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Endocrine Active Chemicals in Breast Cancer Cells: Environmental Impacts on Growth, Signaling and Epigenetic Pathways, and Drug Response

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MELISSA LYNN SOKOLOSKY. Endocrine Active Chemicals in Breast Cancer Cells: Environmental Influences on Growth, Signaling and Epigenetic Pathways, and Drug Response. (Under the direction of DEMETRI SPYROPOULOS).

Abstract

Individuals living in industrialized regions of Westernized societies are exposed to environmental contaminants by many routes, including plastics, personal care items and other consumer products. Endocrine-active chemicals (EACs), many of which are estrogenic, have been detected in human biofluids and breast tissue, warranting investigation of roles in mammary tumorigenesis. This dissertation explores the molecular changes that occur in breast cancer cells upon treatment with relevant human exposure levels of bisphenol-A (BPA), methylparaben (MP), propylparaben (PP), and decabromodiphenyl ether (DBDE), including mixtures thereof with or without 17β-estradiol (E2). Nanomolar (nM) concentrations of these EACs induced viability increases in MCF-7 (ERα+) similar to picomolar (pM) E2 concentrations, but EAC mixtures did not produce additive effects. MDA-MB-231 viability was unaltered by EACs in the absence of E2 but was significantly increased with exposure to five EACs combined with 50 pM E2. To examine ERα gene regulation, transactivation assays in ERα-transfected HepG2 cells confirmed that nanomolar BPA could induce ER-driven transcription, and suggested an additive effect of EAC mixtures. Non-genomic ERα functions were also investigated in breast cancer cells via high-throughput microscopy and quantitative immunofluorescence with the Hermes/WiScan System. MCF-7 cells revealed a 50% increase in phosphorylated (P)-ERα Serine 167 expression after 30-minute exposure to

100 nM PP, followed by alterations in nuclear/cytosolic localization after 24 hours. Signaling/epigenetic pathways were assessed with fifteen additional protein markers. At 100 nM, EACs induced expression of P-ERK, β-catenin, and epigenetic marks at H3K4me2, H3K9acetyl, and H3K27me2, while decreasing levels of AR and LSD1. Similarities and differences in proteomic expression patterns were observed between E2 and EACs. These pathways were further found to influence breast cancer progression, drug response and resistance; EACs were shown here to increase Tamoxifen IC_{50} in MCF-7 cells, but further sensitized them to Doxorubicin toxicity. A proteomic marker shown to undergo nuclear localization changes during observed EAC-altered Tamoxifen responses was P-ERα-S167. After extended low-level EAC exposures (60 days), a four-day EAC-withdrawal was sufficient to reset MCF-7 drug responses to control levels. These results reveal that EACs have diverse cellular mechanisms involving genomic and proteomic ERα effects and signaling/epigenetic alterations, highlighting these EACs as important environmental considerations for breast cancer research, clinical care and prevention.

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Schema 1: Overall Mechanism of Signaling and Epigenetic Pathways Underlying the Observed Phenotypic Effects of EACs in MCF7 Cells

Key to Symbols and Abbreviations

AR: Androgen Receptor

BPA: Bisphenol-A

cE2: Constant 17β-estradiol

DBDE: Decabromodiphenylether

DBP: Dibutyl phthalate

Dox: Doxorubicin

E2: 17β-estradiol

EAC: Endocrine Active Compound/Chemical

ERα: Estrogen Receptor α

ERβ: Estrogen Receptor β

MP: Methylparaben

PP: Propylparaben

PR: Progesterone Receptor

Tam: 4OH-tamoxifen

XE: Xeno-estrogen

Chapter 1: Introduction and Rationale

1.1 Breast Cancer Risk in Developed Regions

After decades of scientific inquiry, the numerous causes and effects involved in breast cancer development have become increasingly clear, yet evermore complex. Both genetics and environment play significant roles in mammary tumors, and in some cases are mutually dependent in promoting their molecular and phenotypic outcomes. Environmental factors can regulate how, when, and where key genes such as oncogenes and tumor suppressors are expressed. Some breast cancers can be attributed to clear heritable causes, such as BRCA2 alterations, although the majority of breast cancers lack a clear genetic basis. According to the 2010 report of the President's Cancer Panel, "the true burden of environmentally induced cancers has been grossly underestimated." In some cases, environmental factors may be more influential drivers of breast cancer than genes, as suggested by a large cohort study of twins that identified only 27% of the breast cancer cases studied to involve heritable factors (Lichtenstein *et al*, 2000). General risk factors for breast cancer include age, parity, and BMI, yet a growing list of environmental exposures are also now consider contributing factors. Further elucidating the complex roles of environmental interactions in breast cancer initiation, progression, and treatment response is vital for effective basic research, clinical care, and preventive approaches.

Global trends for breast cancer yield interesting perspectives on how rapidly evolving lifestyles and environments in developed and developing countries may have

contributed to rising cancer rates over recent decades. Breast cancer was diagnosed in 1.67 million people in 2012, making it the most common cancer in women, as well as counting for 25% of all cancers diagnosed worldwide. More developed regions, including the United States, Europe, and Australia, have recorded increases in breast cancer incidences since data collection began in 1975. Less developed regions, including Thailand, India, and Singapore also experienced gradual increases in breast cancer incidence over that time, although rates remained much lower than that of developed regions (GLOBOCAN, IARC, 2012).

Data from 1995 show a five-fold higher rate of breast cancer incidence within the United States as compared to Thailand. However, 1995 was also when the United States and other developed countries began to report considerable decreases in breast cancer mortality, a trend maintained through 2012, attributable to advances in detection and clinical care. Reductions in mortality were not reported in less-developed regions over the same time period, with the developing world showing the highest ratio of individuals diagnosed with breast cancer succumbing to the disease. The higher rates of survival in developed regions were coupled with higher rates of incidence, with estimated age-standardized rates for 2012 in North America at 92/100,000 individuals, and only 27/100,000 individuals in Eastern Asia and Middle Africa (GLOBOCAN, IARC, 2012).

Marked regional differences in breast cancer incidence around the globe point to the contributions of changing lifestyles and increased environmental exposures to breast cancer risk. Even within the same country/region, breast cancer risk can vary significantly based on degree of urbanization. Relative to rates in the United States and other

Westernized countries, China has a low incidence of breast cancer. Yet, within China, incidence was found to be markedly lower for women living in rural areas as compared to women living in urban settings. Age was also shown to not be the greatest risk factor for breast cancer in Chinese women, as incidence was highest in the 50-54 year age group compared to all older age brackets, based on national cancer statistics from 2005 to 2009. Individuals in younger age groups from urban areas were reported to be diagnosed at rates similar to age groups decades older that resided in rural settings (Fei *et al*, 2015).

1.2 Environmental Estrogens and Breast Cancer Risk

One key regulator of the interplay of environment and genes in breast cancer is the influence of estrogenic compounds under normal and tumorigenic circumstances, especially in the case of estrogen receptor (ER)-positive breast cancers. Expression of ERs in breast cancer cells can orchestrate proliferative responses to endogenous hormonal ligands, such as 17β-estradiol (E2), but also confers susceptibility to exogenous ER-binding compounds. Phenolic hydroxyl (OH) groups of a ligand's structure drive ER binding, and hydrophobic constituents are accommodated and can accelerate receptor binding in certain cases, with regions of the receptor allowing for ligand flexibility (Anstead *et al*, 1997). Due to molecular flexibility in binding, coupled with the prevalence of phenolic OH groups in many chemicals, ERs are considered promiscuous receptors by binding ligands other than E2 and endogenous hormones. Other susceptible targets expressed in breast cells include the progesterone receptor (PR), the androgen receptor (AR), and emerging G-protein coupled receptors and orphan nuclear receptors. An

expanding group of environmental ligands, both natural and synthetic in origin, have shown the ability to bind these receptors and influence hormonally-responsive cells (Sokolosky and Wargovich, 2012).

Exposure to endocrine disrupting chemicals (EACs) is considered nearly ubiquitous for species living within industrialized regions, given the widespread use of EAC-containing items such as plastics, personal care products, and pesticides (Sokolosky and Wargovich, 2012). Common EACs include the plasticizers bisphenol-A (BPA) and dibutyl phthalate (DBP), the preservatives methylparaben (MP) and propylparaben (PP), and the flame retardant decabromodiphenyl ether (DBDE) (Figure 1). Through decades of industrial and consumer use, EACs and other manmade chemicals have been released into soil, air, bodies of water, and inevitably the global food chain. Species at the top, including predators and human consumers, seem to be the most vulnerable to the bioaccumulative effects of EACs (Our Stolen Future, Myers, 1996).

Certain EACs, including BPA and parabens, are classified as xeno-estrogens (XEs) because they exhibit varying degrees of estrogen mimicry and ER binding affinity. Many EACs were originally identified through a large-scale approach for screening compounds for estrogenicity, called the E-SCREEN, which is based on proliferative responses of MCF-7 breast cancer cells due to high levels of ERα expression (Soto *et al*, 1995). The degree of ER activation by EACs depends on their structural similarity to E2, including the presence and positioning of key phenolic OH groups (Figure 1). Compared to the binding affinity of E2, that of EACs including BPA has been shown to be 100-fold to 1000-fold lower. Despite their lowered activation of ERα than E2 at certain concentrations, it may

be possible for complex mixtures of EACs to trigger responses and compete with E2 for receptor binding in some scenarios. Parabens have been shown to interact with the ERα ligand-binding pocket, and early molecular models indicated that phenolic hydroxyls from more than one paraben may bind at once to block E2 binding (Byford *et al*, 2002). Some EACs that are agonists of ERα have also been shown to act as antagonists of the androgen receptor (AR), including BPA (Li *et al*, 2010). This duality in hormone receptor regulation likely contributes to differential tissue responses to EACs based on relative cellular expressions of ERα and AR, among other EAC targets.

1.3 Environmental Estrogens in the Human Body

Biomonitoring studies by the Centers for Disease Control (CDC) and other laboratories have consistently identified the presence of EACs in human samples, with 93-99% of urine samples containing BPA (NHANES Data, 2013-2016). In one study, common forms of paraben were measured in urine samples from couples undergoing fertility treatments, with MP and PP the most commonly detected (respective median concentrations: 112 ug/L and 24.2 ug/L). Gender and racial differences in paraben exposures were also identified, with women showing approximately four to eight-fold higher levels as compared to men, and African Americans showing four-fold higher levels as compared to Caucasians (Smith *et al*, 2012). A study of postmenopausal women found that those with high levels of BPA and mono-ethyl phthalate in their serum also had increased mammographic breast density, but the other phthalates, phenols, and parabens (including PP) tested did not show this association (Sprague *et al*, 2013).

Mixtures of parabens have also been measured directly in human breast tissue, with a study of breast cancer patients undergoing mastectomy revealing 99% of samples to contain one or more quantifiable forms of paraben. Among the 160 samples tested, MP and PP were the most commonly identified, with the median level of total paraben content being 85.5 ng/g tissue, and PP levels significantly higher in the axilla than other regions of the breast (Barr *et al*, 2012). These concentrations were then shown to be functionally-relevant in breast cancer cells, with 12 out of 22 ER+PR+ tumor tissues containing one or more paraben at levels later shown to induce proliferation of MCF-7 breast cancer cells (Charles and Darbre, 2013). Exposure to similar paraben concentrations were also shown to promote anchorage-independent growth of MCF-10A mammary epithelial cells (Khanna and Darbre, 2013). Multiple breast cancer cell lines exposed long-term (20 weeks) to MP or PP in culture were shown to be more migratory and invasive, correlating with indicators of epithelial to mesenchymal transition (Khanna *et al*, 2014). These findings suggest that low-level EAC exposures can lead to accumulation within the human body at levels suspected to be capable of inducing cellular responses related to transformation in breast cells. Wide-scale population exposure to EACs within industrialized regions over time could be linked to increases in breast cancer incidence in these same regions if low-level mixtures of EACs are capable of inducing carcinogenic mechanisms. Parabens have been measured in urine, blood, semen, placenta, and breastmilk, demonstrating that *in utero* and neonatal exposures are occurring (Khanna *et al*, 2014).

1.4 Molecular Changes Associated with EAC Exposures

Despite lower ER-binding affinity, low nanomolar levels of BPA have been shown to induce gene expression profiles in MCF-7 derived cells that are similar, but not identical, to that of E2. With a set of transcripts identified to be unique to BPA exposure, a Venn diagram-like perspective shows transcriptional programs both overlap and digress between endogenous and exogenous estrogens. Even in ER-null cells, BPA was shown to induce transcription, adding support for EAC mechanisms that are ER-independent (Singleton *et al*, 2004).

Given their ability to influence steroid hormone receptors in a wide array of cell types, it is logical to link exposure to EACs with altered ERα and AR activation and signaling in breast cancer cells. These receptors are known to have extensive cellular roles, including participation in canonical signaling pathways. In the context of breast cancer etiology, many of the signaling pathways shown to be directly or indirectly influenced by EACs also have established and emerging roles in breast cancer initiation and progression. Signaling pathways link plasma membrane-bound receptors to nuclear/transcriptional machinery by communicating extracellular events through intracellular networks of regulators such as kinases and cyclins. Different pathways intersect, cross-talk, and perform complex feedback to regulate virtually all cellular functions in an attempt to maintain homeostasis in spite of a dynamic environment. Through its ability to localize within different compartments of a breast cell, ERα can act as a cytoplasmic signaling mediator when membrane-localized (Zivadinovic *et al*, 2005), as well as a transcriptional regulator when found in the nucleus. The estrogenic activity

of EACs extends beyond their ability to bind ERs, as some can perform estrogenic regulation within a cell by influencing ER localization and related signaling pathways.

The β-catenin/Wnt, Ras/Raf/MEK/ERK and Ras/PI3K/Akt/PTEN/mTOR pathways have been extensively studied for their roles in breast cancer (Davis et al, 2014). In normal cells, these pathways are critical for differentiation, metabolism, survival, and growth, but upregulation of these pathways can occur via mutations, epigenetic alterations, and/or enhanced signaling and feedback from other altered proteins. Once a cell has acquired a sufficient number of molecular alterations that promote survival and prevent apoptotic signaling, it can undergo transformation. If tumorigenesis occurs in a mammary stem cell, the resulting malignant cell retains stem-like activity, and is often referred to as a cancer-initiating cell (CIC). Pathways associated with stem cell functions and embryonic development, including Wnt/β-catenin, play roles in morphogenesis of fetal mammary tissue, as well as in homeostasis of adult breast tissue by mammary stem cells (Rangel et al, 2016).

Breast cells can undergo signaling pathway alterations via genetic and proteomic mechanisms in response to EAC exposures. Significant changes in expression of HOXC1 and C6, Wnt5A, Frizzled, TGF-β2 and STAT Inhibitor 2 genes were induced by BPA in MCF-7 cells. These particular genes were unaffected by E2 treatment, although many transcriptional changes were similar between BPA and E2 (Singleton et al, 2006). In another study, MCF-7 cells exposed to BPA upregulated transcription of the cell-cycle promoters, cyclin D1 and p21, which was ERα-dependent (Lee et al, 2012). Other ER target genes including bcl-2 were induced by BPA or E2 in MCF-7 cells, although only BPA

induced Jun and Fas, with differential patterns of microRNA expression also observed (Tilghman *et al*, 2012).

1.5 EACs in *in vitro* and *in vivo* Models

Cell and animal studies suggest that low-level exposure to EACs may be a contributing factor to breast cancer development via the cellular pathways already known to influence etiology. Co-cultures of epithelial and stromal cells were generated from collateral breast tissues collected from breast cancer patients and exposed to BPA. Cellular pathways were induced that led to evasion of apoptosis and cell-cycle deregulation, and these BPA responses were significantly upregulated in breast cells from patients with high-grade tumors and poor outcome (Dairkee *et al*, 2008). Another study from this group examined the effects of EACs on renewable cells from donors at risk for breast cancer yet to develop the disease. Renewable high-risk donor breast epithelial cells (HRBECs) were exposed to BPA at levels found in human blood and placenta, leading to activation of PI3K/Akt/mTOR and downregulation of the tumor suppressor PTEN, which induced proliferation in these cells. When pretreated with BPA or MP, HRBECs were shown to be more resistant to the apoptotic effects of Tamoxifen. These findings showed that EAC exposure can induce pre-malignant changes in benign breast cells taken from donors at risk for breast cancer (Goodson *et al*, 2011). An additional study by this group found that cellular reprogramming of HRBEC-derived cell lines exposed to BPA involved decreased expression of the pro-apoptotic proteins p53, p21, and BAX, and increased ERα to ERβ ratio, PCNA, and cyclin expression. These BPA-induced changes in cellular pathways were sufficient to drive proliferation of all HRBEC cultures exposed,

although the mechanistic and phenotypic effects of BPA could be ameliorated by concurrent exposure to curcumin (a plant-based polyphenol) (Dairkee *et al*, 2013). These findings strongly suggest that EAC-exposure is link to breast cancer development, although effects due to a single compound are not indicative of mixed exposures, some of which can weaken cellular responses to EACs.

1.6 Non-Canonical EAC-mediated Molecular Pathways

Recent studies have identified receptor targets of EACs beyond ERs/ERRs, including the G-protein coupled receptor, GPR30/GPER. In SKBR3 breast cancer cells, as well as cancer-associated fibroblasts lacking ERs, BPA was shown to induce growth and migration through GPR30, linked to activation of the EGFR/ERK cascade (Pupo *et al*, 2012). Therefore, the same canonical signaling pathways can be activated via numerous upstream receptors that are known and emerging targets of EACs. The advent of BPA derivatives and substitutes in recent years has prompted investigation into potential endocrine activity, and like BPA, bisphenol-F and bisphenol-S are also agonists of ER and other nuclear receptors (Molina-Molina *et al*, 2013).

Post-translational modifications (PTMs) like phosphorylation, are rapid signaling events that can lead to localization and functional changes of phosphorylated (or de-phosphorylated) proteins. ERα can undergo PTMs at several residues within its structure, including Serine 104, S106, S118, and 167, all located within the region of the receptor associated with ligand-independent, “non-classical” signaling (Barone at al, 2010). The mutual activation of ERα and cytoplasmic signaling pathways by EACs may be separate events initially, one being binding of a single/multiple ERα by EACs, and another being

activation of GPCRs and/or RTKs. In the absence of receptor binding by an EAC, non-classical ERα activity could be induced by EAC exposure if the receptor undergoes phosphorylation by a kinase activated through upstream EAC-related events.

In one study, BPA inhibited apoptosis in MCF-7 cells without altering total ERα protein levels, leading the authors to conclude that BPA does not act via classical estrogenic routes in these cells (Diel et al, 2002). Instead of increasing total ER expression levels, EACs may exert effects via PTMs of existing ERs thereby influencing receptor localization and activity. One study found low-level BPA (4 nM) sufficient to induce gradual yet extensive nuclear localization of ERα in mammosphere-derived cells, demonstrating that EAC-induced ER-trafficking can occur in stem-like breast cancer cells (Weng *et al*, 2010). Intracellular concentrations of ERα were shown to be influenced similarly by low-level BPA or E2 exposure in MCF-7 cells, initially inducing phosphorylation of ERα at S118 and leading to activity, but total receptor levels within the cell were reduced 24 hours after treatment (La Rosa et al, 2014).

It is well known that ERs rely on accessory proteins for optimal DNA interactions, and these multi-protein complexes determine the extent of ERE transcription (Landel *et al*, 1995). ER binding partners and related transcription factors can be regulated by upstream signaling pathways, many of which cross-talk with or are directly involved with cytoplasmic ER signaling. BPA, genistein, and other EACs, as well as E2, were evaluated for their effects on ERα and ERβ coactivator recruitment and reporter gene expression, yielding evidence of ligand-dependent actions such as enhanced ERβ activity in the presence of certain EACs (Routledge et al, 2000). Ligand-specific effects on ER

coactivators and accessory proteins beyond receptor binding alone seem to strongly dictate cell-type responses based on relative agonistic and antagonistic mechanisms.

1.7 EAC-induced Epigenetic Changes

Epigenetic alterations are also part of a cell's enviromic response, as they ultimately direct the binding of transcription factors regulated by upstream signaling pathways, in order to initiate the appropriate gene expression program. The presence or absence of epigenetic marks (including acetyl and methyl groups) on histones and DNA regulate chromatin structure, unwinding of DNA, and accessibility of the appropriate promoter regions. Enzymes such as methyltransferases and histone deacetylases orchestrate dynamic placement of marks, and like the many proteins involved in EAC-binding and signaling, these epigenetic regulators can also be influenced by EACs (Singh and Li, 2012).

Non-genomic ER signaling was shown to reprogram expression of estrogen-related genes upon estrogen exposure by acting as a rapid way to alter the regulation of epigenetic enzymes, such as the histone methyltransferase EZH2 (Bredfeldt *et al*, 2010). Mammary cells from rodents exposed to BPA in utero showed a greater than 2-fold increase in protein expression of EZH2, along with an increase in trimethylation of histone 3 (H3). This increase in EZH2/trimethyl H3 was also observed in MCF-7 cells treated with BPA, demonstrating that epigenetic reprogramming is one way EACs can influence developing mammary cells and breast cells that are already tumorigenic (Doherty *et al*, 2010). In addition to *in vitro* activation, EZH2 was shown to be induced *in vivo* by E2, BPA, or DES exposure, conferring transcriptional activity in the mammary

glands of ovariectomized rodents (Bhan et al, 2014). In breast epithelial cells, nuclear localization of ERα occurred after low-level BPA exposure and LAMP3 expression was epigenetically silenced, an effect also observed in ER+ breast cancer cells through heightened DNA methylation within the affected gene's CpG islands (Weng et al, 2010).

1.8 Interplay between EACs and Endogenous Estrogens

Production of endogenous estrogens in breast cancer cells may be induced by EAC exposure, and one study found that all levels of MP and PP tested (0.2 nM- 2 uM) induced MCF-7 cell proliferation, with low levels inducing E2 secretion and altered aromatase (CYP19A1) expression. This effect in E2 was not observed in MCF-10A cells, although low-level MP exposure did induce their proliferation (Wróbel and Gregoraszczuk, 2013). Estrogen metabolism may be higher in cells exposed to EAC mixtures, as was observed with breast cancer cell screening assays examining the proliferative effects of EACs combined with steroidal hormones. Although certain mixtures can be additive, upregulation of steroid hormone metabolism may perform negative feedback to keep proliferation in check in the presence of mixtures of estrogens (Silva et al, 2011).

1.9 EACs and Hormonal-based Therapy

Inherent ERα expression in approximately 70% of breast cancers is the rationale for treatment with anti-estrogens such as Tamoxifen. As a Selective Estrogen Receptor Modulator (SERM), Tamoxifen is meant to act as an ERα antagonist in the context of breast cancer by binding the receptor to block E2 activation. Despite knowledge of ER pathways and genetic factors involved, it remains unclear why nearly 50% of patients

experience resistance and relapse. Counter-intuitively, ERα-positive breast cancer cells can stop responding to antiestrogens like Tamoxifen, whether via *de novo* or acquired mechanisms (Arpino et al, 2009). Even if a tumor initially responds to a drug, once a certain proportion of cells develop resistance, the likelihood of relapse and metastasis increase, and more aggressive treatments can led to heightened side effects for a patient.

Inductions of pro-survival, anti-apoptotic phenotypes in cancer cells are molecular drivers of drug resistance, but the role of environmental exposures in inducing these processes is less established. Alteration of signaling pathways including the Ras/PI3K/PTEN/Akt/mTOR cascade is heavily implicated in breast cancer resistance to Tamoxifen and chemotherapy drugs, and MCF-7 cells expressing constitutively active Akt were shown to be more resistant to 4-OHT than control cells (Sokolosky et al, 2011). As mentioned above, numerous EACs like BPA have been shown to activate this same pathway in both mammary epithelial cells and breast cancer cells, which may influence the development of drug resistance. Epigenetic alterations have also been implicated in the development of de novo Tamoxifen resistance in breast tumors, including ER+ types (Raha et al, 2011). Emerging roles of environment factors, in collaboration with established mechanisms, can offer novel perspectives on why breast cancers develop drug resistance and relapse, as well as approaches for avoiding these outcomes.

Endogenous estrogens and Tamoxifen are not the exclusive ligands of ERs, and Tamoxifen most likely competes with other environmentally-derived ligands. Any given cocktail of EACs and drugs could influence complex pharmacological responses in

hormonally-responsive cells, and the basic and clinical science performed to characterize drugs like Tamoxifen did not account for the presence of EAC mixtures. Given that mixtures of endogenous estrogens and EACs could be present in a breast cancer patient on Tamoxifen, the large number of competing ligands could limit the intended effect of endocrine therapy. It was shown that BPA can compete with 4OH-tamoxifen for ER binding, which was able to perform "estrogenic rescue" against drug cytotoxicity in ER+ MCF-7 breast cancer cells (Lewis *et al*, 2000).

The estrogenic properties of EACs stem from their actions on ER isoforms as well as the orphan nuclear receptors known as estrogen related-receptors (ERRs), with ERRγ capable of also binding Tamoxifen. In yeast models of hormone receptor activation, BPA and other phenols including BPA derivatives, were able to overcome the inhibitory effects of Tamoxifen on ERRγ (Li *et al*, 2010). Able to bind ERRγ strongly as an inverse-type antagonist, BPA was shown to maintain the receptor's high basal activation in the presence of 4hydroxy-tamoxifen (4-OHT), attributed to receptor affinity for BPA's phenol groups (Liu *et al*, 2010). In cell models of invasive lobular breast cancer, it was found that ERRγ signaling may be involved in the development of Tamoxifen resistance, with expression being a marker of poor Tamoxifen response (Riggins *et al*, 2008). It was recently suggested that by binding ERRγ as inverse agonists, five common forms of parabens not only play a role in breast carcinogenesis, but also in Tamoxifen response (Zhang *et al*, 2013). The likelihood that breast cancer patients harbor systemic concentrations of these and other EACs at the time of endocrine therapy is an important consideration for clinical care.

1.10 EAC Exposure History and Breast Cancer Susceptibility

Extending beyond time of diagnosis and treatment, it is also likely that breast cancer patients each have personal histories of prior EAC exposure, dating back to the womb and puberty. *In utero* EAC exposure is documented in humans to promote the development of tumors in adulthood, and transgenerational effects have been recorded. Diethylstilbestrol (DES), a morning sickness drug prescribed to pregnant women in the 1970s, was shown later to act as a EAC and has been linked to breast and uterine cancers in the daughters and granddaughters of these women (Greathouse et al, 2012). The two-fold higher breast cancer risk arising from *in utero* DES exposure is thought to involve epigenetic modifications within mammary stem cells (Hilakivi-Clarke, 2014).

Exposure to BPA in pregnant mice has been shown to increase mammary cancer development in adult offspring. Female rats exposed to BPA during lactation and then exposed to the carcinogen DMBA at 50 days of age, developed higher numbers of breast tumors with decreased tumor latency as compared to females that were not exposed to BPA early in life. Even in the absence of DMBA treatment, neonatal/prepubescent BPA exposure led to increased mammary cell proliferation and decreased apoptosis at day 50, supported by increases in SRCs, phosphor-Akt, and erbB3 (Jenkins *et al*, 2009). The ability of fetal BPA exposure to predispose mammary cells to DMBA-induced carcinogenesis was observed in another study in mice, along with the ability of high and low-level BPA to promote growth of MCF-7 breast cancer cells in a second mouse model. The authors concluded that both fetal reprogramming of mammary cells and promotion of established ER+ breast cancers are likely involved in BPA-induced tumorigenesis

(Weber-Lozada and Keri, 2011). Another study showed that pubertal BPA exposure in mice could promote transformation in mammary stem cells, with early cancerous lesions resembling those caused by DMBA exposure (Wang et al, 2014). Fetal exposure to BPA was also found to alter the mammary transcriptome in rodents, with changes involving adipogenesis and focal adhesion within the periductal stroma, dependent upon expression of ERs (Wadia et al, 2013). These studies strongly suggest that early EAC exposures in animals can alter breast cell pathways and influence mammary stroma architecture, thus promoting increased susceptibility to further environmental insults that can promote ER+ tumors (Soto et al, 2013). Furthermore, it was found that epigenetic pathways are involved in BPA-induced mammary changes in rodents, as shown by increased methylation of key histone residues leading to altered gene expression and susceptibility to breast tumors after postnatal exposure (Dhimolea et al, 2014).

1.11 Dose-dependent Response to EACs and Mixtures

Any substance can be toxic at a high enough dose, including water, yet many EACs exert adverse cellular effects at lower doses, often at the level of parts per billion or parts per trillion. The mechanistic and cellular response of cells and tissues to EACs greatly depends on dose, with non-monotonic responses driving differences between higher levels (the standards for toxicological testing) and those considered low-level (more relevant to human exposure) (Vandenberg et al, 2012). In one study, adult transgenic mice overexpressing erbB2 were exposed to a range of BPA doses (2.5-2500 ug/L) in drinking water to investigate the proteomic and phenotypic mechanisms associated with

exposure-related mammary tumor development. Only low doses of BPA increased tumor growth, burden, and metastatic activity, but all doses tested induced mammary cellular proliferation. Mechanistically, 2.5 ug/L BPA phosphorylated numerous influential signaling proteins, including erbB2, erbB3, IGF-1, and Akt, but 2500 ug/L BPA did not. Although high-level BPA exposure did not significantly influence breast tumor development, it was sufficient to raise the apoptotic index of exposed mammary cells (Jenkins et al, 2011). Dynamic, dose-dependent responses in MCF-7 cells to BPA were mapped as 'expressomes' showing differential suppression or induction of numerous BPA-target genes, once again reflecting the non-monotonic mechanisms of EACs (Shioda et al, 2013). Based on these *in vitro* and *in vivo* findings, mixtures of EACs at low nanomolar and micromolar levels could be an important environmental concern for breast cancer patients, as well as breast cancer prevention.

Exposure to EACs could also be linked to breast cancer development in males, with 1% of the male population diagnosed with breast cancer each year in the US (Macon and Fenton, 2013). Rising rates of male breast cancer, which develops in the absence of substantial endogenous E2, suggest that exposure to exogenous estrogens plays a significant etiological role for both genders (Goodson et al, 2011). Whether via endogenous production, oral contraceptives, hormone-replacement therapy or EAC exposure, most individuals encounter a unique combination of estrogenic compounds on a consistent basis. Single-agent studies have been valuable for thoroughly defining specific mechanisms attributed to individual compounds, laying the groundwork for evaluating combinations of EACs. The next step is understanding environmental

influences in the context of realistic human exposures, involving complex mixtures of endogenous hormones and EACs with varying degrees of estrogenicity. One study utilized ER reporter gene and E-SCREEN assays to evaluate mixtures of up to 17 EACs, and found the concentration addition concept adequate to predict mixture effects. When compounds with relatively low estrogenic activity were included in a mixture with more estrogenic EACs, they modulated the mixture effects by reducing the overall cell-based response (Evans et al, 2012). Another group presented a multi-step modeling approach for mixture assessment in which dose addition methods apply when components and mixtures share common dose-response curves through the EPA-guided properties of being toxicologically similar and mixed at fixed proportions across a range of doses (Hertzberg et al, 2013). Cell data and predictive models suggest that exposure to multiple EACs does not always have additive effects as compared to single compounds, additivity and synergism may be possible only with mixtures of certain EACs, and inclusion of EACs with diverse actions may limit the cellular effects of the mixture as a whole.

1.12 Dissertation Research Focus

The body of research thus described provides compelling support for deterministic roles of environmental exposures in breast cancer development, progression, and clinical outcome. The word "environment" can be defined differently depending on context; in relation to the field of cell biology, environment implies any non-genetic factors such as diet/nutrients, pathogen, and chemical exposures (both natural and manmade). This dissertation focuses on the later form of environmental

factor, specifically exposure to common EACs, and how mixtures thereof can influence breast cancer cell phenotypes via genomic and non-genomic signaling mechanisms. By examining multiple EACs and mixtures in multiple breast cancer cell types via multiple quantitative end-points, this study intends to define the environmental responses of breast cancer cells as important considerations for basic research and clinical care. To make the *in vitro* models described herein as relevant as possible to human breast cancer outcomes, low-level EAC concentrations were tested for both short term and extended low-level treatments, with or without estradiol or the drugs doxorubicin and tamoxifen.

Following a Methods chapter, the results of this study are presented within the context of the following aims and objectives:

Specific Aim 1: Determine the effects of EACs and mixtures on breast cancer cell viability.

Specific Aim 2: Investigate the molecular mechanisms of EACs related to genomic and non-genomic ERα functions, signaling pathways and epigenetic marks.

Specific Aim 3: Evaluate the effects of EACs and mixtures on drug response.

Major hypotheses include: treatment of breast cancer cells to EACs and mixtures within human ranges induces differential phenotypic responses via cell-specific molecular mechanisms involving hormone receptors, signaling pathways and epigenetic marks; EAC-induced cellular changes can alter chemo- and hormonal-therapy responses, and effects may be reversible with a period of EAC withdrawal prior to drug treatments.

Chapter 2: Materials and Methods

2.1 Cell Culture and Reagents

The ERα+ MCF-7 and ERα- MDA-MB-231 breast cancer cell lines were purchased from ATCC, cryopreserved at passages 2-3, and used for assays within passages 3-10. The ERα+ luminal breast cancer cell line, SUM44, was a generous gift from the laboratory of Dr. Ethier. All cells were maintained at 37^{o}C with 5% CO_2 in phenol red-free DMEM (Mediatech) containing 10% charcoal/dextran-stripped fetal bovine serum (FBS) (Atlanta Biologicals) to minimize EAC exposure in culture, and 1% penicillin-streptomycin (Lonza). In preparation for experiments, cells were detached with 0.5% trypsin (Mediatech), washed twice with phosphate-buffered saline (PBS), and plated in EDC-minimum media. When specified, phenol-red was present during treatments. Reagents, including BPA, DBDE, DBP, MP, PP, and E2 (Sigma-Aldrich), and 4OH-tamoxifen, doxorubicin, and BEZ235 (SeleckChem) were dissolved in DMSO. Stock solutions were stored at -80 degrees and freshly diluted in EDC-minimum media for experiments, with vehicle concentrations of 0.1% v/v or less.

2.2 Cytotoxicity/MTS Assay

Cells were seeded at 2000 cells/well in flat-bottom, culture-treated 96-well plates (Grenier) and incubated overnight to allow for attachment. The following day, cells were treated in triplicate wells with EACs and/or drugs serially diluted to the indicated concentrations, and incubated for 96 hours. MTS reagent (Promega) was added to the

wells and allowed to metabolize, followed by absorbance readings at 490 nm. At least three biological replicates were performed for each assay.

2.2.1 Coupled Proliferation Staining

To quantitatively assess cellular proliferation in the wells from the proceeding cytotoxicity assay, MTS reagent was removed following absorbance readings and wells were washed three times with PBS. Live cells were fixed within the wells with 3% formaldehyde (Sigma-Aldrich) and washed three times with PBS. Crystal violet was dissolved in PBS and applied to wells to allow for histological staining of cellular content. Wells were washed three times with PBS, and bound crystal violet was solubilized with 10% acetic acid in PBS. Absorbance was measured at 590 nm.

2.3 ESR1 (ERα) Transactivation Assay

The human ESR1 expression plasmid was prepared in the pcDNA3.1 vector (Life Technologies), and a luciferase reporter plasmid was created containing multiple estrogen-responsive elements (EREs), with a pG5luc vector (Promega) including multiple GAL4 binding sites as the reporter construct. Usually lacking ESR expression, HEK293 (hamster kidney) cells were transfected with the ESR1 expression plasmid and ESR-responsive reporter constructs using FuGene transfection reagent (Promega). ERα transactivation and luciferase activity was measured using the Dual-Luciferase Reporter Assay System and Luminometer TD-20/20 (Promega). All transfections and treatments were performed in triplicate.

2.4 Immunofluorescence

Cells were seeded at 5000 cells/well in flat-bottom, culture-treated optical (black) 96-well plates (Corning) and allowed to attach overnight. The following day, wells were treated in triplicate with the indicated reagents and concentrations for time periods ranging from 30 minutes to 24 hours. Wells were then washed three times with PBS, cells were fixed with 3% formaldehyde and solubilized with 0.5% Triton in PBS. Primary antibody staining was performed with antibodies specific for human ER, (Cell Signaling) at the indicated dilutions in PBS. After overnight primary binding, wells were washed three times with PBS, and diluted secondary antibodies, including, were applied for one hour in a darkened room, with plates shaking under foil to prevent light exposure. Wells were washed twice with PBS to remove unbound antibody, and then filled with PBS per microscopic imaging requirements. Plates were imaged using the Hermes automated microscopy system and analyzed with WiScan software (IdeaBiomedical). Parameters for focus and exposure were set for each antibody/protein depending upon cellular location and expression level, such that all images and measurements for the same protein have the same exposure settings for all treatment comparisons. Two images were captured per well treated in triplicate, producing n = 6 images per condition.

2.5 Protein Inhibition via shRNA Knock-down or Small Molecule Inhibitors

To achieve RNAi transfection of cells, lentivirus was produced in 293FT cells which were transfected in Opti-MEM with Lipofectamine 2000, plasmid, and Mission packaging mix (Sigma-Aldrich, St. Louis, MO) under optimal conditions. Collected virus was filtered through a 0.2 um filter before storage at -80° C. All BSL-2 safety protocols were

performed during production, storage, and continued use. Cells were reverse transfected with lentivirus in appropriate growth medium, and polybrene. Virus was removed 24 hours later and cells were fed with media. Cells began selection with antibiotics 48 hours following transfection. Antibiotic concentrations varied with cell lines and were optimized. Generally, 2 ug/ml puromycin, 10 ug/ml blasticidin, and 200 ug/ml hygromycin were efficient for a 4 day selection period before use. The SUM44 cell line requires 3 ug/ml Puromycin selection. Control cells without the addition of lentivirus were plated alongside lentivirus infected cells to ensure the appropriate concentration of antibiotic was used. Cells were continuously maintained in the resistance marker.

PI3K expression was lowered in cells with a small molecule inhibitor, BEZ235, and ERα/GPER30 levels were decreased by the ICI inhibitor.

2.6 Extended Exposure Conditions

To assess the effects of longer term treatments with EACs and tamoxifen, MCF7 cells were cultured in low levels (100 nM) of BPA alone, all five EACs, tamoxifen alone, or EACs plus tamoxifen. Cells were thawed (frozen between passages 3-5), cultured, and passaged in treatment-containing media every four days over 60 days total in culture. Cells well then harvested and subsequently tested by MTS assays with the indicated treatments of E2, BPA, EACs, and tamoxifen. A subset of 60-day culture plates were subjected to a four-day EAC “detox” or drug withdrawal, and then tested.

2.7 Statistics

Averages and standard deviations were calculated and resulting graphs were generated in Microsoft Excel and JMP (SAS Institute). All ungraphed standard deviations

were below 5% of the calculated means of three replicates. This was done to keep graphs as clear as possible in the case of many overlapping lines, and to maintain consistency across line graphs. Unpaired t-tests were performed to identify significant differences. Statistical significance was defined using two-tailed p-values with confidence intervals of 95 or 99%, indicated where applicable with asterisks.

Chapter 3: Results and Discussion- Aim 1

3.1 EDCs alter breast cancer cell behavior

To assess *in vitro* effects of EACs on the growth and viability of different types of breast cancer cells, MCF-7, SUM44, and MDA-MB-231 cells were exposed to EACs for 96 hours. As a positive control for estrogenicity, viability changes in response to E2 were measured over a range of picomolar and nanomolar concentrations. The following EACs were evaluated over a nanomolar to micromolar range: BPA, MP, PP, DBP, and DBDE; step-wise mixtures beginning with MP up to all five EACs were tested, as well as EACs in the presence of a constant level (50 pM) of E2. These ranges were selected to approximate low-level human exposures with concentrations of E2 and EACs that are relevant to breast cell environments (Barr *et al*, 2012).

Shown in Figure 1 are the chemical structures of 17β-estradiol and the five EACs tested. Similarities between compounds are apparent, including molecular size, ring structures, and hydroxyl and methyl groups. Hydroxylated rings are common features of endogenous hormones and synthetic ligands with affinity for ERα (Byford *et al*, 2002). In particular, the molecular arrangement of BPA shares many features with that of estradiol including terminal –OH groups. Given that multiple forms of paraben are relevant for human exposure, both methyl- and propylparaben were evaluated to assess any effects of an additional carbon group on paraben-ERα interactions.

The human breast cancer cell lines included in this study are further characterized in Table 1 by subtype and hormone receptor status. MCF7 cells (ATTC Number HTB-22)

are an ideal *in vitro* model for Luminal A breast cancers that are ERα and PR positive, ERβ negative, and low or negative for HER2 and AR expression. Also ERα and PR-positive, the SUM44 cell line (Riggins *et al*, 2008) is estrogen-responsive like MCF7 cells, but of the Luminal B subtype. To model ERα-negative breast cancer cells, the MDAMB231 line (ATTC Number CRM-HTB-26) is also negative for PR and HER2 expression, making it a triple-negative basal subtype that typically does not respond to estrogenic compounds. MDAMB231 cells are positive for AR, allowing for androgen-responsiveness.

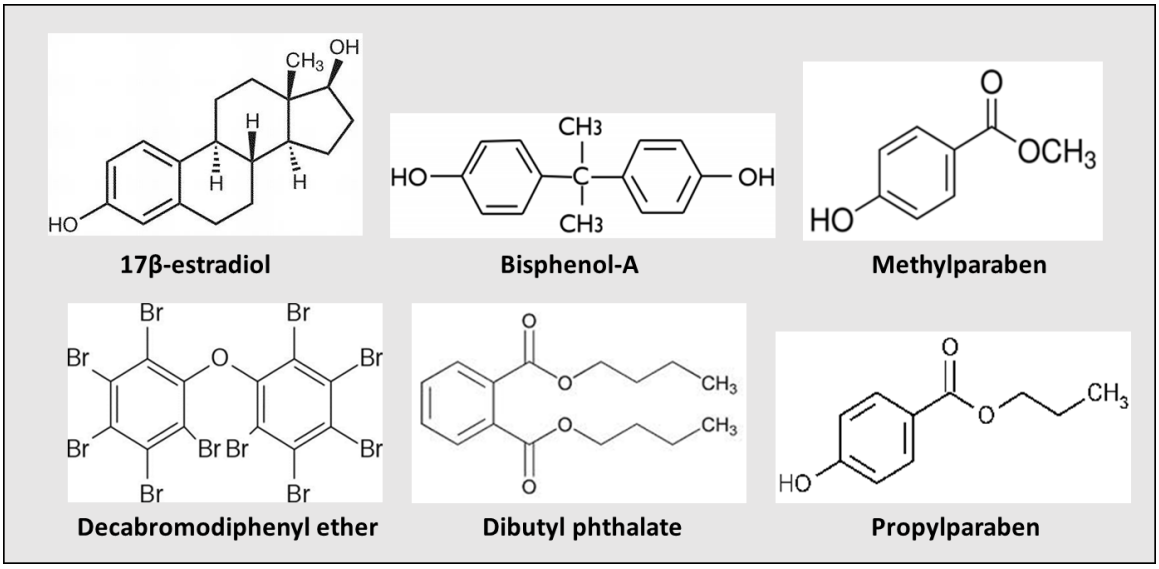


Figure 1: Molecular Structure of E2 and Common EACs. Key structural features for ERα binding and interactions include phenolic rings and –OH groups.

Table 1: Breast Cancer Cell Line Characteristics: subtype and receptor expression.

Cell Lines:	Subtype:	Receptors:				
		ERα	ERβ	PR	HER2	AR
MCF7	Luminal A	+	-	+	-/ low	-
SUM44	Luminal B	+	?	+	+	?
MDA-MB-231	Basal	-	+	-	-/ low	+

3.1.1 ERα+ breast cancer cells display increased viability in the presence of E2 and EACs

To establish viability and growth responses to endogenous estrogens, MCF7, SUM44, and MDAMB231 cells were treated with the concentrations of E2 shown in Figure 2. The two ERα+ lines, MCF7 and SUM44, displayed increased viability across the nanomolar and picomolar range tested. For MCF7 cells, a 40% viability increase occurred at 40 pM E2, and a 30% increase for SUM44 160 pM E2. As expected, MDAMB231 cells (ERα-) did not show elevated viability in the presence of E2 alone. Notably, the lower and mid-range E2 concentrations tested had more pronounced viability effects than higher concentrations.

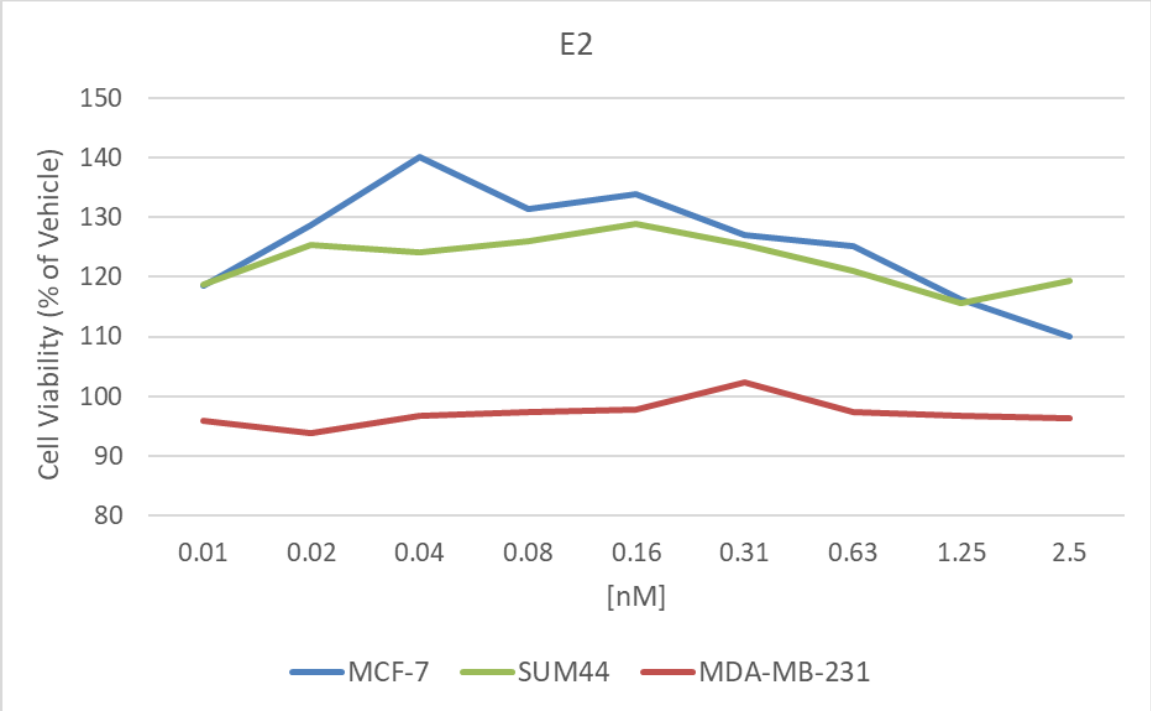


Figure 2: Effects of E2 on Viability of Breast Cancer Cells after 96 Hours. E2 was serially diluted at the indicated concentrations. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

When treated with nM to low uM concentrations of BPA, MCF7 and SUM44 cell viability was found to increase between 10-30% at BPA levels of 40 nM to 1.25 uM, as shown in Figure 3. SUM44 effects were apparent across the BPA range tested, although MCF7 effects were found only at low and mid-range concentrations. At 5 and 10 uM BPA, MCF7 cells do not display viability increases, similar to that of MDAMB231. This suggests that low-level EAC concentrations may be more relevant for certain cell types, and even cells that are ER+ may not respond to EACs at high concentrations. These results support other studies showing low-level EAC concentrations, such as those found in human biofluids and tissues, to be more effective at inducing adverse cellular effects than EACs at the higher levels typically assessed for health effects (Jenkins *et al*, 2011).

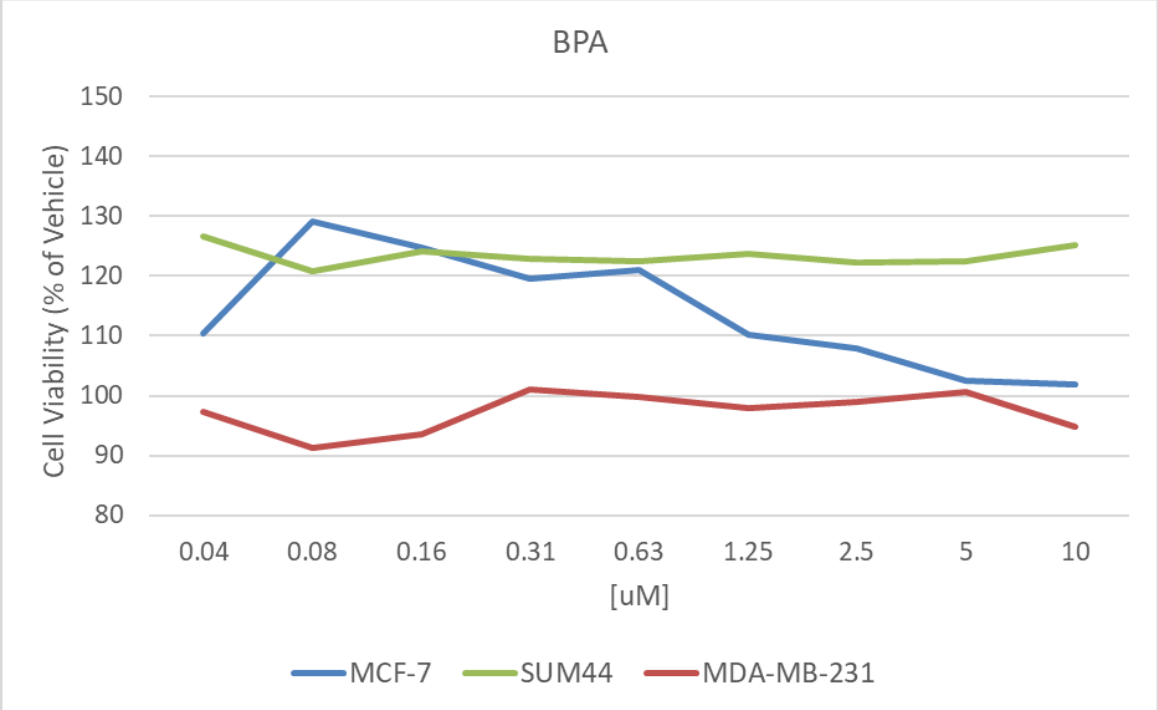


Figure 3: Effects of BPA on Viability of Breast Cancer Cells after 96 Hours. BPA was serially diluted at the indicated concentrations. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

The other EACs were tested individually over the same concentration range, and produced similar results to BPA. In figure 4, MCF7 and SUM44 cells displayed 10-30% viability increases in the presence of PP. Interestingly, the MDAMB231 results shown for PP reveal a minor inhibitory effect at the low-range concentrations tested, which was also seen for E2 and BPA in these ER- cells. As with BPA at 10 uM, PP did not induce viability changes in MCF7 or MDAMB231 cells, but did induce changes at lower levels.

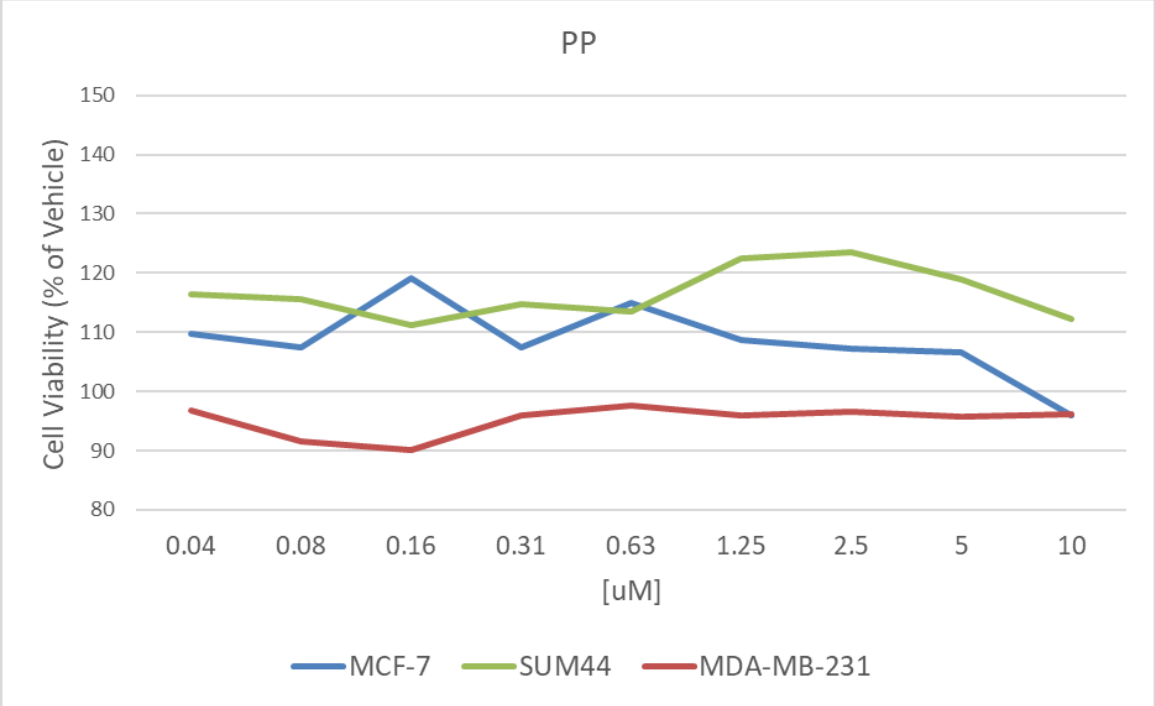


Figure 4: Effects of PP on Viability of Breast Cancer Cells after 96 Hours. PP was serially diluted at the indicated concentrations. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

These baseline measurements were effective at establishing breast cancer cell viability changes in the presence of a single EAC or E2. To more closely model human exposures, all five EACs were combined into a "cocktail" at the concentrations shown in Figure 5. Interestingly, the viability increases observed in ER+ cell lines for single EACs

were not produced in the presence of five EACs. A maximum increase of 10% was measured for MCF7 cells at 630 nM of all EACs. The SUM44 viability increase observed in Figure 3 for BPA alone, was minimized by the addition of four other EACs to BPA. Notably, the minimal increase in MCF7 and SUM44 viability with five EACs was matched by a minor increase in MDAMB231 viability at 1.25 uM of all five EACs tested.

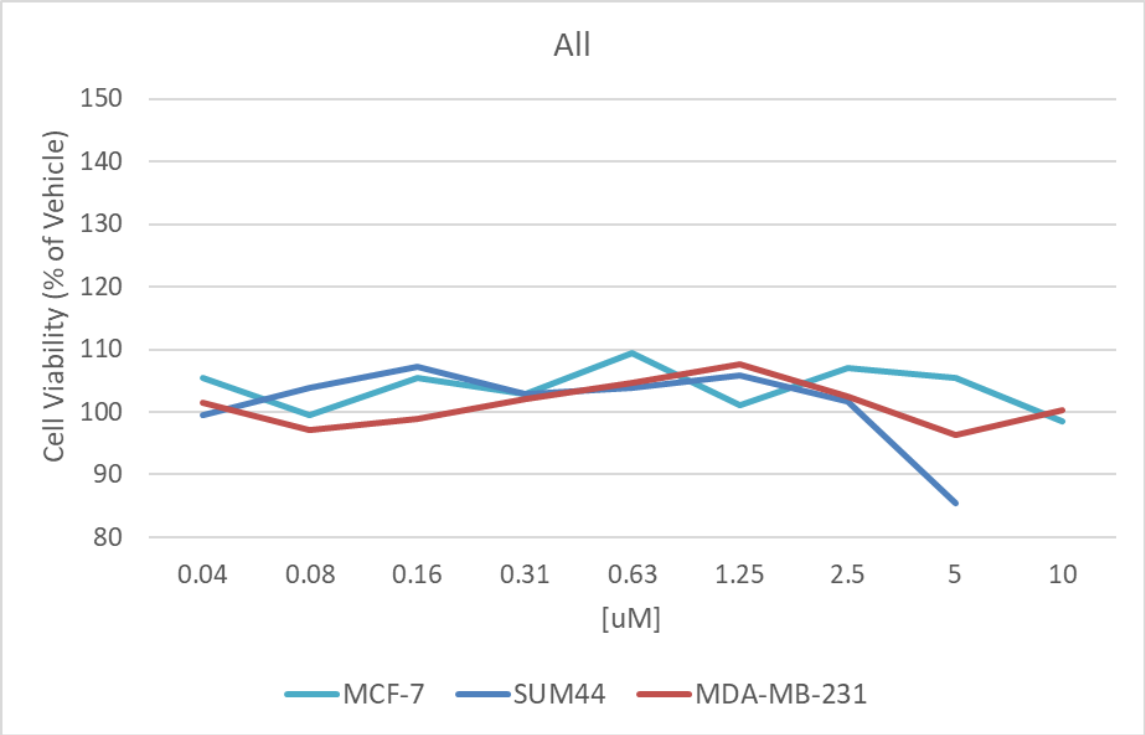


Figure 5: Effects of All EACs on Viability of Breast Cancer Cells after 96 Hours. All five EACs were serially diluted at the same indicated concentrations. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of means.

The MTS method for measuring cell viability is an appropriate assay for assessing cellular metabolic activity and potential cytotoxicity of treatments. It was selected as a common method for comparing both EAC effects and drug responses (Aim 3). Viability is related to cellular health and growth, but does not necessarily indicate cellular proliferation has occurred, as healthy cells may not be actively dividing. To determine if viability measurements could approximate proliferation, plates of treated MCF7 cells were read first for MTS dye production, then immediately washed, fixed and stained for cellular density with crystal violet, and plate/well absorbances were used to measure cell growth.

As shown in Figures 6 and 7, changes in MCF7 viability and proliferation followed similar patterns for E2 and BPA treatments for the lower concentrations tested. However, relative proliferation levels were significantly higher than relative viability levels at the four highest E2 concentrations and two highest BPA concentrations tested, indicating that viability is not always a consistent marker of concurrent proliferation. There is a chance that lower cell viability results from negative feedback via saturation/sequestration of ER activity or mitochondrial toxicity/oxidative stress as a result of higher concentrations of EACs or E2. Given that mitochondrial/viability methods are the standard for assessing drug effects in cancer cells, as well as more efficient and affordable to test in high-throughput, MTS assays were further utilized in this study as a way to assess both EAC and drug responses.

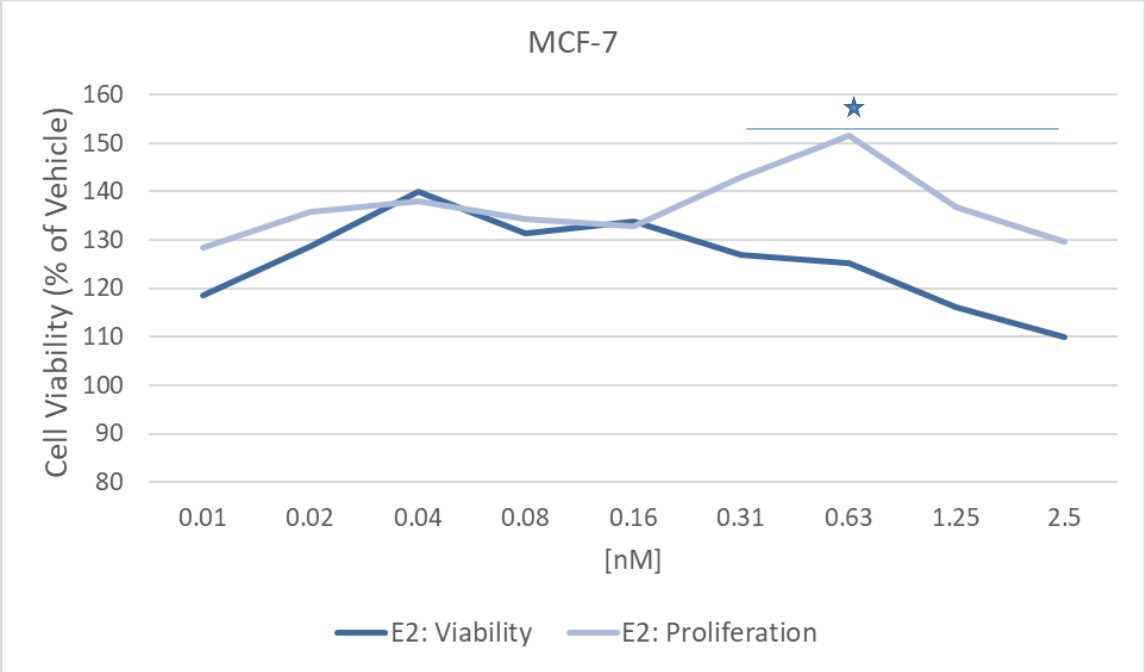


Figure 6: Comparison of MCF7 Viability and Proliferation in the Presence of E2. E2 was serially diluted at the indicated concentrations. Cells assayed by MTS were washed and stained with crystal violet for proliferation readings. Changes are relative to vehicle-control cells (0.1% v/v DMSO). Deviations are within 5% of means; * indicates p <0.05.

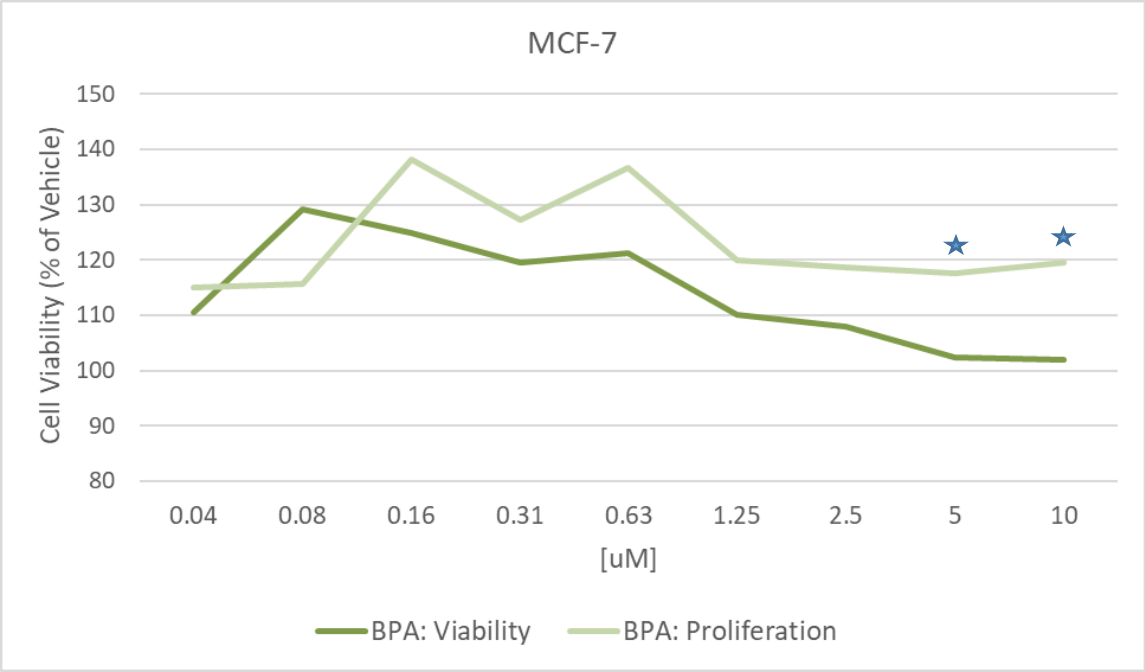


Figure 7: Comparison of MCF7 Viability and Proliferation in Presence of BPA. BPA was serially diluted at the indicated concentrations. Cells assayed by MTS were washed and stained with crystal violet for proliferation readings. Changes are relative to vehicle-control cells (0.1% v/v DMSO). Deviations are within 5% of means; * indicates p <0.05.

3.2 EAC mixtures are not additive

The effects of each of the five EACs tested alone on MCF7 viability are shown in Figure 8. BPA induced the highest increases measured, with MP, PP, and DBP producing 10-20% increases in viability over a nM concentration range. Notably, DBDE treatment led to a 10% reduction in MCF7 viability, suggesting this EAC may have slightly cytotoxic effects despite being endocrine-active. A look at the structures in Figure 1 shows that unlike the other four EACs tested, DBDE contains bromine but not –OH groups, which may explain the different responses observed.

To assess potential additive effects of treatment with multiple EACs at once, MCF7 cells were treated with step-wise combinations of EACs, beginning with the

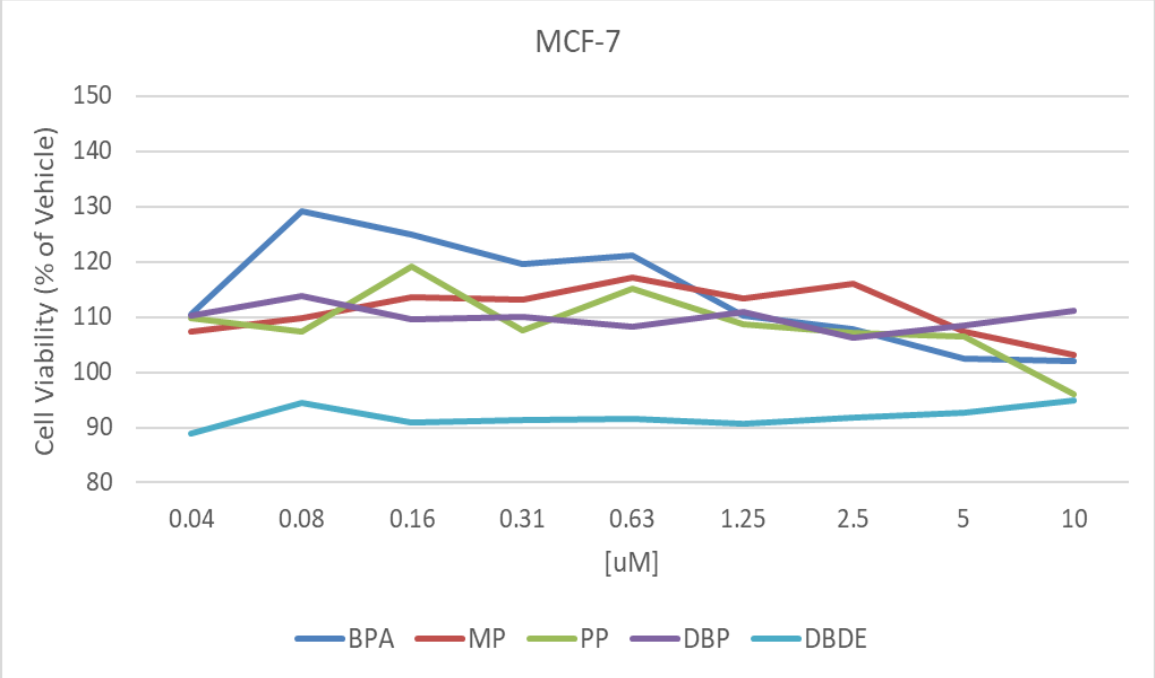


Figure 8: Effects of Single EACs on Viability of MCF7 Cells after 96 Hours. Single EACs were serially diluted at the indicated concentrations. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

lowest molecular weight compound, MP. Figure 9 shows that EAC mixtures did not consistently produce additive viability effects in MCF7 cells, with a combination of all five EACs having minimal effects. To further explore this result at one EAC concentration, viability levels at 1.25 uM were compared for each step-wise addition. As shown in Figure 10, MCF7 viability increased approximately 25% when treated with two EACs and four EACs, but was measured at control levels when cells were treated with three EACs or all five EACs. These results demonstrate that EAC exposures are complex when mixed, do not necessarily follow additive behavior, and display variable results at different concentrations. It is possible that the cellular effects of multiple EACs are not more impactful than the effects of single EACs.

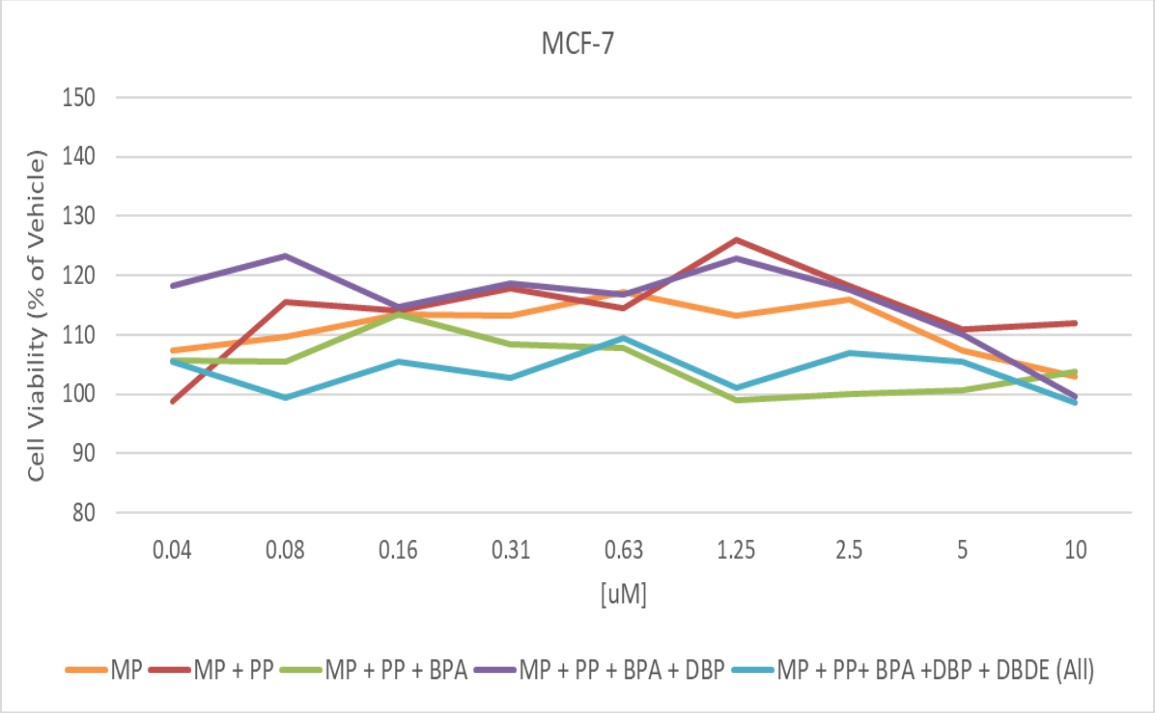


Figure 9: Effects of Step-wise EAC Mixtures on Viability of MCF7 Cells after 96 Hours. EACs and mixtures were serially diluted at the concentrations. Changes are relative to control cells (0.1% v/v DMSO). Standard deviations are within 5% of triplicate means.

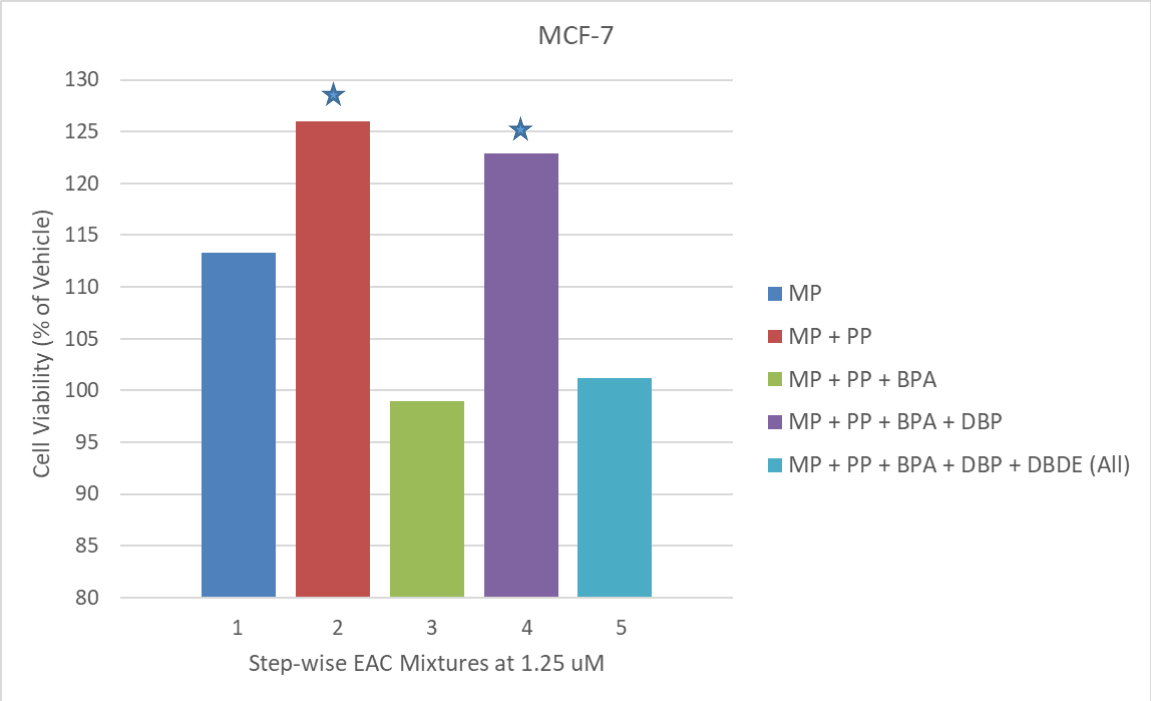


Figure 10: Comparison of EAC Mixtures at 1.25 uM on MCF7 Viability. Changes are relative to vehicle-control cells (0.1% v/v DMSO). Standard deviations are within 5% of triplicate means; * indicates p <0.05.

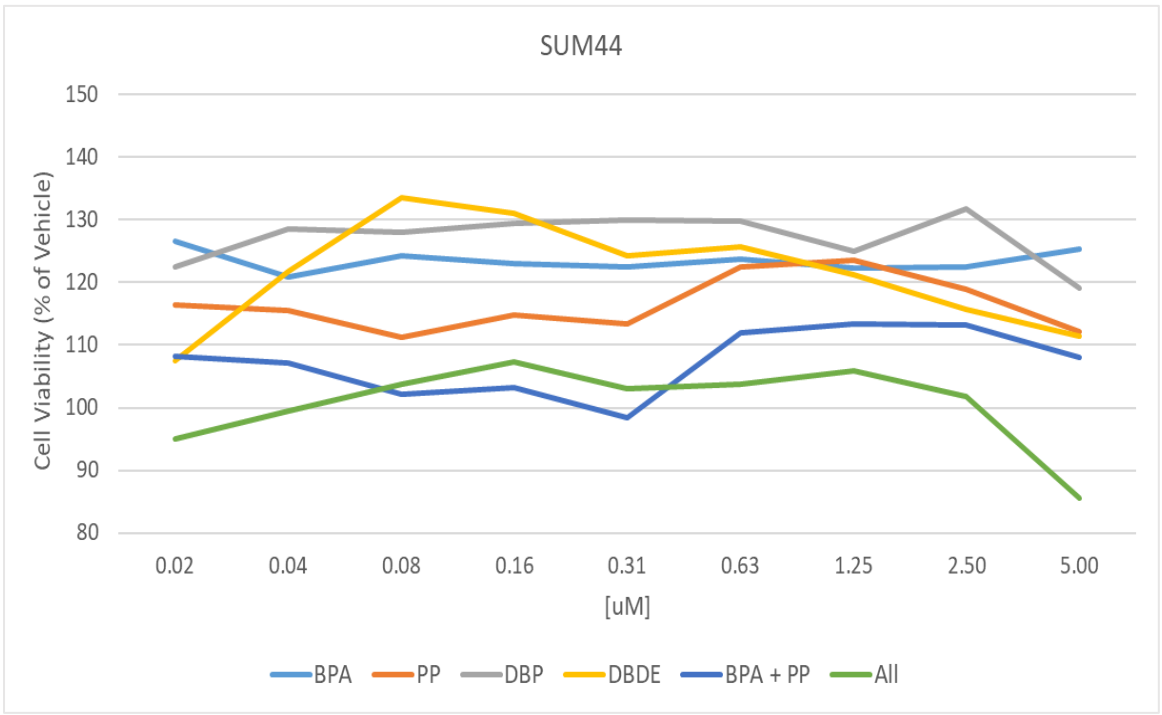


Figure 11: Effects of EACs and Mixtures on Viability of SUM44 Cells after 96 Hours. EACs were serially diluted at the indicated concentrations. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

These findings were further supported by results in SUM44 cells. The mixture of all five EACs did not produce notable viability changes, but EACs tested alone did. Although ER+ like MCF7 cells, there were differences in how SUM44 cells responded to certain EACs. DBP induced a higher increase in SUM44 cells than in MCF7, and instead of displaying cytotoxicity, DBDE elevated SUM44 viability 30% higher at nM concentrations. Interestingly, the five EAC cocktail at 5 uM led to a 15% reduction in relative SUM44 viability.

MDAMB231 cell viability changes were also assessed with each EAC alone, as shown in Figure 12. Except for DBP, the EACs tested did not increase viability, with BPA, MP, PP, and DBDE inducing an approximate 10% reduction in MDAMB231 viability at lower levels. DBP treatment at 310 nM led to a 10% increase in viability, and may cause this response via expression of AR in MDAMB231 cells as shown in Table 1.

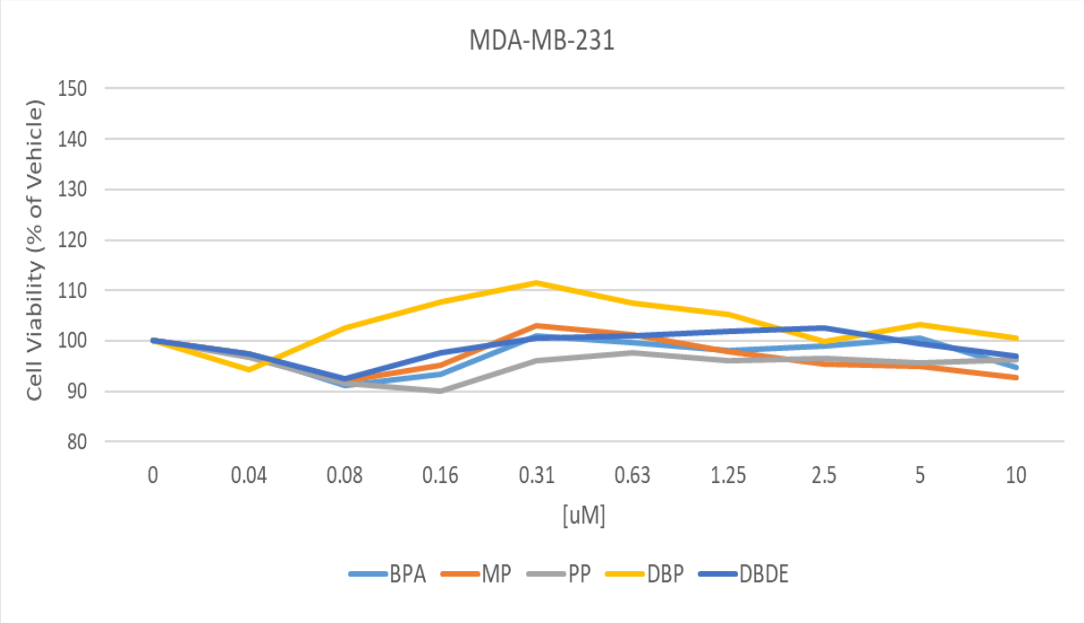


Figure 12: Effects of Single EACs on Viability of MDAMB231 Cells after 96 Hours. Single EACs were serially diluted at the indicated concentrations. Changes are relative to control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

3.3 Presence of E2 alters EAC responses

To further explore effects of mixtures of estrogenic compounds on breast cancer cells, EACs were combined with a constant level of E2, a mixture that breast cancer cells would likely encounter *in vivo*. When treated with MP and constant 50 pM E2, the viability increases observed with MP alone are reduced to vehicle levels when E2 is also present, as shown in Figure 13. Inversely, the minimal viability changes previously observed in the presence of all five EACs become 20% increases when 50 pM E2 is included in the low-level cocktail. The ability of E2 to promote viability in ER+ breast cancer cells appears to depend upon the relative concentrations of other estrogenic compounds in the breast cancer microenvironment.

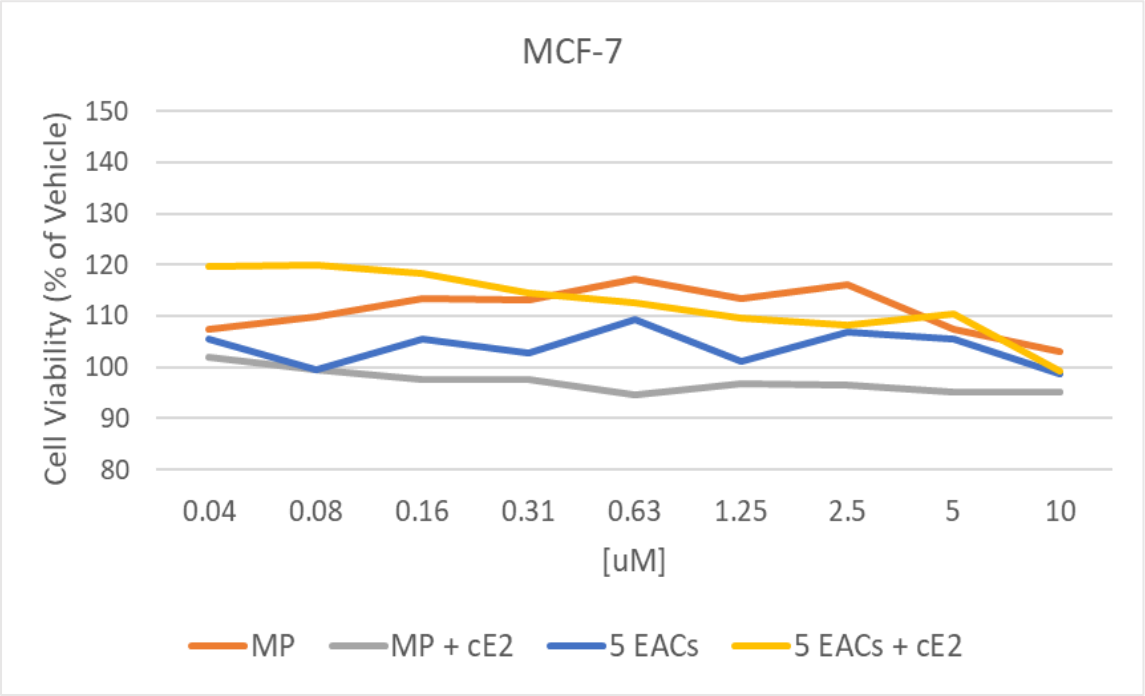


Figure 13: Effects of Constant E2 Combined with EACs on MCF7 Viability. EACs were serially diluted at the indicated concentrations and 50 pM constant E2 was present as indicated (cE2). Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

Additionally, the effect of E2 on ER- breast cancer cell viability appears to be altered by the presence of EACs. Step-wise additions of EACs to MP, up to all five tested, produced minimal changes in MDAMB231 viability in Figure 14. Unexpectedly, the addition of constant 50 pM E2 to MP did induce a 10-20% increase in viability in a breast cancer cell line traditionally thought to be estrogen-independent. These results are shown in Figure 15 along with those for all EACs combined with 50 pM E2. The addition of low-level E2 to a five EAC cocktail increased viability by 20-25% across all EAC concentrations tested, when EACs alone had little to no effect. It is realistic for ER- breast cancer cells to be exposed to both E2 and EACs *in vivo*, and these results suggest that endogenous and synthetic estrogens can alter ER- breast cancer behavior when they are combined.

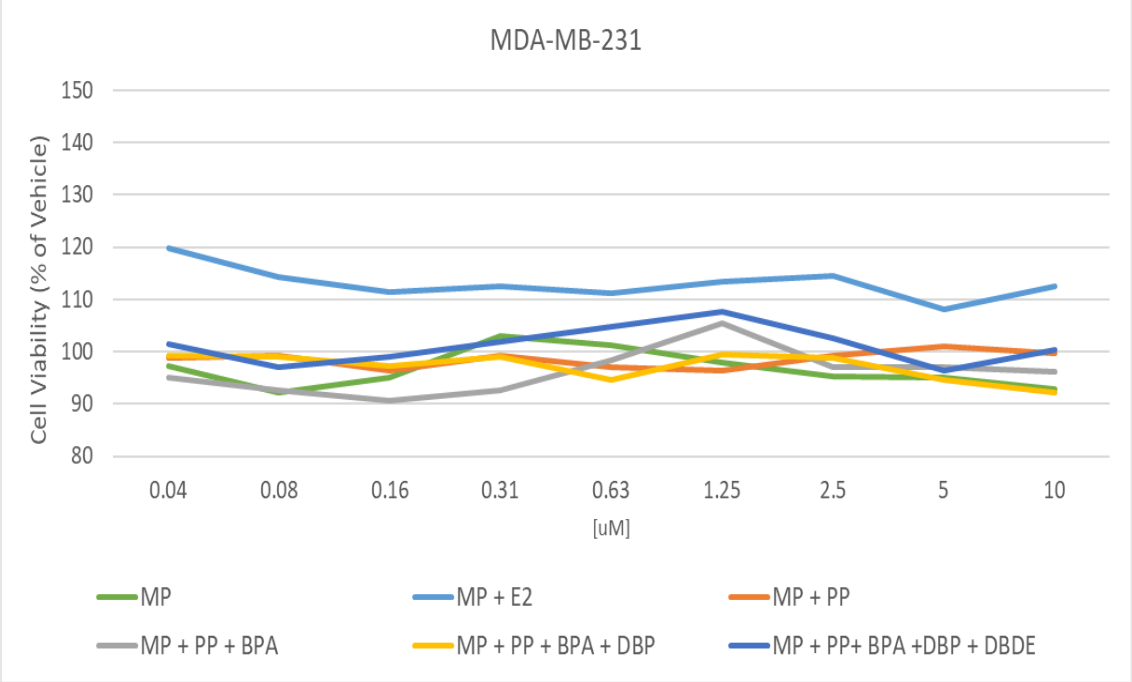


Figure 14: Effects of Step-wise EAC Mixtures on MDAMB231 Viability. EACs were serially diluted at the indicated concentrations and 50 pM constant E2 was present as indicated (cE2). Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

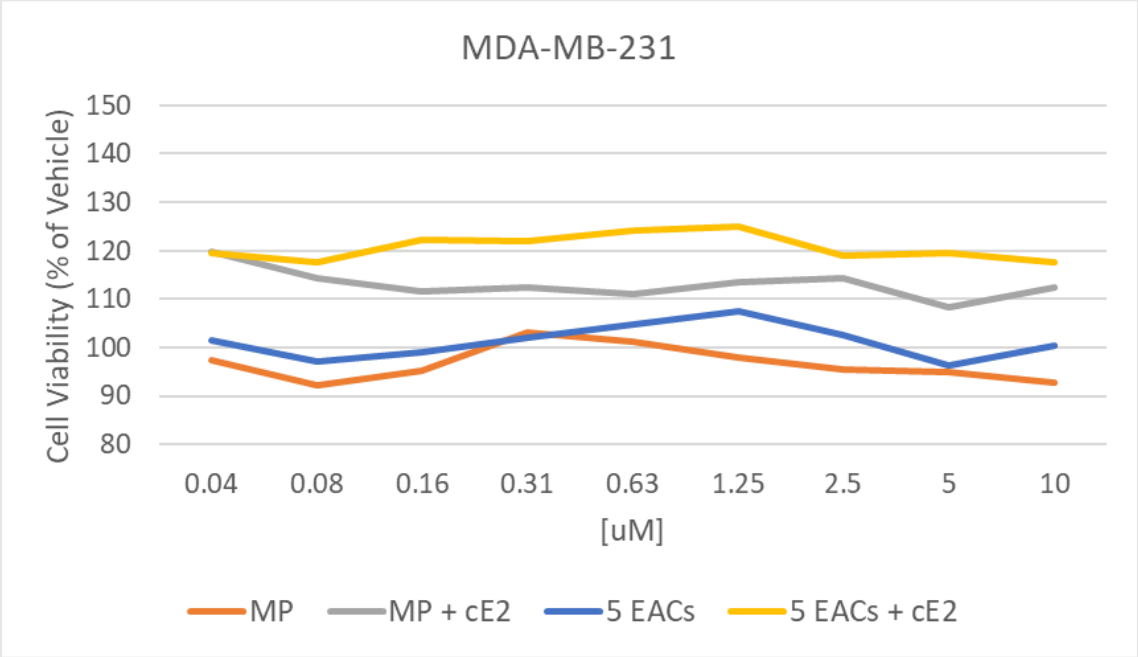


Figure 15: Effects of Constant E2 combined with EACs on MDAMB231 Viability. EACs were serially diluted at the indicated concentrations and 50 pM constant E2 was present as indicated (cE2). Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

These E2-dependent effects were also assessed in SUM44 cells. In Figure 16, E2 results are graphed at nM concentrations on the same axis as EACs at comparable uM concentrations. As stated, SUM44 cells responded to E2 alone but had minimal responses to five EACs combined. When the EAC cocktail and 50 pM constant E2 were combined, SUM44 viability levels were comparable to E2 alone. Given that SUM44 cells are ER+, whether this is a result of the E2 contained in the mixture acting alone or a complex effect from both EAC and E2 actions is not clear. In Figure 17, viability effects are shown for the three cell lines when treated with all five EACs and constant E2. Interestingly, the ER- cell line MDAMB231 displayed viability increases on par with the

ER+ SUM44 cell line when E2 was included. This suggests that rigid definitions of estrogen-responsiveness in breast cancer cells might not apply in every situation.

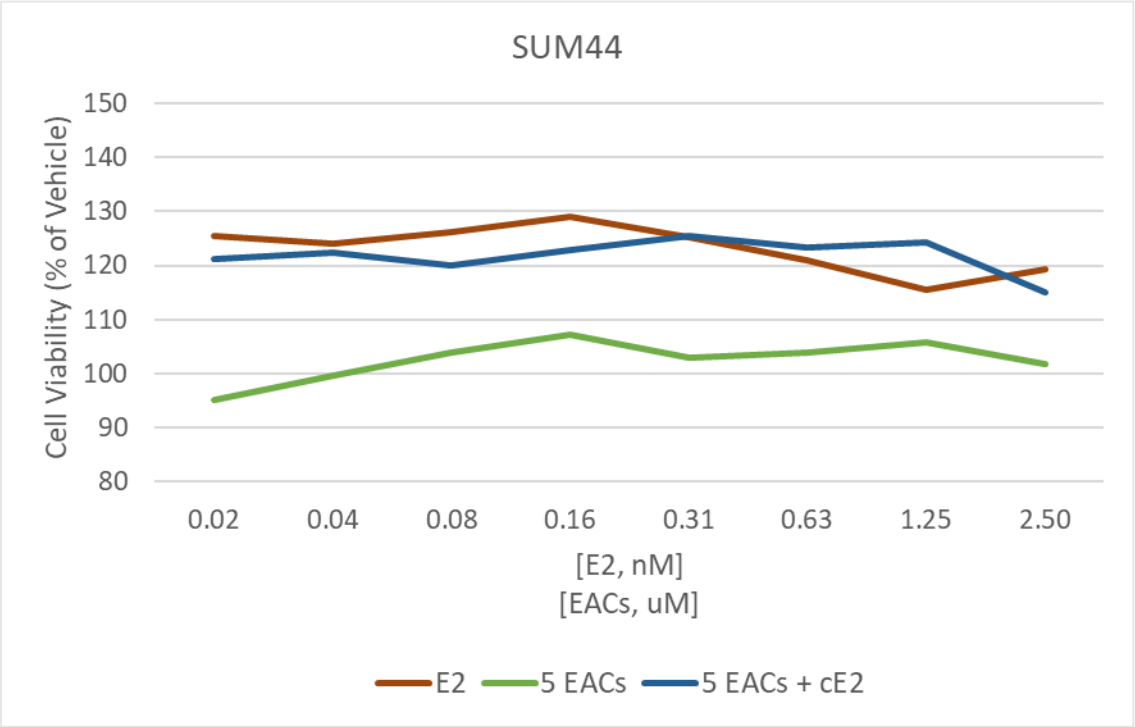


Figure 16: Effects of E2 and Constant E2 combined with EACs on SUM44 Viability. EACs were serially diluted at the indicated concentrations and 50 pM constant E2 was present as indicated (cE2). Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

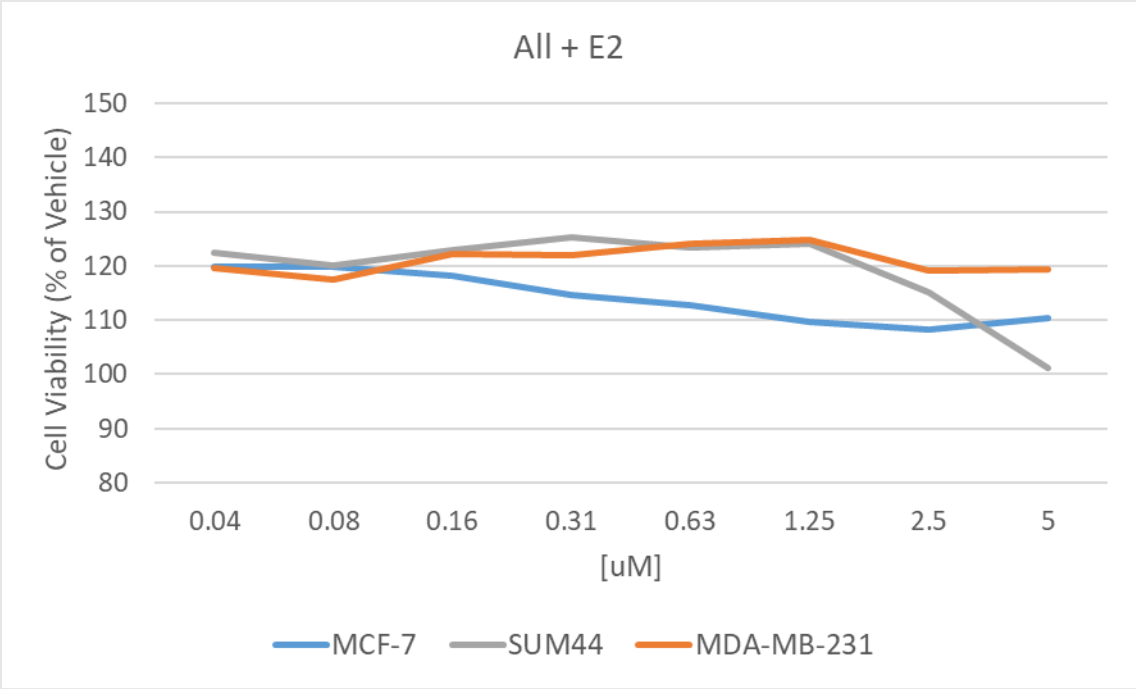


Figure 17: Effects of Five EACs Combined with E2 on Breast Cancer Cell Viability. EACs were serially diluted at the indicated concentrations and 50 pM constant E2 was present as indicated (cE2). Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

3.4 Extended EAC exposures alter subsequent responses

The cellular responses presented above are for 96 hour treatments. Realistically, EAC exposure occurs at low levels over more extensive periods of time. To assess extended EAC exposures in ER+ breast cancer cells, MCF7 cells were cultured and passaged over a period of 60 days in media containing 100 nM BPA or 100 nM each of all five EACs tested. After 60 days, cells were assayed for an additional 96 hours in the presence of a range of E2 and BPA concentrations. As shown in Figure 18, extended low-level BPA treatment induced higher responses to short-term E2 treatment when

compared to cells than were not cultured in BPA prior to E2. This effect was not observed to the same extent for short-term BPA treatment after extended BPA culture.

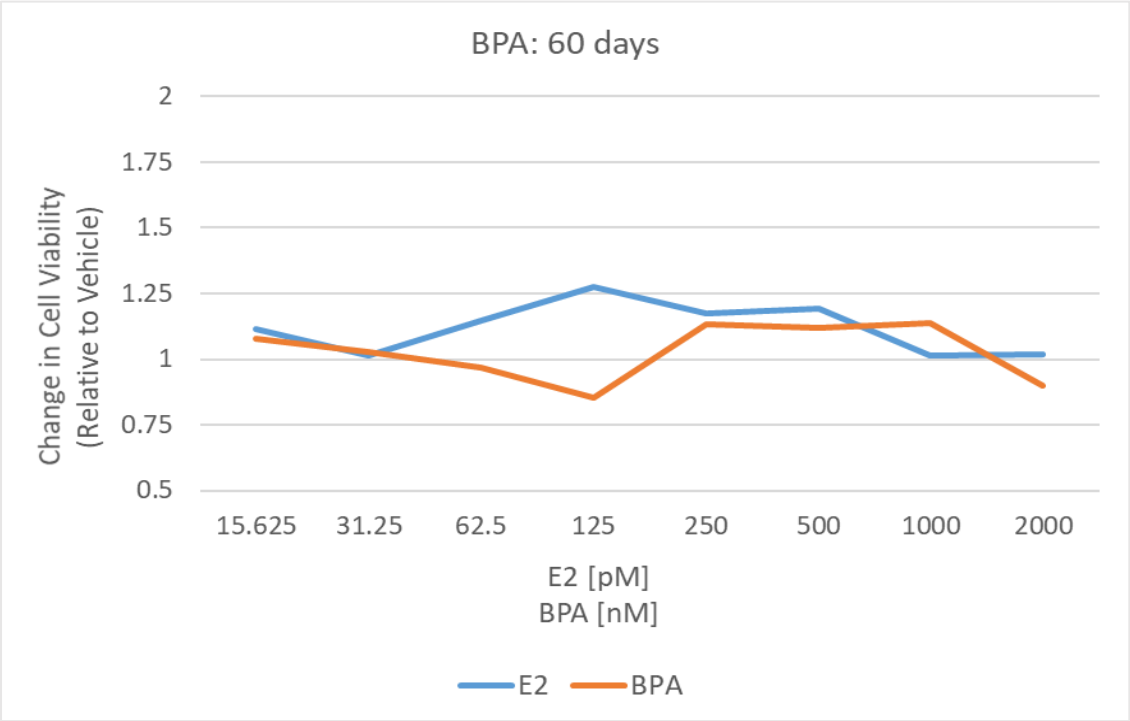


Figure 18: Effects of Extended BPA Treatment on Subsequent E2 and BPA Responses. Cells were cultured in 100 nM BPA for 60 days, and then treated with serially diluted E2 or BPA at the indicated concentrations for 96 hours. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

Short-term E2 and BPA viability responses were also examined after 60-day culture in all five EACs, as shown in Figure 19. After extended exposure to the EAC cocktail, MCF7 cells displayed significant increases in viability after short-term treatments with E2 or BPA. Viability was 50-75% higher in cells that had been cultured in low-level EACs than in cells that had not encountered EACs before E2 and BPA treatments. This suggests that extended low-level exposure of ER+ breast cancer cells to common EACs can prime these cells for more pronounced increases in viability during subsequent encounters with endogenous and synthetic estrogens.

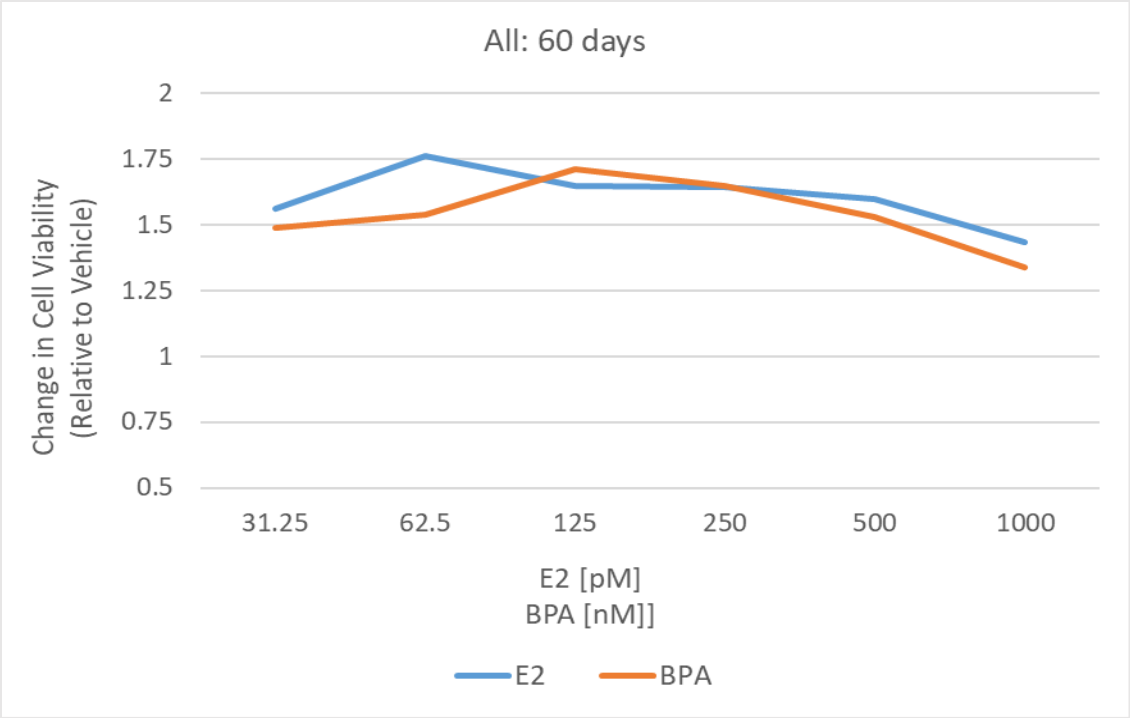


Figure 19: Effects of Extended Treatment with Five EACs on Subsequent E2 and BPA Responses. Cells were cultured in 100 nM of all five EACs for 60 days, and then treated with serially diluted E2 or BPA at the indicated concentrations for 96 hours. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

Chapter 4: Results and Discussion- Aim 2

It has been shown that EACs and E2 exert effects on cellular viability and growth of breast cancer cells. In this chapter, the molecular mechanisms underlying these EAC effects are investigated. Genomic and non-genomic ERα functions, signaling pathways, and epigenetic marks were examined with high-throughput screening methods. To measure genomic ERα activity of EACs and mixtures, transactivation assays were performed with ESR1-transfected HepG2 cells (liver carcinoma, otherwise ERα-). Quantitative immunofluorescence (QIF) sweeps were carried out with Hermes high-throughput microscopy of nearly 20 related proteomic markers including hormone receptors (ERα phosphor-sites included), kinases, transcription factors, epigenetic enzymes, and histone modifications with relevancy to breast cancer. Time courses were examined for key markers at 30 minutes, 2, 8, and 24 hours post-treatments. The influence of PI3K/mTOR in EAC response was tested using small molecule inhibitors, and the role of LSD1 was examined with shRNA knockdown. The effects of ER/GPER inhibition on EAC response was explored via a receptor antagonist.

4.1 ERα transactivation varies with endogenous and synthetic ligands

Relative ERα transcriptional activation levels were compared for E2 alone, BPA alone, and BPA combined with 50 pM E2. As shown in Figure 20, activation of the target receptor increased predictably with E2 concentration, with low nM levels sufficient to induce marked transcriptional activity. Conversely, ERα was activated by BPA within the lower range of concentrations tested, notably 310 nM, but transcriptional activity was

partially inhibited at higher BPA levels, mainly 10uM. These results follow a trend for BPA activity also seen in cell viability effects; lower levels of BPA are more active than higher concentrations, and higher levels may be cytotoxic and/or exert effects beyond ERα transcription regulation. These findings also support other studies showing that low-level EAC concentrations are most relevant for evaluating their effects in cancer cells.

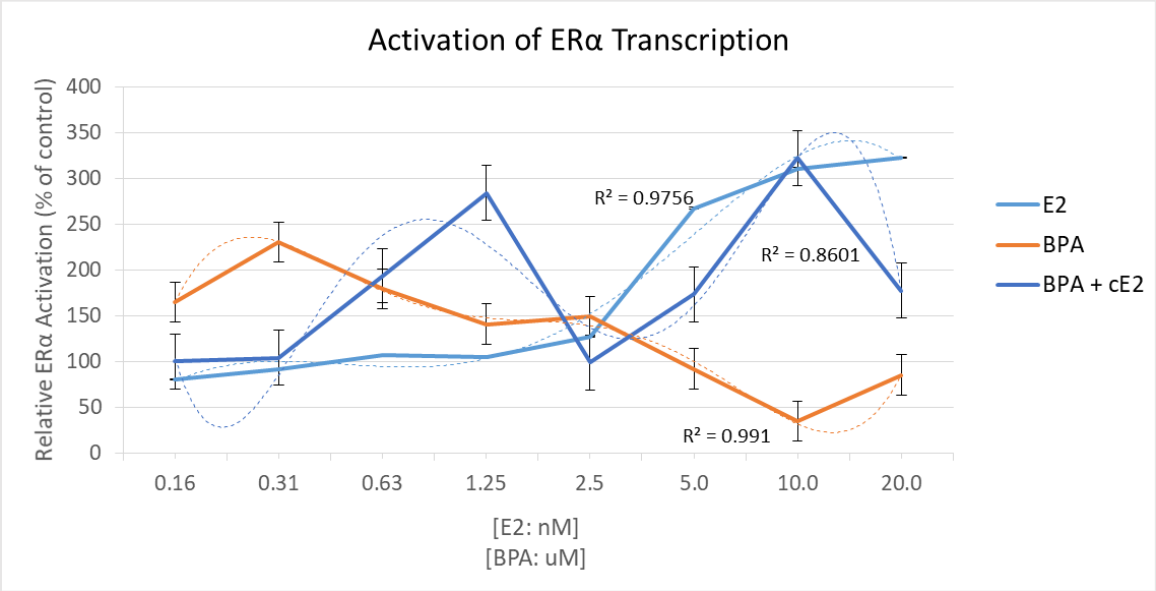


Figure 20: Relative Levels of ERα transactivation induced by E2 and BPA. E2 and BPA were serially diluted to the indicated concentrations. 50 pM constant E2 was present as indicated. Luciferase activity was measured as the level of transcriptional activation at the ERE element. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

When varying concentrations of BPA were combined with 50 pM E2, which does not have substantial activity by itself, ERα activation was altered compared to either compound alone. A non-monotonic dose response curve resulted, as shown in Figure 20, in which lower levels of BPA with constant E2 still activated transcription, and high levels of BPA also activating the receptor when constant low-level E2 is present. This finding is further visualized in Figure 21, in which results for four BPA concentrations are compared

with or without E2. The addition of E2 to 1.25 uM BPA induced a transcriptional response that otherwise did not occur with BPA alone. Whereas 5 and 10 uM BPA alone displayed inhibitory effects on ERα transcription, the addition of E2 reversed this inhibition. This mixture effect was especially apparent at 10 uM BPA, in which transcription increased from only 32% of control levels to 320% when BPA and low pM E2 were combined.

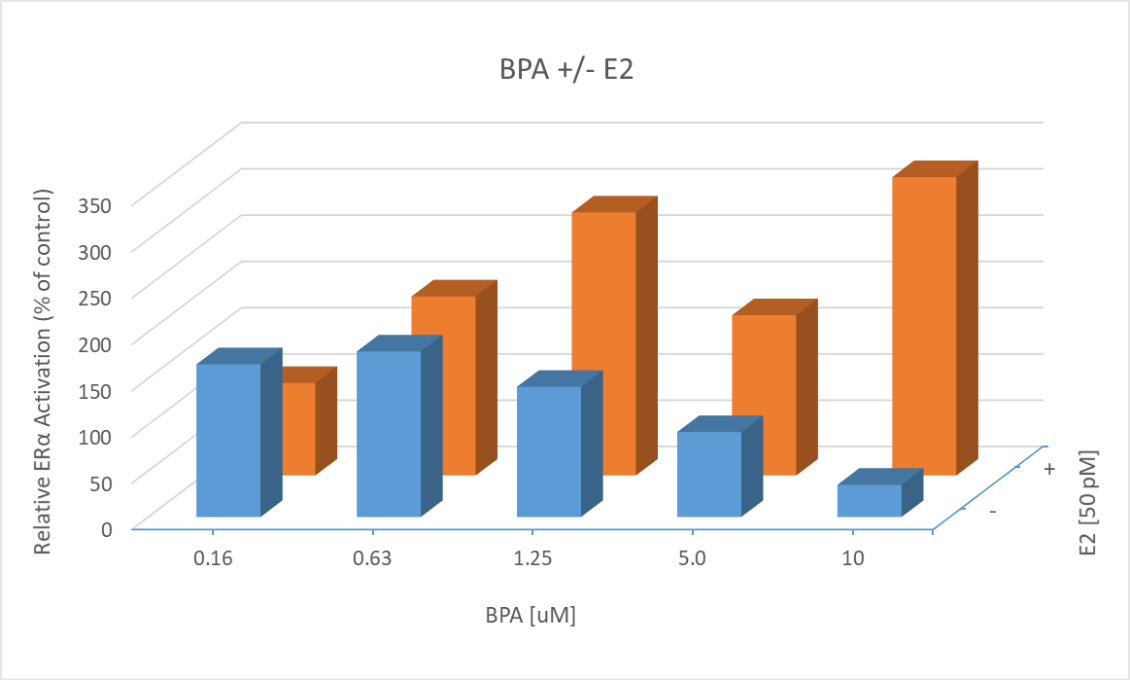


Figure 21: Relative Levels of ERα transactivation induced by BPA with or without E2. E2 and BPA were serially diluted to the indicated concentrations. 50 pM constant E2 was present as indicated. Luciferase activity was measured as the level of transcriptional activation at the ERE element. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

The effects of EAC mixtures with or without E2 are shown in Figure 22. PP alone activated ERα transcription at all concentrations tested, especially within the middle range, where activation levels were comparable to those for low nM concentrations of E2

(Figure 20). This supports previous findings that EACs are typically capable of mimicking ERα binding of E2 at 1000-fold lower affinities, requiring 1000-fold the E2 concentration required (Byford *et al*, 2002). The observed transcriptional activation of PP alone was effectively reduced to control levels with the addition of MP, with an inhibitory effect on receptor activation at 310 and 630 nM MP and PP. However, when BPA was added to MP and PP, the transcriptional response increased to levels higher than single EACs and 1000-fold lower E2 concentrations. When constant E2 was also combined with these three EACs, activation still occurred, but at lower levels than the EAC mixture without E2 added. When constant E2 was tested with only PP, activation was then diminished to inhibitory levels at low PP concentrations, and increased at the two highest PP levels.

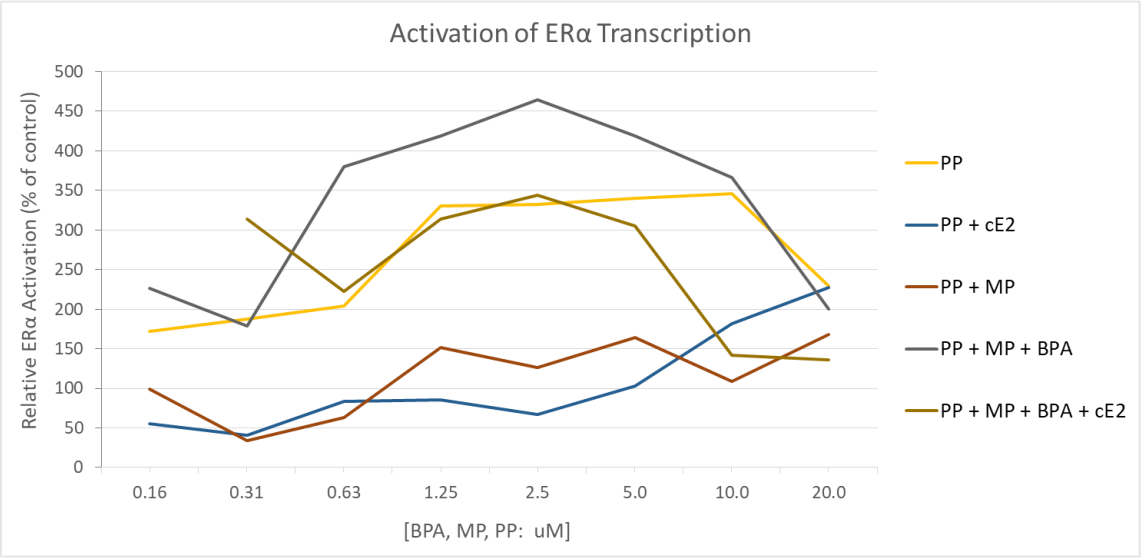


Figure 22: Relative Levels of ERα transactivation induced by EAC Mixtures and E2. EACs were serially diluted to the indicated concentrations. 50 pM constant E2 was present as indicated. Luciferase activity was measured as the level of transcriptional activation at the ERE element. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

When cellular and transactivation results are compared at 1.25 uM EACs, it is apparent that MCF7 mitochondrial activity (viability) responses to single EACs and mixtures did not correlate with ERα transcriptional activation at the same concentration. Whereas MP and PP combined induced an increase in viability, it may not be due to increased ERα transcriptional effects, as shown in Figure 23. Despite displaying slight transcriptional activity, it is possible MP and PP mixed exert cellular effects via other non-mitochondrial mechanisms as well. When MP, PP and BPA are combined at 1.25 uM, MCF7 cells were not relatively more viable, but ERα transactivation was at the highest level produced by any of the treatments tested.

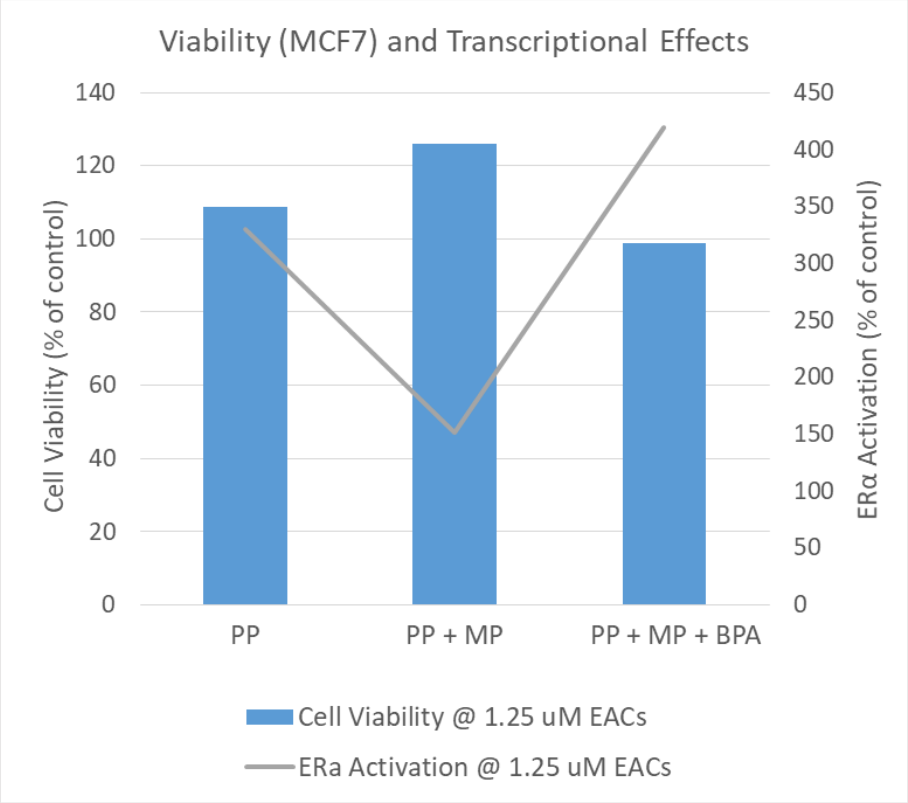


Figure 23: MCF7 Viability Compared to ERα Transactivation Induced by EACs. Cell viability levels were extracted from MTS data. Luciferase activity was measured as the level of transcriptional activation at the ERE element. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

4.2 Proteomic pathways are altered by EACs in breast cancer cells

To explore other potential cellular mechanisms underlying EAC-induced effects, such as epigenetic changes, an initial discovery screen was performed with two common sites of histone methylation in MCF7 cells. Images are shown in Figure 24 with DAPI-stained nuclei and RFP-stained methyl marks overlaid, with EACs at 1 uM in Panel A and 125 nM in Panel B. Expression of di-methylated histone 3 at lysine 4 (H3K4me2) was present at lower levels in a portion of vehicle control cells, ad H3K9me2 was weakly expressed in an even lower number of control cells. When treated with 50 pM E2 for 24 hours, cells underwent strong increases in expression of H3K4me2, with the marker now apparent in all cells pictured. A slight increase in H3K9me2 expression occurred with E2 treatment.

When treated with 1 uM BPA for 24 hours, both epigenetic marks were strongly expressed in nearly all cells, with H3K9me2 levels higher than those for E2. At 125 nM BPA, H3K4me2 expression was comparative to the control, but unlike the control, this marker was then present in nearly all cells. This pattern of expression was also apparent for both concentrations of DBDE and PP tested. Both 1 uM and 125 nM DBP induced similar expression patterns of H3K4me2, despite one concentration being four times higher. An additional EAC tested in the preliminary screen shown was PFOA, which did not induce noticeable increases in H3K4me2 expression at either concentration tested. Although important for human exposures, this molecule was not pursued further, yet included as an example of an EAC that may work via mechanisms distinct from the EACs focused on in this study.

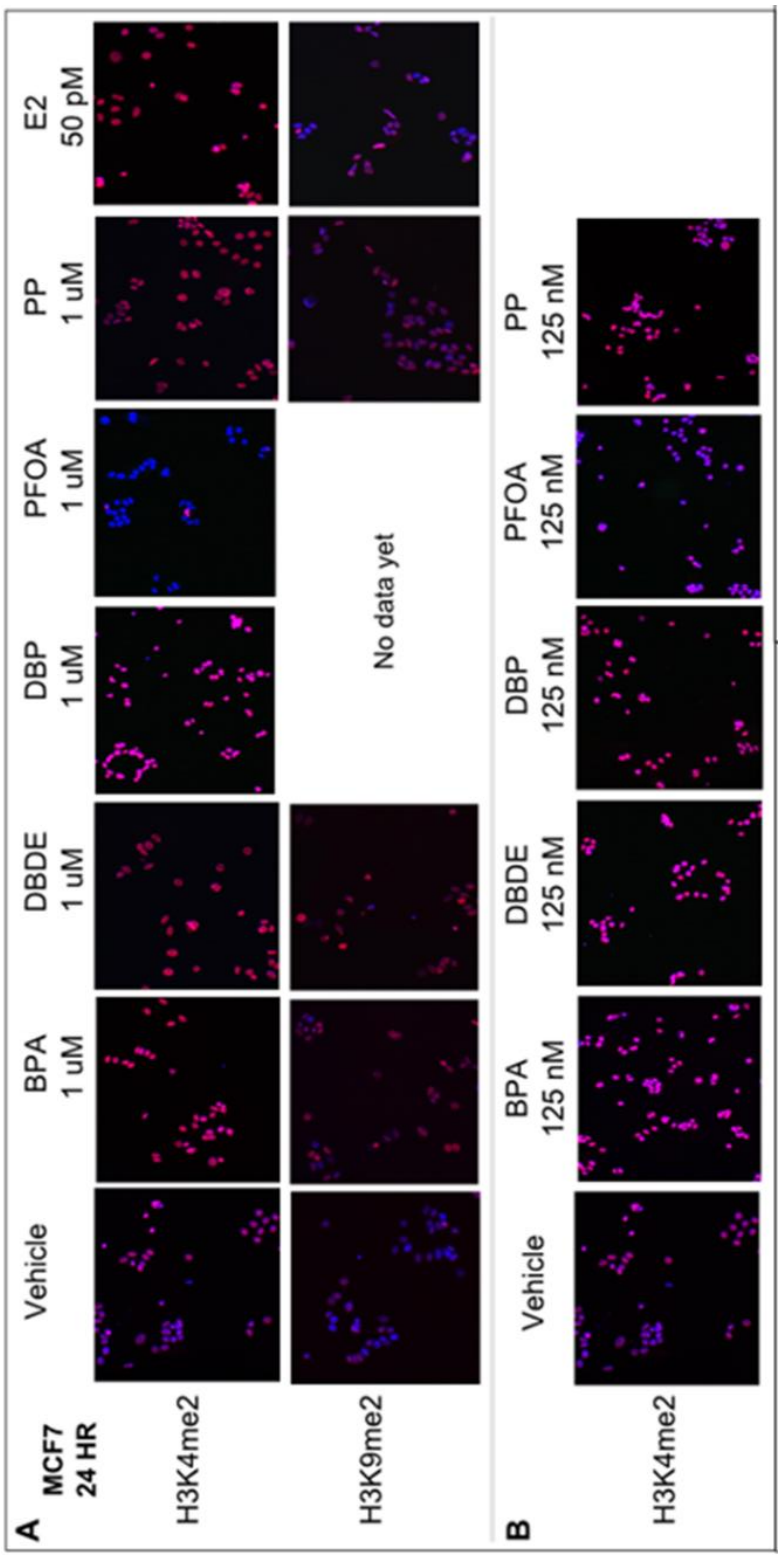


Figure 24: Histone Methylation in MCF7 Cells Treated with EACs for 24 Hours

The observed effects of EACs on MCF7 viability not only involve epigenetic mechanisms, but also early signaling pathway alterations through phosphorylation of ERα. As shown in Figure 25, expression of total ERα (GFP-stained) is increased within 30 minutes of treatment with 50 pM E2 or 100 nM PP. Total ERα appears to be localized mostly within the nucleus, and is expression may be induced at slightly higher levels by 100 nM PP than 50 pM E2, but neither treatment evokes expression in all cells.

The effects of 30 minutes of E2 or PP treatment on phosphorylation of ERα at serine 167 are shown in Figure 26. P-ERα S167 was expressed in control MCF7 cells at moderate levels, localized in nuclei. This expression pattern was increased slightly when cells were treated with 50 pM E2. When exposed to 100 nM PP, P-ERα S167 levels were higher than those for vehicle and E2-treated cells. To quantify expression levels within imaged cells, GFP-related pixels were counted and relative expression levels shown in Figure 27. After 30 minute treatments, P-ERα S167 expression was elevated approximately 15% with E2 and significantly increased by 48% with low-level PP.

Timing of exposure and localization of P-ERα S167 appear to be significant factors in responses of MCF7 cells to estrogenic compounds. Cells exposed to 50 pM E2 or 100 nM PP were assayed for P-ERα S167 expression after 24 hours. As shown in Figure 28, this marker was very weakly expressed in control cells, but markedly expressed 24 hours after E2 treatment, in both nuclear and cytosolic cellular compartments. Cytosolic and nuclear localization of P-ERα S167 was also apparent at for PP treatment, at levels similar to E2-treated cells.

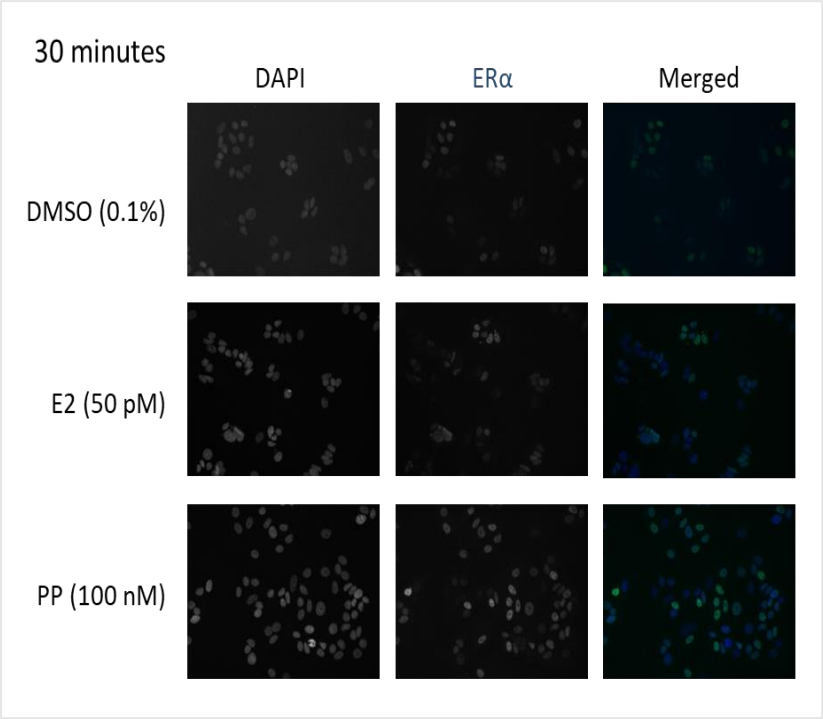


Figure 25: Expression of Total ER in MCF7 Cells Treated with E2 or PP for 30 Minutes

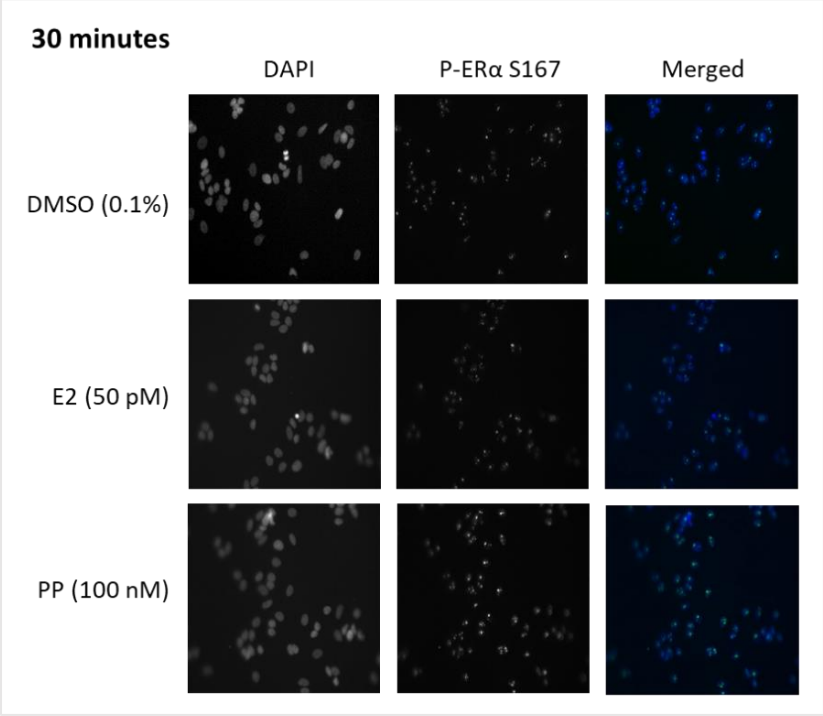


Figure 26: Expression of P-ER S167 in MCF7 Cells Treated with E2 or PP for 30 Minutes

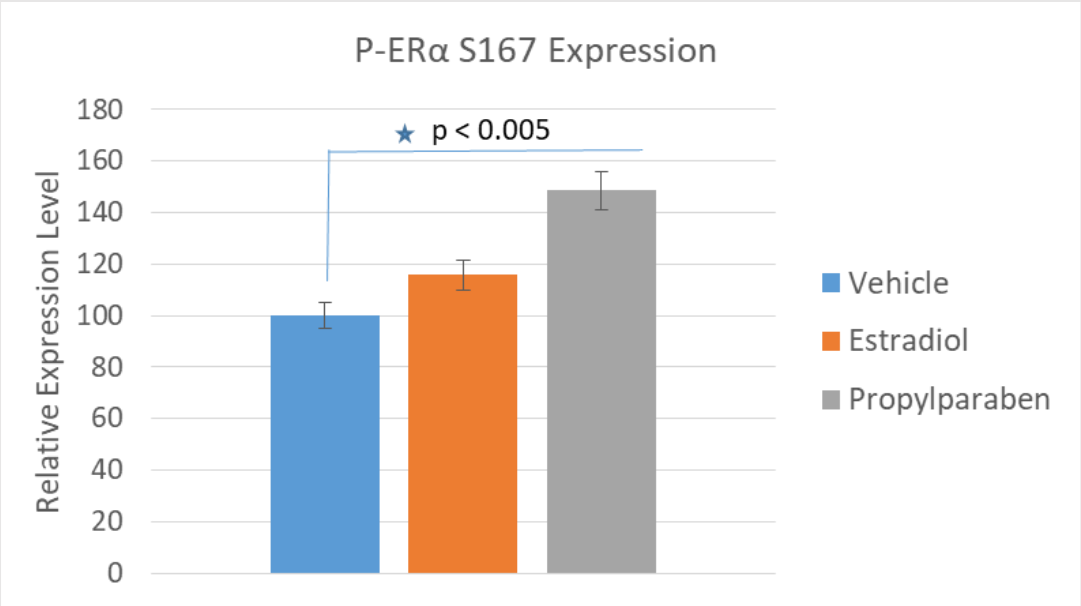


Figure 27: Expression levels of P-ER S167 in MCF Cells Treated with E2 or PP for 30 Minutes. Quantitative values represent pixel counts from 2 images from each of 3 replicate wells= 6 total images analyzed per condition. Changes are relative to vehicle-control cells (0.1% v/v DMSO).

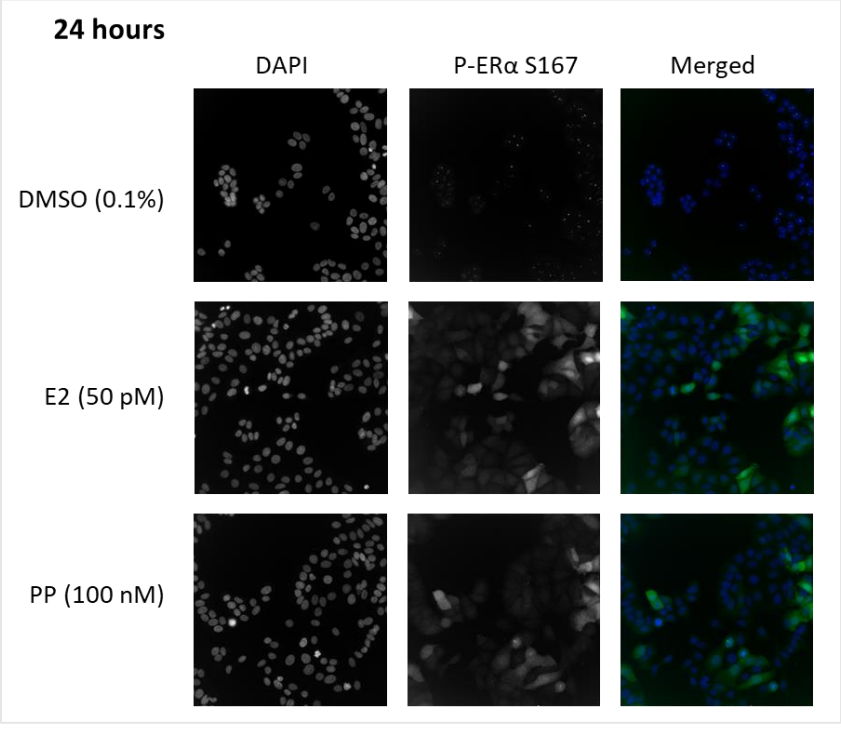


Figure 28: Expression of P-ER S167 in MCF7 Cells Treated with E2 or PP for 24 Hours

To examine the impact of treatment timing on ERα expression, a time course of quantitative-IF analysis was performed after 30 minutes, and 2, 8, and 24 hours of E2 or PP exposure in MCF7 cells. As shown in Figure 29, expression of total ERα, P-ERα S167, and ERα phosphorylated at S104/106 is time-dependent and dynamic. Distinct differences between E2-induced changes and PP-induced effects were also apparent.

To explore additional signaling mechanisms involved in EAC treatment in MCF7 cells, expression of fifteen proteomic markers was assayed by high-throughput immunofluorescence-based scans. Representative images are shown in Figure 30 for hormone receptor expression, including P-ERα S167 and other sites of ERα phosphorylation, AR, PR, and ERR β/γ. After 24 hour treatments with 1 uM BPA, expression of P-ERα S167 was markedly increased in MCF7 cells within cytosolic compartments. Both nuclear and cytosolic expression was apparent in a proportion of imaged cells, and these EAC-induced changes were consistent for both BPA and PP (Figure 28). The other hormone receptor markers examined did not undergo changes in expression with BPA treatment, except for PR, which appears to be slightly downregulated.

Key signaling pathway markers were also examined, including the activated kinases P-ERK and P-Akt, and the transcription factors, β-catenin and c-Myc. As shown in Figure 31, after 24 hours of BPA treatment, MCF7 cells expressed significantly higher levels of P-ERK T202/Y204, but not P-Akt T308. Levels of β-catenin expression were slightly increased by BPA, and localized mainly at cellular membranes. Expression of c-Myc was similar in both control and BPA-treated cells.

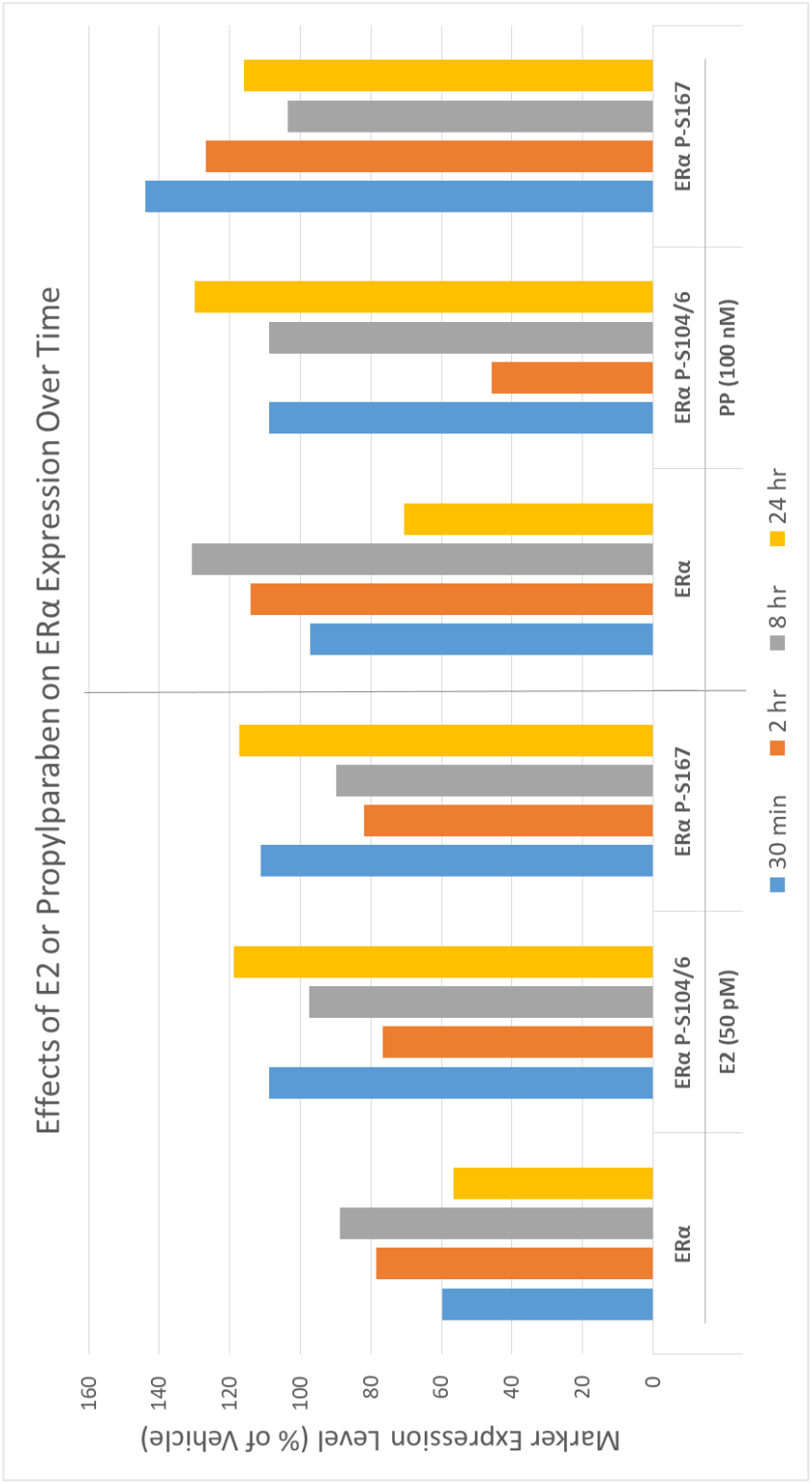


Figure 29: MCF7 Expression Levels for ERα Forms over Time Courses of E2 or PP. Quantitative values represent pixel counts from 2 images from each of 3 replicate wells= 6 total images analyzed per condition. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

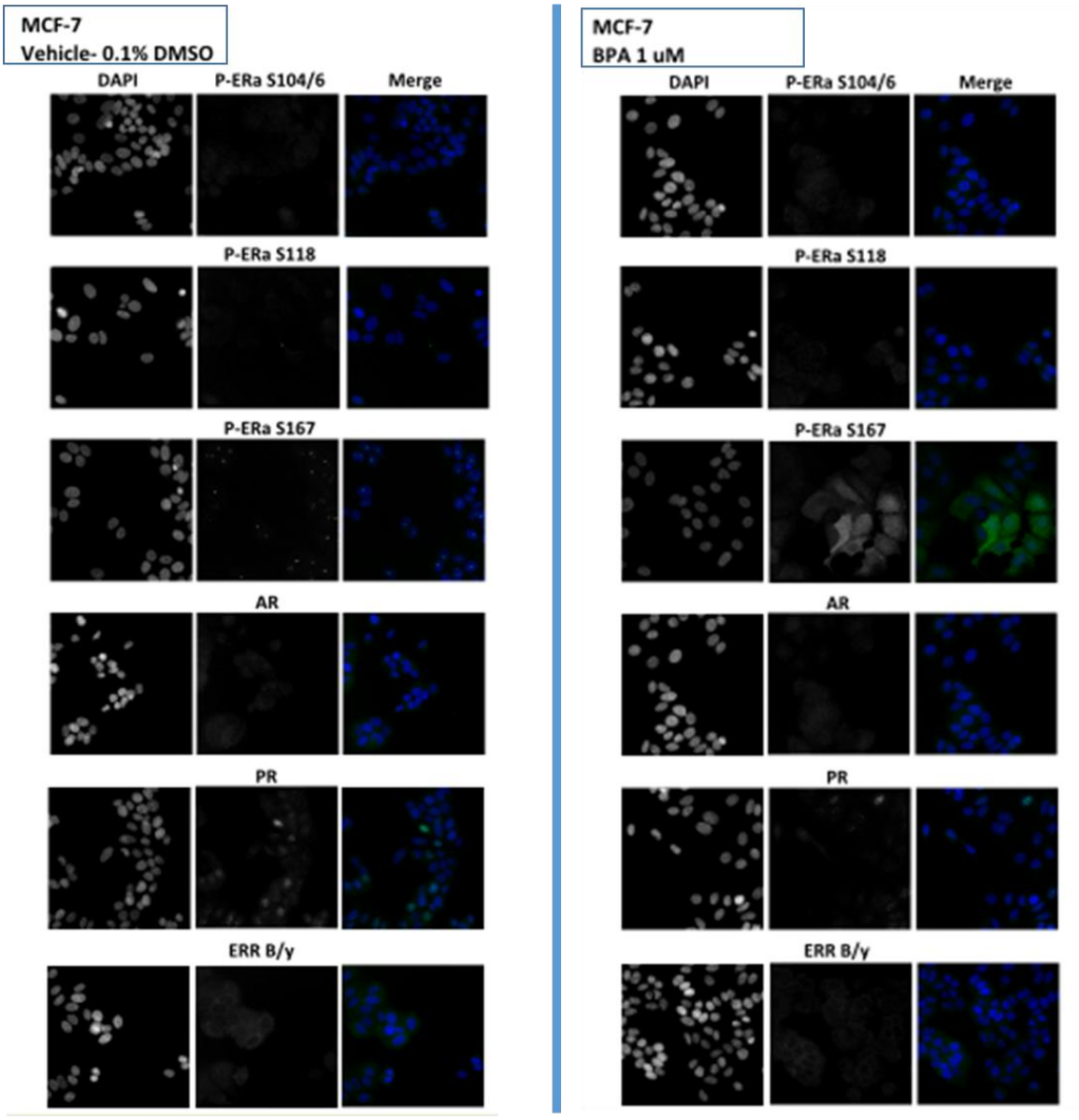


Figure 30: Hormone Receptor Expression in MCF7 Cells with or without BPA Treatment

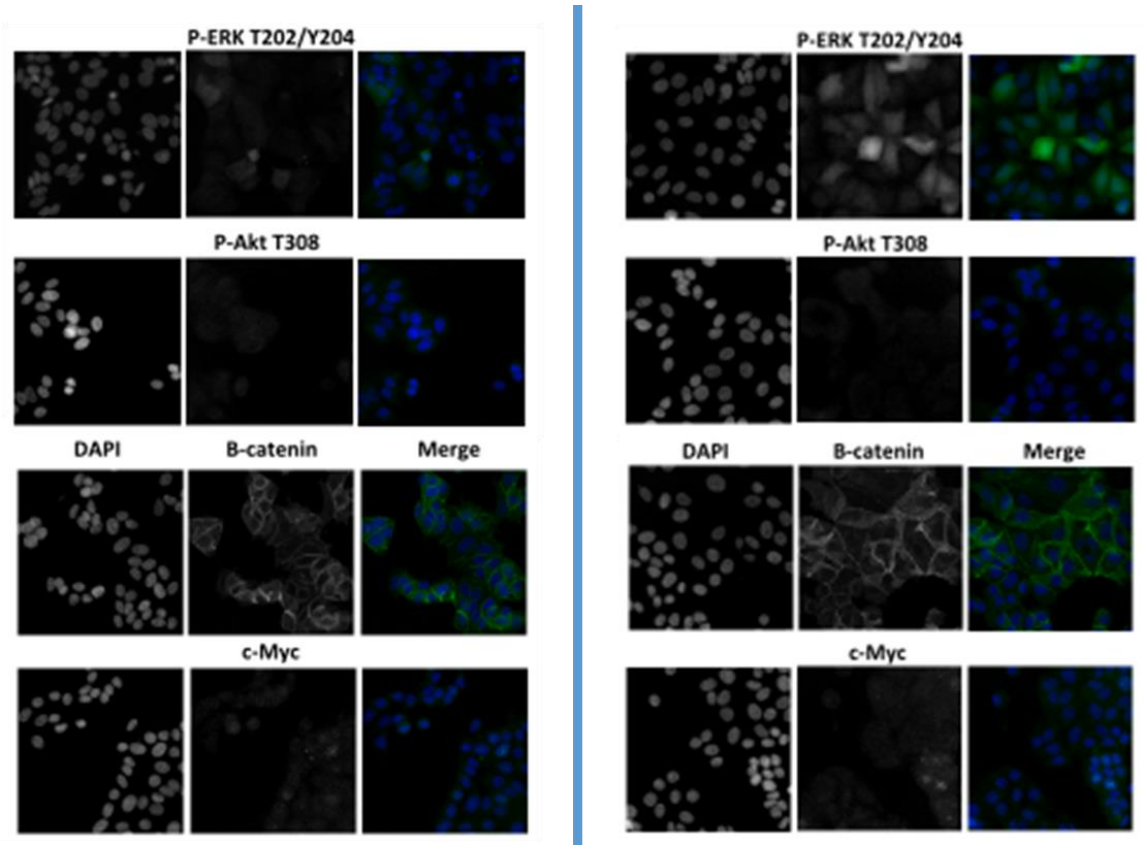


Figure 31: Kinase and Transciption Factor Expression in MCF7 Cells with or without BPA

Treated cells were also stained for key epigenetic enzymes and histone marks, as shown in Figure 32. Consistent with results from Figure 24, treatment with 1 uM BPA for 24 hours led to increased expression of H3K4me2, with a minor increase in H3K9me2. Acetylation of H3K9 also increased, demonstrating that more than one type of epigenetic modification can be induced by EAC-exposure. Dimethylation at lysine 27 of histone 3 (H3K27me2) was weakly induced by BPA, but not expressed in vehicle control cells. Levels of DNMT1 were low in control and BPA-treated cells. LSD1 was moderately expressed in control cells, with a portion of BPA-treated cells showing slightly decreased levels. Given that LSD1 demethylates H3K4 and H3K9, the concurrent increase in these methyl marks is consistent with decreased histone demethylase activity of LSD1. As a

result of examining multiple proteomic markers involved in signaling pathways that cross-talk during breast cancer cellular processes, mechanistic details and patterns of EAC regulation emerged. By quantifying expression levels from IF images, it was possible to create proteomic expression profiles for MCF7 cells treated with E2 and EACs.

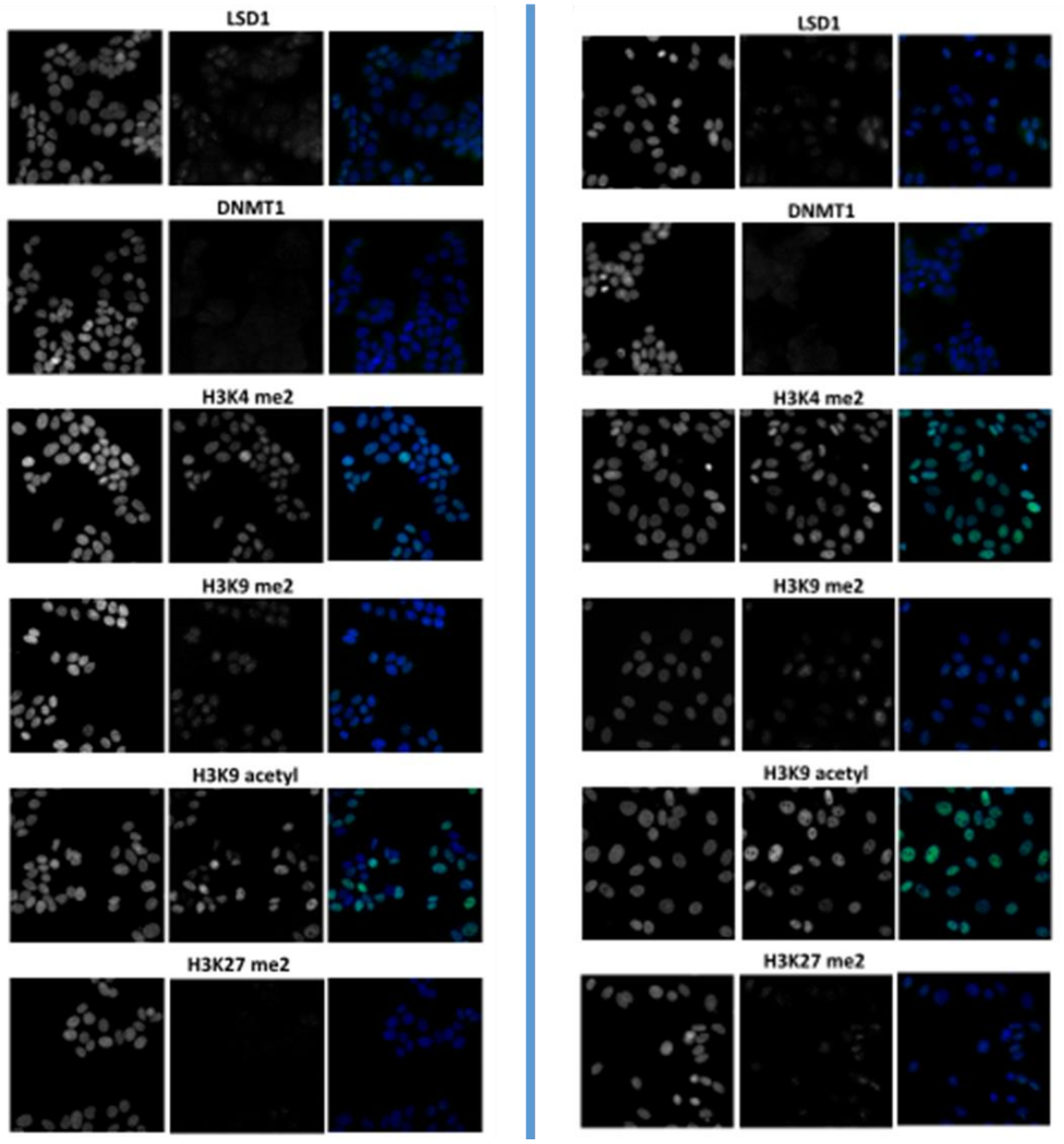


Figure 32: Epigenetic Enzyme and Histone Marker Expression in MCF7 Cells with or without BPA

Figure 33 shows the proteomic expression profile covering the fifteen markers examined for MCF7 cells treated with 50 pM E2 or 1 uM BPA. Patterns of expression induced by E2 or BPA were similar for many markers, but differences in P-ERα S104/106, P-ERα S167, AR and P-ERK T202/Y204 expression emerged. AR expression was reduced when cells were treated with E2, but unaffected by BPA. Although expression of P-ERα S167 and P-ERK T202/Y204 was increased by E2 or BPA, higher expression was observed in BPA-treated cells.

In Figure 34, the proteomic profiles for 1 uM BPA or PP alone, as well as 1 uM BPA and PP combined are shown. Once again, differences emerged for P-ERα, AR, and P-ERK T202/Y204. PP was more effective than BPA or BPA +PP at phosphorylating ERα at multiple serine residues. When combined, BPA and PP had an inhibitory effect on AR expression, whereas BPA alone did not alter AR levels. BPA combined with PP did not induce P-ERK activation at the level of either EAC alone. All three treatments were effective at elevating H3K27me2 levels, with combined BPA and PP resulting in the highest level of expression.

Two concentrations of PP were tested for quantitative IF, and Figure 35 shows results for both 100 nM and 1 uM treatments of PP in MCF7 cells. Despite one concentration being 10-fold greater, both high and low-level PP had unexpectedly similar proteomic profiles. P-ERα expression levels were slightly higher for 1 uM, as well as P-Akt and P-ERK. 100 nM PP was capable of regulating epigenetic marker expression to the same extent as 1 uM PP. A 100 nM concentration of PP is more relevant than 1 uM for modeling low-level human exposure effects.

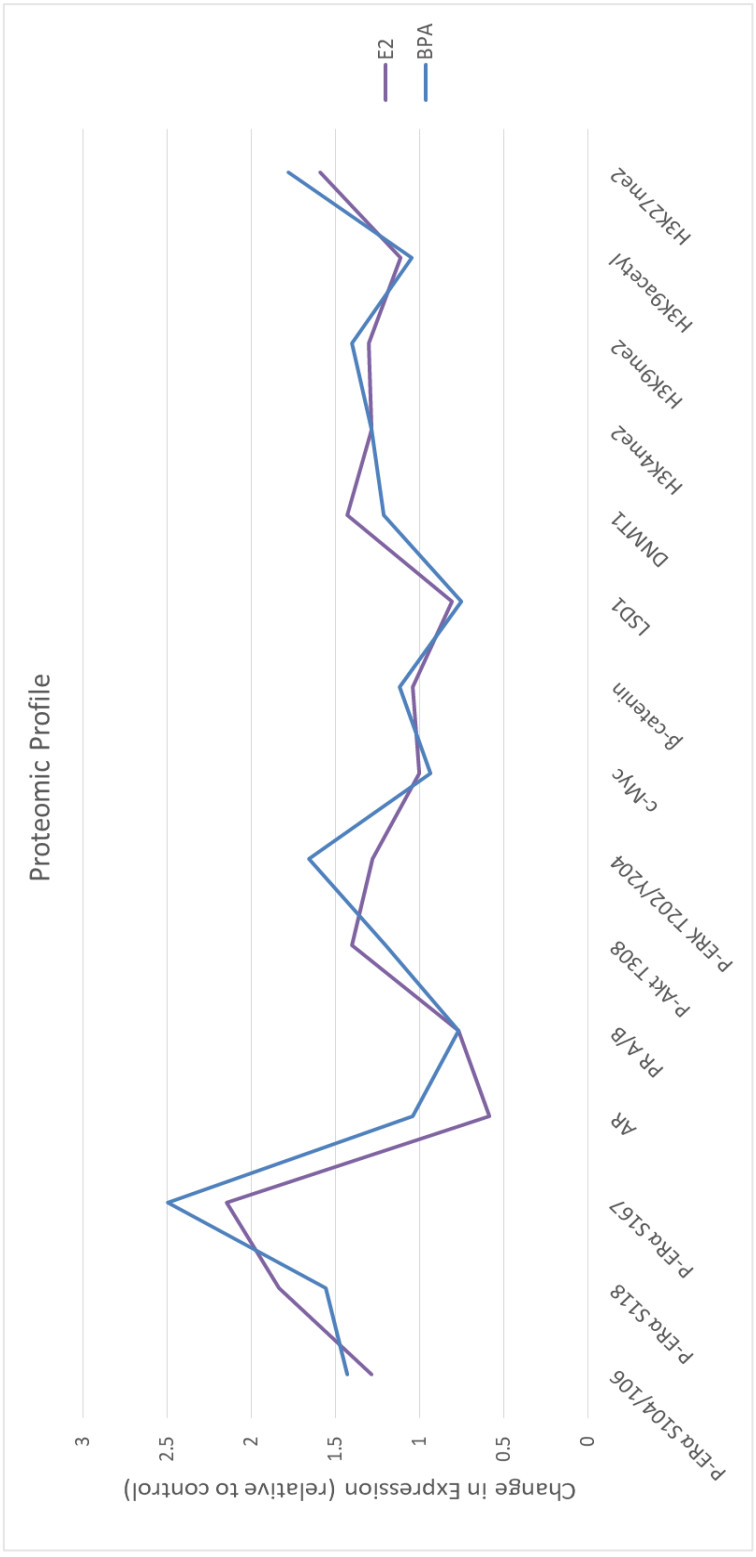


Figure 33: Profile of Proteomic Expression Levels in E2 or BPA-treated MCF7 Cells. Quantitative values represent pixel counts from 2 images from each of 3 replicate wells= 6 total images analyzed per condition. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

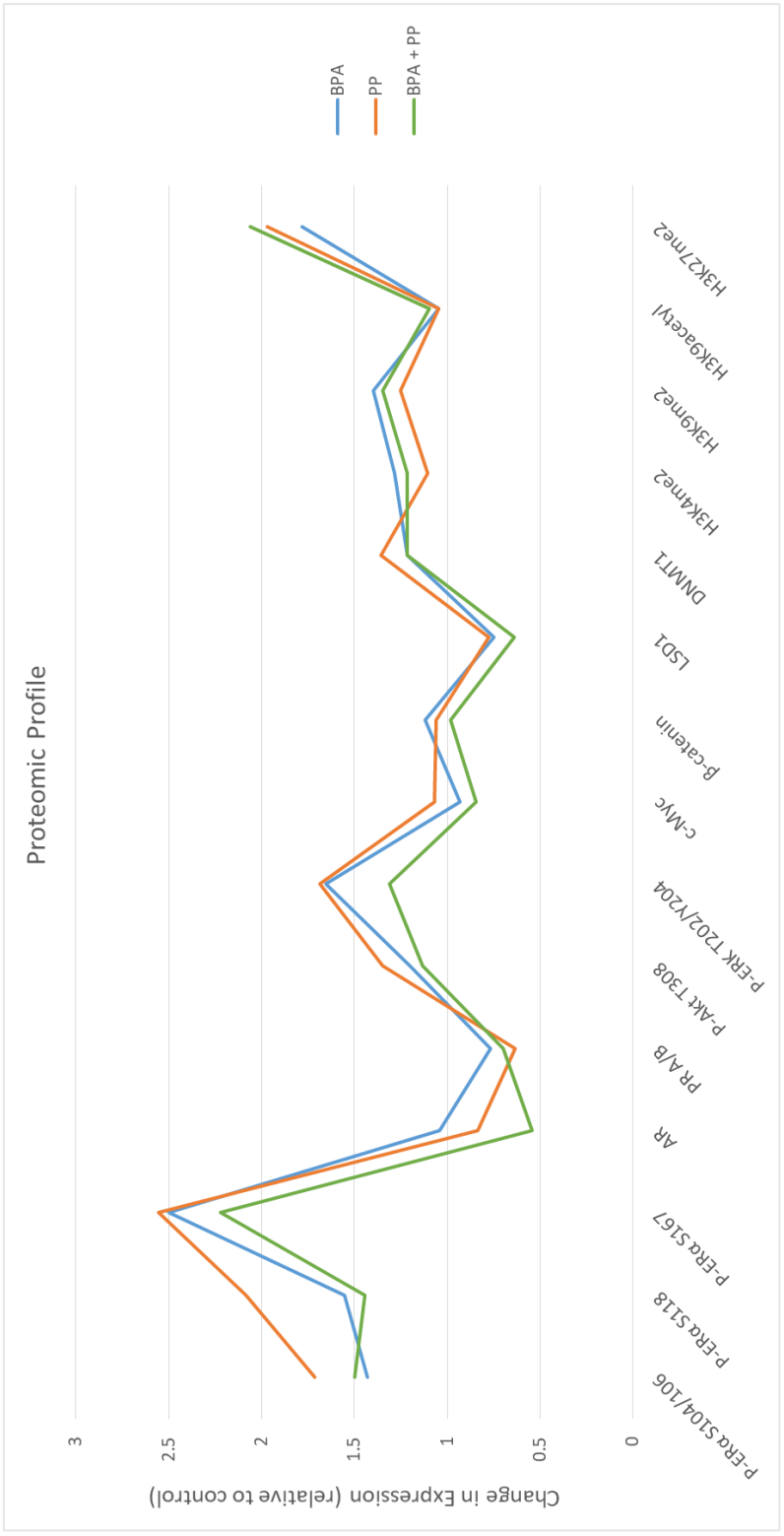


Figure 34: Profile of Proteomic Expression Levels for BPA, PP, or BPA + PP Treatments. Quantitative values represent pixel counts from 2 images from each of 3 replicate wells= 6 total images analyzed per condition. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

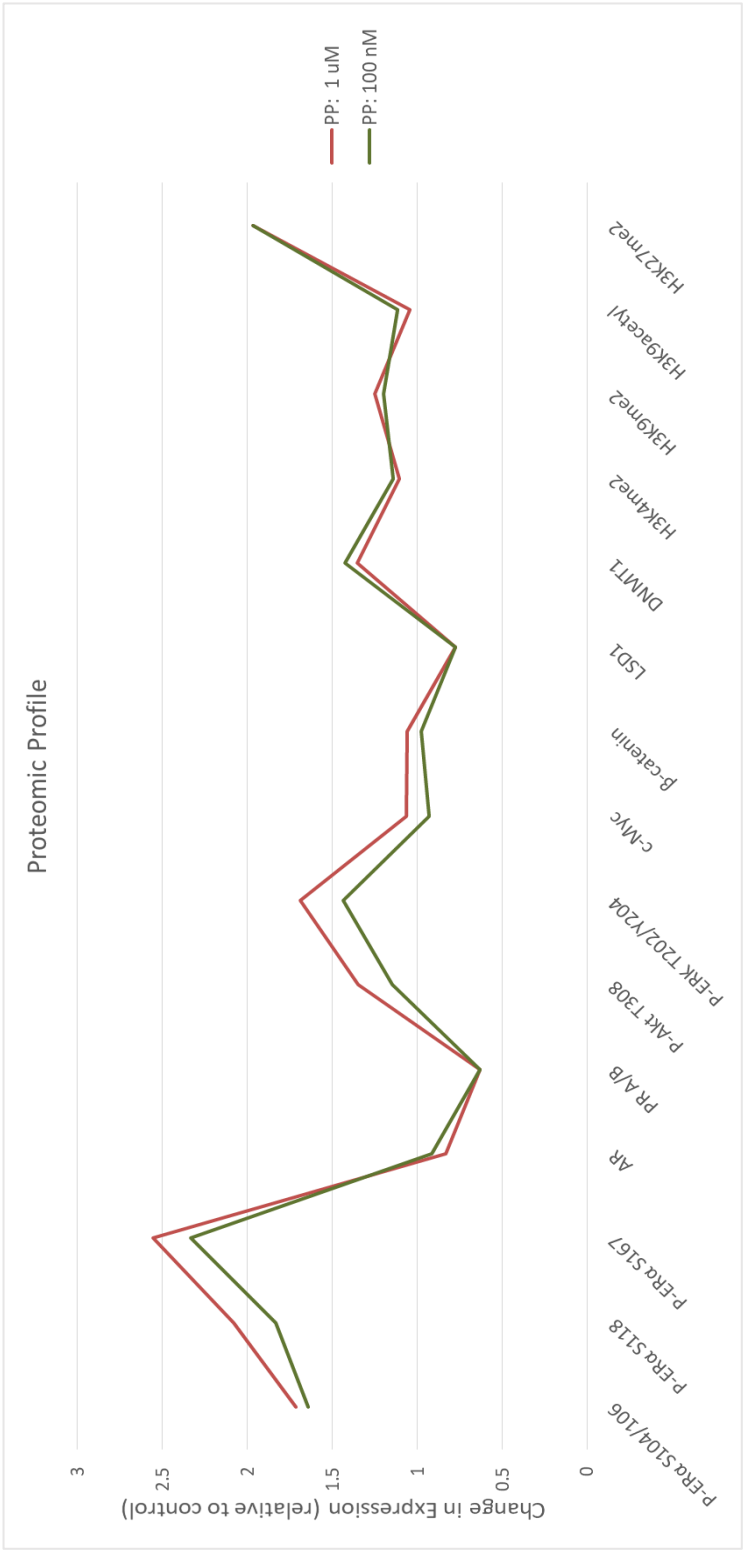


Figure 35: Profile of Proteomic Expression Levels for PP Treatment at 100 nM or 1 uM. Quantitative values represent pixel counts from 2 images from each of 3 replicate wells= 6 total images analyzed per condition. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

Given that these proteins are not linearly expressed or regulated, to move beyond a linear axis for presentation, radar plots can be used to visually summarize these results. The effects of E2 or EACs on protein marker expression, a radar plot is shown in Figure 36 with the fifteen markers tested around a circular axis. A comparison of relative expression levels provides further mechanistic support for treatment-specific regulation of key markers, including multiple forms of P-ERα, AR, P-Akt, P-ERK, and H3K27me2. Similar markers and signaling pathways are regulated by E2 and EACs, but not to the same extent.

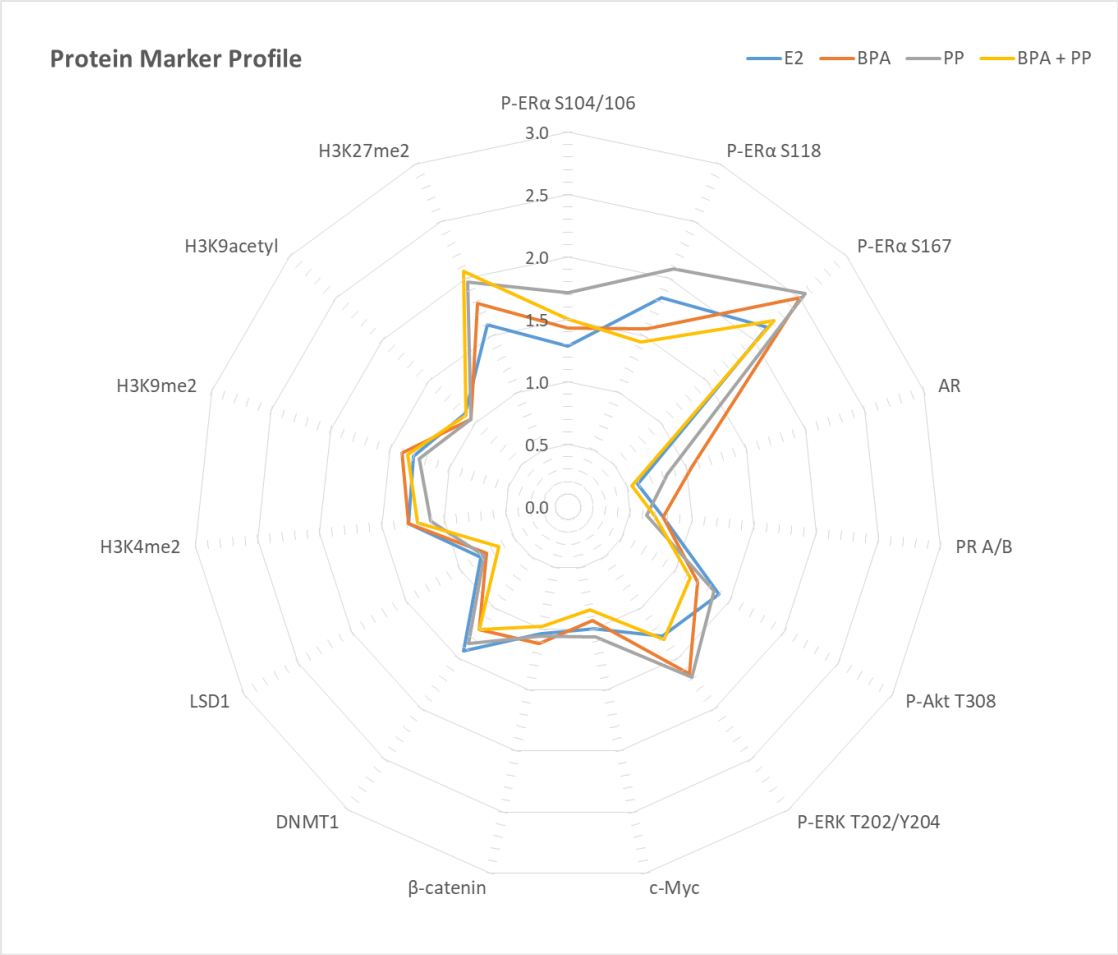


Figure 36B: Radar Plot of Protein Expression Patterns for E2, BPA, PP, or BPA + PP. Quantitative values represent pixel counts from 2 images from each of 3 replicate wells= 6 total images analyzed per condition. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

4.3 LSD1 and PI3K are regulators of E2 and EAC cellular responses

Given that histone methylation changes and LSD1 were shown to be expressed in E2 and EAC-treated MCF7 cells, the role of LSD1 regulation was explored via shRNA knockdown. Compared to LacZ control cells, MCF7 cells lacking LSD1 expression had markedly reduced changes in viability upon E2 or BPA treatment, as shown in Figure 37. To explore the role of LSD1 in regulating cellular responses to mixtures of EACs and E2, LSD1 knockdown cells were evaluated for viability changes in Figure 38. LacZ control cells responded differentially to BPA alone, EACs mixed, and EACs and E2 mixed, as seen in panel A. However, these treatment-related differences were blunted down to control levels in cells lacking LSD1. This implies that LSD1 activity is required for increased mitochondrial activity of ER+ breast cancer cells in the presence of E2 or EACs.

Phosphorylation of Akt was also shown to be involved in regulation of MCF7 cells by E2 and EACs. Akt phosphorylation was investigated further with a small molecule inhibitor (BEZ235) of PI3K, the upstream kinase that phosphorylates Akt on T308 (Davis *et al*, 2014). Given that PI3K is a therapeutic target for breast cancers, BEZ235 cytotoxicity was assessed to choose a non-toxic dose. As shown in Figure 39, cells expressing LSD1 were more susceptible to the effects of PI3K inhibition than LSD1 knockdown cells. Constant 25 nM BEZ235 was then combined with a range of BPA concentrations. The increased viability response to BPA in control cells was attenuated in cells with the PI3K inhibitor. These results further support the roles of LSD1 and P-Akt in EAC-induced effects, and suggests that mechanisms involving both signaling molecules are required for maximum responses in ER+ breast cancer cells.

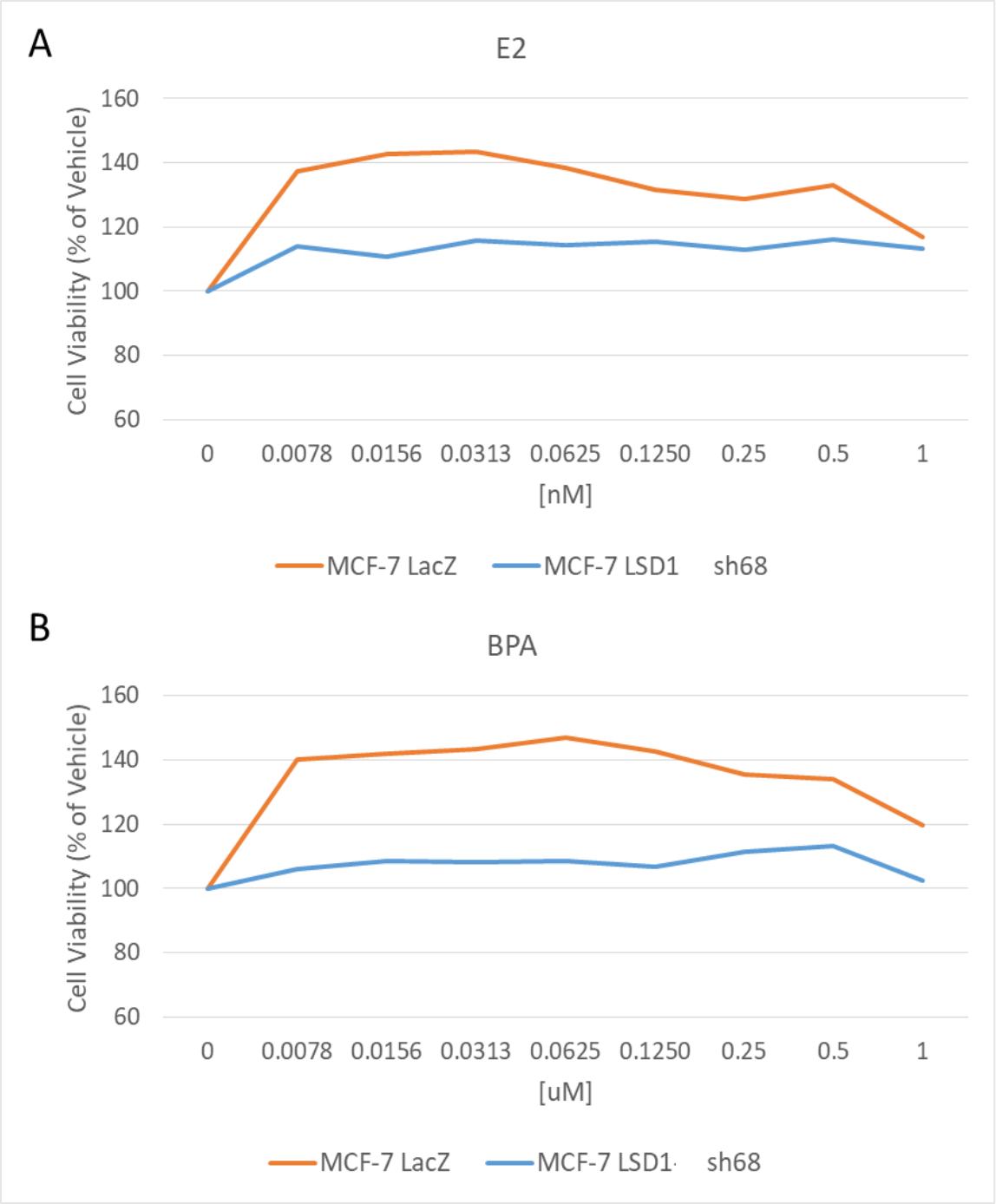


Figure 37: Viability Effects of LSD1 RNAi Knockdown in MCF7 Cells with A: E2 or B: BPA. Cells were transfected for LacZ control expression or LSD1 sh68 knockdown, and treated with serially diluted E2 or BPA at the indicated concentrations for 96 hours. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

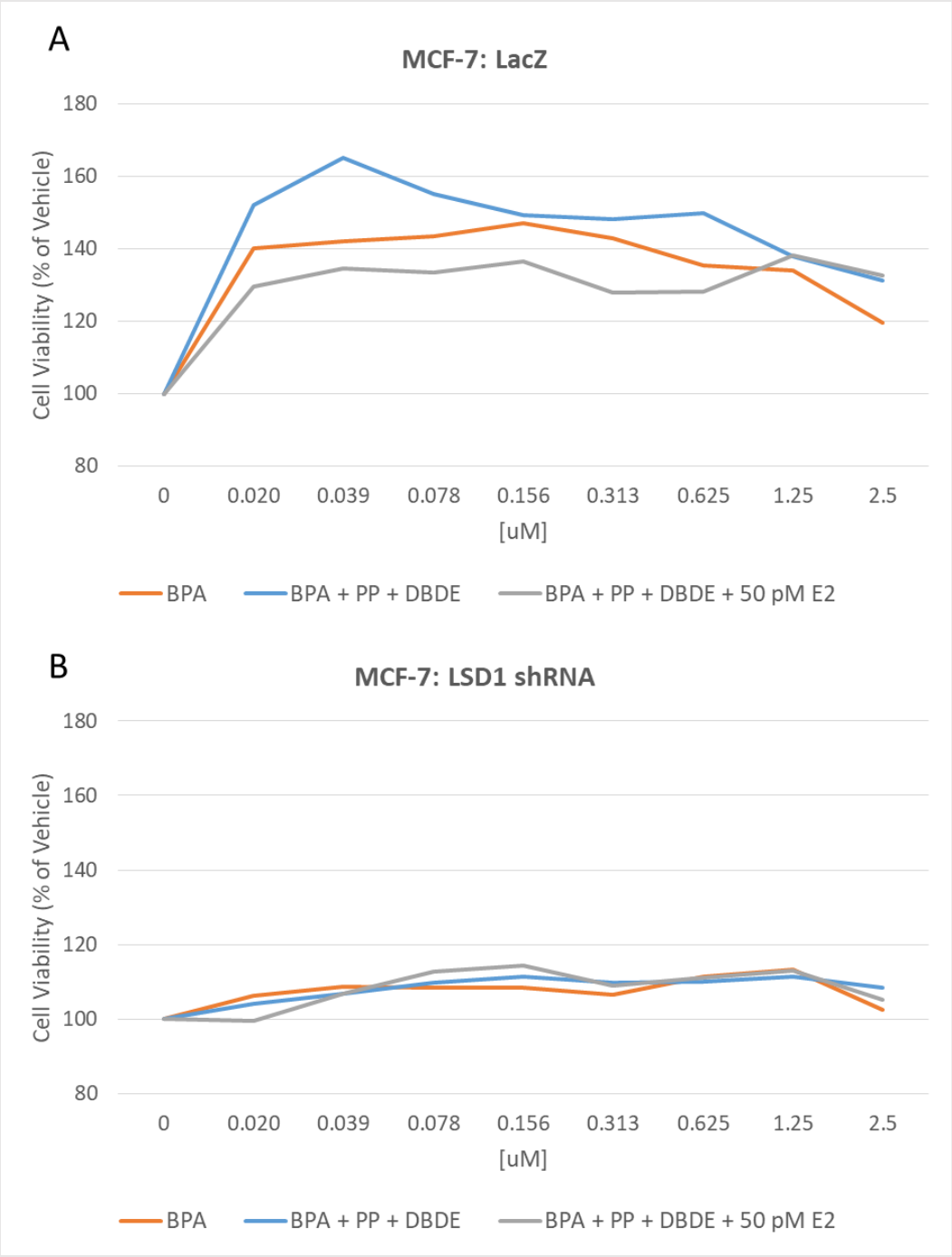


Figure 38: Viability Effects of LSD1 shRNA Knockdown in MCF7 Cells Treated with BPA, three EACs, or EACs; A: LacZ transfected cells, B: LSD1 sh68 cells. Cells were transfected for LacZ control expression or LSD1 sh68 knockdown, and treated with serially diluted EACs at the indicated concentrations for 96 hours. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

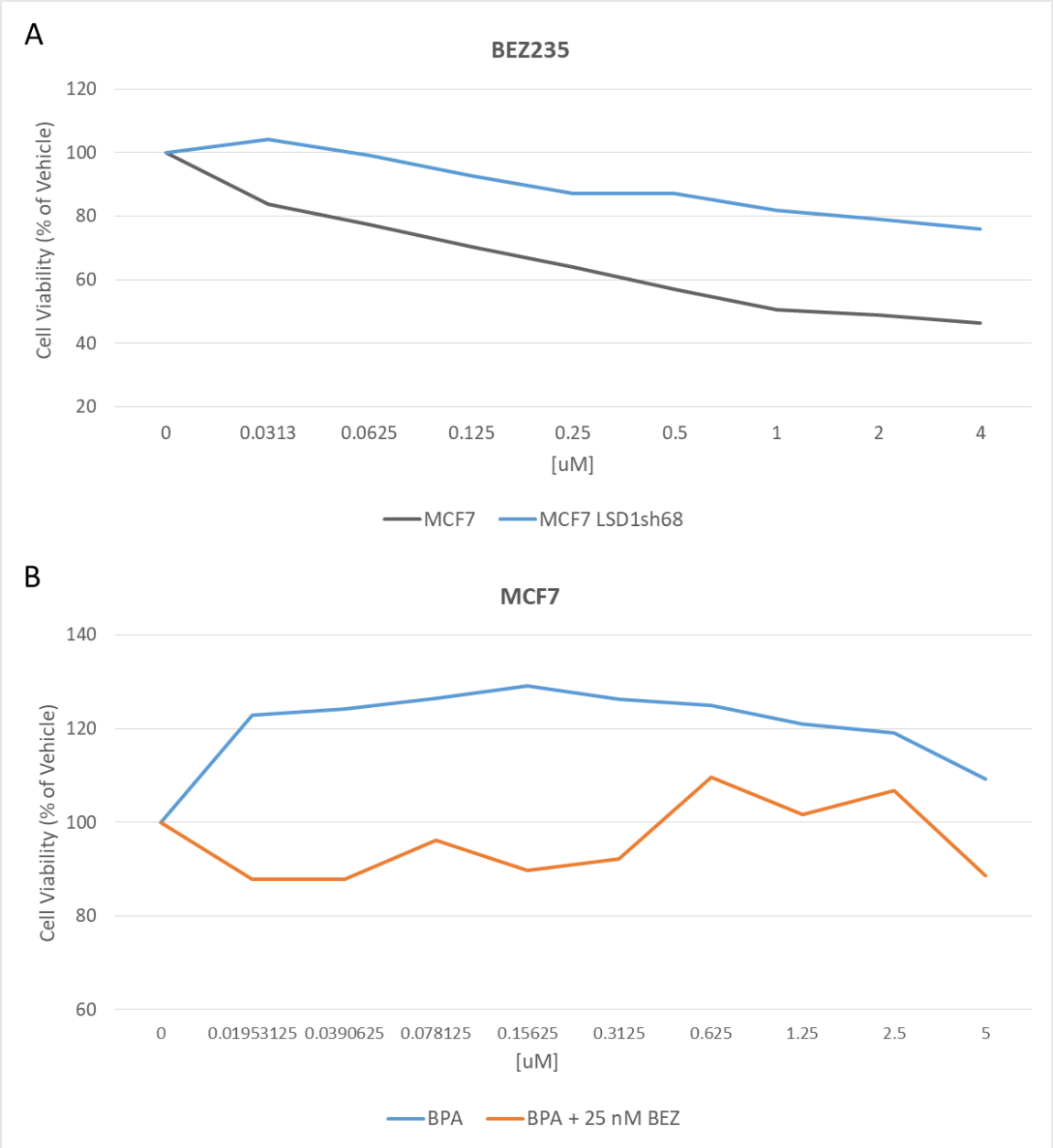


Figure 39: Viability Effects of PI3K Inhibition in MCF7 Cells; A: in LSD1 knockdown cells; cells were transfected for LacZ control expression or LSD1 sh68 knockdown, and treated with serially diluted BEZ235 at the indicated concentrations for 96 hours. B: Non-transfected MCF7 cells were treated with BPA with or without 25 nM BEZ235. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

Chapter 5: Results and Discussion- Aim 3

As shown in Aim 1, EACs affect breast cancer cell viability; the underlying signaling and epigenetic mechanisms were detailed in Aim 2. Given that therapeutic response is vital for clinical care, and drug resistance can emerge in breast cancers, Aim 3 investigated the potential role of EACs in the response of MCF7 and SUM44 cells to chemo- and hormonal-based therapy. Cells were concurrently treated with EACs and 4OH-tamoxifen or doxorubicin for 96 hours, and examined for viability and protein marker expression. To assess the effects of extended EAC treatments, MCF7 cells were cultured in the presence of low-levels of EACs for 60 days. By taking a subset of 60 day-treated cells and removing them for 96 hours from EACs, the effects of "EAC detox" on subsequent responses to EACs and drugs was investigated.

5.1 Doxorubicin sensitivity was increased by EACs in MCF7 cells, but not SUM44

Relative cytotoxicity over a range of doxorubicin concentrations is shown in Figure 40 for SUM44 cells. As expected, doxorubicin was effective at significantly reducing cellular viability. The presence of constant E2, BPA, or all five EACs to doxorubicin did not alter the effects of the drug in this ER+ breast cancer cell line. However, doxorubicin responses were differentially regulated by estrogenic compounds in MCF7 cells, shown in Figure 41. The concentration of doxorubicin required to inhibit cellular viability by 50% (IC_{50}) was calculated for each treatment

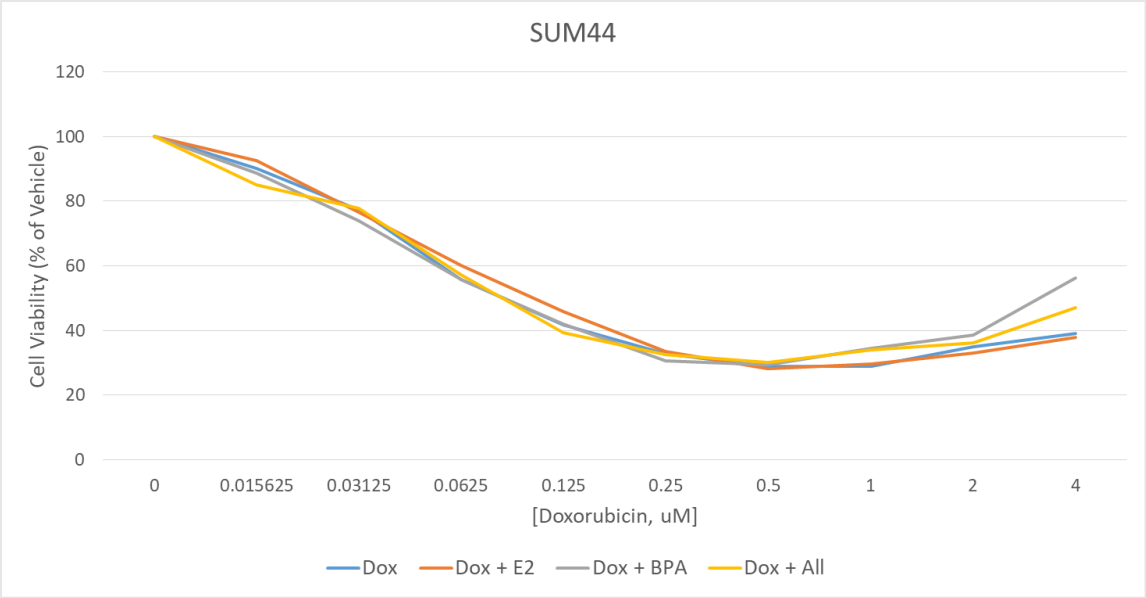


Figure 40: Viability Responses in SUM44 Cells Treated with Doxorubicin Alone or Combined with E2, BPA, or Five EACs. Doxorubicin was serially diluted at the indicated concentrations and combined with 100 nM EACs as indicated. Changes are relative to control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

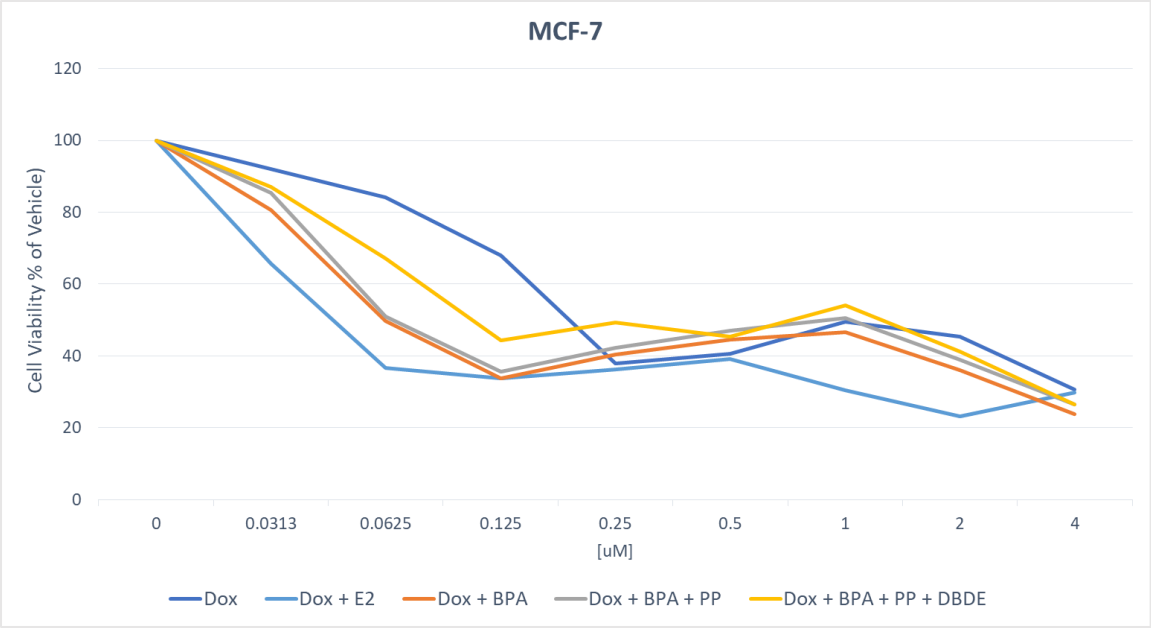


Figure 41: Viability Responses in MCF7 Cells Treated with Doxorubicin Alone or Combined with E2, BPA, BPA + PP, or BPA + PP + DBDE. Doxorubicin was serially diluted at the indicated concentrations and combined with 100 nM EACs as indicated. Changes are relative to control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

condition and shown in Figure 42. Drug alone produced an IC_{50} of 180 nM, while drug combined with 50 pM E2 significantly reduced doxorubicin IC_{50} by 4.5-fold to 40 nM. Doxorubicin combined with 100 nM BPA or a mixture of BPA and PP also resulted in significantly lower IC_{50}s. This effect was slightly attenuated when doxorubicin was combined with BPA, PP and DBDE, but still significantly lower than when cells were treated with doxorubicin alone. The increase in IC_{50} when the drug was treated with two EACs versus three EACs was also significant. These results demonstrate that low levels of natural and synthetic estrogenic compounds can further sensitize certain ER+ breast cancer cells to the cytotoxic effects of doxorubicin.

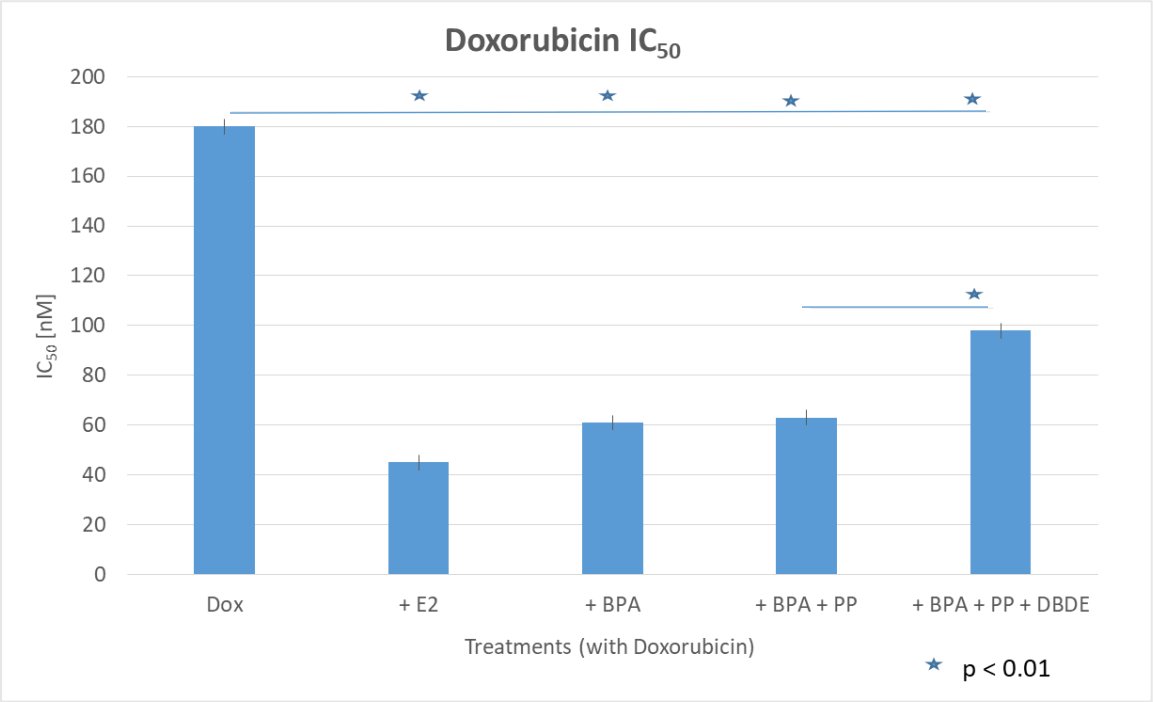


Figure 42: Comparison of Doxorubicin IC_{50}s with or without E2 or EACs in MCF7 Cells. Drugs were serially diluted and combined with 100 nM EACs as indicated. IC50s were extracted from dose-response curves. Changes are relative to control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

5.2 Tamoxifen sensitivity was reduced by EACs in ER+ breast cancer cells

Given that 4OH-tamoxifen is a common therapy for ER+ breast cancers, yet many cancers eventually develop resistance, the potential role of EACs in tamoxifen response was explored. As shown in Figure 43, tamoxifen was not cytotoxic to SUM44 cells, except at the highest concentration tested (4 uM). The addition of constant E2 to tamoxifen produced slight increases in cell viability at low drug concentrations, but not at high. The combination of tamoxifen and low-level BPA increased SUM44 viability by close to 20% over a range of drug concentrations. The addition of multiple EACs to tamoxifen produced a 10-20% viability increase, but not at higher levels of tamoxifen.

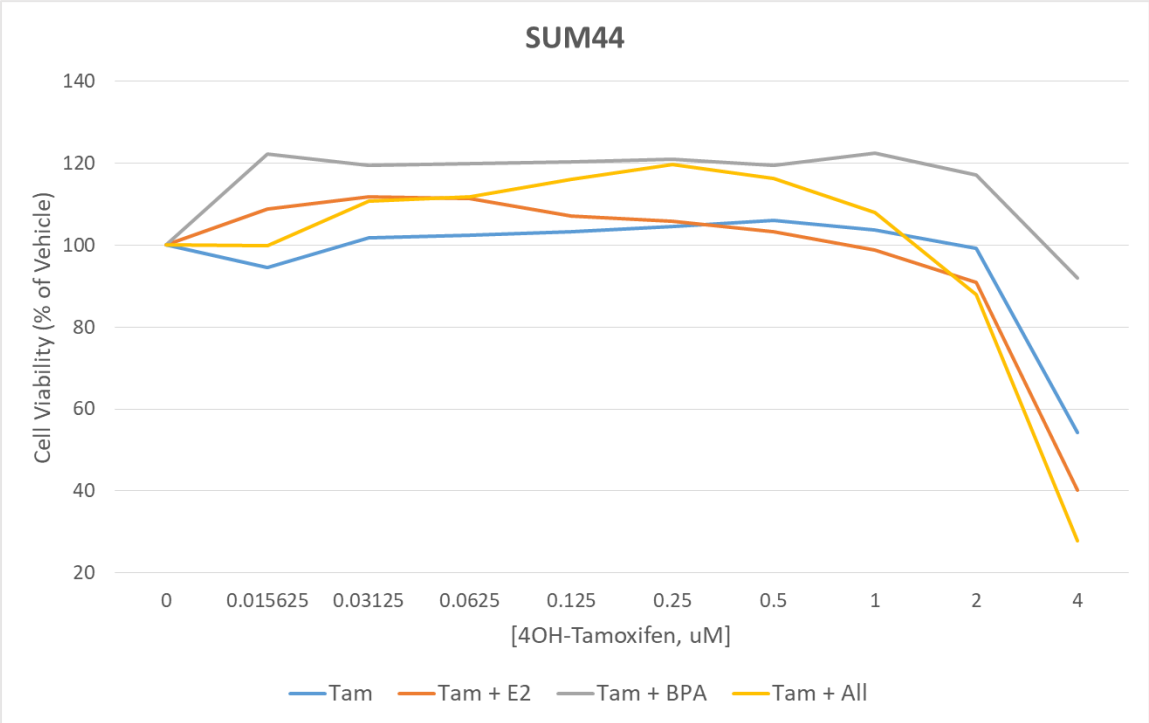


Figure 43: Viability Responses in SUM44 Cells Treated with Tamoxifen Alone or Combined with E2, BPA, or Five EACs. Tamoxifen was serially diluted at the indicated concentrations and combined with 100 nM EACs as indicated. Changes are relative to control cells (0.1% v/v DMSO). Standard deviations are within 5% of triplicate means.

Increased resistance to tamoxifen also occurred in MCF7 cells in the presence of constant levels of E2 or EACs, as shown in Figure 44. The combination of tamoxifen and three EACs produced 20-30% increases in cell viability compared to tamoxifen alone. Increased viability results were similar for tamoxifen combined with low-level E2, BPA, or BPA and PP. Even though tamoxifen is not necessarily cytotoxic, it limits growth of breast cancer cells by blocking ERα activation. Based on these results, the presence of EACs during tamoxifen treatment can led to reduced therapeutic effectiveness by making cells more viable. As earlier transactivation results showed, these effects are likely mediated by interactions between multiple ERα ligands with various binding affinities. The ability of each ligand, including tamoxifen, to bind its target receptor can be altered when tamoxifen, endogenous estrogens, and EACs are combined and competing for the same molecular space.

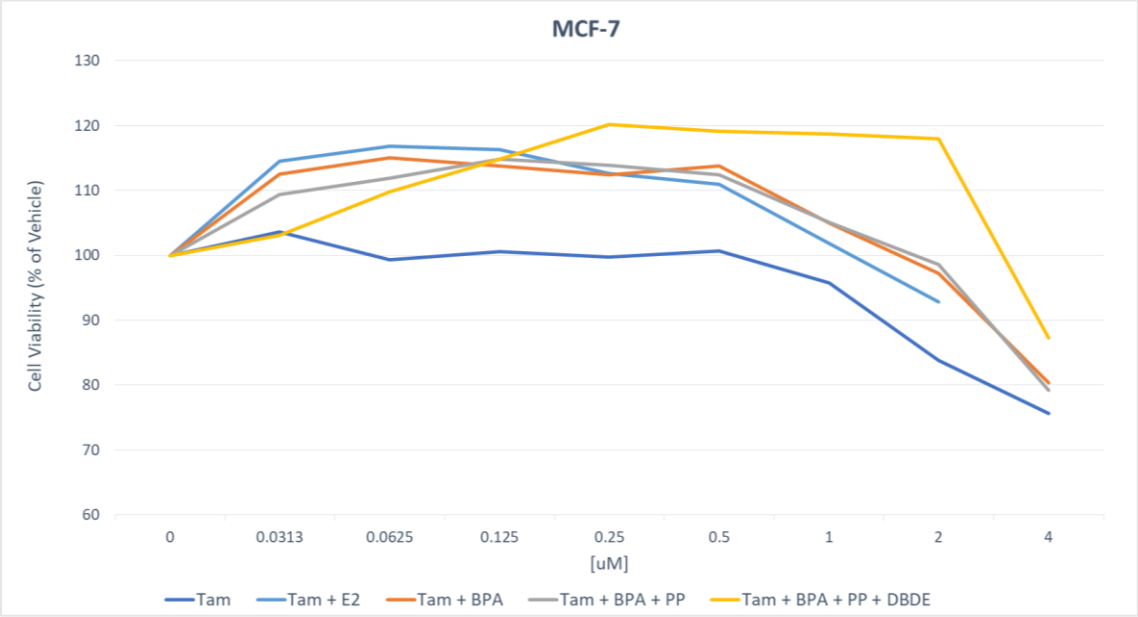


Figure 44: Viability Responses in MCF7 Cells Treated with Tamoxifen Alone or Combined with E2, BPA, BPA + PP, or BPA + PP + DBDE. Tamoxifen was serially diluted at the indicated concentrations and combined with 100 nM EACs as indicated. Changes are relative to control (0.1% v/v DMSO). Deviations are within 5% of triplicate means.

5.3 Proteomic mechanisms underly altered tamoxifen response in MCF7 cells

Given that P-ERα S167 was shown to be an important regulator of EAC reponse in MCF7 cells, this marker was also evaluated for a role in EAC-induced tamoxifen resistance. Shown in Figure 45, P-ERα S167 underwent expression and localizaton changes upon 24 hours of E2 or BPA teatment. When treated with tamoxifen alone, a similar pattern of localization changes occurred, yet P-ERα S167 was not as strongly expressed in nuclei. The presence of BPA with tamoxifen did induce strong nuclear expression in nearly all cells treated. These results suggest that EACs can limit the therapuetic effectiveness of tamoxifen in ER+ breast cancer cells through altered cytosolic and nuclear activity of P-ERα S167.

The proteomic expression profiles for E2, EACs, tamoxifen and tamoxifen with EACs are shown in Figure 46. Consistent with previous aims, the observed cellular effects were mediated largely through P-ERα, AR, P-Akt, P-ERK, and histone methylation changes. The observed effects of various treatments on protein expression in MCF7 cells were analyzed by heirarchal clustering, depicted in Figure 47. A separate cluster, shown in green, consisted of tamoxifen combined with BPA and tamoxifen plus BPA and PP. A 3D plot displaying expression of three key markers, P-ERα S167, AR, and H3K27me2 is shown in Figure 48. The expression patterns for tamoxifen plus EACs (in green) are found in the top left quadrant, while data points for tamoxifen alone and E2 alone are in other quadrants. These findings demonstrate that signaling and epigentic regulation is distinctly different in cells exposed to EACs during tamoxifen treatment, as compared to cells exposed to EACs, E2, or tamoxifen alone.

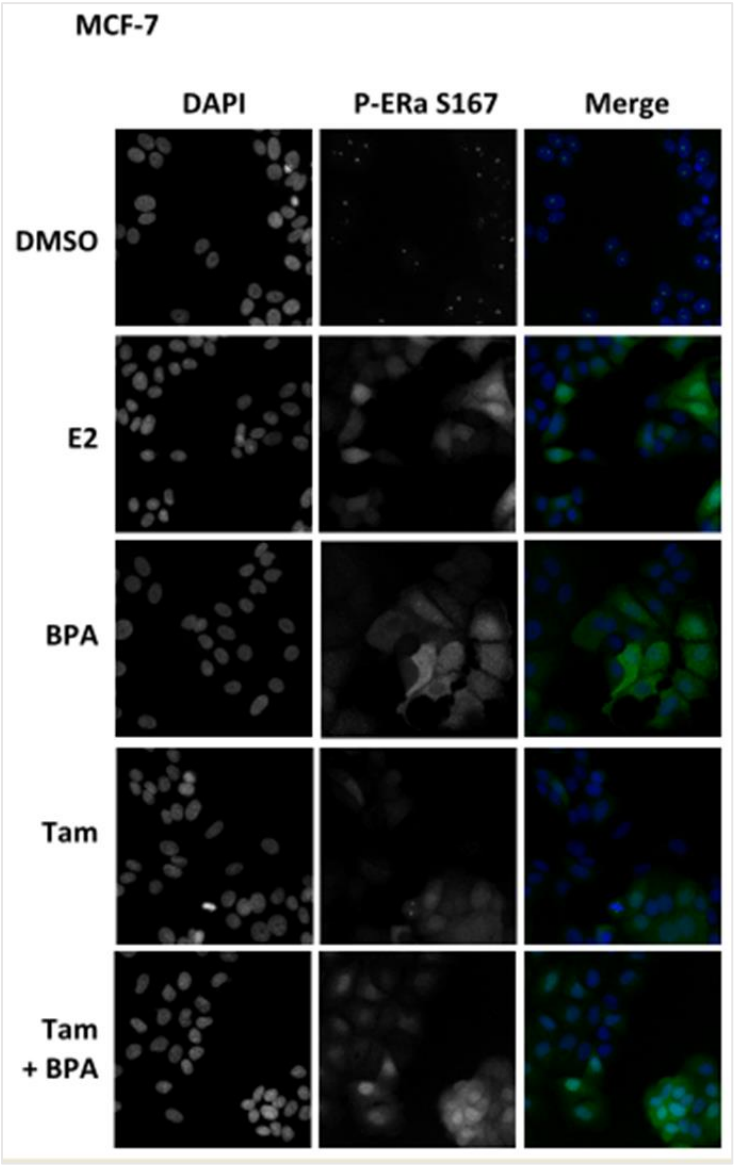


Figure 45: Expression of P-ERα S167 in MCF7 Cells Treated with Tamoxifen or Tamoxifen + BPA after 24 Hours

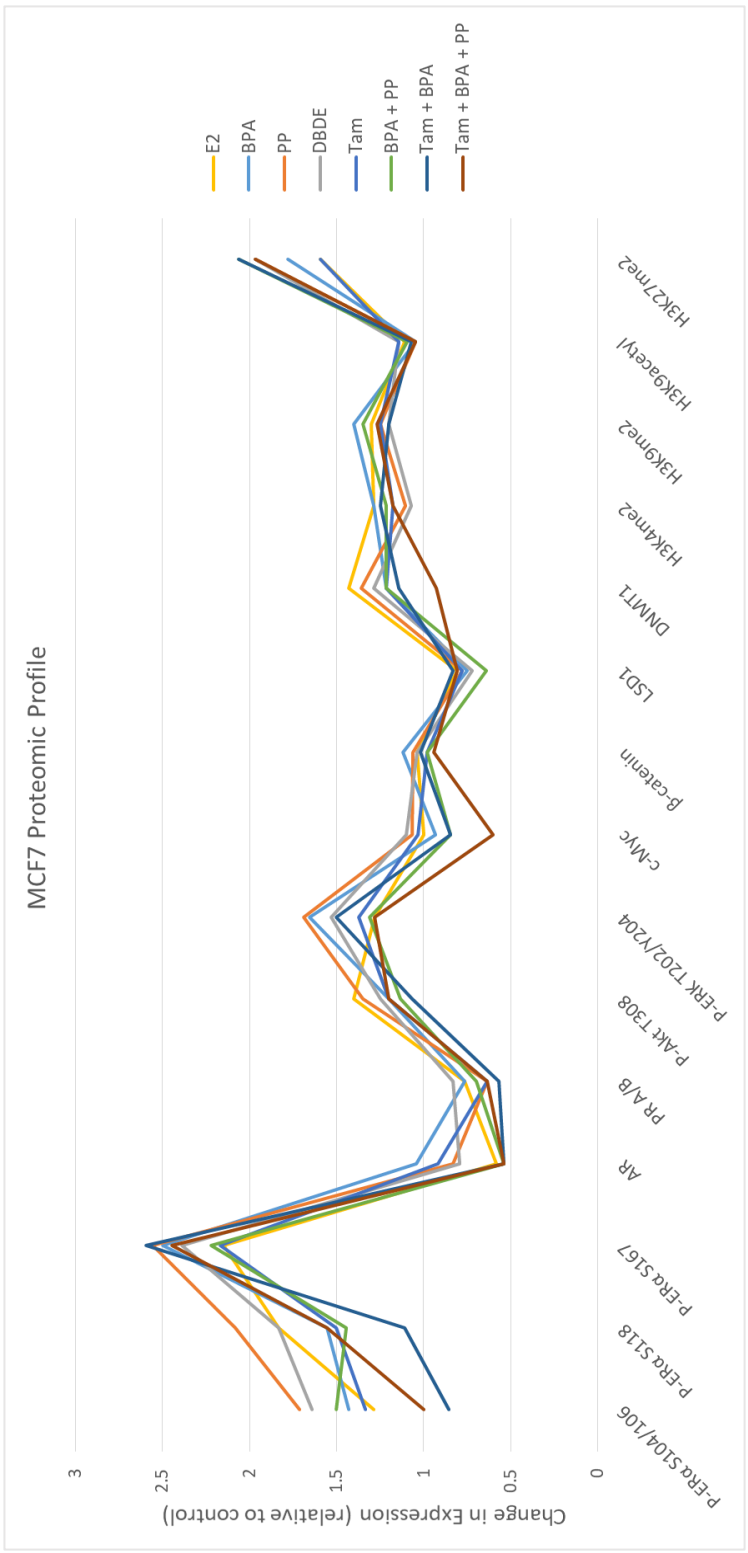


Figure 46: MCF7 Proteomic Expression Profiles Comparing Each Treatment, Inlcuding Tamoxifen with or without EACs. Quantitative values represent pixel counts from 2 images from each of 3 replicate wells= 6 total images analyzed per condition. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means.

Figure 47: Hierarchal Clustering Results for All Treatments Tested in MCF7 Cells

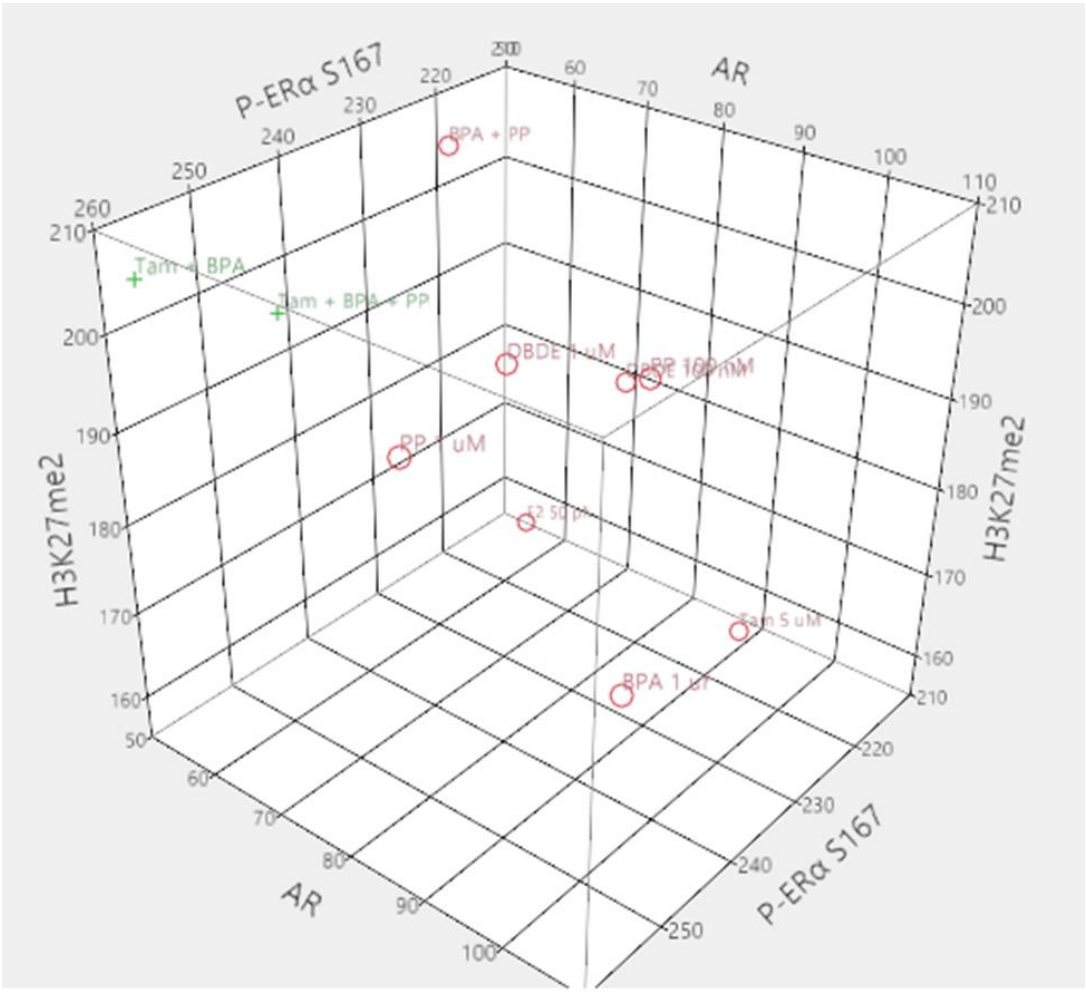


Figure 48: 3D Plot of Protein Expression Levels for Three Key Markers in MCF7 Cells Comparing All Treatments. Markers are P-ERα S167, AR, and H3K37me2, and data points are values from proteomic profiles described in previous figures.

5.4 Extended EAC exposures alter subsequent responses to EACs and drugs

Over four day treatments with EACs, cells were sensitized to doxorubicin, but resistance to tamoxifen was increased. To determine the outcomes of extended periods of low-level EAC exposure on drug response, MCF7 cells were cultured in 100 nM BPA, 100nM all five EACs, 100 nM tamoxifen, or tamoxifen combined with five EACs for 60 days. A subset of cultured cells were then removed from treatments for a 4-day "detox" in culture before further treatments. Extended BPA culture was found to increase doxorubicin sensitivity and increase tamoxifen resistance, shown in Figure 49A. These EAC-induced effects on drug response were attenuated in cells allowed a 4-day detox prior to drug treatment, despite having been previously exposed for 60 days.

Shown in Figure 49B, extended culture in all five EACs also altered drug response by sensitizing cells to doxorubicin. This effect was reversed by EAC withdrawal; cells were more resistant to doxorubicin if they were EAC-free prior to drug treatment, even if they had been cultured in EACs for 60 days before that. The EAC-induced mechanisms that make cells more vulnerable to chemotherapy may also confer survival advantages after EACs are no longer present.

Given that tamoxifen resistance is a common clinical outcome, the effects of extended low-level tamoxifen treatment on subsequent drug responses were evaluated. In Figure 50A, MCF7 cells cultured for 60 days in tamoxifen were more sensitive to doxorubicin than cells without prior tamoxifen culture. This effect was attenuated by a 4-day tamoxifen withdrawal prior to doxorubicin treatment. Extended low-level tamoxifen culture did not increase tamoxifen resistance. When these cells underwent

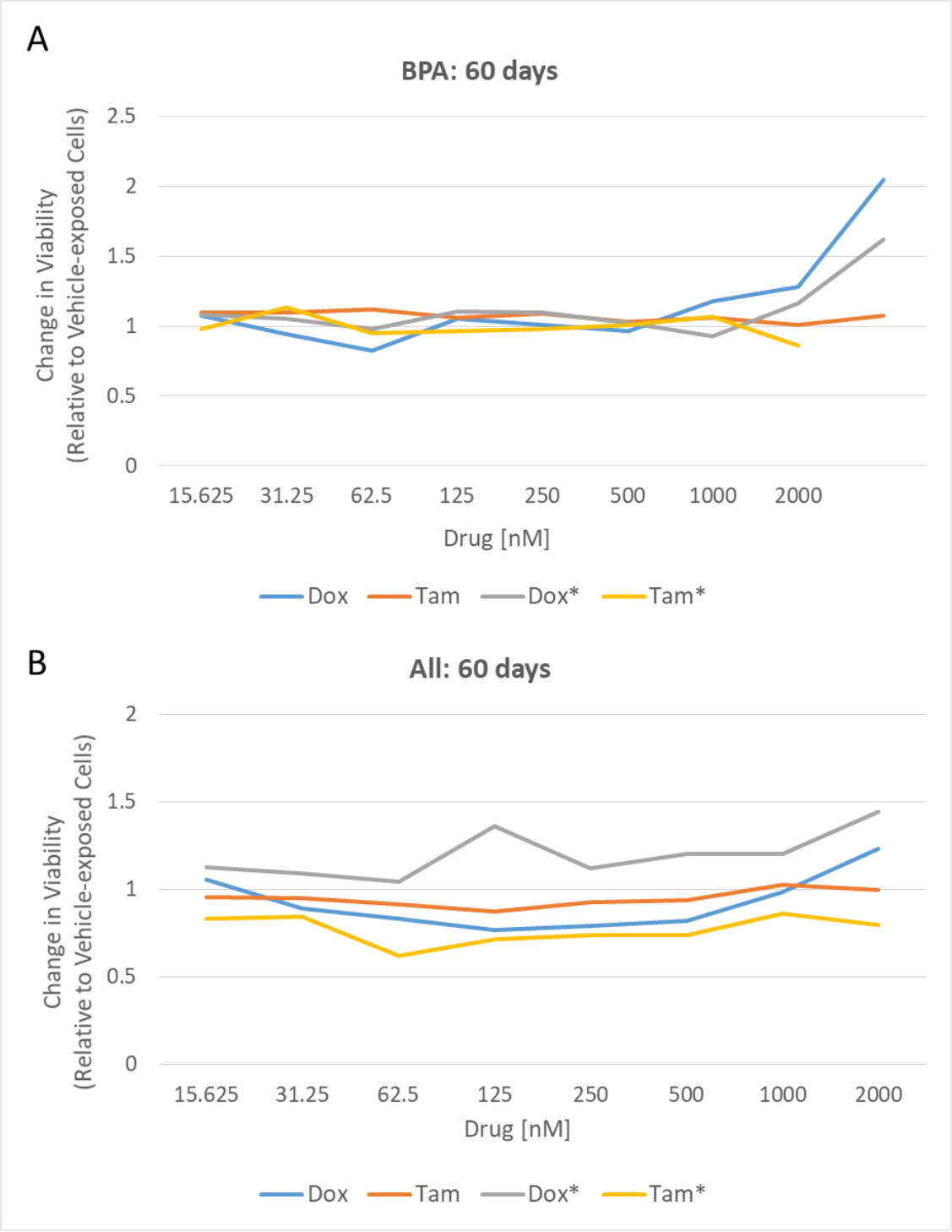


Figure 49: Viability Effects of 60-day Low-level EAC Cultures on Subsequent Doxorubicin and Tamoxifen Responses in MCF7 Cells; A: BPA, B: All Five EACs. Cells were cultured in 100 nM BPA or all five EACs for 60 days, then treated with serially diluted doxorubicin or tamoxifen at the indicated concentrations for 96 hours. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means; * indicates cells that underwent 4-day EAC detox prior to the treatments soon.

4-day tamoxifen withdrawal, they were shown to less viable upon further tamoxifen treatment than cells that had not been cultured in low-level tamoxifen at all. When treated with E2 or BPA alone, tamoxifen-cultured cells were less viable than control cells, suggesting that low-level tamoxifen treatment was effective at downregulating estrogen responsiveness over time.

Results for cells cultured for 60 days in 100 nM tamoxifen plus 100 nM of all five EACs are shown in Figure 51. When EACs were present with tamoxifen in culture, the effects on subsequent drug responses were different from those for tamoxifen culture alone. Cells were much more viable when treated with doxorubicin after extended EAC/tamoxifen culture. Doxorubicin sensitivity was restored close to control levels after four days of EAC/tamoxifen withdrawal. Extended EAC/tamoxifen culture also led to increased viability upon subsequent tamoxifen treatment. Tamoxifen sensitivity in these cells was markedly restored after a 4-day withdrawal from EAC/tamoxifen, rendering them less viable in the presence of tamoxifen than cells that had not been exposed to EACs and tamoxifen prior.

Whereas 60-day treatment in tamoxifen alone was effective at reducing estrogen responsiveness in ER+ breast cancer cells, the addition of five EACs to extended tamoxifen culture made cells more responsive to E2 or BPA (Figure 51B). This model for assessing extended exposures is most realistic for clinical consideration, given that patients on tamoxifen are also likely exposed to EACs concurrently. The intended therapeutic effect on ERα antagonism by tamoxifen may be altered by the presence of EACs, making them suspects in mechanism of acquired tamoxifen resistance.

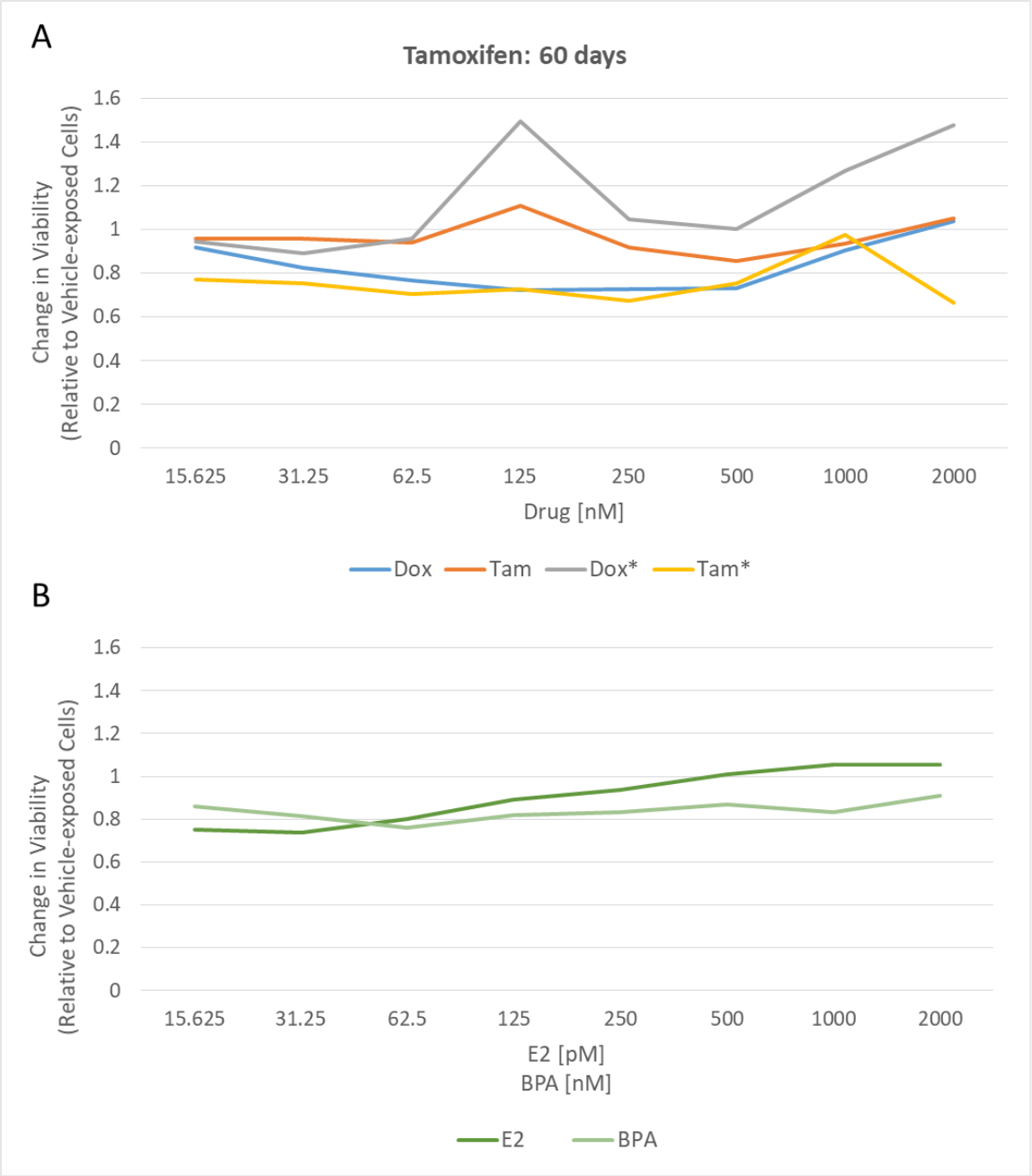


Figure 50: Viability Effects of 60-day Low-level Tamoxifen Culture on Subsequent Responses to A: Doxorubicin or Tamoxifen, and B: E2 or BPA in MCF7 Cells. Cells were cultured in 100 nM tamoxifen for 60 days, and then treated with serially diluted doxorubicin, tamoxifen, E2, or BPA at the indicated concentrations for 96 hours. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means; * indicates cells that underwent 4-day EAC detox prior to the treatments soon.

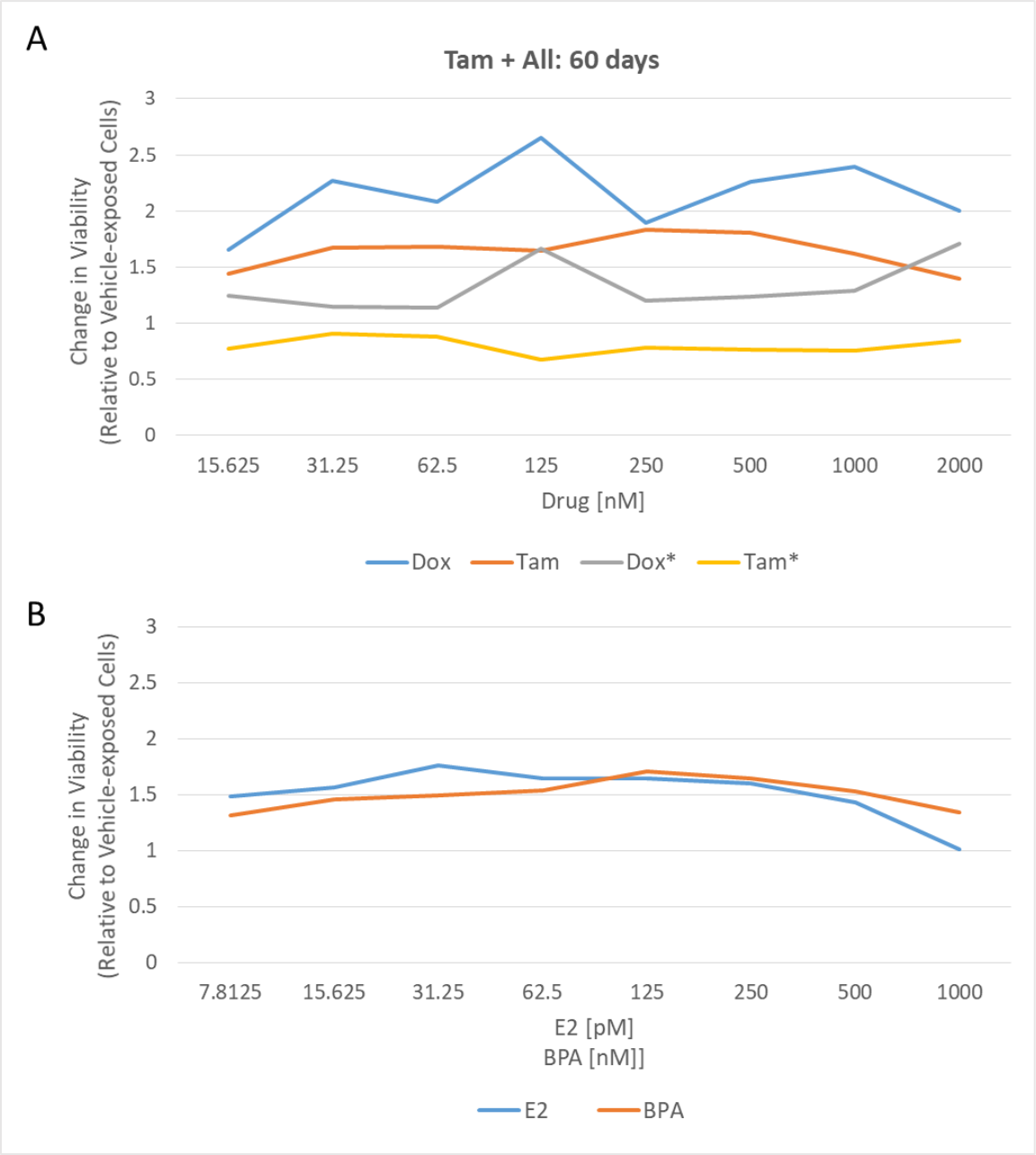
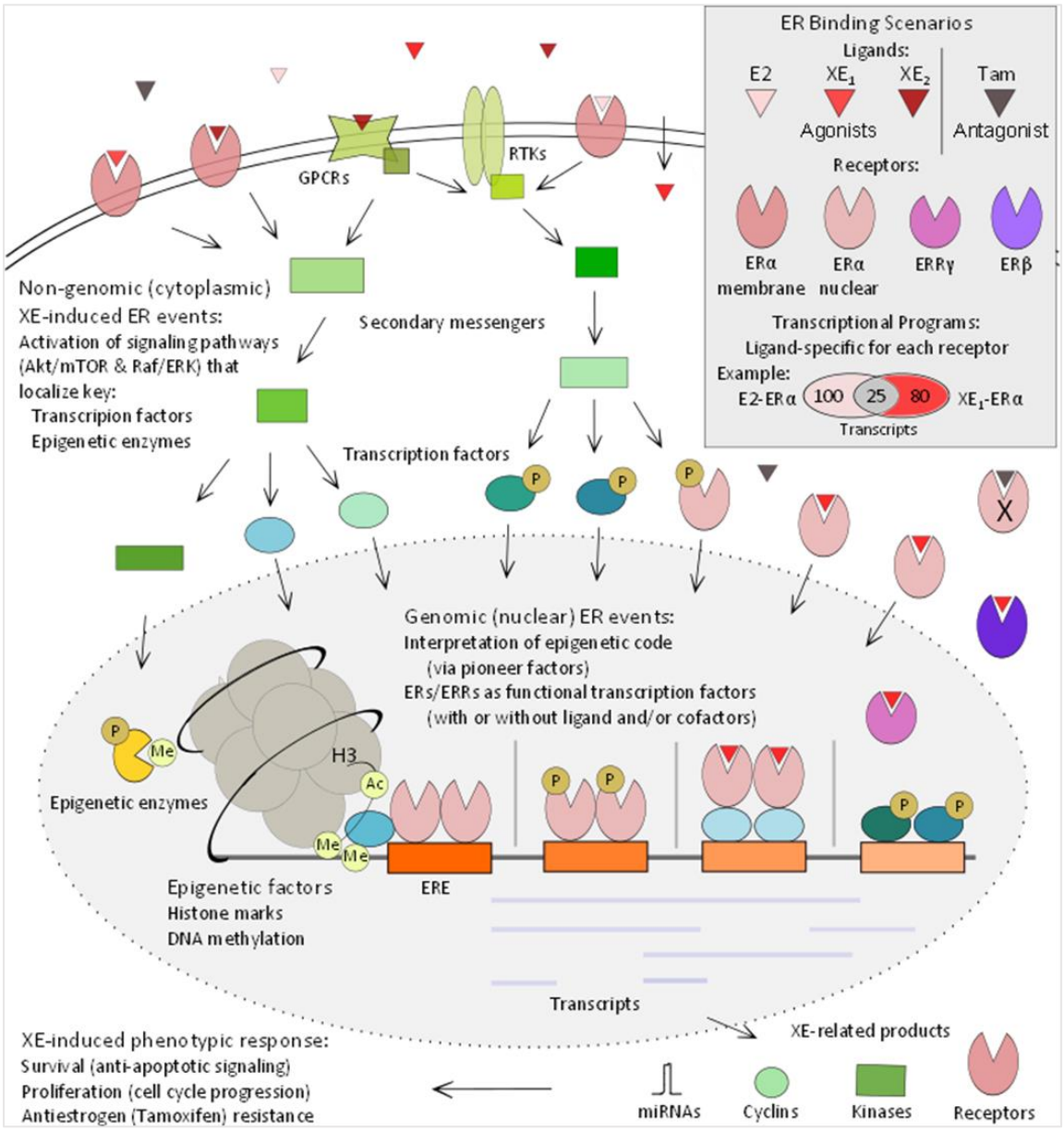


Figure 51: Viability Effects of 60-day Low-level Tamoxifen Culture on Subsequent Responses to A: Doxorubicin or Tamoxifen, and B: E2 or BPA in MCF7 Cells. Cells were cultured in 100 nM tamoxifen and 100 nM of all five EACs for 60 days, and then treated with serially diluted doxorubicin, tamoxifen, E2, or BPA at the indicated concentrations for 96 hours. Changes are relative to vehicle-control cells (0.1% v/v DMSO). All standard deviations are within 5% of triplicate means; * indicates cells that underwent 4-day EAC detox prior to the treatments shown.

Chapter 6: Impact and Future Directions

Research within the last decade has indicated that EACs can play critical roles in the etiology and progression of breast cancers (Sokolosky and Wargovich, 2012). Human exposure is widespread, with EACs measured in urine, blood, semen, placenta, and breastmilk (Khanna *et al*, 2014). To better characterize *in vitro* cellular responses to environmental estrogens, this study explored single EACs and mixtures at concentrations relative to human exposure. Via high-throughput analyses of interrelated pathways known to be involved in breast cancer response to estrogenic compounds, this study links mechanistic information to phenotypic outcomes, including drug effectiveness. By examining multiple cell lines, multiple EAC exposures, and multiple markers and endpoints, this study warrants an examination of these mechanistic pathways within *in vivo* breast cancer models.

A proposed mechanism for ER+ breast cancer cell responses to EACs, specifically xeno-estrogens (XEs), is shown in Schema 1. Theoretically, a mixture of ERα ligands, including endogenous hormones such as E2, combinations of low-level EACs/XEs, and tamoxifen could be present in a breast cancer microenvironment. Multiple receptor types at the cell membrane, including hormone receptors like ERα and ERβ, G-protein coupled receptors such as GPER, and receptor tyrosine kinases can be activated by XEs. Via receptor binding and/or intracellular transport, XEs can then activate secondary signaling messengers in the cytoplasm, including kinase pathways such as P-Akt/mTOR and Raf/P-MEK/P-ERK.



Schema 1: Overall Mechanism of Signaling and Epigenetic Pathways Underlying the Observed Phenotypic Effects of EACs in MCF7 Cells; in this schematic, EACs are indicated with the abbreviation 'XE' for xeno-estrogen to reference their estrogenic activities

These phosphorylated kinases can regulate downstream transcription factors such as β-catenin and c-Myc, some of which localize in the nucleus, along with activated hormone receptors such as P-ERα S167 and ERRγ. Gene transcription effects are mediated there by epigenetic enzymes including DNMT3 and LSD1, the latter of which can later histone lysine methyl marks at H3K4, H3K9, and H3K27. Epigenetic marks are then interpreted and accessible ERE regions of DNA are bound by transcription factors, in some cases assisted by cofactors. Previous work also found epigenetic regulators to be influenced by EACs (Singh and Li, 2012).

The resulting XE-induced mRNA transcripts are translated into additional cellular regulators including protein kinases and receptors, or maintained as miRNAs. Gene and protein expression profiles for E2 and EACs/XEs are similar but not identical, yielding a Venn diagram-like perspective for the effects of endogenous and exogenous estrogens on breast cancer cells. These dynamic, time-dependent, and parallel mechanisms could explain the genomic and phenotypic responses observed in ER+ breast cancer cells in this study.

Effects of a single compound are not indicative of mixed exposures, some of which can weaken cellular responses to EACs. Although certain mixtures can be additive, upregulation of steroid hormone metabolism may perform negative feedback to keep proliferation in check in the presence of mixtures of estrogens (Silva *et al*, 2011). When compounds with relatively low estrogenic activity were included in a mixture with more estrogenic EACs, they modulated the mixture effects by reducing the overall cell-based response (Evans *et al*, 2012).

This work demonstrates that EAC responses are dose-dependent and mixture-dependent in certain cell types. Although it is valid for basic research to understand the effects of single EACs, it is important to further explore mixture effects in an effort to model realistic clinical scenarios. This study also demonstrates the importance of assessing EACs at low-levels (nanomolar concentrations) relevant to those measured in human biofluids at ng/ml levels and breast tissues at ng/g (Barr *et al*, 2012). These concentrations were functionally-relevant in previous *in vitro* studies, in which MCF7 cells were shown to proliferate in the presence of nanomolar levels of parabens, with these levels set based on paraben concentrations measured in human ER+PR+ tumor biopsy tissues (Charles and Darbre, 2013).

In most cases in this study, low-level EAC treatments produced more pronounced viability effects than higher levels. Low-level EACs were also just as effective as higher levels at regulating key signaling and epigenetic mechanisms. Despite 1000-fold lower ERα-binding affinity, low nanomolar levels of PP were shown here to induce proteomic expression profiles in MCF7 cells that may result from mechanisms other than acting as a ligand. The estrogenic activity of EACs extends beyond their ability to bind ERα and into abilities to regulate PTMs of ERα, allowing EACs to regulate P-ERα localization and related signaling pathways. Through its ability to localize within different compartments of a breast cell, ERα can act as a cytoplasmic signaling mediator when membrane-localized (Zivadinovic *et al*, 2005), as well as a transcriptional regulator when found in the nucleus.

EACs can influence ERα activity, as well as activity of other related receptors and kinases at many points along the mechanism shown in Schema 3, and the outcomes

depend on timing, dose, and location of exposure. The low-level effects observed in this study can be compared to an *in vivo* breast cancer study in which different BPA doses were tested, but only low doses increased tumor growth, burden, and metastatic activity in rodents. Mechanistically, 2.5 ug/L BPA phosphorylated erbB2, erbB3, IGF-1, and Akt, but 2500 ug/L BPA did not. High-level BPA exposure did not significantly influence breast tumor development (Jenkins *et al*, 2011).

The β-catenin/Wnt, Ras/Raf/MEK/ERK and Ras/PI3K/Akt/PTEN/mTOR pathways play well-established roles in breast cancer (Davis, Sokolosky *et al*, 2014), and this study shows that EACs can influence these pathways. Alteration of signaling pathways including the Ras/PI3K/PTEN/Akt/mTOR cascade is heavily implicated in breast cancer resistance to tamoxifen and chemotherapy drugs (Sokolosky *et al*, 2011). Counter-intuitively, ER+ breast cancer cells can stop responding to antiestrogens like tamoxifen, whether via *de novo* or acquired mechanisms (Arpino *et al*, 2009). Epigenetic alterations have also been implicated in the development of *de novo* tamoxifen resistance in breast tumors, including ER+ types (Raha *et al*, 2011). It was shown that BPA can compete with 4OH-tamoxifen for ER binding, the effect of which was described as "estrogenic rescue" against drug cytotoxicity in MCF7 cells (Lewis *et al*, 2000). The mechanistic results of this present study strongly support roles for EAC-induced signaling and epigenetic alterations in the development of tamoxifen resistance in breast cancer cells.

These results are relevant to previous findings in studies of clinical breast cancer samples. BPA upregulated evasion of apoptosis and cell-cycle deregulation in breast cells from patients with high-grade tumors and poor outcome (Dairkee et al, 2008). BPA treatment

activated P-Akt in MCF7 cells in the present study, a mechanism also found in a study of high-risk donor breast epithelial cells (HRBECs) that underwent activation of PI3K/Akt/mTOR and increased proliferation in the presence of low-level BPA. When pretreated with BPA or MP, HRBECs were shown to be more resistant to the apoptotic effects of tamoxifen (Goodson *et al*, 2011).

These findings suggest that EAC-exposure may play roles in *in vivo* breast cancer development, and future studies in both animal models and clinical settings are warranted. Given the mechanistic basis for effects of EACs in cells presented here, these high-throughput proteomic screens can be applied to primary cultures of treated human breast cancer cells, followed by mammary cells from exposed rodents. If key markers consistently emerge as playing roles in EAC-induced effects, they should then be examined in breast tissue and tumor biopsies from patients undergoing clinical care and drug treatments.

In time and with more research, it may be valid to explore lifestyle modifications in breast cancer patients, involving minimizing and preventing further EAC exposures. Although this study presents preliminary *in vitro* results for the effects of EAC-detox on tamoxifen response, it was notable to find that cells could overcome acquired tamoxifen resistance with only a four-day withdrawal period before drug treatments. Given that almost half of ER+ breast cancer patients on tamoxifen eventually develop resistance, and the same signaling pathways identified in this study are implicated in acquired tamoxifen resistance (Sokolosky *et al*, 2011), it is possible that EAC exposure plays a vital role in clinical response to therapy. By continuing mechanistic research, moving to

animal studies, followed by potential translational applications, it is possible to educate scientists and physicians about EACs. With a concerted effort from experts in many fields, the roles of environmental exposures in human health and disease will become increasingly clear, along with awareness for cancer patients and the general public. By understanding the underlying genetic and environmental causes of cancer, we can more effectively develop treatments. Most importantly, we can use this knowledge to focus on preventing disease before it even begins.

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