in the overall screen (●), and the overall $Z'$ value for the entire screen are denoted above.
Figure 2.2. Comparison of the effects of well-established ERα-Inhibitors and a toxic compound on luciferase reporters. Treatment of cells with well-established ERα-Inhibitors and a toxic compound significantly inhibited (A) (ERE)_3-firefly luciferase and (B) SV40-renilla luciferase activity. T47D cells were maintained for 3 days in 10% CD-FBS prior to transfecting cells with 0.4 μg of (ERE)_4-firefly Luciferase and 2.5 ng SV40-renilla luciferase for 24 hours. Cells were treated after transfection for 24 hours with a ethanol-vehicle control (-E2), 1 nM 17β-Estradiol (E_2), or a combination of 1 nM E_2 and either 1 μM 4-Hydroxy-Tamoxifen (TAM), 1 μM Raloxifene (RAL), 1 μM ICI 182,780/Fulvestrant (ICI), 1 μM TPSF, or 10 μM of Compound 45 (toxic compound). Cells were harvested and assayed for firefly and renilla luciferase activity. Data represents the mean ± S.E.M. for three separate experiments.
Figure 2.3. Primary screening data for the 75 representative compounds selected for further characterization. For each compound, percent inhibition of DHT-AR-stimulated PSA-luciferase activity (black bars) and E$_2$-ER$\alpha$-stimulated ERE-luciferase activity (grey bars) is shown.
Figure 2.4 Strictly standardized mean difference (SSMD) scores for the effects of the 75 selected small molecules. SSMD score on (A) E2-ERα-induced (ERE)3-luciferase and (B) DHT-AR-induced ARE-luciferase. SSMD values of ±1, ±2, and ±3 correlate with d+ probability boundaries of 0.5, 0.95, and 0.975 respectively (17). SSMD scores less than -2 reflect statistically significant reductions in luciferase activity at the 95% confidence level. The population value of SSMD was used to organize small molecules into categories based on their strength of inhibition, as described previously (17).
Figure 2.5. Summary of assays used to evaluate inhibitors of ERα-mediated gene expression as a surrogate marker for inhibitors of E2-dependent growth. (A) Flow
chart showing the breakdown by category of compounds screened for reporter activity and growth inhibition. (B) Summary of the classes of compounds identified. (C) Compounds were further assessed for (from top to bottom) their ability to inhibit ERE-luciferase activity (T47D-KBluc), growth of ERα MDA-MB-231 breast cancer cells (MDA-MB-231), E2-ERα-dependent proliferation of ERα+ T47D, and MCF-7 breast cancer cells. Each assay was carried out in triplicate, and mean inhibition levels are denoted above. Numerical data is in Table 1.

Figure 2.6. Characterization of representative inhibitors. (A) Structures of the compounds that were tested selected. (B) Dose-response curves for inhibition of ERE-luciferase by selected compounds. T47D-KBluc cells were maintained for 3 days in 10% CD-FBS and then incubated for 24 hours in the presence of 10 nM E2 and the indicated concentrations of the test compounds. Cells were harvested and assayed for luciferase activity.
Figure 2.7. Dose-response studies of the effects of the inhibitors on cell proliferation. Dose response studies were used to evaluate the effect on the E2-ERα-dependent proliferation of MCF-7 and T47D cells and on the growth of ERα negative MDA-MB-231 for (A) Compound 4, (B) Compound 45, (C) Compound 14, and (D) Compound 11. Cells received the indicated concentrations of each inhibitor in DMSO. After 3.5 days cell number was quantified by MTS. ERα+ cell lines were also treated with ICI 182,780, a known ERα antagonist. Mean percent growth inhibition is representative of triplicate assays ± S. E.M.
Figure 2.8. Effect of the 3 inhibitors on ERα levels and on the induction of pS2 mRNA. The effects of the three compounds on the level of ERα was evaluated in Western blots of extracts from (A) T47D cells and (B) MCF-7 cells. ICI 182,780 (ICI) and TPSF, known down-regulators of ERα were used as controls. (C) Compounds were tested for their ability to inhibit E2-ERα induction of pS2 mRNA. pS2 mRNA levels were quantified by qRT-PCR, following 24-hour treatment of T47D-KBluc cells with 10 nM E2 and 5 μM of compound 4.
Table 2.1. Percent inhibition and S.E.M. values for the seventy-five small molecules further characterized. The classification of each compound is denoted using the following symbols: "L" denotes a "Lead" inhibitor, "S" denotes a "Cell Selective" inhibitor, "NG" denotes a "No growth" inhibitor, "TA" denotes a "Toxic" inhibitor, and "T-231" denotes a "Toxic-231 Only" inhibitors.

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References


Abstract

In response to cell stress, cancer cells often activate the endoplasmic reticulum (EnR) stress sensor, the unfolded protein response (UPR). Little was known about the potential role in cancer of a different mode of UPR activation; anticipatory activation of the UPR prior to accumulation of unfolded protein or cell stress. We show that estrogen, acting via estrogen receptor α (ERα), induces rapid anticipatory activation of the UPR, resulting in increased production of the antiapoptotic chaperone BiP/GRP78, preparing cancer cells for the increased protein production required for subsequent estrogen-ERα induced cell proliferation. In ERα containing cancer cells, the estrogen, 17β-estradiol (E2) activates the UPR through a phospholipase C γ (PLCγ)-mediated opening of EnR IP₃R calcium channels, enabling passage of calcium from the lumen of the EnR into the cytosol. siRNA knockdown of ERα blocked the estrogen-mediated increase in cytosol calcium and UPR activation. Knockdown or inhibition of PLCγ, or of IP₃R, strongly

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2 This chapter appeared in its entirety in *Oncogene*. Andruska N, Zheng X, Yang X, Helferich WG, Shapiro DJ. (2014) Anticipatory estrogen activation of the unfolded protein response is linked to cell proliferation and poor survival in estrogen receptor α-positive breast cancer. Oncogene. 33 (advance online publication, September 29, 2013. This article is reprinted with the permission of the publisher.
inhibited the estrogen-mediated increases in cytosol calcium, UPR activation and cell proliferation. $E_2$-ER$\alpha$ activates all three arms of the UPR in breast and ovarian cancer cells in culture and in a mouse xenograft. Knockdown of ATF6$\alpha$, which regulates UPR chaperones, blocked estrogen induction of BiP and strongly inhibited $E_2$-ER$\alpha$ stimulated cell proliferation. Mild and transient UPR activation by estrogen promotes an adaptive UPR response that protects cells against subsequent UPR-mediated apoptosis. Analysis of data from ER$\alpha$ positive breast cancers demonstrates elevated expression of a UPR gene signature that is a powerful new prognostic marker tightly correlated with subsequent resistance to tamoxifen therapy, reduced time to recurrence and poor survival. Thus, as an early component of the $E_2$-ER$\alpha$ proliferation program, the mitogen estrogen, drives rapid anticipatory activation of the UPR. Anticipatory activation of the UPR is a new role for estrogens in cancer cell proliferation and resistance to therapy.

**Introduction**

Estrogens, acting via estrogen receptor $\alpha$ (ER$\alpha$), stimulate cell proliferation and tumor growth (1-3). The importance of estrogens and ER$\alpha$ in breast cancer is illustrated by the central role of endocrine therapy targeting estrogens and ER$\alpha$ in treatment of ER$\alpha^+$ breast cancer (1-5). To help fold and sort the increased protein required for estrogen-ER$\alpha$ induced cell proliferation, cells must increase chaperone levels. The endoplasmic reticulum (EnR) stress sensor, the unfolded protein response (UPR) monitors and maintains protein-folding homeostasis (6, 7). The UPR responds to misfolded proteins, or other forms of stress, by activating three signal transduction
pathways, which reduce protein production and increase EnR protein-folding capacity. Protein production is regulated by autophosphorylation of the stress-activated transmembrane kinase, PERK (6, 7). P-PERK phosphorylates eukaryotic initiation factor 2α (eIF2α), resulting in transient inhibition of protein synthesis. The other UPR arms initiate with proteolytic activation of the transcription factor ATF6α, leading to increased chaperone production and activation of the EnR splicing factor IRE1α, which alternatively splices the transcription factor XBP1, leading to production of active spliced-XBP1, increased protein folding capacity and altered mRNA decay and translation (6, 7).

The UPR is usually inactive in normal cells, but is overexpressed in several cancers (8). Chronic UPR activation leads to increased expression of EnR chaperones, such as BiP (GRP78/HSAP5), p58IPK and calreticulin that facilitate protein folding and promote survival, proliferation, angiogenesis, and resistance to chemotherapy and endocrine therapy (9-12). In the widely studied “reactive mode", the UPR in tumor cells is activated in response to accumulation of stress from rapid cell division, hypoxia and therapy. A few studies in immune cells describe a different type of UPR activation; in this “anticipatory mode", the UPR is activated in the absence of EnR stress and prior to the accumulation of unfolded proteins (13, 14). We explored whether estrogen induces anticipatory activation of the UPR in the absence of EnR stress, increasing protein folding capacity prior to the increased protein production and protein folding load that accompanies activation of the genomic estrogen-ERα cell proliferation program.

Previous studies of the UPR and of estrogen-ERα action focused on the estrogen-
inducible UPR gene, XBP1. XBP1 binds to and activates ERα; XBP1 expression is associated with tamoxifen resistance in ERα+ breast cancer (15-18).

The plasma membrane enzyme phospholipase Cγ (PLCγ) hydrolyzes PIP2 to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). We show that the mitogen estrogen, 17β-estradiol (E2), acting through a rapid extranuclear action of ERα, elicits a PLCγ-mediated opening of EnR IP3R calcium channels, increasing cytosol calcium and triggering anticipatory activation of each arm of the UPR. Opening the IP3R calcium channel and activating the ATF6α arm of the UPR, resulting in BiP induction, are important for subsequent E2-ERα induced cell proliferation. Consistent with an important role in cancer for anticipatory activation of the UPR, analysis of data from ~1,000 ERα+ breast cancer patients demonstrates that elevated expression of a UPR gene signature is tightly correlated with subsequent resistance to tamoxifen therapy, time to tumor recurrence and poor survival.

Materials and Methods

Cell Culture and Reagents

Cell culture medium and conditions were previously described (19-21). MCF-7, T47D, and T47D-kBluc cells were obtained from the ATCC. Drs. S. Kaufmann and K. Korach provided PEO4 cells and BG-1 cells, respectively. E2, 4-OHT, U73122, 2-APB, and tunicamycin were from Sigma Aldrich. ICI 182,780 was from Tocris Biosciences and ryanodine was from Santa Cruz Biotechnology. Phospho-eIF2α (#3398), eIF2α.
(5324), Phospho-PERK (#3179), PERK (#5683), and BiP (#3177) antibodies were from Cell Signaling. Pan-IP3R (sc-28613), XBP1 (sc-7160), and ERα (sc-56836) antibodies were from Santa Cruz Biotechnology. Other antibodies used were ATF6α (Imgenex) and β-Actin (Sigma).

**Cell Proliferation Assays**

Cells proliferation assays were carried out as described (19-21).

**Protein Synthesis**

Protein synthesis was evaluated by measuring incorporation of 35S-Methionine into newly synthesized protein. Cells were incubated in 96 well plates for 20 minutes with 3 μCi of 35S-methionine per well (PerkinElmer), lysed, and clarified by centrifugation. The appropriate volume, normalized to total protein, was spotted onto Whatman 540 filter paper discs and immersed in cold 10% TCA and washed in 5% TCA. Trapped protein was solubilized and filters counted.

**Calcium Imaging**

Cytoplasmic Ca2+ concentrations were measured using the calcium-sensitive dye, Fluo-4 AM (22, 23). Cells were grown on 35 mm-fluorodish plates (World Precision Instruments) for two days prior to experiments. Cells were loaded with 5 μM Fluo-4 AM (Life Technologies) in buffer (140 mM NaCl, 4.7 mM KCl, 1.13 mM MgCl2, 10 mM...
HEPES, 10 mM Glucose, pH = 7.4) for 30 minutes at 37 °C. The cells were washed three times with this buffer and incubated with either 2 mM or 0 mM CaCl2 for 10 minutes. Images were captured for one minute to determine basal fluorescence intensity, and then the appropriate treatment was added. Measurements used a Zeiss LSM 700 confocal microscope with a Plan-Four 20X objective (N.A. = 0.8) and 488-nM laser excitation (7% power). Images were obtained through monitoring fluorescence emission at 525 nM, and analyzed with AxioVision and Zen software (Zeiss).

**Luciferase Assays, qRT-PCR, and siRNA Transfections**

Reporter gene assays and qRT-PCR were previously described (19, 20). siRNA knockdowns were performed using DharmaFECT1 Transfection Reagent and 100 nM ON-TARGETplus non-targeting pool or SMARTpools for ERα (ESR1), PLCγ (PLCG1), PERK (EIF2AK3), ATF6α (ATF6), XBP1, or pan-IP3R (Dharmacon). The pan-IP3R SmartPool consisted of three individual SmartPools, each at 33 nM, directed against each isoform of the IP3R (ITPR1, ITPR2, and ITPR3).

**MCF-7 Xenograft**

Experiment were approved by the Institutional Animal Care Committee (IACUC) of the University of Illinois at Urbana-Champaign. The MCF-7 cell mouse xenograft model has been described previously (24). Estrogen pellets (1 mg:19 mg estrogen:cholesterol) were implanted into 30 athymic female OVX mice at 7 weeks of age. Three days later, 1 million MCF-7 human breast cancer cells suspended in...
matrigel were subcutaneously injected into two sites on each flank, for a total of 4 tumors per mouse. When average tumor size reached 17.6 mm$^2$, E2 pellets were removed and a lower dose of E2 in sealed silastic tubing (1:31 estrogen:cholesterol, 3 mg total weight) was implanted. When average tumor size reached 23.5 mm$^2$, 15 mice retained E2 silastic tubes (+E2 group) and 15 mice received silastic tubes containing only cholesterol (-E2 group). Tumors were measured every 4 days with a caliper. Tumor cross sectional area was calculated as $(a/2)^2(b/2)*3.14$, where a and b were the measured diameters of each tumor. On termination of the experiments mice were euthanized and tumors were excised.

**Tumor Microarray Data Analysis**

Analysis was performed using several publically available tumors cohorts. ER$\alpha$ and UPR gene expression profiles of histologically normal breast epithelium (GSE20437) (25) were compared to IDC tumors from ER$\alpha^+$ breast cancer patients (GSE20194). ER$\alpha$ and UPR correlation analysis was performed on 278 invasive ductal carcinoma samples (GSE20194) (26). A “UPR Gene Signature” was constructed to carry out risk prediction analysis. The UPR gene signature was evaluated for its ability to predict: (i) tumor relapse in 261 early-stage ER$\alpha^+$ breast cancers (GSE6532),37 (ii) tumor relapse in 474 ER$\alpha^+$ patients receiving solely tamoxifen therapy for 5 years (GSE6532, GSE17705) (27, 28), and (iii) overall survival in a mixed-cohort of 236 breast cancer patients (GSE3494) (29). Microarray data analysis was performed using BRB ArrayTools (version 4.2.1) and R software version 2.13.2. Gene expression values from
CEL files were normalized by use of the standard quantile normalization method (30). Pearson correlation tests and Spearman log rank tests were used to determine gene expression correlation coefficients. Wald tests were used to test whether UPR genes were predictive of tumor recurrence and overall survival. Univariate and multivariate hazard ratios were estimated using Cox regression analysis. Covariates statistically significant in univariate analysis were further assessed in multivariate analysis. A patient was excluded from multivariate analysis, if data for one or more variables were missing. Risk prediction using the UPR gene signature was carried out using the supervised principle components method (31), and visualized using Kaplan-Meier plots and compared using log-rank tests.

**Statistical Analysis**

Calcium measurements are reported as mean ± SE. All other data is reported as mean ± S.E.M. Two-tailed student’s t-test used for comparisons between groups. One-way ANOVA followed by Fisher’s LSD or Tukey’s post hoc test used for multiple comparisons. P < 0.05 was considered significant.

**Results**

*Estrogen Activates all 3 Arms of the UPR*

To evaluate the ability of E2-ERα to activate the UPR, we focused on production of spliced and modified proteins that result from activating the three arms of the UPR
E₂ rapidly activated the IRE1α arm of the UPR, as shown by increases in spliced-XBP1 (sp-XBP1) mRNA in T47D and MCF-7 breast and PEO4 ovarian cancer cells (Fig. 2A and B), and by induction of downstream sp-XBP1 targets, SERP1 and ERDJ (Fig. 3A) (32). The antiestrogens ICI 182,780/Faslodex/fulvestrant (ICI) and 4-hydroxytamoxifen, (4-OHT), which compete with E₂ for binding to ERα, blocked the E₂-mediated increase in sp-XBP1 (Fig. 2A). Consistent with E₂-ERα activating the IRE1α arm of the UPR, RNAi knockdown of ERα blocked E₂-induction of sp-XBP1 mRNA (Fig. 2C), and induction of GREB1 by nuclear E₂-ERα (Fig. 3B).

We next assessed whether estrogen activates the ATF6α arm of the UPR. ATF6α is a 90 kDa protein (p90-ATF6α) that translocates from the EnR to the Golgi in response to stress, where it undergoes proteolytic cleavage to its active 50 kDa form (p50-ATF6α) (Fig. 1B) (6, 7, 33). Increased ATF6α proteolysis in T47D cells and PEO4 cells demonstrates that E₂-ERα transiently activates the ATF6α arm of the UPR (Fig. 2D and Fig. 3C). Since pretreatment with ICI, abolished the E₂-mediated increase in p50-ATF6α, this effect is mediated through ERα (Fig. 2D). Active cleaved ATF6α regulates induction of BiP and other EnR chaperones (33, 34). Consistent with this, ATF6α knockdown in T47D cells blocked BiP induction (Fig. 2E). BiP increases EnR protein folding capacity, contributing to resolution of the stress, and helps reverse UPR activation; likely preventing the cytotoxicity that would result if UPR activation was sustained. Consistent with its antiapoptotic role, in several cancers, elevated levels of BiP are associated with a poor prognosis (9). Estrogen rapidly induced BiP mRNA in breast and ovarian cancer cells (Fig. 2F), leading to a 2.3-fold increase in BiP protein (Fig. 2G). RNAi knockdown of ERα prevented E₂-induction of BiP mRNA (Fig. 2H).
PERK activation leads to inhibition of protein synthesis (Fig. 1C). Surprisingly, E\textsubscript{2} induces a rapid and transient increase in PERK phosphorylation (Fig. 4A), resulting in increased phosphorylation of eIF2\textsubscript{α} (Fig. 4B) and a modest transient decline in overall protein synthesis (Fig. 4C). Consistent with p-PERK catalyzing formation of p-eIF2\textsubscript{α}, PERK knockdown inhibited formation of p-eIF2\textsubscript{α} (Fig. 4D). Consistent with E\textsubscript{2} acting through ER\textsubscript{α}, ICI inhibited E\textsubscript{2}-stimulated phosphorylation of PERK and eIF2\textsubscript{α} and largely reversed the E\textsubscript{2}-mediated inhibition of protein synthesis (Fig. 4A, B, and C). PERK activation leads to ATF4 expression, and we observed a transient increase in ATF4 expression (Fig. 4E). However, the proapoptotic protein CHOP was not induced because mild and transient activation of PERK does not induce CHOP (Fig. 3D, Fig. 4F) (35). Together, this data demonstrates that E\textsubscript{2}, acting through ER\textsubscript{α}, activates all three UPR arms.

**E\textsubscript{2}-ER\textsubscript{α} Rapidly Increases Cytosol Ca\textsuperscript{2+} by a PLC\textsubscript{γ}-mediated Opening of the EnR IP\textsubscript{3}R Ca\textsuperscript{2+} Channel, Activating the UPR**

Rapid UPR activation by E\textsubscript{2}-ER\textsubscript{α} suggested accumulation of unfolded protein was not triggering UPR activation. Some UPR activators, such as thapsigargin, rapidly activate the UPR by depleting Ca\textsuperscript{2+} stores in the lumen of the EnR, increasing intracellular Ca\textsuperscript{2+}. To test whether E\textsubscript{2} rapidly alters cytosol Ca\textsuperscript{2+}, we monitored cytosol calcium using the sensor dye Fluo-4 AM. In the presence or absence of extracellular Ca\textsuperscript{2+}, estrogen produced a rapid and transient increase in fluorescence in T47D breast cancer cells (Fig. 5A and B). Since E\textsubscript{2} increases cytosol Ca\textsuperscript{2+} when there is no
extracellular Ca\(^{2+}\), and the EnR lumen is the major Ca\(^{2+}\) store available to increase cytosol Ca\(^{2+}\), E\(_2\) is acting by depleting the EnR Ca\(^{2+}\) store. Estrogen also increased cytosol calcium in PEO4 ovarian cancer cells (Fig. 6). Inhibition of the IP\(_3\)R channel with 2-APB, which locks the IP\(_3\)R Ca\(^{2+}\) channels closed, and RNAi knockdown of the three isoforms of the IP\(_3\)R channels (Fig. 5C), abolished the rapid E\(_2\)-ER\(\alpha\)-mediated increase in cytosol Ca\(^{2+}\) (Fig. 5A, B, and D). In contrast, high concentration ryanodine (Ry), which closes the ryanodine receptor (RyR) Ca\(^{2+}\) channels, did not block the increase in cytosol Ca\(^{2+}\) (Fig. 5A and B). We next assessed whether Ca\(^{2+}\)-release was necessary for UPR activation using 2-APB and ryanodine individually, or in combination. 2-APB, but not ryanodine, inhibited E\(_2\)-ER\(\alpha\) activation of the PERK arm of the UPR, as shown by inhibition of formation of p-eIF2\(\alpha\) (Fig. 7A). RNAi knockdown of IP\(_3\)R (Fig. 5C) blocked E\(_2\)-induced Ca\(^{2+}\) release (Fig. 5D), activation of the IRE1\(\alpha\) arm of the UPR (Fig. 7B), and blocked E\(_2\)-induction of BiP (Fig. 5C), which is a commonly used surrogate readout for UPR activation.

We next tested the possibility that activation of PLC\(\gamma\), which hydrolyzes PIP\(_2\) to DAG and IP\(_3\), plays a role in E\(_2\)-mediated opening of the IP\(_3\)R Ca\(^{2+}\) channels. Treating T47D cells with the PLC\(\gamma\) inhibitor, U73122, or siRNA knockdown of PLC\(\gamma\), abolished the rapid E\(_2\)-ER\(\alpha\)-mediated increase in cytosol Ca\(^{2+}\) (Figure 5E and F; Fig 8). Since PLC\(\gamma\) mediates E\(_2\)-dependent opening of the IP\(_3\)R Ca\(^{2+}\) channels and calcium release (Fig. 5F), we examined the effect of siRNA knockdown of PLC\(\gamma\) on E\(_2\)-ER\(\alpha\)-dependent activation of the UPR. siRNA knockdown of PLC\(\gamma\) blocked E\(_2\)-ER\(\alpha\) activation of the ATF6\(\alpha\) arm of the UPR, as shown by a reduction in p50-ATF6\(\alpha\), and inhibition of BiP induction (Fig. 5E).
To evaluate the role of ERα in the E2-mediated increase in cytosol calcium, we performed siRNA knockdown. In T47D cells, RNAi knockdown of ERα, in the absence of extracellular Ca^{2+}, prevented E2-stimulated calcium release (Figure 5G and H). PLCγ is on the inner leaflet of the plasma membrane and the E2-ERα-mediated increase in cytosol Ca^{2+} occurs in <2 min. Thus, the E2-ERα-mediated increase in intracellular Ca^{2+} that leads to UPR activation is a rapid, extranuclear action of ERα at the plasma membrane.

The UPR and E2-ERα Action in E2-ERα Stimulated Cell Proliferation

We explored the role of Ca^{2+}-release from the EnR in promoting E2-ERα induced gene expression, UPR activation, and subsequent cell proliferation. Consistent with a possible role for intracellular Ca^{2+} in E2-ERα action (36), chelating intracellular Ca^{2+} with BAPTA-AM blocked E2-stimulated cell proliferation (Fig. 9A). In T47D cells, PLCγ or IP₃R knockdown, or locking IP₃R with 2-APB, strongly inhibited the increase in cytosol Ca^{2+} (Figure 5A, B, D and F), UPR activation (Figure 5C and E, Fig. 7), and E2-ERα stimulated cell proliferation (Fig. 9A and B). However, IP₃R knockdown did not inhibit E2-dependent down-regulation of ERα or E2-induction of GREB1 or pS2 mRNA (Figure 9C; Fig. 10B) (37, 38). Similarly, 2-APB did not abolish E2-ERα induced expression of stably transfected ERE-luciferase in T47D cells, while 2-APB and Ry together, strongly inhibited reporter gene expression (Fig. 9D). This suggests there are different intracellular Ca^{2+} requirements for E2-ERα-mediated UPR activation and E2-ERα-mediated gene expression. Importantly, the IP₃R knockdown data uncouples UPR
activation from E2-ERα-mediated gene expression, and demonstrates that blocking UPR activation is sufficient to inhibit estrogen-stimulated cell proliferation.

We next evaluated the role of E2-induction of EnR chaperones in E2-ERα-stimulated cell proliferation. Knockdown of PLCγ or IP3R strongly inhibited E2-induction of BiP and E2-ERα-stimulated cell proliferation (Fig. 5C and E, Fig. 9A). Knockdown of the primary UPR regulator of EnR chaperones, ATF6α, also strongly inhibited E2-induction of BiP and E2-ERα-stimulated cell proliferation (Fig. 2E and 9A). Thus, UPR activation and subsequent induction of EnR chaperones plays an important role in E2-ERα-stimulated cell proliferation.

We further evaluated the effects of PLCγ, IP3R, ATF6α, XBP1, and PERK knockdown on E2-stimulated proliferation of MCF-7 cells (Fig. 11). Knockdown of the ATF6α and XBP1 arms of the UPR produced 40% declines in E2-stimulated in cell proliferation, while PERK knockdown had no effect (Fig. 10E). IP3R knockdown produced a 50% decline in E2-ERα-stimulated MCF-7 cell proliferation (Fig. 9E). This is consistent with the 40% decline in proliferation following 2-APB treatment (Fig. 10C), which did not fully abolish E2-induction of pS2 and GREB1 mRNA (Fig. 9F; Fig. 10D). Targeting IP3R in MCF-7 cells produced less dramatic inhibition of E2-ERα-stimulated cell proliferation compared to T47D cells or BG-1 ovarian cancer cells (Fig. 9A, B, E; Fig. 10C and E). Knockdown of PLCγ in MCF-7 cells nearly abolished E2-ERα-stimulated cell proliferation (Fig. 9E). Together, this data demonstrates that weak anticipatory activation of the UPR, resulting in induction of chaperones, plays an important role in E2-ERα-stimulated cell proliferation. This novel E2-ERα pathway leading to cancer cell proliferation is shown (Fig. 9G).
**E₂-ERα Action Increases Levels of UPR Sensors and Downstream Targets**

We investigated whether E₂-ERα facilitates UPR activation by inducing the sensors that trigger activation of the three UPR arms. E₂ rapidly induced mRNAs encoding sensors for all 3 UPR arms and the chaperones BiP and GRP94 (Fig. 12A). These were early responses, usually visible within 2 hours. Although some responses declined at later times, estrogen produced sustained increases in resident chaperones and some UPR components, such as eIF2α (Fig. 12A).

**E₂-ERα-regulated Gene Expression and UPR Activation are Correlated In Vivo**

To assess in vivo relevance, we used growing MCF-7 tumors receiving estrogen and regressing MCF-7 tumors receiving only cholesterol vehicle (Fig. 12B) and compared expression of classical measures of E₂-ERα activity to markers of UPR activation (24). In the +E₂ tumors, the markers for E₂-ERα activity, pS2 and GREB1 mRNAs (37, 38), were induced 12-fold and 17-fold and all three UPR arms were moderately activated (Fig. 12C and D). Consistent with activation of the IRE1α arm of the UPR, active sp-XBP1 increased 3-fold while inactive XBP1 declined (Fig. 12D). Consistent with E₂-activation of the ATF6α arm of the UPR, +E₂ tumors displayed 2.0 and 1.8-fold increases in BiP and GRP94 mRNAs, respectively (Fig. 12D). Levels of CHOP and GADD34 mRNA were 2.1-fold and 1.4-fold higher in the +E₂ group, respectively, indicating weak activation of the PERK arm (Fig. 12D). While levels of
primary UPR sensors IRE1α and PERK were reduced in these tamoxifen-sensitive tumors, their immediate targets eIF2α and sp-XBP1 were increased (Fig. 12D).

To assess UPR activity early in ERα⁺ breast cancer development, we compared E₂-ERα activity and UPR pathway activity in samples of histologically normal breast epithelium and invasive ductal carcinoma (IDC). Compared to normal epithelium from IDC patients, IDC samples displayed elevated levels of ERα mRNA and E₂-ERα induced pS2 and GREB1 mRNAs, and reduced levels of E₂-ERα downregulated IL1-R1 mRNA (Fig. 12E). IDC samples displayed elevated SERP1 mRNA, a marker for IRE1α activation (32); CHOP and GADD34, which are markers of PERK activation; and BiP and GRP94 chaperones, which are markers of ATF6α activation (Fig. 12F). These data suggest UPR activation occurs very early in tumor development.

Using data from an independent cohort of 278 ERα⁺ breast cancers we explored whether expression of ERα mRNA and protein, or E₂-ERα-regulated genes, correlates with expression of UPR genes. Expression of several UPR genes displayed highly significant correlation with expression of ERα and ERα-target genes (Table 1).

Prior Estrogen Activation of the UPR Protect Cells from Subsequent Exposure to Cell Stress

Weakly activating, non-toxic, concentrations of the UPR activator, tunicamcytin (TUN), elicit an adaptive stress response that increases EnR chaperones, and renders cells resistant to subsequent exposure to an otherwise lethal concentration of tunicamycin (35, 39). Consistent with weak E₂ activation of the UPR, E₂ induces a 2.3-
fold increase in BiP protein compared to a 5.5-fold increase in BiP following maximal UPR activation by a lethal concentration of tunicamycin (Fig. 2G and Fig 13). We tested whether prior exposure of T47D cells to E2, or a low concentration of tunicamycin, altered the concentration of tunicamycin required to subsequently induce substantial cell death. Pre-treating cells with estrogen or TUN had nearly identical effects; each elicited an ~10 fold increase in the concentration of tunicamycin required to induce apoptosis (Fig. 14A). Thus, the E2-induced weak anticipatory activation of the UPR both facilitates tumor cell proliferation and is a potential mechanism by which estrogen might protect ERα+ breast tumors against subsequent apoptosis due to hypoxia, nutritional deprivation and therapy.

**A UPR Gene Signature Predicts Clinical Outcome in ERα Positive Breast Cancer**

To explore UPR activation as a potential prognostic marker in ERα+ breast cancer, we developed a UPR gene signature consisting of genes encoding components of the UPR pathway and downstream targets of UPR activation (Table 2). Using data from 261 ERα+ breast cancer patients, each assigned to a high- or low-genomic UPR grade, we observed reduced time to relapse for patients overexpressing the UPR signature (hazard ratio (HR) = 5.5, 95% CI: 3.1-9.8) (Fig. 15A and B). To evaluate the UPR signature in patients undergoing tamoxifen therapy, samples collected from 474 ERα+ breast cancer patients, prior to starting 5-years of tamoxifen therapy, were assigned to low, medium, or high UPR risk groups. Increased prior expression of the UPR gene signature was tightly correlated with subsequent reduced time to recurrence.
(Figure 14B and D; Fig. 15C). Hazard ratios increased from 2.2 to 3.7 for the medium and high-risk groups, respectively, suggesting that recurrence risk is sensitive to levels of the UPR gene signature (Fig. 14B). The UPR index provides prognostic information beyond current clinical covariates. In a cohort of 236 ERα+ breast cancer patients, UPR overexpression was strongly predictive of reduced survival (HR 2.69, 95% CI: 1.3-5.6), over and above clinical covariates alone (tumor grade, node involvement, tumor size and ERα status) (Fig. 14C and D; Fig. 15D). Thus, the UPR index is a powerful prognostic gene signature in ERα+ breast cancer with predictive power to stratify patients into high and low risk groups.

Discussion

In contrast to the well-studied “reactive mode” of UPR activation that occurs in response to endoplasmic reticulum stress, there are few studies of UPR activation that anticipates the future need for increased capacity to fold and sort proteins, and occurs in the absence of endoplasmic reticulum stress (7). Anticipatory UPR activation is observed in B-cell differentiation where UPR activation in plasma cells precedes the massive production and secretion of immunoglobulins (13, 14). Because the signals responsible for anticipatory activation of the UPR are largely unknown, it is poorly understood.

In the absence of cell stress or misfolded proteins, the mitogen, estrogen, acting via ERα, triggers anticipatory activation of the UPR in breast and ovarian cancer cells. In less than 2 minutes, E2-ERα triggers PLCγ-mediated opening of EnR IP3R calcium
channels and release of Ca\textsuperscript{2+} into the cytosol. This increase in cytosol Ca\textsuperscript{2+} stimulates activation of all three arms of the UPR and is required for E\textsubscript{2}-ER\textalpha-stimulated cell proliferation.

Anticipatory activation of the UPR by E\textsubscript{2}-ER\textalpha enhances EnR protein folding capacity, and thereby primes cells to meet the higher protein folding and sorting demands that characterize the later growth phases of the cell cycle. The major EnR chaperone BiP, plays a central role in EnR homeostasis, protein processing, and UPR signaling. Since BiP knockdown stimulates UPR activation and promotes EnR stress-induced apoptosis (10, 40), and cells undergoing E\textsubscript{2}-mediated apoptosis have lower levels of chaperones (41), we assessed the consequences of abrogating the expansion of EnR protein-folding capacity by blocking anticipatory activation of the UPR. PLC\gamma, IP\textsubscript{3}R or ATF6\textalpha knockdown blocked E\textsubscript{2}-induction of BiP and inhibited E\textsubscript{2}-ER\textalpha-stimulated proliferation of T47D cells. While IP\textsubscript{3}R knockdown nearly abolished E\textsubscript{2}-ER\textalpha-stimulated Ca\textsuperscript{2+}-release from the EnR, and this blocked UPR activation, it did not inhibit E\textsubscript{2}-ER\textalpha-mediated gene expression. Thus, inhibition of E\textsubscript{2}-ER\textalpha-stimulated UPR activation and chaperone induction is sufficient to inhibit E\textsubscript{2}-ER\textalpha-stimulated cell proliferation. Using 2-APB and ryanodine together, or chelating intracellular calcium with BAPTA, completely abrogated the increase in intracellular calcium, and blocked E\textsubscript{2}-ER\textalpha-regulated gene expression. Based on the inhibitor and knockdown data, we hypothesize that very small increases in intracellular calcium are sufficient to enable E\textsubscript{2}-ER\textalpha-regulated gene expression and that somewhat larger increases in intracellular calcium are likely required for E\textsubscript{2}-ER\textalpha activation of the UPR. E\textsubscript{2}-ER\textalpha induces a substantial increase in intracellular calcium, which may promote coordination between the nucleus and
endoplasmic reticulum, and couple activation of the E₂-ERα genomic program with UPR activation and expansion of the EnR protein-folding capacity.

We further validated the importance of this novel extranuclear pathway of E₂-ERα action using MCF-7 cells to assess how knockdown of each pathway component affects E₂-ERα stimulated cell proliferation. PERK knockdown produced a 20% in E₂-ERα-stimulated cell proliferation, and may be required to fully activate the ATF6α arm of the UPR (42). Knockdown of the XBP1 or ATF6α produced a 40% decline in E₂-ERα-stimulated cell proliferation. IP₃R knockdown produced an even larger reduction in E₂-ERα stimulated cell proliferation, while PLCγ knockdown had the largest effect. Thus, anticipatory activation of the UPR plays an important role in E₂-ERα dependent proliferation of cancer cells.

As expected (1, 3), IDC tumor samples exhibited increased ERα expression and activation compared to normal breast epithelial tissue. Consistent with a role for the UPR in this proliferative phase of early tumor development, increased UPR expression and activation was observed in IDC tumor samples. This suggests that increased UPR expression occurs early in tumor development, long before detection, diagnosis, and the initiation of treatment.

Activation of the UPR by E₂-ERα exerts a long-term impact on the pathology of ERα positive breast cancer. Weak activation of the UPR by estrogen, or by tunicamcyin, elicits an adaptive response that protects cells from subsequent exposure to higher levels of cell stress. We explored whether the effects of E₂-ERα on the UPR correlated with clinical resistance to tamoxifen therapy. Increased UPR activation and elevated expression of UPR components were predictive of a poor response to tamoxifen-
therapy, shorter time to recurrence, and decreased overall survival. If UPR expression promotes resistance to tamoxifen therapy, some UPR genes should exhibit differential regulation in our tamoxifen-sensitive MCF-7 tumors (24), compared to their expression in the tamoxifen-resistance gene signature. Supporting this view, several genes encoding UPR components were E$_2$-downregulated in tamoxifen-sensitive MCF-7 tumors, but elevated in the human tumors expressing the tamoxifen-resistance gene signature (PERK, p58$^{IPK}$).

For ER$\alpha^+$ breast cancers resistant to endocrine therapies, an important objective is development of more specific biomarkers that predict therapeutic response and identification of new therapeutic targets. The UPR is a new biomarker and therapeutic target in ER$\alpha^+$ breast cancer; validated through mechanistic studies in culture, a mouse xenograft, and bioinformatics analysis of patient tumor samples. Anticipatory estrogen activation of the UPR is a novel extranuclear action of ER$\alpha$, a previously undescribed early component of the estrogen-ER$\alpha$ cell proliferation program and a new paradigm by which estrogens may influence tumor development and resistance to therapy.
Figure 3.1. Endoplasmic reticulum (EnR) stress activates the three arms of the UPR. (A) EnR stress induces the oligomerization, autophosphorylation, and activation of the transmembrane endoribonuclease, IRE1α. Activated IRE1α removes an intron from full-length XBP1 (fl-XBP1) mRNA, producing spliced-XBP1 (sp-XBP1) mRNA, which is subsequently translated into active sp-XBP1 protein. sp-XBP1 enhances the protein-folding capacity of the EnR, increases turnover of misfolded proteins by inducing EnR-associated degradation (ERAD) genes, and alters mRNA decay and translation (6, 7). ERDJ and SERP1 are commonly used readouts of IRE1α activation (32, 43, 44). (B) EnR stress activates the transmembrane protein, activating transcription factor 6 α (ATF6α). Full-length ATF6α (p90-ATF6α) translocates from the EnR to the Golgi Apparatus, where it is cleaved by site-1 and site-2 proteases, resulting in the release of a 50-kDa ATF6α (p50-ATF6α) fragment into the cytosol. p50-ATF6α enters the nucleus and induces several UPR genes including BiP, GRP94, calreticulin, and other EnR chaperones (34). (C) EnR stress induces the oligomerization, autophosphorylation, and activation of the transmembrane kinase PERK (6, 7). P-PERK phosphorylates eukaryotic initiation factor 2α (eIF2α), leading to general inhibition of protein synthesis and a reduction in the endoplasmic reticulum protein folding load. However, increased eIF2α phosphorylation also leads to preferential translation of certain mRNA, including the transcription factor, ATF4 (6, 7). Increased translation of ATF4 induces the transcription factor CHOP, which induces GADD34 and several pro-apoptotic genes. Inhibition of protein synthesis is normally reversed by inactivating PERK and dephosphorylating eIF2α. p58IPK binds PERK, inhibiting PERK activation, and GADD34 forms a phosphatase complex with protein phosphatase 1 (PP1), which dephosphorylates eIF2α (45-47).
Figure 3.2. E2-ERα activates the IRE1α and ATF6α arms of the UPR in breast and ovarian cancer cells, resulting in the induction of the major EnR chaperone, BiP. 

(A) qRT-PCR comparing the effect of estrogen (E2), ICI 182,780 (ICI) and 4-hydroxytamoxifen (4-OHT) on E2-ERα induction of spliced-XBP1 (sp-XBP1) in T47D breast cancer cells (n = 3; -E2 set to 1). Different letters indicate a significant difference among groups (p < 0.05) using one-way ANOVA followed by Tukey’s post hoc test. 

(B) qRT-PCR showing the effect of E2-ERα on sp-XBP1 mRNA in MCF-7 breast and PEO4 ovarian cancer cells (n = 3; -E2 set to 1). P-values testing for significance between indicated group and -E2 group. 

(C) RNAi knockdown of ERα abolishes E2-induction of sp-XBP1 in MCF-7 cells (n = 3). Cells treated with 100 nM non-coding control (NC) or ERα siRNA SmartPools for 3 days, followed by E2 treatment for the indicated times. 

(D) Western blot analysis showing full-length 90 kDa ATF6α (p90-ATF6α) and proteolytically cleaved 50 kDa ATF6α (p50-ATF6α) in E2-treated T47D breast cancer cells. 

(E) RNAi knockdown of ATF6α blocks E2-induction of BiP in T47D cells. Cells treated with 100 nM non-coding control (NC) or ATF6α siRNA SmartPool for 3 days, followed by E2 treatment for 4 hours. 

(F) qRT-PCR showing the effect of E2 on BiP mRNA in MCF-7 cells and in PEO4 ovarian cancer cells (n = 3; -E2 set to 1). 

(G) Western blot analysis of BiP protein levels in MCF-7 cells treated with E2. The fold-change in BiP protein levels is shown below each lane and was determined by quantifying BiP and β-Actin signals, and calculating the ratio of BiP/β-Actin (t=0, [-E2], set to 1). 

(H) RNAi knockdown of ERα abolishes E2-induction of BiP in MCF-7 cells (n = 3). Cells treated with 100 nM non-coding control (NC) or ERα siRNA SmartPools for 3 days, followed by E2 treatment for the indicated times. Concentrations: E2, 1 nM (A, D), 10 nM (B, C, E-H); ICI, 1 μM (A, D); 4-OHT, 1 μM (A). Data is mean ± S.E.M. * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 3.3. E2-ERα induces the UPR. (A) E2-ERα stimulates induction of downstream transcriptional targets of spliced-XBP1, SERP1 and ERDJ.(32, 43, 44) The increase in SERP1 and ERDJ mRNA coincides with increased splicing of XBP1 mRNA, which together indicate that E2-ERα stimulates activation of the IRE1α-arm of the UPR. -E2 treatment set to 1. P-values testing for significance between indicated group and -E2 group. (B) ERα knockdown abolishes E2-induction of GREB1 (growth regulated by estrogen in breast cancer 1) mRNA, which is a well-established transcriptional target of E2-ERα (38, 48). (C) E2-ERα activates the ATF6α arm of the UPR in PEO4 ovarian cancer cells. The increase in the level of p50-ATF6α (p50-ATF6α) demonstrates activation of the ATF6α arm of the UPR. Ug-ATF6α band represents the unglycosylated or underglycosylated precursor of p90-ATF6α, which has been described previously (49). (D) qRT-pCR analysis of CHOP mRNA following treatment of MCF-7 cells with E2, or the UPR activator tunicamycin (TUN). Concentrations: E2, 10 nM; TUN, 10 μg/mL. Data is mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001; ns, not significant.
Figure 3.4. E2-ERα activates the PERK arm of the UPR. Western blot analysis showing (A) p-PERK and total PERK levels and (B) p-eIF2α levels and total eIF2α levels in T47D cells treated with ICI 182,780 (ICI) or a vehicle control for 2 hours, followed by treatment with 10 nM 17β-estradiol (E2) (n = 3). Numbers below each lane are the ratio of p-PERK/PERK or p-eIF2α/eIF2α normalized to the vehicle-treated control. (C) Protein synthesis in ERα+ T47D breast cancer cells treated with ICI 182,780 (ICI) or a vehicle control for 2 hours, followed by treatment with 10 nM 17β-estradiol (E2) (n = 3). P-values testing for significance between indicated groups and -E2 samples. (D) PERK knockdown inhibits downstream phosphorylation of eIF2α in T47D cells. (E) Western blot analysis of ATF4 following treatment of T47D cells with E2, or the UPR activator tunicamycin (TUN). (F) qRT-pCR analysis of CHOP mRNA following treatment of T47D cells with E2. Brackets denote pre-treatment with ICI for 2 hours. Concentrations: E2, 1 nM (A-F); ICI, 1 μM (A, B, C); TUN, 10 μg/mL (E). Data is mean ± SEM. * p < 0.05; ** p < 0.01; *** p < 0.001; ns, not significant.
Figure 3.5. Estrogen stimulates the release of calcium from the endoplasmic reticulum, and this calcium release is necessary for UPR activation. (A) Effects of 300 nM estrogen (E2) on cytosolic calcium levels in T47D breast cancer cells conditioned in the presence (2 mM CaCl₂) or absence (0 mM CaCl₂) of extracellular calcium, or cells pre-treated with 2-APB or ryanodine (Ry) for 30 minutes in the absence of extracellular calcium (0 mM CaCl₂). Visualization of intracellular Ca²⁺ using Fluo-4 AM. Colors from basal Ca²⁺ to highest Ca²⁺: Blue, green, red, white. (B) Graph depicts quantitation of cytosolic calcium levels in ERα⁺ T47D breast cancer cells treated with E₂ in the presence or absence of extracellular calcium, and in cells pre-treated with 2-APB or ryanodine (Ry) in the absence of extracellular calcium (n = 10 cells). E₂ was added at 60 sec, and fluorescence intensity prior to 60 sec was set to 1. (C) Western blot analysis of IP₃R and BiP protein levels following treatment of T47D cells with either 100 nM non-coding (NC) siRNA or a IP₃R SmartPool, followed by treatment with E₂ (+E2) or ethanol-vehicle (-E2) for 4 hours. IP₃R smartpool contained 33 nM siRNA directed against each isoform of IP₃R Ca²⁺-channel. (D) Quantitation of cytosolic Ca²⁺ levels in response to E₂, following treatment of T47D cells with 100 nM non-coding (NC) siRNA or IP₃R siRNA SmartPools for 3 days (n = 10 cells) (E) Western blot analysis of PLCγ, BiP, and ATF6α protein levels after treatment of T47D cells with 100 nM non-coding (NC) siRNA or PLCγ siRNA SmartPool, followed by treatment with E₂ (+E2) or ethanol-vehicle (-E2) for 4 hours. (F) Quantitation of cytosolic Ca²⁺ levels in response to E₂, following treatment of T47D cells with 100 nM non-coding (NC) siRNA or PLCγ siRNA SmartPool for 3 days. (G) Western blot analysis of ERα protein levels after treating T47D cells with either 100 nM non-coding (NC) siRNA or ERα siRNA SmartPool,
followed by treatment with E2 (+E2) or ethanol-vehicle (-E2) for 4 hours. (H)
Visualization and quantitation of cytosolic Ca\textsuperscript{2+} levels in response to E\textsubscript{2} after ER\textalpha knockdown in T47D cells. Concentrations: E\textsubscript{2}, 300 nM (A, B, D, F, H), 1 nM (C, E, G); 2-APB, 200 \textmu M (A, B); ryanodine, 200 \textmu M (A, B). Graphical data is mean ± SE (n = 10).
Figure 3.6. Estrogen increases intracellular calcium levels in ERα+ PEO4 ovarian cancer cells. Effect of 300 nM E₂ on intracellular calcium levels in ERα+ PEO4 ovarian cells. Cells visualized with the Ca²⁺ sensitive dye Fluor-4. Low levels of basal [Ca²⁺] are blue and then green, whereas higher levels of [Ca²⁺] are seen as red, with the highest levels white. The trace represents relative signal intensity averaged from 10 cells. E₂ was added at 60 sec, and fluorescence intensity prior to 60 sec was set to 1. Data is mean ± S.E.
Figure 3.7. Blocking Ca\textsuperscript{2+}-release from the EnR through the IP\textsubscript{3}R Ca\textsuperscript{2+} channel prevents estrogen-dependent activation of the IRE1\textalpha and PERK arms of the UPR. (A) Pre-blocking the IP\textsubscript{3}R Ca\textsuperscript{2+}-channel with 2-APB prevents estrogen-dependent activation of the PERK arm of the UPR in T47D cells. Western blot analysis showing p-eIF2\textalpha and total eIF2\textalpha levels in T47D breast cancer cells pre-treated for 30 minutes with 2-APB and/or ryanodine (Ry) or a vehicle control, followed by treatment with E\textsubscript{2} for 30 minutes. Numbers below each lane are the ratio of p-eIF2\textalpha/eIF2\textalpha with the vehicle-treated control set at 100. (B) IP\textsubscript{3}R knockdown abolishes E\textsubscript{2}-induction of sp-XBP1 mRNA in T47D cells. Cells were treated with non-coding control (NC) or IP\textsubscript{3}R siRNA SmartPools for 3 days, followed by E\textsubscript{2} treatment for 4 hours. Data is mean ± SEM. Letters indicate a significant difference among groups (p < 0.05) using one-way ANOVA followed by Tukey’s post hoc test. Concentrations: E\textsubscript{2}, 10 nM; 2-APB, 200 \textmu M; Ryanodine, 200 \textmu M.
Figure 3.8. Treatment of T47D cells with the PLCγ inhibitor, U73122, blocks E₂-stimulated calcium release. Graph depicts quantitation of cytosolic calcium levels in ERα⁺ T47D breast cancer cells pre-treated with DMSO vehicle or U73122, followed by treatment with 300 nM E₂ in the absence of extracellular calcium (n = 10 cells). E₂ was added at 60 sec, and fluorescence intensity prior to 60 sec was set to 1. Data is mean ± S.E.
Figure 3.9. E2-ERα induced calcium release from the EnR into the cytosol is important for E2-ERα mediated gene expression and E2-ERα stimulated cell proliferation. (A) E2-ERα stimulated proliferation of T47D breast cancer cells treated 100 nM non-coding (NC), PLCγ, IP3R, or ATF6α siRNA SmartPools (n = 6). (B) E2-ERα stimulated proliferation of T47D breast cancer cells treated with ryanodine (Ry), 2-APB, or both inhibitors (Ry + 2-APB) for 4 days (n = 5). (C) qRT-PCR analysis of effects of IP3R knockdown on E2-ERα induction of GREB1 mRNA in T47D cells (n = 3). Western blot shows ERα protein levels after treatment of T47D cells with 100 nM non-coding (NC) siRNA or IP3R siRNA, followed by treatment with E2 (+E2) or ethanol vehicle (-E2) for 4 hours. (D) ERE-luciferase activity in kBluc-T47D breast cancer cells treated with E2 and either ryanodine (Ry), 2-APB, or both inhibitors for 24-hours (Ry + 2-APB) (n = 4). (E) E2-ERα stimulated proliferation of MCF-7 breast cancer cells treated 100 nM non-coding (NC), PLCγ, IP3R, ATF6α, XBP1, or PERK siRNA (n = 6). (F) qRT-PCR analysis of effects of ryanodine (Ry), 2-APB, or both inhibitors (Ry + 2-APB) on E2-ERα induction of pS2 mRNA in MCF-7 cells (n = 3). (G) Model of E2-ERα acting through the UPR to influence breast tumorigenesis. “•” denotes cell number at day 0. Concentrations: E2, 100 pM (A-F); 2-APB, 200 μM (B, D, F); Ryanodine, 100 μM (B, D, F). Data is mean ± SEM. Different letters indicate a significant difference among groups (p < 0.05) using one-way ANOVA followed by Tukey’s post hoc test. ns, not significant (p > 0.05).
Figure 3.10. $E_2$-ER$\alpha$ induced calcium release from the EnR into the cytosol is important for $E_2$-ER$\alpha$ mediated gene expression and $E_2$-ER$\alpha$ stimulated cell proliferation. (A) Effects of the intracellular calcium chelator BAPTA-AM on $E_2$-ER$\alpha$ stimulated cell proliferation ($n = 5$). MCF-7 cells were treated with 10 $\mu$M BAPTA-AM for 3 days. (B) qRT-PCR analysis of effects of IP$_3$R knockdown on $E_2$-ER$\alpha$ induction of pS2 mRNA in T47D cells ($n = 3$). (C) $E_2$-ER$\alpha$ stimulated proliferation of MCF-7 breast cancer cells treated with ryanodine (Ry), 2-APB, or both inhibitors (Ry + 2-APB) for 4 days ($n = 5$). (D) qRT-PCR analysis of effects of ryanodine (Ry), 2-APB, or both inhibitors (Ry + 2-APB) on $E_2$-ER$\alpha$ induction of GREB1 mRNA in MCF-7 cells ($n = 3$). (E) $E_2$-ER$\alpha$ stimulated proliferation of BG-1 ovarian cancer cells treated with ryanodine (Ry), 2-APB, or both inhibitors (Ry + 2-APB) for 4 days ($n = 5$). “•” denotes cell number at day 0. Data is mean ± SEM. Letters indicate a significant difference among groups ($p < 0.05$) using one-way ANOVA followed by Tukey’s post hoc test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant. Concentrations: $E_2$, 100 pM; 2-APB, 200 $\mu$M; Ryanodine, 200 $\mu$M.
Figure 3.11. Western blot analysis of UPR proteins following siRNA knockdown in MCF-7 cells. Western blot analysis of (A) PLCγ, (B) pan-IP₃R, (C) ATF6α, (D) XBP1, and (E) PERK protein levels following treatment of MCF-7 cells with either 100 nM non-coding SmartPool siRNA or 100 nM SmartPool siRNA directed against the protein of interest. The IP₃R SmartPool consisted of three individual siRNAs SmartPools targeting each isoform of the IP₃R Ca²⁺-channel (ITPR1, ITPR2, ITPR3).
Figure 3.12. E2-ERα activity and UPR activity are correlated in vivo. (A) qRT-PCR analysis of levels of mRNAs for each arm of the UPR after treatment of MCF-7 cells with 10 nM E2 for the indication times (n = 3). (B) MCF-7 tumor growth in the presence or absence of estrogen in athymic mice. All mice were treated with estrogen to induce tumor formation. On “Day 0”, E2 in silastic tubes was replaced with silastic tubes containing only cholesterol in the –E2 group (n = 15), while silastic tubes were retained in the +E2 treatment group (n = 15). qRT-PCR analysis of (C) classical E2-ERα regulated genes and (D) the UPR in mouse tumors collected after 24 days of exposure to estrogen (+E2) or vehicle-control (-E2) (n = 15). Relative mRNA levels of (E) classical E2-ERα regulated genes and (F) the UPR pathway in patient samples of normal breast epithelium taken from patients undergoing reduction mammoplasty (RM) (n = 18),
histologically normal breast epithelium taken from patients diagnosed with invasive ductal carcinoma (IDC) (n = 9), and carcinoma epithelium taken from IDC patients (n = 20). p-values represent comparisons to –E2 groups (A, C, D) or to histologically normal breast epithelium from patients who underwent reduction mammoplasty (e, f). Data is mean ± SEM. * P < 0.05; ** P < 0.01; ***P < 0.001; ns, not significant.
Figure 3.13 The UPR Activator, tunicamycin, induces BiP. Time course analysis of BiP protein levels following tunicamycin (TUN) treatment. Densiometric analysis performed by normalizing BiP protein levels to β-Actin.
Figure 3.14. Anticipatory activation of the UPR by estrogen protects cells from subsequent cell stress, and expression of the UPR gene signature predicts relapse-free and overall survival in ERα positive breast tumor cohorts. (A) Weak anticipatory activation of the UPR with estrogen or tunicamycin protects cells from subsequent UPR stress. T47D cells were maintained in 10% CD-FBS for 8 days and treated with either 250 ng/ml tunicamycin (TUN), 100 pM E2, or ethanol/DMSO-vehicle (Untreated). E2, TUN, or the vehicle control were removed from medium, and cells were harvested in 10% CD-calf serum and treated with the indicated concentrations of tunicamycin. Data is mean ± SEM (n = 6). Different letters indicate a significant difference among groups (p < 0.05) using one-way ANOVA followed by Fisher’s LSD post hoc test. (B) Relapse-free survival as a function of the UPR gene signature for patients with ERα+ breast cancer who subsequently received tamoxifen alone for 5 years. Interquartile range used to assign tumors to risk groups, representing UPR activity from high to low. Hazard ratios are between low and medium and low and high
UPR groups (n = 474). (C) Overall survival as a function of the UPR signature and clinical covariates (node status, tumor grade, ERα-status, tumor size). p-value is testing for significance between the combined model (UPR gene signature and clinical covariates) versus the covariates only model (multivariate analysis) (n = 236). (D) Univariate and multivariate Cox regression analysis of the UPR signature, clinical covariates, and classical estrogen-induced genes for time to recurrence and survival (n.s., not significant). Median used to classify tumors into high and low risk groups.
Figure 3.15. The UPR genomic index is a new biomarker that predicts relapse free and overall survival of breast cancer patients. (A) Relapse rate of 261 ERα positive breast tumors, classified by expression levels of the UPR gene signature, plotted by the Kaplan-Meier method. Tumor samples were analyzed on both, U133A and U133B gene chips. The table below denotes univariate and multivariate Cox regression hazard ratios and p-values for the UPR gene signature and other clinical covariates (tumor grade, tumor size, node status). (B) UPR genes independently predictive of relapse (p < 0.05) in gene expression profiles obtained from 277 ERα+ positive breast cancers (27). Kaplan-Meier plots and Cox regression hazard analysis for this tumor cohort is displayed in Figure 15A. (C) UPR genes independently predictive of relapse in 474 gene expression microarrays taken from ERα-positive breast cancer patients prior to the initiation of tamoxifen-therapy (27, 28). Kaplan-Meier analysis and Cox regression hazard analysis for this tumor cohort are displayed in Figure 14B and Figure 14D, respectively. (D) UPR genes predictive of survival in 236 gene expression microarrays from breast cancer patients (29). Kaplan-Meier plots and Cox regression hazard analysis for this tumor cohort is displayed in Figure 14C and Figure 14D, respectively. All Kaplan-Meier plots assessing UPR risk prediction were computed using leave-one-out cross-validation. UPR signature genes shown in the tables are listed with their respective univariate Cox hazard ratio and p-value test the hypothesis if expression data is predictive of relapse or overall survival.
Table 3.1. Expression of UPR genes is positively correlated with expression of ERα and ERα-regulated target-genes. Correlations between the UPR and ERα protein levels (ERα), ERα mRNA levels (ESR1), or transcriptional activity of E2-ERα. E2-ERα transcriptional activity was assessed using downstream target genes of E2-ERα (pS2, GREB1) (38, 48, 50). Analysis carried out on a cohort of 278 breast cancer patients (GSE20194) (26), which consisted of 164 ERα positive tumors and 114 ERα negative tumors. Quantitation of ERα protein was by IHC. Pearson correlation coefficients and parametric p-values are shown in the table. “n.s.” indicates that no significant correlation was observed. While expression of UPR genes is correlated with ERα levels and expression of ERα-regulated genes, the UPR index is not simply a surrogate marker for ERα activity. In multivariate analysis, the UPR index, but not ERα, or classical ERα-regulated genes, exhibits a statistically significant increase in hazard ratio (Fig. 14D). Also, UPR index exhibits predictive power to stratify patients into high and low risk groups above ERα status (Fig. 14C). Thus, while active ERα is important for expression of the UPR signature, it’s the UPR signature not ERα level or activity that is predictive of reduced time to recurrence and reduced survival.

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### Table 3.2. UPR gene signature.
The table shows the genes used to construct the UPR gene signature. HUGO Gene Nomenclature Committee (HGNC) approved names for each gene are shown in parenthesis. UPR genes independently predictive either of relapse free or overall survival ($p < 0.05$) were used to construct the UPR gene signature, which was then used to carry out risk prediction analysis.

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References


CHAPTER 4

AN ESTROGEN RECEPTOR α INHIBITOR ACTIVATES THE UNFOLDED PROTEIN RESPONSE, BLOCKS PROTEIN SYNTHESIS AND INDUCES TUMOR REGRESSION

Abstract

Recurrent estrogen receptor α (ERα) positive breast and ovarian cancers are often therapy-resistant. Using screening and functional validation, we identified BHPI, a potent non-competitive small molecule ERα biomodulator that selectively blocks proliferation of drug-resistant ERα positive breast and ovarian cancer cells. In a mouse xenograft model of breast cancer, BHPI induced rapid and substantial tumor regression. While BHPI potently inhibits nuclear estrogen-ERα-regulated gene expression, BHPI is effective because it elicits sustained ERα-dependent activation of the endoplasmic reticulum (EnR) stress sensor, the unfolded protein response (UPR) and persistent inhibition of protein synthesis. BHPI distorts a newly described action of estrogen-ERα, mild and transient UPR activation. In contrast, BHPI elicits massive and sustained UPR activation, converting the UPR from protective to toxic. In ERα+ cancer cells, BHPI rapidly hyperactivates plasma membrane PLCγ, generating IP₃, which opens EnR IP₃R calcium channels, rapidly depleting EnR Ca²⁺ stores. This leads to activation of all three arms of the UPR. Activation of the PERK arm, stimulates phosphorylation of eukaryotic initiation factor 2α (eIF2α), resulting in rapid inhibition of protein synthesis. The cell attempts to restore EnR Ca²⁺ levels, but the open EnR IP₃R calcium channel leads to an
ATP-depleting futile cycle, resulting in activation of the energy sensor AMPK and phosphorylation of eukaryotic elongation factor 2 (eEF2). eEF2 phosphorylation inhibits protein synthesis at a second site. BHPI’s novel mode of action, high potency, and effectiveness in therapy-resistant tumor cells, make it an exceptional candidate for further mechanistic and therapeutic exploration.

Introduction

Estrogens, acting via estrogen receptor α (ERα), stimulate tumor growth (1-3). Approximately 70% of breast cancers are ERα positive and most deaths due to breast cancer are in patients with ERα+ tumors (2, 4). Endocrine therapy using aromatase inhibitors to block estrogen production, or tamoxifen and other competitor antiestrogens, often results in selection and outgrowth of resistant tumors. Epithelial ovarian cancer usually presents at an advanced stage (5). Although 30-70% of these tumors are ERα positive (1), endocrine therapy is largely ineffective (6-8). After several cycles of combination chemotherapy using taxanes and platinum, tumors recur as resistant ovarian cancer (5). More than half of ovarian cancer patients die within 5 years (9).

Non-competitive ERα inhibitors targeting this unmet therapeutic need including DIBA, TPBM, TPSF, and newly described LRH-1 inhibitors that reduce ERα levels, show limited specificity, require high concentrations (>5 μM) and have usually not advanced through preclinical development (10-13). These non-competitive ERα inhibitors and competitor antiestrogens are primarily cytostatic and act by preventing estrogen-ERα action; therefore, they are largely ineffective in therapy-resistant ERα containing cancer cells that no longer require estrogens and ERα for growth.
To target the estrogen-ERα axis in therapy-resistant cancer cells, we developed (14) and implemented an unbiased pathway-directed screen of ~150,000 small molecules. We identified ~2,000 small molecule biomodulators of 17β-estradiol (E2)-ERα induced gene expression, evaluated these biomodulators for inhibition of E2-ERα-induced cell proliferation and performed simple follow-on assays to identify inhibitors with a novel mode of action. Here, we describe BHPI, our most promising small molecule ERα biomodulator. BHPI non-competitively interacts with ERα, resulting in two independent effects: Sustained inhibition of protein synthesis that blocks growth and ultimately kills many ERα+ breast, ovarian and endometrial cancer cells and rapid suppression of E2-ERα-regulated gene expression.

In response to stress cancer cells often activate the endoplasmic reticulum (EnR) stress sensor, the unfolded protein response (UPR). We recently showed that as an essential component of the E2-ERα proliferation program, estrogen induces a different mode of UPR activation, a weak anticipatory activation of the UPR prior to increased protein folding loads that accompany cell proliferation. This weak and transient E2-ERα-mediated UPR activation is protective (15). BHPI distorts this normal action of E2-ERα and induces a massive and sustained ERα-dependent activation of the UPR, converting UPR activation from cytoprotective to cytotoxic.

Materials and Methods

Cell Culture and Reagents
MCF-7, T47D, T47D-kBluc, HCC-1500, ZR-75-1, MCF10A, MDA MB-231, CAOV-3, OVCAR-3, IGROV-1, ES2, ECC-1, HeLa, PC-3, DU145, H1793, A549, MEF, and HepG2 cells were obtained from the ATCC. Dr. E. Wilson provided HeLa-AR13 cells, Dr. K. Korach provided BG-1/MCF-7 cells, Dr. B.H. Park provided MCF10AER IN9 cells, Dr. R. Schiff provided BT-474 cells, and E. Alarid provided MCF7ERαHA cells. Prior to experiments, to deplete cells of estrogens in the serum and medium, ERα positive cell lines were maintained for 4 days in medium supplemented with phenol red-free charcoal-dextran (CD) treated serum.

**Chemical Libraries and Screening**

The small molecule libraries screened were: The ~150,000 small molecule Chembridge MicroFormat small molecule library, the ~10,000 small molecule University of Illinois Marvel library developed by Drs. K. Putt and P. Hergenrother (16), and the ~2,000 small molecule NCI diversity set obtained from NIH. High throughput screening for small molecule inhibitors of endogenous E₂-ERα induced expression of the stably transfected (ERE)₃-luciferase reporter in T47D-KBluc cells, was carried out using the assay we recently described (14).

**Cell Proliferation Assays**

Cells were resuspended in the following media and plated in 96 well plates at the indicated densities: MCF-7 (10% CD-calf, 1,000 cells); MCF7ERαHA (10% CD-calf,
1,000); T47D (10% CD-calf, 2,000); T47D-kBluc (10% CD-FBS, 1,000); HCC-1500 (10% CD-FBS, 1,000); BT-474 (10% CD-calf, 2,000); ZR-75-1 (10% CD-calf, 2,000); MCF10AER IN9 (2% CD-FBS, 1,000); MCF10A (2% CD-FBS, 1,000); MDA MB-231 (10% FBS, 1,000); BG-1 (5% FBS, 250); CaOV-3 (10% CD-CALF, 2,000); OVCAR-3 (10% CD-FBS, 2,000); IGROV-1 (10% FBS, 1,000); ES2 (10% FBS, 1,000); ECC-1 (5% CD-FBS, 1,000); Ishikawa (10% CD-calf, 2,000); HeLa (10% FBS, 1,000); PC-3 (10% CD-FBS, 1,000); DU145 (10% FBS, 1,000); 201T (10% FBS, 2,000); 273T (10% FBS, 1,000); H1793 (5% FBS, 2,000); A549 (10% FBS, 1,000); HepG2 (10% CD-FBS, 1,000), MEF (10% FBS, 2,000). The medium was replaced with treatment medium the following day, and plates were incubated at 37°C in 5% CO₂ for 4 days except for BT-474, BG-1/MCF-7 and Ishikawa which were incubated for 6 days and ZR-75-1 cells which were incubated for 7 days. Treatment solutions were replaced every two days.

Cell number was determined from MTS assays using CellTiter 96 Aqueous One Solution Reagent (Promega). For each cell line, cell number was calculated from a standard curve of the number of plated cells versus A₄₉₀.

**ATP Measurements**

To measure ATP levels, cells were lysed and ATP luminescence levels were measured using an ATPlite Luminescence Assay kit (PerkinElmer, MA). ATP released from cells was quantified from a standard curve of ATP standards versus luminescence.

**Luciferase Assays**
Reporter gene assays were carried out, as previously described (14, 17). Briefly, cells were switched to 10% CD-FBS for four days prior to experiments, and plated at a density of 50,000 cells/well in 24-well plates. The medium was replaced the next day with medium containing the test compounds, with or without hormone, incubated for 24 hours and luciferase assays were performed using Bright Glow reagent (Promega, WI).

**qRT-PCR**

RNA was extracted using a QiaShredder kit (Qiagen) for cell homogenization, and purified with the RNAeasy mini-kit (Qiagen, CA). cDNA was prepared from 0.5 μg of RNA by reverse transcription using a DyNAmo cDNA synthesis kit (Finnzymes, Finland). Quantitative PCR assays were performed on samples from 3 independent sets of cells (biological triplicate). Reactions contained 10 ng of cDNA and 50 nM forward and reverse primers in 15 μl and were carried out using Power SYBR Green PCR Mastermix (Applied Biosystems). The fold change in expression of each gene was calculated using the ∆∆Ct method with the ribosomal protein 36B4 used as the internal control, as described previously (11, 14, 15).

**Chromatin Immunoprecipitation**

MCF-7 cells were stripped of estrogens for 3 days in 5% CD-FBS. Cells were pretreated with 1 μM BHPI or DMSO (0.1%) as a control for 105 minutes, and then were treated with either 10 nM E2 or an ethanol-vehicle control (0.1%) for 45 minutes. ChIP was carried out essentially, as previously described (17).
siRNA Transfections

siRNA knockdowns were performed using DharmaFECT1 Transfection Reagent and 100 nM ON-TARGETplus non-targeting pool or SMARTpools for ERα (ESR1), PLCγ (PLCG1), PERK (EIF2AK3), or pan-IP3R (Dharmacon). The pan-IP3R SmartPool consisted of three individual SmartPools, each at 33 nM, directed against each isoform of the IP3R (ITPR1, ITPR2, and ITPR3). To knockdown ERα, MCF10AER IN9 were treated for 16 hours with either human ERα SMARTpool (ESR1) siRNA or Non-targeting Control Pool siRNA. Cells were treated with transfection complex for 16 hours, and medium was replaced with DMEM/F12, supplemented with 2% CD-FBS. ERα knockdown at the mRNA and protein level was assessed every 24 hours following transfection. The effects of BHPI on protein synthesis following ERα knockdown were assessed 3-days post-knockdown by treating cells with either 0.1% DMSO loading control or 100 nM BHPI for the indicated times and protein synthesis was then assessed by measuring 35S-Methionine incorporation. Knockdowns of PERK, IP3R, and PLCγ were performed by maintaining MCF-7 cells in MEM containing 5% CD-FBS for 4 days prior to plating cells in serum-free MEM. Cells were treated with transfection complexes for 16 hours and medium was replaced with MEM, supplemented with 10% CD-calf serum. The effects of BHPI on protein synthesis or calcium signaling were assessed 3-days post-knockdown.

Immunoblotting
Western blotting was carried out as previously described (14, 15, 18). The following antibodies were used: ERα [6F11] antibody (Biocare Medical, CA), Phospho-eIF2α (Ser51) (#3398; Cell Signaling Technology), eIF2α (#5324; Cell Signaling Technologies, MA), Phospho-eEF2 (#2331; Cell Signaling Technology, MA), eEF2 (#2332; Cell Signaling Technology, MA), Phospho-p44/42 MAPK (#4370; Cell Signaling Technology, MA), p44/42 MAPK (#4695; Cell Signaling Technology, MA), Phospho-PERK (#3179; Cell Signaling Technology, MA), PERK (#5683; Cell Signaling Technology, MA), ATF6α (Imgenex, CA), Phospho-AMPKα (#2535; Cell Signaling Technology, MA), AMPKα (#2603; Cell Signaling Technology, MA), Phospho-AMPKβ1 (#4181; Cell Signaling Technology, MA), AMPKβ1/2 (#4150, Cell Signaling Technology, MA), Phospho-Acetyl-CoA Carboxylase (#3661; Cell Signaling Technology, MA), Acetyl-CoA Carboxylate (#3676; Cell Signaling Technology, MA), Phospho-IP₃R (#8548; Cell Signaling Technology, MA), IP₃R (#8568; Cell Signaling Technology, MA), Pan-IP₃R (sc-28613; Santa Cruz, CA), Phospho-PLCγ (#2821; Cell Signaling Technology, MA), PLCγ (#5690; Cell Signaling Technology, MA), BiP (#3177; Cell Signaling Technology, MA), p58IPK (#2940; Cell Signaling Technology, MA), laminin A/C (Santa Cruz, CA), β-Actin (Sigma, MO), and α-Tubulin (Sigma, MO). Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and chemiluminescent immunodetection with an ECL Detection Kit (GE Healthcare, NJ), and were visualized using a PhosphorImager.

**Nuclear-cytoplasmic Distribution of ERα**
MCF-7 cells were pre-treated with 1 μM BHPI or DMSO (0.1%) for 30 minutes, followed by 2 hours with or without E₂. Nuclear and cytoplasmic extraction was carried out on ~6 million cells/treatment using a NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoScientific). Lamin A/C and α-Tubulin, were used as nuclear and cytoplasmic markers, respectively.

**Protein Synthesis**

Protein synthesis rates were evaluated by measuring incorporation of ³⁵S-Methionine into newly synthesized protein. Cells were plated at a density of 10,000 cells/well in 96-well plates. Cells were incubated for 30 minutes with 3 μCi of ³⁵S methionine (PerkinElmer, MA) per well at 37°C. Cells were washed two times with PBS, and lysed using 30 μL of RIPA buffer. Cell lysates were collected in microfuge tubes and clarified by centrifugation at 13,000 x g for 10 min at 4°C. Samples were normalized to total protein, and the appropriate volume of sample was spotted onto Whatman 540 filter paper discs and immersed in cold 10% TCA. The filters were washed once in 10% TCA and 3 times in 5% TCA and air dried. Trapped protein was then solubilized and the filters were counted.

**Calcium Imaging**

Cytoplasmic Ca²⁺ concentrations were measured using the calcium-sensitive dye, Fluo-4 AM. The cells were grown on 35 mm-fluorodish cell culture plates (FD35-
100, World Precision Instruments) for two days prior to imaging experiments. The cells were loaded with 5 μM Fluo-4 AM (Life Technologies, CA) in HEPES-based buffer (140 mM NaCl, 4.7 mM KCl, 1.13 mM MgCl₂, 10 mM HEPES, 10 mM Glucose, pH = 7.4) for 30 minutes at 37° C before measurement of intracellular calcium. The cells were washed three times with HEPES buffer to remove extracellular Fluo4-AM dye and incubated with either 2 mM CaCl₂ or 0 mM CaCl₂ for 10 minutes to complete de-esterification of the dye. Confocal images were obtained for one minute to determine basal fluorescence intensity, and then the appropriate treatment was added. Confocal images were captured using a Zeiss LSM 700 confocal system, Plan- Four 20X objective (N.A. = 0.8) and scanned at a resolution of 512x512 pixels (780ms/min). To minimize photo-bleaching and photo-toxicity of samples, the laser power was reduced to 7%. For fluorescence measurements, the cells were excited at 488 nm, and the emission was collected at 525 nm. Images were acquired and analyzed with AxioVision and Zen software (Zeiss). Calcium traces were generated by normalizing fluorescence to basal fluorescence intensity. Data presented as mean ± standard error (n = 10 individual cells).

**Protease Sensitivity Assays**

ERα LBD (N304-S554) containing an N-terminal 6-His tag, was expressed and purified as described previously (19), and stored in Tris-HCl buffer (50 mM Tris-HCl pH 8.0, 10% glycerol, 2 mM DTT, 1 mM EDTA, and 1 mM Na₃VO₄). Purified ERα LBD protein (10 μg) was incubated with 500 nM E₂ for 20 min at 37° C. Samples were then
treated with either DMSO vehicle, BHPI (1 µM) or inactive Compound 8 (1 µM) and incubated for 20 min at 37°C. For partial protease cleavage, the binding mixture was added with/without protease K at a concentration of 7.5 ng protease K per µg protein. After incubation for 10 min at 22°C, the digestions were terminated by addition of SDS sample buffer buffer. The denatured samples were analyzed on a 15% SDS-PAGE gel and visualized by coomassie blue staining.

**Intrinsic Fluorescence Spectroscopy**

The stock solution of full-length ERα was diluted to 400 nM in a Tris-Buffer (50 mM Tris/HCl pH8.0, 150 mM KCl, 2 mM DTT, 1 mM EDTA, and 10% glycerol). The intrinsic fluorescence measurements were carried out using Varian Cary Eclipse Fluorescence Spectrophotometer in a 10 mm quartz cuvette. The excitation and emission slits were set at 5 nm. Tryptophan fluorescence was measured using an excitation wavelength of 295 nm. Emission spectra were collected from 310-380 nm. All spectra were conducted at 37°C. E₂ (500 nM), BHPI (500 nM), or inactive compound 88 (500 nM) was added and incubated at 37°C for 10 min, and then the ERα emission spectra were recorded. All the spectra were corrected for baseline in the absence of E₂.

**Colony Formation Assays**

Assays to assess anchorage-independent cell proliferation in soft agar were carried out as previously described (3). Each treatment condition was evaluated on five independent sets of cells. Culture medium was changed every 3 days. Colonies were
visible after 2 weeks, and total colonies were counted at Day 21 using a dissecting microscope. Photographs of colonies were taken using a Zeiss AxioImager2 imaging system at 5X magnification.

Mouse xenograft

All experiments were approved by the Institutional Animal Care Committee (IACUC) of the University of Illinois at Urbana-Champaign. The MCF-7 cell mouse xenograft model has been described previously (20, 21). At least 12 animals, with 2-4 tumors per animal, were required per experimental group to maintain significant statistical power to detect >25% difference in tumor growth rates. Briefly, estrogen pellets (1 mg:19 mg estrogen:cholesterol) were implanted into 60 athymic female OVX mice, which were 7 weeks of age. Three days after E₂ pellet implantation 1 million MCF-7, human breast cancer cells per site in matrigel were subcutaneously injected at 2 sites in each flank for a total of 4 potential tumors per mouse. When the average tumor size reached 17.6 mm² (4.7 by 4.7 mm), E₂ pellets were removed and a lower dose of E₂ in sealed silastic tubing (1:31 estrogen:cholesterol, 3 mg total weight) was implanted in the same site. When the average tumor size reached 23.5 mm² (5.5 by 5.5 mm), mice were divided into 4 groups with tumor size normalized: E₂ group, no treatment control (NC) group, B_10 group and B_1/B_15 group. E₂ silastic tubes in the NC group were removed, while E₂ silastic tubes in the E₂, B_10, and B_1/B_15 groups were retained. The E₂ and NC group received intraperitoneal injection every other day with 10 ml/kg vehicle (2% DMSO, 10% Tween-20, and 88% PBS). The B_10 group received 10 mg/kg BHPI by intraperitoneal injection every other day. The B_1/B_15 group received
1 mg/kg BHPI by intraperitoneal injection every other day for 14 days. Since this extremely low BHPI dose had no effect, (average tumor cross-sectional area ~45 mm$^2$) they then received 15 mg/kg BHPI every day for another 10 days. Food intake and body weight were measured every 4 days and food intake is presented as grams/day. Tumors were measured every 4 days with a caliper. Tumor cross sectional area was calculated as $(a/2)\times(b/2)\times3.14$, where $a$ and $b$ were the measured diameters of each tumor. On termination of the experiments mice were euthanized and the tumors were excised and weighed. 2 of 60 mice were removed during the course of the study, one that failed to form tumors and the other due to unrelated illness. No tumors were excluded from analysis, and blinding was not performed.

**IP$_3$ Quantitation**

MCF-7 cells were incubated for 10 minutes in 100 nM E$_2$, 10 μM BHPI or vehicle. Intracellular IP$_3$ levels were determined by extracting the cells, and determining IP$_3$ levels in an assay based on competition with radiolabeled IP$_3$ for binding to a recombinant fragment of IP$_3$R containing the IP$_3$ binding site. Unlabeled IP$_3$ provided a standard for the competition assays. 1.5x10$^6$ MCF-7 cells were incubated with ice-cold 1 M trichloroacetic acid (TCA) containing 1 mM EDTA on ice for 15 min. After centrifugation, the supernatant was collected and incubated for 15 min. at room temperature. The TCA solution was removed by adding two volumes of 1,1,2-Trichloro-1,2,2-trifluoroethane (TCTFE)-triocylamine solution. The TCTFE-solution was prepared by mixing 3:1 (v/v) of TCTFE and triocylamine (Sigma). Unlabeled IP$_3$, labeled, IP$_3$ and the unlabeled IP$_3$R fragment were from Perkin Elmer (Waltham, MA) and were used
largely according to the supplier’s directions. Briefly, unlabeled IP₃ standards or cell extracts were incubated with the working receptor/tracer solution at 1:4 (v/v) for 1 hr. at 4°C. The samples were sedimented by centrifugation at 2,000xg for 20 min and the supernatant was discarded. The pellet was suspended in 0.15 M NaOH. After 15 min. at room temperature, the samples were mixed with 5 ml of Pico-Fluor Plus scintillation fluid (Perkin Elmer, Watham, MA) and radioactivity determined by scintillation counting. IP₃ levels in biological samples were calculated from the standard curve generated using a range of unlabeled IP₃ concentrations.

**EDC Dendrimer**

The EDC dendrimer was prepared and used as previously described (22).

**Statistical Analysis**

Calcium measurements reported as mean ± S.E. All other pooled measurements are represented as mean ± S.E.M. Two-tailed student t-tests or one way ANOVA with post-hoc Fisher’s LSD tests were used to evaluate significance, considering p < 0.05 as statistically significant.

**Results**

*BHPI is Effective in Drug-resistant ERα+ Breast and Ovarian Cancer Cells*
BHPI, (Fig. 1A), strongly inhibited E₂-ERα induction of an estrogen response element (ERE)-luciferase reporter and had no effect on androgen induction of an androgen response element (ARE)-luciferase reporter (Fig. 1B). We tested 9 compounds closely related to BHPI. One compound was as potent as BHPI, some had lower potency and several had negligible ability to inhibit E₂-ERα induced cell proliferation (Fig. 1C). With nanomolar potency, BHPI remained the focus of our studies. E₂-ERα stimulates cell proliferation and metastatic potential in most breast cancers, and likely in ovarian, endometrial and cervical cancers (1). We therefore investigated BHPI’s effect on proliferation in therapy-sensitive and therapy resistant ERα⁺ breast, ovarian and endometrial cancer cells. BHPI completely inhibited proliferation of ERα⁺ breast (Fig. 2 A, E, F and G), endometrial (Fig. 2C) and ovarian (Fig. 2B, H, and I) cancer cells, and had no effect in counterpart ERα⁻ cell lines (Fig. 2D). At 100-1,000 nM, BHPI completely blocked proliferation in diverse drug-resistant cell lines: 4-hydroxytamoxifen (4-OHT)-resistant ZR-75-1 breast cancer cells (Fig. 2E); tamoxifen and fulvestrant/ICI 182,780 (ICI)-resistant BT-474 cells (Fig. 2F) (23); epidermal growth factor (EGF) stimulated T47D breast cancer cells, which are resistant to 4-OHT, ICI and raloxifene (RAL) (Fig. 2G); Caov-3 ovarian cancer cells, which are resistant to 4-OHT, ICI and cisplatin (Fig. 2H) (24), and multidrug resistant OVCAR-3 ovarian cancer cells, which are resistant to 5 μM ICI (Fig. 2I) and to paclitaxel, cisplatin and other anticancer drugs (25, 26). BHPI blocked proliferation in all 15 ERα⁺ cell lines and at 10 μM had no effect on proliferation in all 12 ERα⁻ cell lines tested (Fig. 3). Furthermore, BHPI blocked anchorage-independent growth of MCF-7 cells in soft agar (Fig. 4).
**BHPI Induces Tumor Regression**

We next evaluated BHPI in a mouse xenograft model using MCF-7 cell tumors (21). For each tumor, cross-sectional area at Day 0 (~45 mm²) is set to 0%. Control (vehicle injected) and BHPI treated mice were continuously exposed to estrogen. After daily IP injections for 10 days, the tumors in the vehicle treated mice exhibited continued robust growth (Fig. 5, red bars). While BHPI at 1 mg/kg every other day was ineffective (Fig. 6A), initiation of 15 mg/kg daily BHPI treatment resulted in rapid regression of 48/52 tumors (Fig. 5, blue bars). BHPI easily exceeded the goal of >60% tumor growth inhibition proposed as a benchmark more likely to lead to clinical response (27). Furthermore, BHPI, at 10 mg/kg every other day, ultimately stopped tumor growth and final tumor weight was reduced ~60% compared to controls (Fig. S6A and B). BHPI was well tolerated; BHPI-treated and control mice exhibited similar food intake and weight gain (Fig. S6C and D).

**BHPI is an ERα-dependent Inhibitor of Protein Synthesis**

Surprisingly, BHPI greatly reduced protein synthesis in ERα+ cancer cells (Fig. 7A and Fig. 8). If BHPI inhibits protein synthesis through ERα, it should only work in ERα+ cells, and ERα overexpression should increase its effectiveness. BHPI inhibited protein synthesis in all 15 ERα+ cell lines, with no effect on protein synthesis in all 12 ERα- cell lines (Fig. 7A and Fig. 8). BHPI does not inhibit protein synthesis in ERα negative MCF-10A breast cells, but gains the ability to inhibit protein synthesis when ERα is stably expressed in isogenic MCF10A ER In9 cells (Fig. 7B) (28). Notably, BHPI
loses the ability to inhibit protein synthesis when ERα in the stably transfected cells is
knocked down with siRNA (Fig. 7C), or is degraded by ICI (Fig. 7D). Furthermore,
increasing the ERα level in MCF7ERαHA cells (29, 30), stably transfected to express
doxycycline-inducible ERα, progressively increased BHPI inhibition of protein synthesis
(Fig. 7E). BHPI does not work by activating the estrogen binding protein GPR30. BHPI
has no effect on cell proliferation (Fig. 3) or protein synthesis (Fig. 8) in HepG2 cells that
contain functional GPR30 (31) and activating GPR30 with G1, did not inhibit protein
synthesis (Fig. 9A and B). Thus, ERα is necessary and sufficient for BHPI to inhibit
protein synthesis.

**BHPI Inhibits Protein Synthesis by a PLCγ-mediated Opening of the Inositol Triphosphate Receptor (IP3R) Ca2+ Channel, Activating the PERK Arm of the UPR**

Inhibiting mTOR signaling did not strongly inhibit protein synthesis (Fig. 9C),
suggesting BHPI is unlikely to work through mTOR. We next investigated whether BHPI
inhibits protein synthesis by activating the PERK arm of the UPR. There are three UPR
arms. The transmembrane kinase PERK is activated by autophosphorylation. P-PERK
phosphorylates eukaryotic initiation factor 2α (eIF2α), inhibiting translation of most
mRNAs (Fig. 10A) (32, 33). The other arms of the UPR initiate with ATF6α activation
(Fig. S10B), leading to increased protein folding capacity and activation of IRE1α, which
alternatively splices XBP1, producing active spliced (sp)-XBP1 (Fig. S10C) (32, 33). In
ERα+ MCF-7 and T47D cells, but not in ERα− MDA-MB-231 cells, BHPI rapidly inhibited
protein synthesis (Fig. 11A) and in parallel increased eIF2α phosphorylation (Fig. 11B
and C, Fig. 12A). Downstream readouts of eIF2α phosphorylation, CHOP and GADD34 mRNAs, were rapidly induced by BHPI (Fig 11D and E). Consistent with BHPI inducing phosphorylation of eIF2α through activation and autophosphorylation of PERK, p-PERK was increased 30 minutes after BHPI treatment (Fig. 11F and Fig. 12A). RNAi knockdown of PERK (Fig. 12B) abolished BHPI inhibition of protein synthesis at 30 minutes and strongly inhibited BHPI-stimulated eIF2α phosphorylation (Fig. 12C and D). Since PERK knockdown blocks eIF2α phosphorylation, BHPI is not inhibiting translation by activating other upstream kinases that phosphorylate eIF2α. Furthermore, BHPI rapidly activates the ATF6α and IRE1α arms of the UPR, as shown by increased cleaved p50-ATF6α and sp-XBP1 (Fig. 12E and 12F).

To explore how BHPI activates the UPR, we examined inhibition of protein synthesis by known UPR activators. Thapsigargin and Ionomycin, which simulate cell stress and activate the UPR by release of Ca^{2+} from the lumen of the ENR into the cytosol (32, 33), but not UPR activators that work by other mechanisms, elicited the rapid and near quantitative inhibition of protein synthesis seen with BHPI (Fig. 13A).

To test whether BHPI alters intracellular Ca^{2+}, we monitored intracellular Ca^{2+} with the calcium sensitive dye Fluo-4 AM. In MCF-7 cells, BHPI produced a large and sustained increase in intracellular Ca^{2+} in the presence of extracellular Ca^{2+}, and a large transient increase in intracellular Ca^{2+} in the absence of extracellular calcium (Fig. 13B). Time-dependent changes in cytosol calcium in BHPI-treated MCF-7 cells were quantitated (Fig 13B). Since BHPI elicits a large increase in cytosol Ca^{2+} when there is
no extracellular Ca\(^{2+}\), BHPI is acting by depleting the Ca\(^{2+}\) store in the EnR. BHPI had no effect on intracellular Ca\(^{2+}\) in ER\(\alpha^*\) HeLa cells (Fig. 13C).

We next identified the EnR Ca\(^{2+}\) channel that opens after BHPI treatment. The inositol triphosphate receptor (IP\(_3\)R) and ryanodine (RyR) receptors are the major EnR Ca\(^{2+}\) channels. Treatment with 2-APB, which locks the IP\(_3\)R Ca\(^{2+}\) channels closed, but not closing the RyR Ca\(^{2+}\) channels with high concentration ryanodine (Ry), abolished the rapid BHPI-ER\(\alpha^*\)-mediated increase in cytosol Ca\(^{2+}\) and inhibition of protein synthesis (Fig. 14A and B). Furthermore, RNAi knockdown of IP\(_3\)R (Fig. 14C) abolished the BHPI-mediated increase in cytosol Ca\(^{2+}\) and inhibition of protein synthesis (Fig. 14D and E). IP\(_3\)R Ca\(^{2+}\) channels are also modulated through protein kinase A (PKA), but BHPI did not induce PKA-dependent IP\(_3\)R-Ser\(^{1756}\) phosphorylation (34) (Fig 14F).

**BHPI Strongly Activates Phospholipase C \(\gamma\) (PLC\(\gamma\)), Producing Inositol 1,4,5-triphosphate (IP\(_3\))**

IP\(_3\) is produced when the activated phosphorylated plasma membrane enzyme, PLC\(\gamma\), hydrolyzes PIP\(_2\) to diacylglycerol (DAG) and IP\(_3\). Supporting a role for PLC\(\gamma\), siRNA knockdown of PLC\(\gamma\) (Fig, 14G), abolished the BHPI-mediated increase in cytosol Ca\(^{2+}\) (Fig. 14H) and BHPI inhibition of protein synthesis (Fig. 14E), and the PLC\(\gamma\) inhibitor U73122 abolished the BHPI-ER\(\alpha^*\) increase in cytosol Ca\(^{2+}\) (Fig. 14H). Confirming PLC\(\gamma\)'s role, BHPI induces rapid PLC\(\gamma\)-Tyr\(^{783}\) phosphorylation (Fig. 14I), and strongly increased IP\(_3\) levels (Fig. 14J and K). Supporting the idea that BHPI acts by
distorting the newly described weak E\textsubscript{2}-ER\textsubscript{\alpha} activation of the UPR (15), BHPI induced a much larger increase in IP\textsubscript{3} levels than E\textsubscript{2} (Fig. 14K).

Rapid BHPI activation of plasma membrane PLC\textsubscript{\gamma} indicates UPR activation is an extranuclear action of BHPI-ER\textsubscript{\alpha}. Interestingly, PLC\textsubscript{\gamma} and ER\textsubscript{\alpha} coimmunoprecipitate (35). Furthermore, compared to cells expressing basal ER\textsubscript{\alpha}, overexpression of ER\textsubscript{\alpha} in MCF7ER\textsubscript{\alpha}HA cells further increased IP\textsubscript{3} levels in response to BHPI (Fig. 14J).

Consistent with extranuclear ER\textsubscript{\alpha}-dependent activation of the UPR, an estrogen-dendrimer conjugate (EDC) that cannot enter the nucleus (22), induced sp-XBP1, but not nuclear estrogen-regulated genes (Fig 15).

**BHPI Inhibits E\textsubscript{2}-ER\textsubscript{\alpha}-regulated Gene Expression and Likely Interacts with ER\textsubscript{\alpha}**

Since BHPI hyperactivates the UPR by distorting a normal action of E\textsubscript{2}-ER\textsubscript{\alpha}, it should interact with ER\textsubscript{\alpha}. Consistent with BHPI binding to E\textsubscript{2}-ER\textsubscript{\alpha}, BHPI, but not an inactive close relative, Compound 8 (Fig. 1C), significantly altered the fluorescence emission spectrum of purified ER\textsubscript{\alpha} (Fig. 16A). We also tested whether BHPI alters the sensitivity of purified ER\textsubscript{\alpha} ligand-binding domain (LBD) to protease digestion. Addition of BHPI followed by cleavage with proteinase K revealed a 15 kDa band in BHPI treated ER\textsubscript{\alpha} LBD that was nearly absent in the LBD treated with DMSO or Compound 8 (Fig. 16B).

Since BHPI interacts with ER\textsubscript{\alpha} and distorts an extranuclear action of E\textsubscript{2}-ER\textsubscript{\alpha}, we tested whether, independent of its ability to inhibit protein synthesis and activate the
UPR, BHPI would also modulate nuclear E\textsubscript{2}-ER\textsubscript{\alpha}-regulated gene expression. At early times when BHPI inhibited E\textsubscript{2}-ER\textsubscript{\alpha} induction of pS2 mRNA, neither inhibiting protein synthesis with CHX, nor activating the UPR with Tunicamycin (TUN) (Fig. 17A) inhibited induction of pS2 mRNA (Fig. 16C). BHPI inhibited E\textsubscript{2}-ER\textsubscript{\alpha} induction of pS2, GREB1, XBP1, CXCL2, and ERE-luciferase in ER\textsubscript{\alpha}\textsuperscript{+} MCF-7 and T47D cells (Fig. S17 B-F), and blocked E\textsubscript{2}-ER\textsubscript{\alpha} down-regulation of IL1-R1 and EFNA mRNA (Fig. 17E and G). BHPI is not a competitive ER\textsubscript{\alpha} inhibitor. Increasing the concentration of E\textsubscript{2} by 1,000 fold abolished the ability of the competitor antiestrogen ICI to inhibit gene expression, with no effect on BHPI inhibition of E\textsubscript{2} induction of pS2 mRNA (Fig. 16D). Moreover, BHPI did not compete with E\textsubscript{2} for binding to ER\textsubscript{\alpha} (Fig. 18A). Since BHPI inhibits E\textsubscript{2}-ER\textsubscript{\alpha} induction and repression of gene expression, BHPI acts at the level of ER\textsubscript{\alpha} and not by a general inhibition or activation of transcription.

BHPI did not alter ER\textsubscript{\alpha} protein levels or nuclear localization (Fig. 18B and C). Chromatin immunoprecipitation (ChIP) showed that BHPI strongly inhibited E\textsubscript{2}-stimulated recruitment of ER\textsubscript{\alpha} and RNA polymerase II to the pS2 and GREB1 promoter regions (Fig. 16E and Fig. 18D). Consistent with BHPI inducing an ER\textsubscript{\alpha} conformation exhibiting reduced affinity for gene regulatory regions, ten-fold overexpression of ER\textsubscript{\alpha} in MCF7ER\textsubscript{\alpha}HA cells abolished BHPI inhibition of induction of GREB1 mRNA (Fig. 16F). BHPI still kills these cells because ER\textsubscript{\alpha} overexpression enhances BHPI inhibition of protein synthesis (Fig. 7E). Taken together, our data provides compelling evidence BHPI is a new type of biomodulator, altering both nuclear and extranuclear actions of ER\textsubscript{\alpha}. 

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BHPI Rapidly Depletes Intracellular ATP Stores and Activates AMPK

BHPI treatment results in rapid depletion of EnR Ca\(^{2+}\). To restore EnR Ca\(^{2+}\), the cell activates SERCA (Sarco/Endoplasmic Reticulum Ca\(^{2+}\)-ATPase) pumps, which catalyze ATP-dependent transfer of Ca\(^{2+}\) from the cytosol into the lumen of the EnR. Since BHPI opens the IP\(_3\)R Ca\(^{2+}\) channel, Ca\(^{2+}\) pumped back into the EnR lumen by SERCA flows back into the cytosol. This futile cycle rapidly depletes intracellular ATP, resulting in activation of AMP-activated protein kinase (AMPK) by AMPK\(\alpha\)-Thr\(^{172}\) phosphorylation (Fig. 19A and B). Moreover, the AMPK target, acetyl CoA-carboxylase (ACC) is rapidly phosphorylated (Fig. 19B). Since Thapsigargin, which depletes EnR Ca\(^{2+}\) by inhibiting SERCA pumps, had no effect on ATP levels (Fig. 19A) and did not increase levels of p-AMPK\(\alpha\) and p-ACC (Fig. 20A), ATP depletion, rather than increased cytosol Ca\(^{2+}\) is responsible for AMPK activation. Importantly, pre-blocking SERCA-pumps with Thapsigargin, abolished the BHPI-induced decline in ATP levels and phosphorylation of AMPK\(\alpha\) (Fig. 19A).

BHPI Blocks UPR Inactivation by Targeting a Second Site of Protein Synthesis Inhibition

In ER\(\alpha^+\), but not ER\(\alpha^-\) cells, after ~2 hours, BHPI phosphorylates and inactivates eukaryotic elongation factor 2, (eEF2) (Fig. 19C, Fig. 20B and C). eEF2 phosphorylation is regulated by a single Ca\(^{2+}\)/calmodulin-dependent kinase, eukaryotic elongation factor 2 kinase (CAMKIII/eEF2K). eEF2K is inhibited by mTORC1-p70\(^{S6K}\) and ERK-p90\(^{RSK}\)
through eEF2K-Ser\textsuperscript{366} phosphorylation and activated by Ca\textsuperscript{2+}/calmodulin and AMPK (36, 37). BHPI increases cytosol Ca\textsuperscript{2+} and activates AMPK, but inhibiting AMPK did not inhibit eEF2 phosphorylation (Fig. 20D). BHPI also rapidly induces a transient increase in ERK1/2 activation (Fig. 20E and F), which stimulates ERK-p90\textsuperscript{RSK} and mTORC1-p70\textsuperscript{S6K} activation (32). Together, these pathways induce eEF2K-Ser\textsuperscript{366} phosphorylation (Fig. 19D), and prevent increases in p-eEF2 for \~1 hour after BHPI treatment (Fig. 19C and Fig. 20G). Consistent with this, blocking ERK activation with U0126 prevented BHPI from producing transient declines in eEF2 phosphorylation through inactivation of eEF2K (Fig. 20G).

UPR activation normally induces chaperones, which are critical for resolving EnR stress and inactivating the UPR. Thus, conventional UPR activators induce BiP and p58\textsuperscript{IPK} chaperones (Fig. 21A and B), promoting transient eIF2\textalpha phosphorylation and inhibition of protein synthesis (Fig. 20A; Fig. 21C and D). In contrast, BHPI blocks induction and reduces levels of BiP and p58\textsuperscript{IPK} protein (Fig. 19E), leading to sustained UPR activation and long-term inhibition of protein synthesis (Fig. 3 and Fig. 11B). While BHPI blocked increases in p58 protein (Fig. 19E), it robustly induced p58 mRNA (Fig. 19F), suggesting that inhibiting protein synthesis at the second site prevented translation of chaperone mRNAs required to resolve UPR activation.

**Discussion**

BHPI and estrogen share the same ER\textalpha-dependent pathway for UPR activation: Activation of PLC\gamma producing IP\textsubscript{3}, opening of the IP\textsubscript{3}R Ca\textsuperscript{2+} channels, release of EnR
Ca\textsuperscript{2+}, and activation of the PERK, IRE1\textgreek{a} and ATF6\textgreek{a} arms of the UPR (Fig. 22). We recently reported that as an early component of the proliferation program, E\textsubscript{2}-ER\textgreek{a} weakly and transiently activates the UPR. We showed that E\textsubscript{2}-ER\textgreek{a} elicits a mild and transient activation of the PERK arm of the UPR, while simultaneously increasing chaperone levels and protein folding capacity by activating the IRE1\textgreek{a} and ATF6\textgreek{a} arms of the UPR (15). BHPI distorts this normal action of E\textsubscript{2}-ER\textgreek{a} by increasing the amplitude and duration of UPR activation. Compared to E\textsubscript{2}, BHPI hyperactivates PLC\textgreek{g}, producing much higher IP\textsubscript{3} levels, Ca\textsuperscript{2+}-release from the EnR, and UPR activation. BHPI inhibits protein synthesis by strongly activating the PERK arm of the UPR. Knockdown of ER\textgreek{a}, PLC\textgreek{g}, IP\textsubscript{3}R and PERK blocked BHPI inhibition of protein synthesis. While BHPI activates the IRE1\textgreek{a} and ATF6\textgreek{a} UPR arms, BHPI prevents the synthesis of chaperones required to inactivate the UPR by acting at later times to inhibit protein synthesis at a second site. Because the cell attempts to restore EnR Ca\textsuperscript{2+} while the IP\textsubscript{3}R Ca\textsuperscript{2+} channels remain open, BHPI rapidly depletes ATP (Fig. 22), resulting in activation of AMPK. Working together, long-term inhibition of protein synthesis, ATP depletion and AMPK activation enable BHPI to block cell proliferation, and often kill, ER\textgreek{a}\textsuperscript{+} cancer cells. Supporting BHPI targeting PLC\textgreek{g} and the UPR through ER\textgreek{a}, independent of its effects on the UPR, BHPI inhibits E\textsubscript{2}-ER\textgreek{a}-mediated induction and repression of gene expression.

BHPI and E\textsubscript{2} activation of plasma membrane-bound PLC\textgreek{g}, resulting in increased IP\textsubscript{3}, is an extranuclear action of ER\textgreek{a}. Increasing the level of ER\textgreek{a}, increased IP\textsubscript{3} levels. Consistent with ER\textgreek{a} and PLC\textgreek{g} interaction, they coimmunoprecipitate (35). BHPI and E\textsubscript{2}
induce Ca\textsuperscript{2+} release in 1 min., too rapidly for action by regulating nuclear gene
expression (15). Furthermore, a membrane-impermeable estrogen-dendrimer induces
the UPR marker sp-XBP1, but not nuclear E\textsubscript{2}-ER\textalpha-regulated genes.

The UPR plays important roles in tumorigenesis, therapy resistance, and cancer
progression (15, 38). Moderate and transient UPR activation by E\textsubscript{2} and other activators
promotes an adaptive stress response, which increases UPR expression and confers
protection from subsequent exposure to higher levels of cell stress (15, 39, 40). In
contrast, sustained UPR activation triggers cell death. Since most current anticancer
drugs inhibit a pathway or protein important for tumor growth or metastases, most UPR
targeting efforts use the same strategy and focus on inactivating a protective stress
response by inhibiting UPR components (41). UPR overexpression in cancer is
associated with a poor prognosis (15), suggesting that sustained lethal hyperactivation
of the UPR by BHPI represents a novel alternative anticancer strategy.

BHPI can selectively target cancer cells, because its targets, ER\textalpha and the UPR,
are both overexpressed in breast and ovarian cancers (15, 30, 38, 42). Cells expressing
low levels of ER\textalpha, more typical of non-transformed ER\textalpha containing cells, such as PC-3
prostate cancer cells, were less sensitive to BHPI inhibition of protein synthesis (Fig. 3),
while doxycycline-treated MCF7ER\textalphaHA cells expressing very high levels of ER\textalpha
exhibited near complete inhibition of protein synthesis (Fig. 7E). Consistent with low
toxicity, in the xenograft study, BHPI-treated mice showed no evidence of gross toxicity.

Despite a role for ER\textalpha in gynecological cancers, most ovarian cancer cells show
little dependence on estrogens for growth and endocrine therapy is largely ineffective.
Other non-competitive ER\(\alpha\) inhibitors have not demonstrated effectiveness in these cells. BHPI is highly effective in several breast and ovarian cancer drug-resistance models and extends the reach of ER\(\alpha\) biomodulators to gynecologic cancers that do not respond to current endocrine therapies. BHPI’s effectiveness in a broad range of ER\(\alpha\)-containing breast, ovarian and endometrial cancer cells is consistent with recent findings demonstrating that female reproductive cancers exhibit common genetic alterations and might respond to the same drugs (43), and with our finding that E\(_2\)-ER\(\alpha\) weakly activates the UPR in breast and ovarian cancer cells (15).

With its sub-micromolar potency, effectiveness in a broad range of therapy resistant cancer cells, ability to induce substantial tumor regression and unique mode of action, BHPI is a promising small molecule for therapeutic evaluation and mechanistic studies.
Figure 4.1. BHPI and structurally related compounds selectively inhibit estrogen-dependent cell proliferation and E$_2$-ER$_{\alpha}$ mediated gene expression. (A) Structure of BHPI (3,3-bis(4-hydroxyphenyl)-7-methyl-1,3-dihydro-2H-indol-2-one). (B) Dose response studies of the effect of BHPI on 17$\beta$-estradiol (E$_2$) induction of ERE-luciferase activity in ER$_{\alpha}$ positive T47D-kBluc breast cancer cells (black bars) and for dihydrotestosterone-androgen receptor (DHT-AR) induction of prostate specific antigen (PSA)-luciferase in ER$_{\alpha}$ negative HeLaA6 cells (open bars). HeLaA6 cells are stably transfected to express AR and a PSA-luciferase reporter. Data is the mean ± SEM (n = 3 sets of cells). (C) Inhibition of cell proliferation in ER$_{\alpha}^{+}$ T47D breast cancer cells by BHPI and structurally related compounds.
Figure 4.2. BHPI selectively inhibits proliferation of ERα+ cancer cells sensitive or resistant to drug therapy. BHPI inhibits proliferation of ERα+ (A) MCF-7 breast, (B) PEO4 ovarian, and (C) ECC-1 endometrial cancer cells with no effects on (D) counterpart ERα- cancer cells. Effects of BHPI on proliferation of drug-resistant cells: Tamoxifen- and ICI-resistant (E) ZR-75-1 cells and (F) BT-474 breast cancer cells. (G) T47D cells treated with 1 μM BHPI or competitor antiestrogens (4-OHT, RAL, ICI) in the presence or absence of E2 and/or EGF. Proliferation of (H) cisplatin resistant Caov-3 ovarian cancer cells and (I) multi-drug resistant OVCAR-3 ovarian cancer cells treated with BHPI, or the antiestrogens 4-OHT or ICI. Concentrations: E2, 1 nM (E, G, H) or 10 nM (A-C, F, I); EGF, 50 ng/mL (G); ICI, 1 μM (E, G, H), 5 μM (I); 4-OHT, 1 μM (E, G, H); RAL, 1 μM (G) “•” denotes cell number at day 0. Hatched bars denote antiestrogens (4-OHT, RAL, or ICI). Cell proliferation is expressed as mean ± SEM (n = 6).
Figure 4.3. BHPI selectively inhibits cell proliferation in ERα positive cancer cells. Effects of BHPI on cell proliferation in 15 ERα positive (colored bars) and 12 ERα negative (black bars) cell lines. Cell lines are grouped by tissue of origin (breast, ovary, cervix, prostate, lung and liver). “•” on each graph denotes the number of cells at the start of the experiment. Most cell proliferation studies were for 3 or 4 days in 10 nM E2. Since we recently found that our BG-1 cells are genetically identical to MCF-7 cells, that data is presented as BG-1/MCF-7. Data is the mean ± SEM (n = 6).
Figure 4.4. BHPI inhibits anchorage-independent growth of MCF-7 cells in soft agar. 5,000 MCF-7 cells were plated into top agar. Cells were treated with medium containing DMSO (vehicle) and either, 10 nM E2 or Ethanol (vehicle), or 1 μM BHPI and 10 nM E2. Medium was changed every 3 days. After 21 days, colonies were counted and photographed at 5x magnification. For each treatment, the bar graph represents the average of the total number colonies per well with a diameter >0.5 mm. Data is the mean ± SEM (n = 6).
Figure 4.5. BHPI induces tumor regression in a mouse xenograft. Change in tumor cross sectional area in mouse MCF-7 xenografts after 10 days of daily IP injections of either 15 mg/kg BHPI (blue) or vehicle control (red). Tumors had an average starting cross-sectional area of ~45 mm$^2$. For each tumor, area at day 0 was set to 0% change.
Figure 4.6. BHPI inhibits tumor growth in a mouse xenograft model of breast cancer and is not toxic. (A) MCF-7 tumor growth in athymic mice was monitored by measuring tumor diameter with a caliper every 4 days. The E2 and NC group received vehicle injection, while the B_10 group was injected with 10 mg/kg BHPI every other day. The B_1/B_15 group received the extremely low dose of 1 mg/kg BHPI every other day for 14 days. This very low dose of BHPI had no effect on tumor growth. They then received 15 mg/kg BHPI every day until the end of the study (* denotes change in dosage). Tumor size was represented as tumor cross sectional area (mm$^2$). Each tumor was analyzed individually, and data are expressed as mean ± SEM (n = 52). (B) Mice were sacrificed and tumor weights were recorded. Data is expressed as mean ± SEM (n = 52) and analyzed using one way ANOVA with post hoc Fisher’s LSD test. Different letters indicate significant differences between groups (p <0.05). (C) Mouse body
weight, measured every 4 days after initiation of drug injection. Data expressed as mean ± SEM (n = 13). (D) Mouse food intake, measured every 4 days after initiation of drug injection. Data expressed as mean ± SEM (n = 13). BHPI treatment had no effect on body weight or food intake and was therefore not overtly toxic.

Figure 4.7. BHPI selectively inhibits protein synthesis in ERα positive cancer cells by activating PLCγ, depleting endoplasmic reticulum Ca^{2+}, and activating the UPR. (A) Protein synthesis in BHPI-treated ERα+ and ERα− cells (n = 4). CHX, cycloheximide. (B) ERα is sufficient to make a cell sensitive to BHPI inhibition of protein synthesis. Protein synthesis in parental ERα− MCF10A cells and ERα expressing MCF10A_{ER IN9} cells (n = 4). (C) RNAi knockdown of ERα abolishes BHPI inhibition of protein synthesis. Protein synthesis in MCF10A_{ER IN9} cells treated with non-coding (NC) siRNA or ERα siRNA SmartPool followed by 100 nM BHPI (n = 4). (D) Protein synthesis and immunoblot analysis of ERα protein levels in MCF10A_{ER IN9} cells pre-treated with 1 μM ICI for 24 hours to degrade ERα, followed by treatment with 100 nM BHPI (n = 4). (E) Residual protein synthesis (untreated cells are set to 100%) after treatment with 1 μM BHPI in Doxycycline-treated MCF7ERαHA cells expressing increasing levels of ERα (n = 6). Western blot shows ERα levels in each sample.
Figure 4.8. BHPI selectively inhibits protein synthesis in ERα positive cells.

Comparison of ERα protein levels and the effects of BHPI treatment on protein synthesis in 23 cell lines. The number of samples was too large to run on a single gel and the data is from 3 identically processed gels. Protein synthesis was determined by incorporation of 35S-methionine into protein. Incorporation with no added BHPI was set to 100%. In general, protein synthesis in cells expressing moderate or high levels of ERα was robustly inhibited by 100 nM BHPI (yellow bars), while 10,000 nM BHPI (orange bars), the highest concentration tested, had very little or no effect on protein synthesis in ERα negative cells. Cells expressing low levels of ERα, more typical of non-transformed ERα containing cells, such as PC-3 prostate cancer cells, were much less sensitive to BHPI inhibition of protein synthesis. As a control, cycloheximide (CHX) potently inhibited protein synthesis in all the cell lines. Data is the mean ± SEM (n = 4).
Figure 4.9. Activation of the estrogen binding protein, GPR30, or inhibition of mTOR, have minimal effects on protein synthesis. Effects of the GPR30 activator, G1, on (A) MCF-7 and (B) BG-1/MCF-7 cells. Cells were plated at 10,000 cells/well, the indicated concentrations of G1 (0-100 nM) were added for the times indicated, and 35S-Methionine incorporation was used to assess rates of protein synthesis. Protein synthesis with no added G1 was set to 100%. (C) The effects of rapamycin (RAP) on protein synthesis in MCF-7 cells in the absence of growth factors (blue bars), or in the presence of 10 nM E2 (red bars) or 10 μg/ml Insulin (green bars). Inhibition of mTORC1 with rapamycin blocks insulin-dependent increases in protein synthesis (green bars) and substantially blocks estrogen-dependent increases in protein synthesis (red bars). In contrast, BHPI and cycloheximide (CHX) treatment elicit near-quantitative inhibition of protein synthesis, far below baseline levels of protein synthesis (blue bars). Data is mean ± SEM (n = 4).
Figure 4.10. Endoplasmic reticulum (EnR) stress activates the three arms of the UPR. (A) EnR stress induces the oligomerization and phospho-activation of the transmembrane kinase PERK. P-PERK phosphorylates eukaryotic initiation factor 2α (eIF2α), leading to inhibition of protein synthesis and a reduction in the endoplasmic reticulum protein folding load (32, 33). Reduced protein synthesis increases levels of the transcription factor, ATF4. ATF4 induces the transcription factor CHOP, which induces GADD34 and several pro-apoptotic genes. (B) EnR stress promotes the translocation of the transmembrane protein, p90-ATF6α, from the EnR to the Golgi Apparatus, where it encounters proteases that liberate the N-terminal fragment of ATF6α (p50-ATF6). p50-ATF6 increases the protein-folding capacity of the EnR by inducing EnR-resident chaperones, including BiP and GRP94 (32, 33, 44). (C) EnR stress induces the oligomerization and phospho-activation of the transmembrane protein, IRE1α (32, 33, 45). Activated IRE1α removes an intron from full-length XBP1 (fl-XBP1) mRNA, producing spliced (sp)-XBP1 mRNA, which is subsequently translated into sp-XBP1 protein (sp-XBP1). sp-XBP1 increases the protein-folding capacity of the EnR and turnover of misfolded proteins by inducing EnR resident-chaperone protein genes (BiP, HEDJ, SERP1), EnR-associated degradation (ERAD) genes and alters mRNA decay and translation (32, 33).
Figure 4.11. In ERα positive cell lines, BHPI rapidly inhibits protein synthesis by activating the PERK arm of the UPR. (A) Time course of BHPI inhibition of protein synthesis. ERα positive MCF-7, T47D, and BG-1/MCF-7 cells were incubated for the indicated times in 1 μM BHPI. Set to 100% was incorporation of 35S-methionine into protein at time = 0. Data is mean ± SEM (n = 4 sets of cells). At 30 min. in BHPI, 35S-methionine incorporated into protein was reduced by ~50%. (B) In the presence [+E2], or absence (-E2) of estrogen, BHPI increases p-eIF2α (Ser-51) in ERα+ MCF-7, BG-1/MCF-7, and T47D cells. In the absence of estrogen, BHPI increases eIF2α phosphorylation in ERα+ MCF-7 cells. (C) BHPI does not increase p-eIF2α in ERα negative MDA MB-231 cells. Since the UPR activator tunicamycin (TUN) increased p-eIF2α in these cells, the absence of BHPI induced phosphorylation of eIF2α in the MDA MB-231 cells was not due to the inability of UPR activation to induce eIF2α phosphorylation. (D) Induction of CHOP and GADD34 mRNA in MCF-7 cells following treatment with 1 μM BHPI, as determined by qRT-PCR. (E) Induction of CHOP mRNA
in BG-1/MCF-7 cells following treatment with 1 μM BHPI. Increased levels of ATF4 induce the transcription factor, CHOP, which then induces GADD34. Increased phosphorylation of eIF2α results in translational upregulation of the transcription factor, ATF4. ATF4 contains short, inhibitory upstream open reading frames (uORFs), which normally inhibit translation of ATF4 mRNA (10). Under conditions of reduced eIF2α availability, the inhibitory uORFs are skipped, allowing ATF4 translation. (F) Time course of phosphorylation of PERK (Thr-980) and total PERK protein levels following treatment with BHPI in MCF-7 cells. PERK-Thr<sup>960</sup> phosphorylation serves as a marker of PERK activation.
**Figure 4.12. BHPI selectively inhibits protein synthesis in ER\(\alpha\) positive cancer cells by activating all three arms of the UPR.** (A) Time course of phosphorylation of PERK and eIF2\(\alpha\) following BHPI treatment of MCF-7 cells. (B) Western blot analysis of PERK protein levels following treatment of MCF-7 cells with either 100 nM non-coding (NC) or PERK siRNA SmartPool. (C) Protein synthesis and (D) eIF2\(\alpha\) phosphorylation after 4-day treatment of MCF-7 cells with either 50 nM non-coding (NC) siRNA or PERK siRNA, followed by treatment with BHPI (n = 4). (E) Effects of BHPI on levels of spliced-XBP1 mRNA (sp-XBP1). -E2 samples set to 1 (n = 3). (F) Western blot analysis showing full-length (p90-ATF6\(\alpha\)) and cleaved p50-ATF6\(\alpha\) in BHPI-treated cells and effect of BHPI on levels of spliced-XBP1 mRNA (sp-XBP1). Data is mean ± S.E.M.
Figure 4.13. BHPI activates the UPR through depletion of endoplasmic reticulum calcium stores in ERα+ MCF-7 breast cancer cells, but not in ERα- HeLa cervical cancer cells. (A) Protein synthesis in MCF-7 cells treated for 2 hours with UPR activators (n = 6). Effect of BHPI and Thapsigargin (THG) on intracellular calcium levels in (B) ERα+ MCF-7 breast cancer cells and (C) ERα- HeLa cervical cells. Although BHPI has no effect, HeLa cells remain sensitive to Thapsigargin. Cells visualized with the Ca^{2+} sensitive dye Fluo-4 AM. Low levels of basal [Ca^{2+}] are blue and then green, whereas higher levels of [Ca^{2+}] are seen as red, with the highest levels white. Trace
represents calcium following treatment with Thapsigargin or BHPI. Intensity was normalized to the basal signal, which was set to 1. Data is mean ± SEM (n = 10).

Figure 4.14. Effects of BHPI on IP₃R, IP₃, and PLCγ. (A) BHPI increases intracellular calcium levels. Visualization of intracellular Ca²⁺ using Fluo-4 AM; BHPI (1 μM) was added to MCF-7 cells at 30 sec. Color scale from basal Ca²⁺ to highest Ca²⁺: blue, green, red, white. (B) Inhibiting opening of the endoplasmic reticulum IP₃R Ca²⁺ channel abolishes BHPI inhibition of protein synthesis. The ryanodine and IP₃R Ca²⁺ channels were pre-blocked with 100 μM ryanodine (RyR) and 100 μM 2-amino propyl-benzoate (2-APB), respectively, followed by 70 nM BHPI for 3 hours (n = 4). (C) Western blot analysis of pan-IP₃R protein levels after treatment of MCF-7 cells with either 100 nM non-coding (NC) SmartPool siRNA or 100 nM SmartPool IP₃R siRNA. (D) Quantitation of cytosolic Ca²⁺ levels after treating MCF-7 cells with either 50 nM non-coding (NC)
siRNA, pan IP$_3$R siRNA SmartPool, followed by treatment with BHPI (n = 10). IP$_3$R SmartPool contained equal amounts of three individual SmartPools directed against each isoform of IP$_3$R. (E) Effects of BHPI on protein synthesis in MCF-7 cells treated with either 100 nM NC siRNA, pan-IP$_3$R siRNA, or PLC$_\gamma$ siRNA SmartPool (n = 4). (F) Time course of phosphorylation of the IP$_3$R Ca$^{2+}$-channel and total IP$_3$R following treatment with BHPI. Phosphorylation of IP$_3$R at Ser-1756 by cyclic AMP-dependent protein kinase A (PKA) regulates the activity of the IP$_3$R Ca$^{2+}$-channel. While BHPI had no effect, the MCF-7 cells contain a functional protein kinase A pathway since the protein kinase A activators, IBMX and Forskolin, increased phosphorylation of IP$_3$R. (G) Western blot shows PLC$_\gamma$ protein levels following treatment of MCF-7 cells with either 100 nM non-coding SmartPool siRNA or 100 nM SmartPool PLC$_\gamma$ siRNA. (H) Effects of BHPI on cytosol Ca$^{2+}$ following either PLC$_\gamma$ knockdown or blocking PLC$_\gamma$ activation with U73122. Western blot shows PLC$_\gamma$ protein levels following treatment of MCF-7 cells with either 100 nM non-coding SmartPool siRNA or 100 nM SmartPool PLC$_\gamma$ siRNA. (I) Effects of BHPI on phosphorylation and activation of PLC$_\gamma$. Phosphorylation of PLC$_\gamma$ at Tyr-1756 regulates the activity of PLC$_\gamma$. MCF-7 cells were treated for 10 min. with 1 μM BHPI. (J) Quantitation of intracellular IP$_3$ levels following treatment of MCF-7 cells for 10 min. with E2 or BHPI (n=3) (K) Effects of overexpressing ER$\alpha$ on BHPI-induced increases in IP$_3$ levels. ER$\alpha$ in MCF7ER$\alpha$HA cells was induced with DOX as described in Figure 3E. IP$_3$ levels were determined 10 min. after treatment with 1 μM BHPI. Data is mean ± SEM (n=3). Data is mean ± S.E.M. Different letters indicate a significant difference among groups (p < 0.05) using one-way ANOVA followed by Tukey’s post hoc test.
Figure 4.15. Effects of estrogen-dendrimer-conjugate (EDC) on UPR activation and E2-regulated genes. Comparison of effects of 17β-estradiol (E2) and estrogen-dendrimer-conjugate (EDC) on the ability of ERα to activate (A) GREB1, (B) PI-9, and (C) Fos expression. These are classical estrogen-regulated genes. (D) Comparison of the effects of E2 and EDC on the ability to induce spliced-XBP1 (sp-XBP1), which is a widely used marker of UPR activation (see UPR model in Figure S7C).
Figure 4.16. BHPI interacts with ERα and inhibits E2-ERα regulated gene expression. (A) Fluorescence emission spectra of full-length ERα in the presence of E2 and either DMSO, 500 nM BHPI, or 500 nM of the BHPI-related inactive Compound 8 (C8). (B) ERα LBD was subjected to proteinase K digestion in the presence of DMSO vehicle, C8, or BHPI. Bands were visualized by Coomassie-staining. (C) qRT-PCR showing pS2 mRNA in MCF-7 cells pre-treated for 0.5 hours with BHPI, cycloheximide (CHX), Tunicamycin (TUN), Thapsigargin (THG), or DMSO, followed by treatment with or without E2 for 2 hours. (D) BHPI is a non-competitive ERα inhibitor. qRT-PCR showing pS2 mRNA in MCF-7 cells treated with BHPI or the competitive inhibitor ICI, and low (1 nM) or high (1,000 nM) E2. (E) ChIP showing effect of BHPI on recruitment of E2-ERα (green bars) and RNA polymerase II (RNAP, yellow hatched bars) to the promoter region of pS2. (F) qRT-PCR showing GREB1 mRNA levels in MCF7ERαHA cells after 1 day + or – doxycycline (DOX), pre-treated for 30 minutes with BHPI or DMSO, followed by 4 hours with or without E2. Concentrations: E2, 500 nM (A and B), 10 nM (C–F); BHPI, 500 nM (A) or 1 μM (B–F); C8, 500 nM (A) or 1 μM (B); CHX, 10 μM; THG, 1 μM; TUN, 10 μg/ml. Data is mean ± SEM (n = 3). * P < 0.05, ** P < 0.01, ***P < 0.001, compared with +E2 samples. n.s., not significant.
Figure 4.17. BHPI inhibits E₂-ERα regulated gene expression. (A) Comparison of the effects of BHPI, the protein synthesis inhibitor cycloheximide (CHX), and the UPR activator Tunicamycin (TUN) on protein synthesis. Since protein synthesis was robustly inhibited by CHX after 120 minutes, this time was used in the gene expression studies. Effects of BHPI on E₂-ERα induction of (B) pS2, (C) GREB1 and (D) CXL2 mRNAs and (E) XBP1 in MCF-7, T47D and BG-1/MCF-7 cells. (F) Dose response studies of the effect of BHPI on E₂-ERα induction of ERE-luciferase activity in ERα positive T47D-kBluc breast cancer cells (black bars) and for dihydrotestosterone-androgen receptor (DHT-AR) induction of prostate specific antigen (PSA)-luciferase in ERα negative HeLaA6 cells (open bars). HeLaA6 cells are stably transfected to express AR and a PSA-luciferase reporter. Supporting specificity of BHPI, it did not inhibit DHT-AR induction of ARE-luciferase. BHPI blocks E₂-ERα down-regulation of EFNA1 in MCF-7 cells (E) and IL1-R1 in T47D cells (G). Data is mean ± SEM (n = 3).
Figure 4.18. BHPI is a non-competitive inhibitor that reduces binding of E₂-ERα to gene regulatory regions. BHPI does not compete with estrogens for binding to ERα. (A) Competitive radioligand binding assay comparing the ability of E₂ and BHPI to compete with [³H] estradiol (E₂) for binding to ERα. The relative binding affinity (RBA) of BHPI for the estrogen-binding pocket of ERα was determined using 0.2 nM [³H] E₂ and a range of BHPI concentrations. RBA values were determined from the competitive radiometric binding assay (22, 46). Values are expressed as percentages relative to the affinity of the standard, E₂ = 100%. (B) Western blots showing that at early times after treatment with 1 μM BHPI, ERα protein levels are nearly unchanged. (C) Western blot showing that treatment of MCF-7 cells with 1 μM BHPI does not inhibit nuclear
localization of ERα. α-Tubulin and lamin A/C were controls for the cytoplasmic and nuclear fractions, respectively. ERα protein levels and nuclear localization were assessed 2 hours after treatment, which was the same time used to assess endogenous mRNA levels of E₂-ERα regulated genes via qRT-PCR. (D) ChIP shows BHPI inhibits recruitment of E₂-ERα (black bars) and RNA polymerase II (RNAP, hatched bars) to the GREB1 promoter region. Data is mean ± SEM (n = 3). * Significant at (p < 0.05).
Figure 4.19. BHPI depletes intracellular ATP stores, activates AMPK, and inhibits protein synthesis at a second site. (A) Inhibiting SERCA pumps with Thapsigargin (THG) prevents BHPI from reducing intracellular ATP levels. Western blot showing effect of THG (1 μM) or BHPI (1 μM) treatment of MCF-7 cells on AMPKα Thr\textsuperscript{172} phosphorylation. ATP levels in MCF-7 cells treated with 1 μM BHPI, or 1 μM BHPI and 1 μM THG (n = 5). (B) Western blot analysis of the time course of AMPKα (Thr-172),
AMPKβ (Ser-108), acetyl CoA carboxylase (ACC) (Ser-79) phosphorylation in BHPI-treated MCF-7 cells. AMPKα-Thr\textsuperscript{172} and AMPKβ-Ser\textsuperscript{108} phosphorylation are required for AMPK activation. (C) Western blot analysis of eEF2 phosphorylation (Thr-56) over time in BHPI-treated ERα\textsuperscript{+} MCF-7 cells. (D) Western blot analysis showing the time course of decreasing eEF2K (Ser-366) phosphorylation in BHPI-treated MCF-7 cells. Ser-366 dephosphorylation activates eEF2K. (E) qRT-PCR analysis showing changes in p58\textsuperscript{IPK} mRNA and Western blot analysis showing p58\textsuperscript{IPK} and BiP protein after treatment with BHPI (n = 3). -E\textsubscript{2} set to 1.
Figure 4.20. Conventional UPR activators do not induce phosphorylation of eEF2, but induces transient eIF2α phosphorylation, transient inhibition of protein synthesis, and induction of chaperones. (A) Analysis of the time course of Thapsigargin (THG) effects on phosphorylation of eIF2α (Ser-51), AMPKα (Thr-172), ACC (Ser-79), and eEF2 (Thr-56). Unlike BHPI, Thapsigargin does not induce phosphorylation of eEF2 but induces transient phosphorylation of eIF2α. Western blots of the time course of BHPI effects on phosphorylation of eEF2 (Thr-56) in (B) ERα positive T47D and (C) ERα negative HeLa cells. T47D Cells were pre-treated with 10 nM E2 for 24-hours. eEF2 is essential for protein synthesis, and eEF2-Ser56
phosphorylation inactivates eEF2, blocking the elongation step of protein synthesis. The positive controls, Forskolin (FOR) and Rottlerin (ROT) induce robust eEF2 phosphorylation, demonstrating eEF2 retains the capacity for phosphorylation in HeLa cells. (D) Inhibiting AMPK phosphorylation and activation with ST-609 did not block BHPI-stimulated phosphorylation of eEF2. Effects of BHPI on Thr-202/Thr-204 phosphorylation of p44/p42 MAPK (p-ERK) in ERα positive (E) MCF-7 cells and (F) T47D cells. Activation of p44/p42 MAPK promotes the phosphorylation and inactivation of eEF2K. The classical ERK activator, EGF (20 ng/ml), served as a positive control for ERK1/2 phosphorylation. As a control, cells were treated with 10 μM U0126 for the indicated times. U0126 inhibits the upstream kinase MEK1/2, inhibiting ERK1/2 phosphorylation. UO126 pre-treatment was for 2 hours. (G) Effects of blocking ERK activation with U0126 on BHPI-induced phosphorylation of eEF2. By inhibiting the ERK pathway, UO126 allows eEF2K to be active and the reduced activation seen at 0.5 and 1 hours due to BHPI-induced ERK activation is abolished.
Figure 4.21. The UPR activator, Tunicamycin, reversibly activates the UPR. Induction of BiP and p58IPK normally helps resolve UPR stress and reverses UPR activation. Western blot analysis showing the time course of Tunicamycin (TUN) induction of BiP (A) and p58IPK (B) in MCF-7 cells. Data in panel A is from ((47), supplementary figures) (C) Western blot analysis of phosphorylation of eIF2α following TUN treatment. TUN induces transient phosphorylation of eIF2α. (D) Time course of TUN inhibition of protein synthesis. Consistent with transient phosphorylation of eIF2α and resolution of UPR stress, protein synthesis begins to recover at 4 hours after treatment with TUN. Data is mean ± SEM (n = 3). (A-D) 24-hour pre-treatment with 10 nM E₂.
Figure 4.22. Model of BHPI. In ERα+ cancer cells, BHPI rapidly hyperactivates plasma membrane PLCγ, generating IP₃, which opens EnR IP₃R calcium channels, rapidly depleting EnR Ca²⁺ stores. This stimulates activation all 3 arms of the UPR. Activating the PERK arm of the UPR leads to phosphorylation of eukaryotic initiation factor 2α (eIF2α) and potent inhibition of protein synthesis. The cell attempts to restore EnR Ca²⁺ levels, but the open EnR IP₃R calcium channel leads to an ATP-depleting futile cycle, resulting in activation of the energy sensor AMPK and phosphorylation of eukaryotic elongation factor 2 (eEF2). Inhibiting protein synthesis at a second site prevents synthesis of chaperones and other proteins that resolve UPR stress. Independent of BHPI’s effects on protein synthesis, BHPI inhibits E₂-ERα regulated gene expression. Together, these effects promote cell death in ERα+ cancer cells.
References


CHAPTER 5

DISCUSSION

The importance of estrogens (E₂), acting through estrogen receptor α (ERα), in stimulating the proliferation and metastases of most breast cancers is demonstrated by the successful use of aromatase inhibitors, tamoxifen and other endocrine therapies (1-4). Yet, the development of resistance to tamoxifen and to aromatase inhibitors is a prevalent problem in breast cancer treatment (2). Selection and outgrowth of breast cancers resistant to endocrine therapy is common (2, 5, 6), and most deaths due to breast cancer are in patients with ERα⁺ tumors (5). While 30-70% of epithelial ovarian cancers are ERα positive (1), most tumors display de novo resistance to endocrine therapy (7-9). Therapeutic options for these resistant tumors are poor and nearly two-thirds of ovarian cancer patients die within 5 years (10). The presence of ERα in most therapy-resistant breast and gynecologic cancers suggested the presence of additional modes of ERα action potentially targetable with small molecules.

To identify new small molecule inhibitors that target the estrogen-ERα axis in cancer cells resistant to current therapies, we developed and implemented an unbiased pathway-directed screen of ~150,000 small molecules. This type of unbiased screen asks the cell to tell us what interactions and pathways are susceptible to targeting by small molecules, using a readout of inhibition of E₂-ERα-mediated gene expression. Candidate inhibitors were then evaluated in more detail for the ability to inhibit estrogen-dependent growth of ERα⁺ MCF-7 breast cancer cells, with little or no effect on the growth of ERα⁻ MDA-MB-231 cells. The ~40 most potent inhibitors were further tested
for specificity using non-tumorigenic, ER\textsubscript{α} MCF-10A human mammary epithelial cells. The screening conditions were designed to minimize the possibility that our small molecule hits would be ligands that compete with estradiol for binding in the ligand-binding pocket of ER\textsubscript{α}. Since competitors would be expected to show reduced inhibition of ER\textsubscript{α} action at high concentrations of 17\textbeta-estradiol, we tested ~40 of the most potent hits for the ability to inhibit ER\textsubscript{α}-mediated transactivation in the presence of 1 nM estradiol and 500 nM E\textsubscript{2}. None of the inhibitors appeared to function as competitive ligands. 23 compounds, comprising 18 distinct structural families, specifically inhibited the estrogen dependent growth of MCF-7 cells with IC\textsubscript{50}s \textless 1 \textmu M. Inhibitors with different modes of action exhibited a broad range of potency, with highly potent inhibitors targeting several different sites.

From the screen, the selective non-competitive ER\textsubscript{α} inhibitor, BHPI, was identified and became the candidate lead compound. BHPI blocks cell proliferation and kills tamoxifen and fulvestrant-resistant ER\textsubscript{α\textsuperscript{+}} cancer cells, with no effect on ER\textsubscript{α\textsuperscript{−}} cells. In a mouse xenograft, BHPI induced rapid and substantial regression of large pre-existing tumors. BHPI’s effectiveness in a broad range of ER\textsubscript{α}-containing breast, ovarian and endometrial cancer cells is due to its ability to target the unfolded protein response (UPR) pathway. The UPR is inactive in most normal cells, but is commonly overexpressed in advanced-stage and highly metastatic cancers (11-14). While standard UPR activators are nonspecific and highly toxic, BHPI selectively targets ER\textsubscript{α\textsuperscript{+}} cancer cells by activating the UPR through the same pathway used by E\textsubscript{2}-ER\textsubscript{α} to weakly activate the UPR. In effect, BHPI distorts this normal action of E\textsubscript{2}-ER\textsubscript{α} to kill
most ERα+ cancer cells. Since E2-ERα does not appear to activate the UPR in normal mammary cells, BHPI selectively targets the cancer cells and not normal ERα+ cells. While UPR activation is normally transient, due to its mechanism of UPR activation, BHPI rapidly depletes intracellular ATP stores, activating the metabolic energy sensor, AMPK, and establishes a second site of protein synthesis inhibition that prevents the production of proteins required to shut off the UPR. This results in sustained UPR activation, inhibition of protein synthesis, and ultimately cell death.

BHPI also restores sensitivity of multidrug-resistant ovarian cancer cells to therapeutically relevant concentrations of paclitaxel. Taxanes, are among the most commonly used cytotoxic drugs used in breast and ovarian cancer chemotherapy (15, 16). Taxanes promote microtubule assembly and inhibit tubulin disassembly, which promotes apoptosis of cancer cells (17-19). While most epithelial ovarian cancer initially respond to taxanes, most advanced-stage cancers become resistant (20, 21).

Overexpression of membrane efflux pumps, such as P-glycoprotein (MDR1/ABCB1), is the primary mechanism for paclitaxel resistance (22-25). We tested if BHPI could restore paclitaxel sensitivity to multidrug-resistant ERα+ OVCAR-3 ovarian cancer cells, which overexpress MDR1/P-glycoprotein (26, 27). Treating cells with therapeutically relevant concentrations of paclitaxel (100 nM-10 μM) (28), did not kill OVCAR-3 cells. However, treating cells with 1 μM BHPI and 10 nM paclitaxel led to a >80% reduction in cell number. A future direction of this work will involve trying to decipher how BHPI restores taxane sensitivity in multi-drug resistant cells, and to assess whether targeting this pathway can serve as a viable avenue for targeting multi-drug resistant cancer cells.
BHPI offers the potential to significantly increase the reach of endocrine therapies for breast and gynecologic cancer. Previously reported non-competitive ERα inhibitors have usually not advanced through preclinical development. The inhibitors DIBA, TPBM, TPSF, and newly described LRH-1 inhibitors that reduce ERα levels, show limited ERα+ specificity, require >5 μM to inhibit cell proliferation and effectiveness has not been demonstrated in therapy resistant ERα+ ovarian cancer cells (29-31). In contrast, BHPI is far more potent and specific, is effective in breast and ovarian cancer cells resistant to antiestrogens and chemotherapy, and at a modest dose of 15 mg/kg/day induced rapid and substantial tumor regression. Our finding that BHPI is effective in a broad range of ERα containing breast, ovarian and endometrial cancer cells is consistent with recent findings demonstrating these cancers exhibit common genetic alterations, and suggesting they might respond to the same drugs (32).

Using BHPI as a small molecule probe, we identified a novel pathway of ERα action that appears to be ubiquitously activated by estrogens in ERα cancer cells. Our findings that estrogen activates the UPR (33), along with a recent study showing that VEGF also weakly activates the UPR (34), together propose a completely new mode of UPR activation in cancer. Prior to these studies, little was known about the potential role in cancer of a different mode of UPR activation involving anticipatory activation of the UPR prior to accumulation of unfolded protein or cell stress. This work has shown that estrogen, acting via estrogen receptor α (ERα), induces rapid anticipatory activation of the UPR, resulting in increased production of antiapoptotic chaperones, which prepares cancer cells for the increased protein production required for subsequent estrogen-ERα induced cell proliferation. In ERα containing cancer cells, estrogen activates the UPR
through a phospholipase C \(\gamma\) (PLC\(\gamma\))-mediated opening of EnR IP\(_3\)R calcium channels, enabling passage of calcium from the lumen of the EnR into the cytosol. Estrogen-ER\(\alpha\) activates all three arms of the UPR in breast and ovarian cancer cells in cell culture and in a mouse xenograft. In addition, these studies have shown that mild and transient UPR activation by estrogen promotes an adaptive UPR response that protects cancer cells from undergoing apoptosis when subsequently exposed to otherwise lethal levels of stress. This finding suggest that the biological relevance of estrogen-dependent activation of the UPR lies in its ability to protect cancer cells from subsequent exposure to environmental stresses that include hypoxia, drug therapy, and radiation.

This mechanism of estrogen action via anticipatory activation of the UPR is a new paradigm by which estrogens may influence tumor growth, progression and resistance to therapy. The UPR index we identified is an exceptionally robust prognostic marker. For example, in a cohort of 474 ER\(\alpha^+\) breast cancer patients treated 5 years with tamoxifen and followed for 15 years, ~70% of patients with a low UPR index at diagnosis, experienced relapse-free survival, while fewer than 10% of those with a high UPR gene index experienced relapse-free survival (HR=3.7 Log Rank P <0.01). Importantly, the UPR index exhibits predictive power in multivariate analysis over and above current clinical covariates, such as tumor size and node involvement. Future work will address whether this UPR gene signature has the potential to serve as a diagnostic marker. Future studies will be geared toward trying to visualize UPR activation in mouse xenograft tumors, and whether UPR activation can be used to monitor tumor metastases to distant sites. The idea that prior activation of the cancer cell’s stress response pathway is a prognostic marker predicting aggressive, metastatic, therapy-resistant breast cancers is
a novel concept and potentially could represent a new prognostic biomarker, diagnostic marker and therapeutic target.

Future work also aims to address whether anticipatory activation of the UPR extends beyond estrogen to other cancer mitogens. Recent work suggests that the mitogen, epidermal growth factor (EGF), acting through EGFR, also weakly activates the UPR through a PLCγ-dependent mechanism. It is well established that EGFR is overexpressed in a number of human malignancies including lung, pancreatic, brain, bladder, and breast cancer. EGF receptors are expressed in most breast cancers, but are overexpressed in 50% of triple-negative (ERα, PR, and HER2/neu) breast cancers and frequently overexpressed in highly-aggressive invasive breast cancer (IBC) (35-37). Furthermore, EGFR overexpression is associated with larger tumor size, poor differentiation, and poor clinical outcome (38-40). Our present data suggests that EGF may also similarly drive expression of the UPR, which ultimately makes cells more resistance to conventional therapies in cancer treatment.

Our surprising finding that UPR activation is important for subsequent E2-ERα regulation of gene expression is supported by a very recent report that activation of the UPR using a regulated system for production of unfolded protein enhances E2-ERα induced gene expression (41). Very rapid anticipatory activation of the UPR is essential for subsequent E2-ERα induced cell proliferation. Several very recent reports suggest this newly described role for the UPR is conserved across species and classes of mitogens. The insect steroid hormone ecdysone elicits anticipatory activation of the UPR. This ecdysone-ecdysone receptor-mediated activation of the UPR is required for ecdysone to stimulate metamorphosis and cell proliferation (42). Moreover, the peptide
hormone mitogens VEGF elicit anticipatory activation of the UPR as part of its pathway of action (34). Since several other mitogens, including fibroblast growth factor (FGF), phosphorylate and activate PLC\(_{\gamma}\), it is probable that they also elicit anticipatory activation of the UPR through this pathway (p-PLC\(_{\gamma}\)\(\rightarrow\) IP\(_3\)\(\rightarrow\) IP\(_3\)-IP\(_3\)R\(\rightarrow\) intracellular Ca\(^{2+}\)). Thus, anticipatory activation of the UPR appears to be a new type of cell proliferation checkpoint. If cells cannot activate the UPR to ready themselves for proliferation, they do not proceed through the cell proliferation program.

Activation of the UPR that anticipates future stress is both a new pathway for activation of the unfolded protein response and a new role for the UPR. Previously, the UPR was known to be activated by sensors that sense unfolded protein and other stressors and respond by triggering UPR activation. This is sometimes termed the “reactive” mode for UPR activation. The PLC\(_{\gamma}\) mediated pathway for UPR activation we identified describes a new “anticipatory” mode of UPR activation that readies cells for subsequent stress they will encounter as they proliferate.

Classically, a new pathway related to cancer is identified and proposed as a potential drug target, and in subsequent studies a lead small molecule inhibitor might be identified. We turn this approach 180° and start with unbiased screening to generate a large data set from which we choose a lead small molecule inhibitor whose properties in simple follow on assays suggest it is likely to exhibit an unexplored mode of action. Then we progress from identification of the inhibitors mode of action to the relation of that action to previously undescribed normal actions of its target. For a well-studied protein, such as ER\(_{\alpha}\), it was unclear whether this approach could identify new pathways and biomarkers and promising new drug candidates. These studies demonstrate the
potential of small molecules identified from a large database generated by targeted cell-based high throughput screening, to reveal and validate new pathways of action, therapeutic drug targets, and small molecule therapeutic candidates; even in a system as intensively studied as ERα positive breast and ovarian cancer.

References


