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PREVENTING ESTROGEN RECEPTOR ALPHA-POSITIVE BREAST CANCER OUTGROWTH WITH THE USE OF HORMONE REPLACEMENT THERAPY

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ABSTRACT

Menopause occurs in all women, typically between the ages of 45 and 55. As a result of menopause, women often experience undesirable symptoms such as hot flashes, vaginal dryness and atrophy, and osteoporosis. These symptoms can be alleviated with hormone replacement therapy (HRT). The Women's Health Initiative (WHI) trial concluded that PremPro, a HRT formulation that combines conjugated equine estrogens (CE) with medroxyprogesterone acetate, increases the risk of breast cancer. Over the years, the number of women taking HRT has dramatically decreased due to the perceived risk based mainly on the results of the WHI trial. Follow-up studies suggest that breast cancer cases from PremPro treatment were primarily due to the outgrowth of undetectable occult tumors, not the formation of new disease. Duavee, a more recent form of HRT that combines CE and bazedoxifene (BZA), a selective estrogen receptor modulator (SERM) and degrader (SERD), has been approved by the FDA for treatment of hot flashes and to reduce the risk of osteoporosis. More importantly, this CE+BZA mixture not only relieves symptoms associated with menopause, but it also does not stimulate the breast or endometrium. Several preclinical studies suggest that CE+BZA might be protective in the breast; however, the mechanism of action of this new combination therapy is not known. Our goal, therefore, is to elucidate the underlying molecular mechanisms by which CE+BZA differentially affects estrogen receptor alpha (ERα) action in the mammary gland, using transcriptome and cistrome analysis in breast cancer cell lines. RNA-Seq and ChIP-Seq studies suggest that CE+BZA increases pathways related to cell death and decreases activation of pathways related to cell proliferation and survival. BZA was found to exert its action through the aryl hydrocarbon receptor (AHR) pathway, further clarifying the role of AHR in breast cancer. We are also studying the effects of CE+BZA on early mammary cancer progression in the estrogen-sensitive polyoma middle T antigen (PyMT) transgenic mouse model and have observed that treatment with CE+BZA delayed the onset of tumors and decreased their rate of growth. Mice treated with CE+BZA also survived longer. In addition, CE+BZA was able to decrease the rate of tumor

growth in an ERα-positive patient-derived xenograft (PDX) mouse model. An improved understanding of the molecular mechanisms of CE+BZA action in hormone-sensitive breast cancer cell and animal models should have important implications for women considering HRT and for physicians prescribing it.

CHAPTER I: INTRODUCTION AND BACKGROUND

Menopause

Menopause occurs in all women as part of the natural aging process. Studies suggest that in developed nations, the median age of menopause is approximately 48 to 52 years of age, and around 49 years in the United States.[1,2] Menopause onset is caused by cessation of hormone production by the ovaries and is defined by loss of menstruation.[3] Due to a decrease of estradiol levels in the body, many women experience vasomotor symptoms (commonly known as hot flashes), vaginal dryness and atrophy, osteoporosis, difficulty sleeping and fatigue. All menopausal symptoms have a negative effect on quality of life. Hot flashes are the symptom that is most bothersome to women and about 50% of women report symptoms.[4] Women experience hot flashes for an average of five years after menopause, and up to ten years in about one third of women.[5] Both pharmaceutical and homeopathic treatments are available to alleviate various menopausal symptoms, including hot flashes.

Breast Cancer

Women of menopausal age and older are also at a higher risk for developing breast cancer. In the United States, breast cancer has the highest incidence of any cancer in women; it is estimated that about 268,600 women will be diagnosed with breast cancer in 2019 alone. Breast cancer is also the second-leading cause of cancer-related death in women in the United States and it is estimated that about 41,760 women will die due to breast cancer in 2019.[6] Still, breast cancer survival rates have improved in the United States over the past few decades due to screening and earlier detection; studies show that survival at five and years post diagnosis is 91% and 86%, respectively.[7] Recurrence, unfortunately, can occur in patients at a rates of 10-20%

within ten years.[8,9] Most breast cancers, over 75%, express estrogen receptor alpha (ERα) and have a relatively good prognosis.[10] ER-positive breast cancer treatment depends on the grade and stage of disease, but usually involves a combination of surgery, radiation and endocrine therapy. In addition to diagnosed cases, autopsy studies estimate that 7% of women between the ages of 40 and 80 have undiagnosed occult ductal carcinoma in situ (DCIS, a non-invasive form of breast cancer) and about 1% of women have invasive carcinoma.[11–13]

Estrogen Receptors

ER-positive breast cancers account for over 75% of all breast cancer cases.[10] Estrogen receptors are members of the nuclear receptor family of transcription factors and contain distinct domains: DNA-binding domain, ligand binding domain, and two activation function regions (AF1 and AF2) where co-factor interactions occur.[14,15] There are two estrogen receptors, ERα and ERβ, which are the products of two distinct genes that are located on different chromosomes (6q25.1-q25.2 and 14q23.2-q23.3, respectively), and several isoforms of both have also been described.[15–17] ERα and ERβ are expressed in tissues throughout the body, however their expression is often at different levels, depending on the tissue. The two ERs have distinct, nonredundant physiological roles and can even have opposing actions on the same gene.[15,18] Of the two receptors, ERα has been much better studied and is the focus of this study as well.[19,20] Also, by convention, ERα expression is used to identify breast cancers as ER-positive. Binding of ligand facilitates translocation of ERα to the nucleus where it binds as a dimer to DNA and/or chromatin and modulates transcription. Transcriptional activation, or repression, requires binding of co-regulators, which are recruited by ERα and can function as co-activators or co-repressors. A consensus DNA binding sequence for ERα has been determined (GGTCA), although ERα binding is not limited to this sequence.[21,22] ERα itself can interact with other transcription factors

to modulate gene expression. ERα can also signal without ligand being present through phosphorylation of the receptor and subsequent dimerization and binding to DNA.[15] Although 17β-estradiol (E2) is the most potent estrogen produced in the body, many ligands have been discovered and some are used as therapeutics: tamoxifen, bazedoxifene (BZA), conjugated equine estrogens (CE), fulvestrant, etc. Throughout a woman's life, E2 levels cycle during menstruation and decrease during menopause. Most menopausal symptoms occur due to the decreased level of E2.[3,22]

ER-positive breast cancer is most often treated with antiestrogen or estrogen ablation therapy. The current first line therapy for ER-positive breast cancer in post-menopausal women is an aromatase inhibitor.[23–25] Aromatase inhibitors such as anastrozole, letrozole and exemestane, inhibit the enzymatic conversion of testosterone to estradiol.[15] Tamoxifen, a selective estrogen receptor modulator (SERM) was a first line therapy for many years prior to aromatase inhibitors. Tamoxifen is an effective treatment due to its action as an antagonist in the breast and an agonist in the bone. In the endometrium, tamoxifen is a partial agonist, therefore there is a small risk of endometrial cancers for women taking it.[26,27] ER antagonists, such as fulvestrant, are also used to treat ER-positive breast cancer. Fulvestrant is a selective estrogen receptor degrader (SERD), often referred to as a pure ER antagonist, which promotes degradation of ER upon binding. Although various therapies exist, resistance to therapy is unfortunately seen in patients. One such pathway of resistance is though ERα mutations in the ligand binding domain.[28]

Hormone Replacement Therapy

For women going through menopause, hormone replacement therapy (HRT) can be used to relieve vasomotor symptoms and improve quality of life. CE has been used for hormone

replacement therapy for many years, as a single agent (Premarin) as well as in combination with medroxyprogesterone acetate (MPA) in PremPro.[29,30] CE can only be used in women who have had a hysterectomy due to stimulation of endometrial tissue. CE contains a mixture of estrogenic components that can bind to ERα and ERβ (Table 1). 17β-estradiol (E2), the primary biological ligand for ER, makes up less than one percent of the total CE mixture but is still one of the ten most abundant components. The unique mechanism of action exerted by CE is most likely a result of combined activities of all its components, and may be tissue specific, like that of selective estrogen receptor modulators (SERMs). Biochemical and cell-based studies have shown that each of the top ten CE components can induce a different conformation of ERα, thereby recruiting a different set of ERα co-regulators.[31] Gene regulation studies have suggested that CE components have SERM-like effects because CE components share more differentially regulated genes with SERMs than E2 does.[32]

PremPro, an HRT formulation that is still in use, contains conjugated equine estrogens (CE) and medroxyprogesterone acetate (MPA). As previously mentioned, CE alone can relieve menopausal symptoms, but stimulates the endometrium, leading to an increased risk of endometrial cancer. MPA is able to counterbalance the increase of endometrial cell proliferation by inhibiting several E2-stimulated pathways, and decreasing endometrial stimulation.[33,34] For women taking HRT, concerns arose after the Women's Health Initiative (WHI) trial found that long-term use of CE+MPA was linked to a significant increase in breast cancer mortality.[35,36]

WHI trial

In 2002, the WHI study revealed that the then standard of care CE+MPA (PremPro) was associated with an increased incidence and higher risk of mortality due to breast cancer.

Compound	% of mixture
Estrone	48
Equilin	24
17α-Dihydroequilin	15
8,9-Dehydroestrone	4.3
17α-Estradiol	3.8
17β-Dihydtoequilin	1.8
Equilenin	1.1
17β-Estradiol	0.68
17α-Dihydroequilenin	0.45
17β-Dihydroequilenin	0.3

Table 1: Top compounds in CE mixture

However, women who had undergone a hysterectomy and could therefore take CE alone experienced a protective effect in the breast: there was a small, but significant decrease in breast cancer incidence and mortality.[37–39] The reason why CE has a protective effect in the breast is not known; some hypothesize that CE components may have both agonistic and antagonistic effects.[40] Since the results of the WHI trials were reported, many women have stopped using HRT both on their own accord and based on the advice of their physician.[38,41]

Studies suggest that the increase in tumors seen in the WHI trial was due to tumor outgrowth, not initiation. Autopsy studies on women 40 to 80 years of age, who died of unrelated causes, report that an average of seven percent have undiagnosed, or occult, breast cancer.[11–13,42,43] Most likely, these lesions are not detectable due to their small size; mammography cannot detect tumors smaller than approximately one centimeter in diameter. Santen and colleagues suggest that tumor doubling time ranges from ten to over 700 days, with a median of 200 days.[42,43] Based on their model, they were able to closely mimic the results of the WHI trial: women receiving placebo had a breast cancer incidence of about 2.38%, while those in the CE+MPA group had an incidence of 2.99%.[42] Based on the time it took for the tumors to be detected, Santen and colleagues suggest that CE+MPA was actually stimulating the outgrowth of tumors that existed before the start of the trial, not initiating new tumors.

CE+BZA combination therapy (Duavee)

Due to the significant decrease in HRT use after the WHI trials, a need arose and exists to this day for a new form of HRT that will alleviate menopausal symptoms without stimulating the breast or endometrium. Tissue selective estrogen complexes (TSECs), which often combine an estrogenic component with a selective estrogen receptor modulator (SERM), have been in

development for some time. Duavee, a combination of CE and the SERM bazedoxifene (BZA), was approved by the Federal Drug Administration in 2013 for the treatment of symptoms associated with menopause. Classical SERMs, like BZA, tamoxifen and raloxifene, can act as estrogen agonists or antagonists. This agonist or antagonist state depends on the tissue, relative expression of ERα and ERβ, conformational changes of the receptor upon ligand binding, and differential expression of receptor coregulators. SERMs alone can inhibit osteoporosis and act as antagonists in the breast, but they do not alleviate hot flashes, vaginal atrophy or sleep disturbances. In fact, SERMs can contribute to these problems.[31,32,44,45] BZA can bind both ERα and ERβ in multiple tissues: breast, bone, uterus, and vascular endothelium. BZA is an agonist in the bone and an antagonist in the breast, similar to other classical SERMs. Additionally, BZA does not stimulate endometrial tissue.[40]

Studies on the CE+BZA combination have been performed both *in vitro* and *in vivo*. The *in vitro* studies were primarily done in MCF-7 cells, an ERα-positive breast cancer cell line. These studies concluded that BZA indeed halted the stimulatory effects of CE by inhibiting cell proliferation, increasing apoptosis, decreasing DNA synthesis and gene expression. When compared to other SERMs (raloxifene and lasofoxifene), BZA was more effective at inhibiting gene expression upregulated by CE.[44] Genes that were downregulated by CE were found to be unaffected by BZA, leading authors to believe that BZA and CE target different pathways.[32,46,47] Studies in ovariectomized mice determined that BZA inhibited CE-induced proliferation and ductal branching better than other SERMs.[44] BZA was also able to block estrogen-stimulated gene expression and tumor growth in nude mice with MCF-7 xenografts.[48] In addition, preclinical studies were performed in ovariectomized cynomolgus macaques, in which BZA was able to

antagonize the stimulatory effects of CE on the breast (Ki67 expression, proliferation, cell cycle progression and ERα activity), and BZA treatment alone was most similar to vehicle treatment.[49]

Clinical trials have tested the effects of CE+BZA in women. The Selective estrogens, Menopause, And Response to Therapy (SMART) trials evaluated the effect of CE+BZA in healthy postmenopausal women with an intact uterus. The results of these trials included a decreased number and severity of hot flashes, increased bone density, as well as significant improvements in sleep and overall quality of life.[50–54] These trials, which also revealed a low incidence of endometrial hyperplasia,[55,56] showed that CE+BZA combination therapy was a safe and effective treatment for hot flashes.

Due to the short duration of the SMART trials, it was not possible to determine the long-term safety of CE+BZA in the breast and it was not possible to speculate on breast cancer risk. Additional studies are needed to test the hypothesis that CE+BZA does not increase the incidence of breast cancer and that it may in fact decrease breast cancer risk. In collaboration with other institutions, we are participating in a window of opportunity clinical trial to compare the effect of CE+BZA on breast tissue in women undergoing surgical therapy for ductal carcinoma in situ (DCIS). This trial enrolls postmenopausal women who are diagnosed with DCIS and will undergo surgery. The women are randomized to receive either placebo or CE+BZA for 28±7 days prior to surgery and several endpoints will be evaluated: Ki-67, epithelial and stromal markers, ER expression, quality of life, as well as others.

In the study presented here, CE+BZA has been studied in preclinical models that include cell lines and mouse models. The molecular mechanism of action of CE, BZA and the combination has been evaluated in cell lines through whole genome transcriptomic and cistromic

analysis. The effect of CE, BZA and the combination have also been evaluated in various mouse models to determine effect on tumor growth and survival.

It is essential to better understand the underlying mechanisms of CE+BZA action in ER-positive breast cancer cells as well as its effects on breast cancer development. The results of this study, in conjunction with the window of opportunity clinical trial, will allow women and physicians to make a more educated choice about the appropriate course of action in treating menopausal symptoms. This study also sheds light on the mechanism of action of CE+BZA and provides women with a safer means to alleviate menopausal symptoms. Other drugs, such as tamoxifen and raloxifene, have already been approved for reducing the risk of breast cancer.[57] However, because these drugs elicit and/or exacerbate many menopausal symptoms, they are not ideal and have not been widely accepted for this use. Thus, CE+BZA treatment fills an unmet need for a drug combination that will both alleviate menopausal symptoms and reduce the risk of breast cancer. It is also possible that CE+BZA can be used as a treatment for breast cancer prevention in premenopausal women who have been identified as being at a higher risk for breast cancer, or as HRT in women who are breast cancer survivors.

CHAPTER II

DUE TO ITS ACTION AS AN ANTAGONIST, CE+BZA ALTERS ERα BINDING TO DNA, THEREBY DECREASING GENE EXPRESSION AND CELL PROLIFERATION

Background

Previous studies on CE and BZA have made progress into understanding their mechanisms of action, yet these studies have had limited scope. In one study, various SERMs were evaluated for their ability to antagonize CE-mediated breast cancer cell proliferation, in which BZA was most effective. BZA was also found to suppress signaling pathways upregulated by CE, including those involved in cell cycle regulation, growth hormones and growth factors. Signaling pathways downregulated by CE treatment were, surprisingly, not affected by BZA treatment. Additionally, BZA shared more differentially regulated genes with CE than with E2, suggesting that some of the components present in the CE mixture may possess SERM-like behaviors.[32] BZA was able to block the effects of CE on cell proliferation, overall cell growth, gene expression and protein expression.[46] BZA was also able to inhibit the growth of tamoxifen-resistant cells, and therefore can be used as a therapy in patients with advanced breast cancer.[47] The mechanism of action of CE+BZA has not been evaluated on a global transcriptome level and the global ERα occupancy has not been determined upon CE and BZA treatment. It is essential to understand the molecular mechanism of CE+BZA to confidently treat women suffering from menopausal symptoms in a safe manner.

Results

In order to understand the mechanism of action of CE+BZA, studies using cell lines were performed. Both ERα-positive (MCF7, T47D and ZR-75) and ERα-negative (C4-12, MDA-MB-

231) cell lines were used in this study (Figure 2.1A). Cells were treated with 10nM CE and 400nM BZA to resemble the approximate one to forty ratio of the compounds, respectively, in the Duavee dosage for patients. Cell growth was evaluated using an IncuCyte live cell imager to determine if treatment had any effect on proliferation. Growth of MCF7 cells labeled with nuclear green fluorescent protein (GFP) was assessed by counting green cells; it was found that cells grew faster upon E2 treatment, and slower upon BZA or CE+BZA combination treatment (Figure 2.1B). MDA-MB-231 cell growth, also assessed by counting cells expressing nuclear GFP, was not significantly affected by any treatment (Figure 2.1C). C4-12 cells were evaluated for confluence and no difference was observed between treatments over time (Figure 2.1D).

I next evaluated gene expression in MCF7 cells upon treatment with E2, CE and/or BZA. ERα-responsive genes are often evaluated for their activation to determine if compounds activate ERα and E2 is most often used as a positive control[58]. The genes evaluated in this study were E2F1, GREB1, PDZK1, PGR and TFF1, due to their role in breast cancer. The E2F1 gene, the expression of which is activated by E2 treatment, encodes a transcription factor that regulates many cell-cycle associated genes and is necessary for proliferation of breast cancer cells.[59] The GREB1 gene responds early to E2 treatment and when expression is suppressed, E2-induced proliferation decreases.[60] PDZK1 is indirectly stimulated by E2 and is required for E2-dependent growth of MCF7 cells.[61] PGR encodes the progesterone receptor, a target of ER, which increases proliferation in breast cancer.[62] TFF1, upon E2 treatment, enhances breast cancer growth and proliferation, as well as migration and invasion.[63,64] After 24-hour treatment, both E2 and CE increased expression of all ERα-responsive genes tested, while BZA and combination treatment decreased gene expression, sometimes below the vehicle amount (Figure 2.2). A similar pattern was observed in T47D and ZR-75 cells (Figure 2.3 and 2.4). In ER-negative C4-12 and MDA-

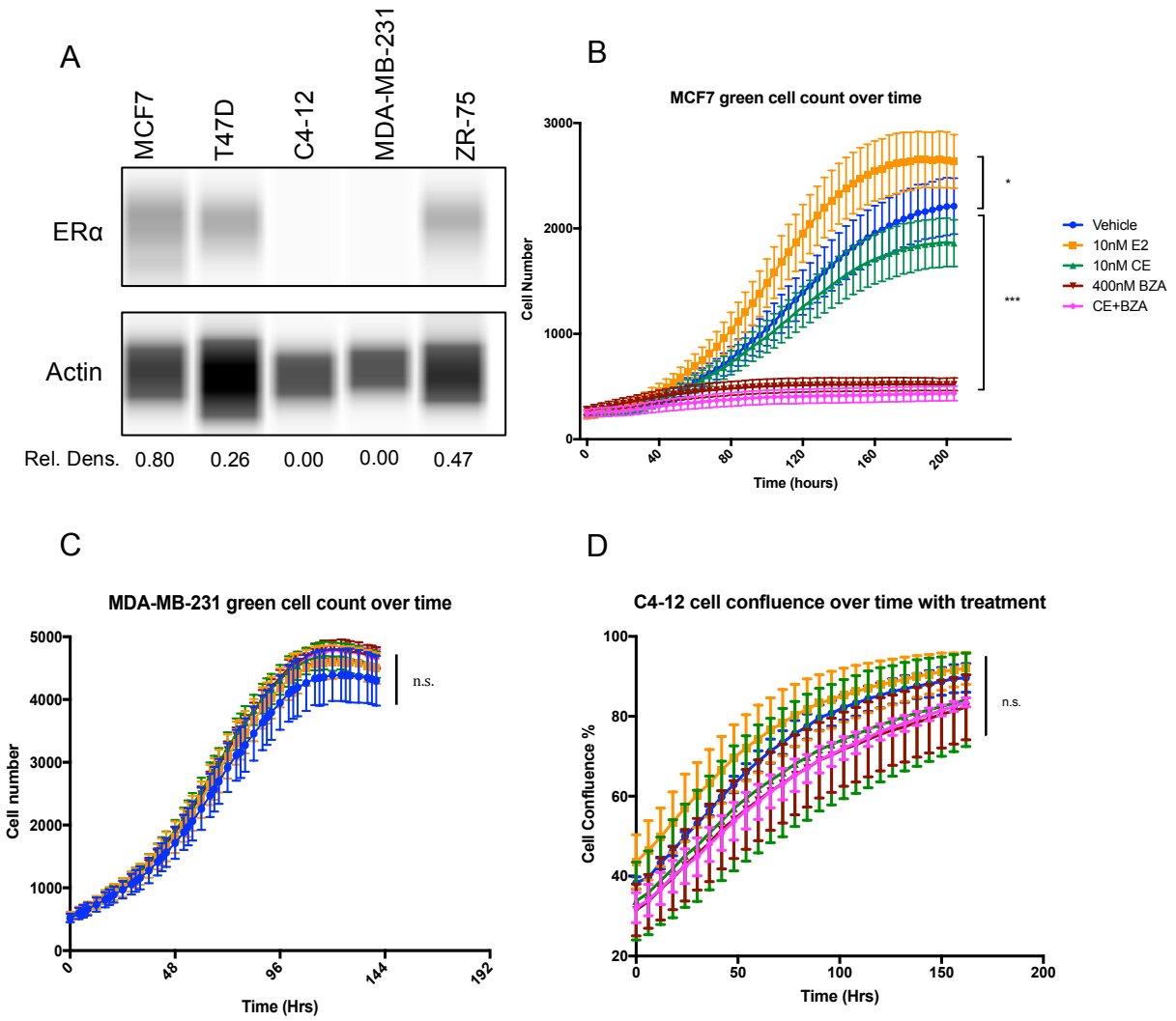


Figure 2.1 CE+BZA treatment slows growth of ER-positive, but not ER-negative cell lines. (A) Western blot showing ERα and actin expression in MCF7, T47D, C4-12, MDA-MB-231 and ZR-75 cell lines along with relative density for each ERα band. (B) MCF7 nuclear GFP cell count over time (hours) upon treatment with vehicle, E2, CE, BZA and CE+BZA. (C) MDA-MB-231 nuclear GFP cell count over time (hours) upon treatment with vehicle, E2, CE, BZA and CE+BZA. (D) C4-12 cell confluence over time (hours) upon treatment with vehicle, E2, CE, BZA and CE+BZA. Data are represented as mean ± SEM; *p<0.05, **p<0.01 by Student's *t* test.

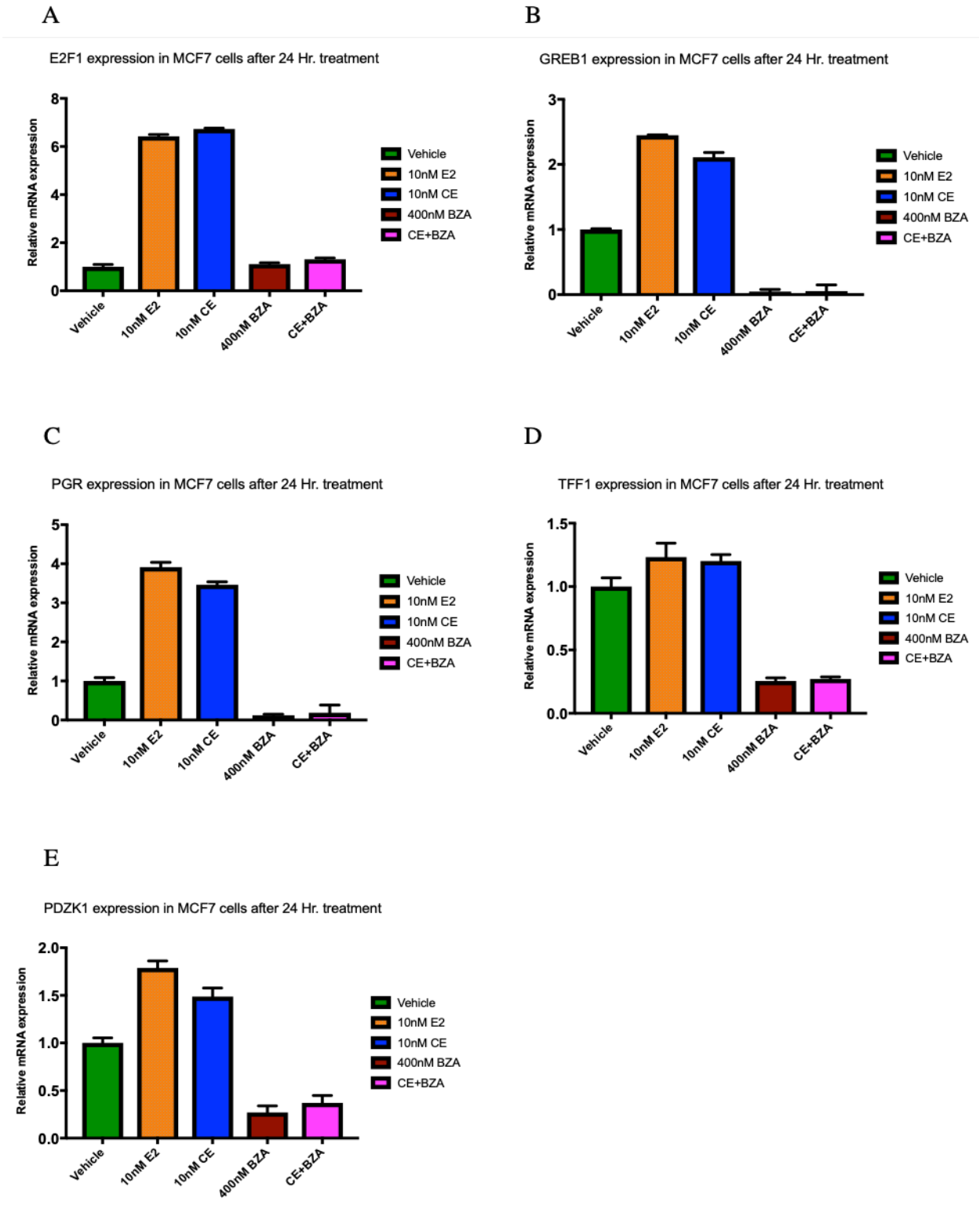


Figure 2.2 Gene expression in MCF7 cells upon 24 hour treatment. 10nM E2 and 10nM CE increased expression of ERα-responsive genes E2F1, GREB1, PGR, TFF1 and PDZK1, while 400nM BZA and CE+BZA decreased expression, compared to vehicle in MCF7 cells. Results from one representative experiment shown.

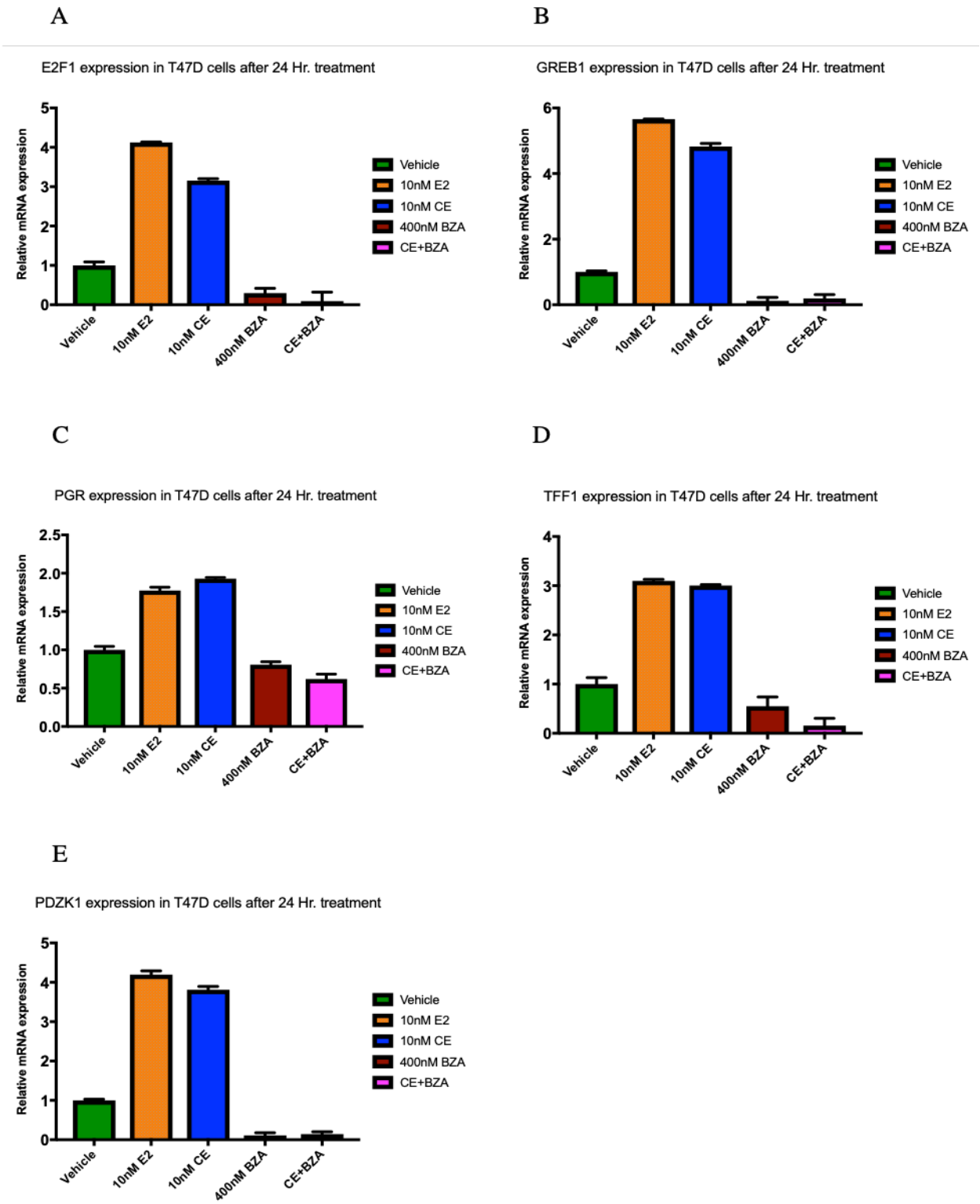


Figure 2.3 Gene expression in T47D cells upon 24-hour treatment. 10nM E2 and 10nM CE increased expression of ERα-responsive genes E2F1, GREB1, PGR, TFF1 and PDZK1, while 400nM BZA and CE+BZA decreased expression, compared to vehicle in T47D cells. Results from one representative experiment shown.

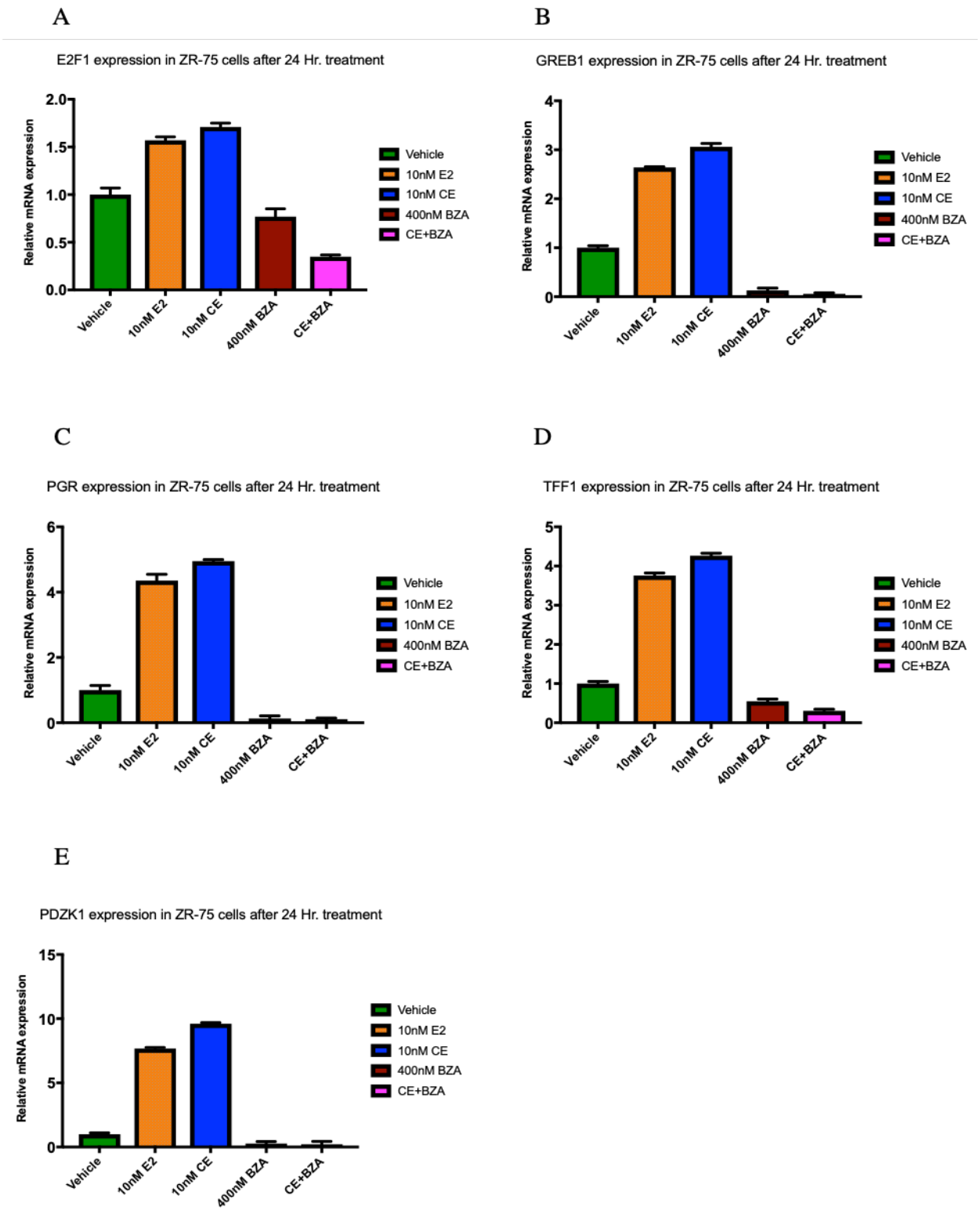


Figure 2.4 Gene expression in ZR-75 cells upon 24-hour treatment. 10nM E2 and 10nM CE increased expression of ERα-responsive genes E2F1, GREB1, PGR, TFF1 and PDZK1, while 400nM BZA and CE+BZA decreased expression, compared to vehicle in ZR-75 cells. Results from one representative experiment shown.

MB-231 cell lines, there was no significant difference observed in gene expression between treatment groups (Figure 2.5).

To determine whether ERα was required for signaling, gene expression was evaluated in MCF7 cells after ERα knockdown. siRNA knockdown reduced ERα expression in MCF7 cells by approximately 65% (Figure 2.6A). In MCF7 cells treated with siRNA against ERα, gene expression was greatly decreased in E2- and CE-treated cells, compared to siRNA scrambled (SCR) control (Figure 2.6B-D). Conversely, BZA and CE+BZA-treated cells exhibited higher gene expression upon siRNA treatment (Figure 2.6B-D). These data suggest that ERα is required for regulating gene expression upon CE and BZA treatment in MCF7 cells.

BZA has been reported to promote partial ERα degradation in MCF7 cells[47]. A time course study was performed to evaluate the expression of ERα at various time points in MCF7 cells. It was determined that BZA did indeed decrease ERα protein expression over time (Figure 2.7D). E2 also induced a decrease in ERα expression over time (Figure 2.7B). CE decreased ERα protein expression at 8 and 24 hours (Figure 2.7C) and CE+BZA treatment resulted in a notable decrease of ERα protein at 24 hours (Figure 2.7E).

Next, ERα occupancy was evaluated at target gene loci. In MCF7 cells, chromatin immunoprecipitation (ChIP) experiments showed that ERα occupancy was higher after E2 and CE treatment, compared to BZA and CE+BZA treatment, for GREB1, TFF1 and PDZK1 loci (Figure 2.8).

MCF7 cells were used for both RNA-seq and ChIP-seq studies. Cells were treated with Vehicle, E2, CE and/or BZA for the studies. Sequencing data was analyzed by Wen-Ching Chan at the University of Chicago Center for Research Informatics. RNA-seq analysis was able to

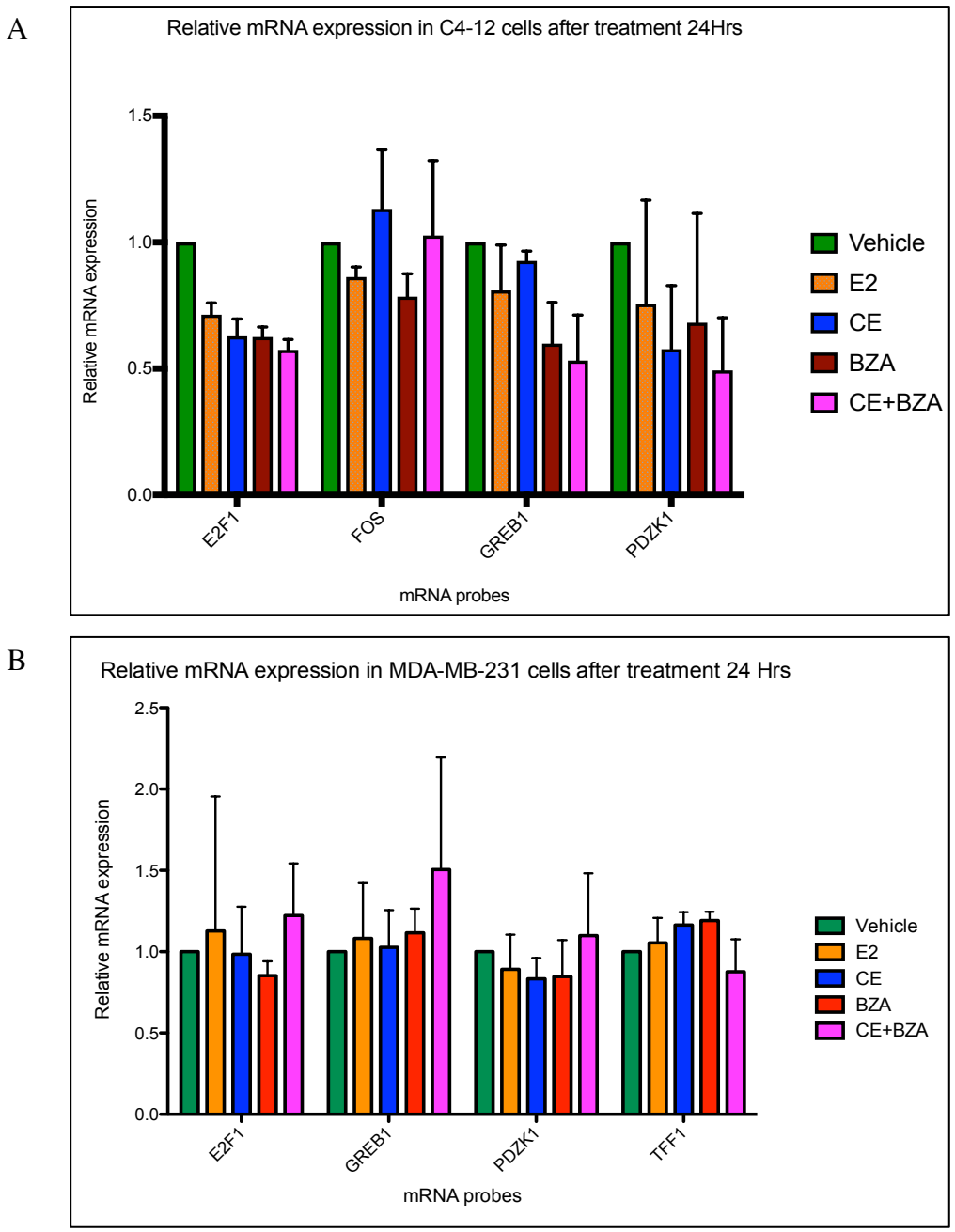


Figure 2.5 CE+BZA does not alter gene expression in ERα-negative cell lines. ER-negative C4-12 (A) and MDA-MB-231 (B) cell lines did not exhibit altered gene expression upon treatment with 10nM E2, 10nM CE, 400nM BZA or CE+BZA, as compared to vehicle after 24 hours. Results from one representative experiment shown.

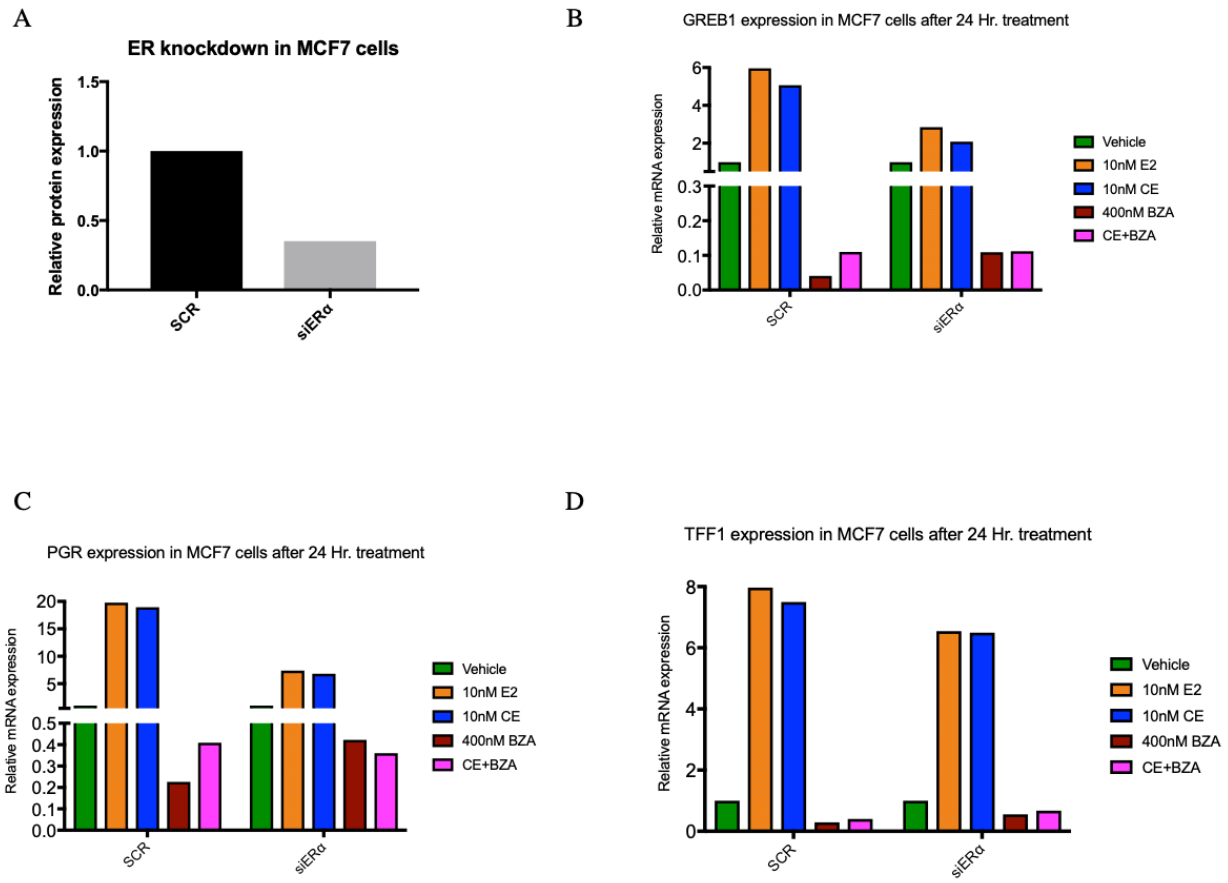


Figure 2.6 ERα is required for CE and BZA. siRNA knockdown in MCF7 cells showed an approximate 60% protein decrease of ERα. (A) ERα knockdown resulted in decreased gene expression in E2- and CE-treated cells. Conversely, ERα knockdown resulted in a higher level of gene expression in BZA- and CE+BZA-treated cells. Results from one representative experiment shown (B-D).

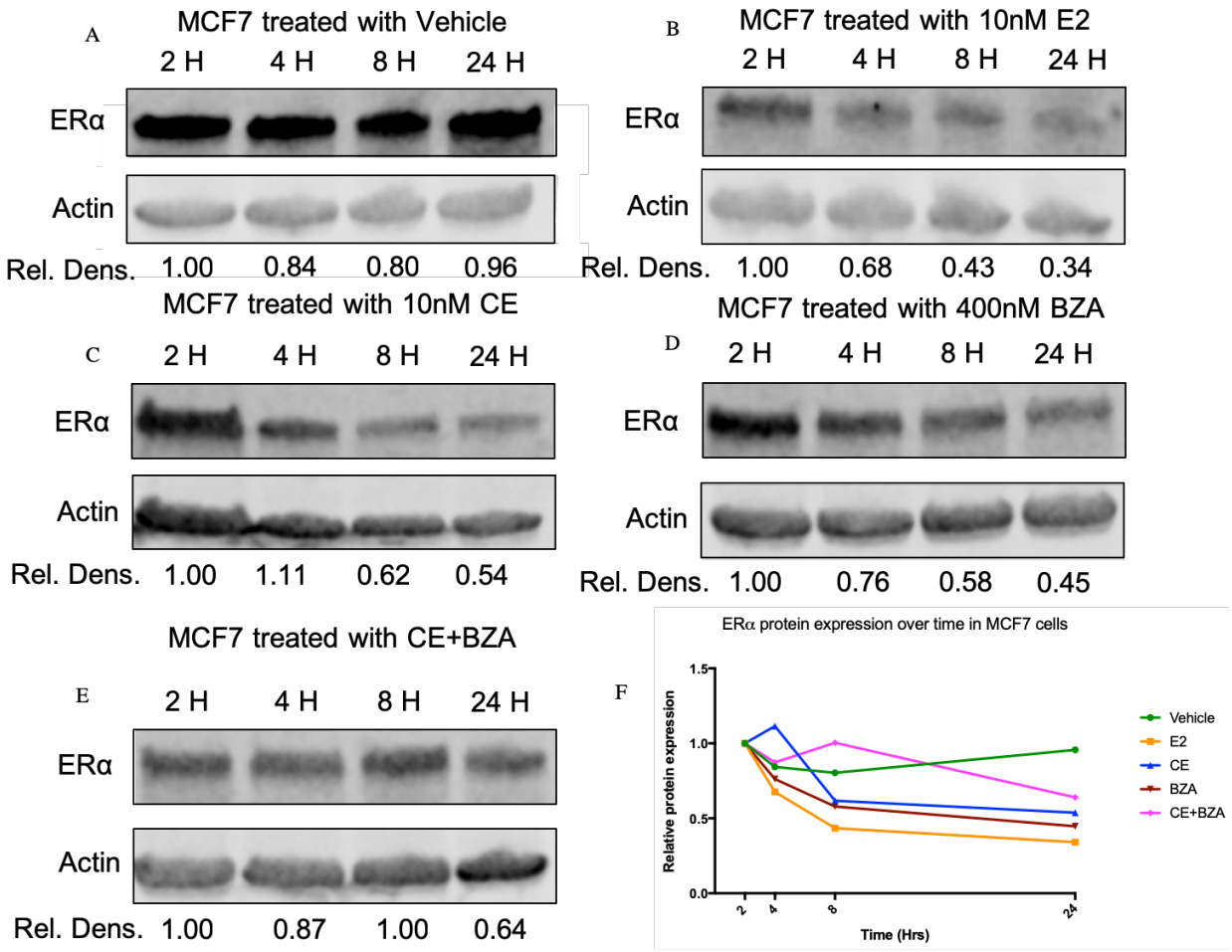


Figure 2.7 Relative ERα protein expression in MCF7 cells with treatment over time. Vehicle treatment did not alter ERα expression over time (A). E2, CE, BZA and CE+BZA treatment all decreased ERα expression over the course of 24 hours (B-E). Graphical representation of relative densities for vehicle, E2, CE, BZA and CE+BZA treatments over time (F).

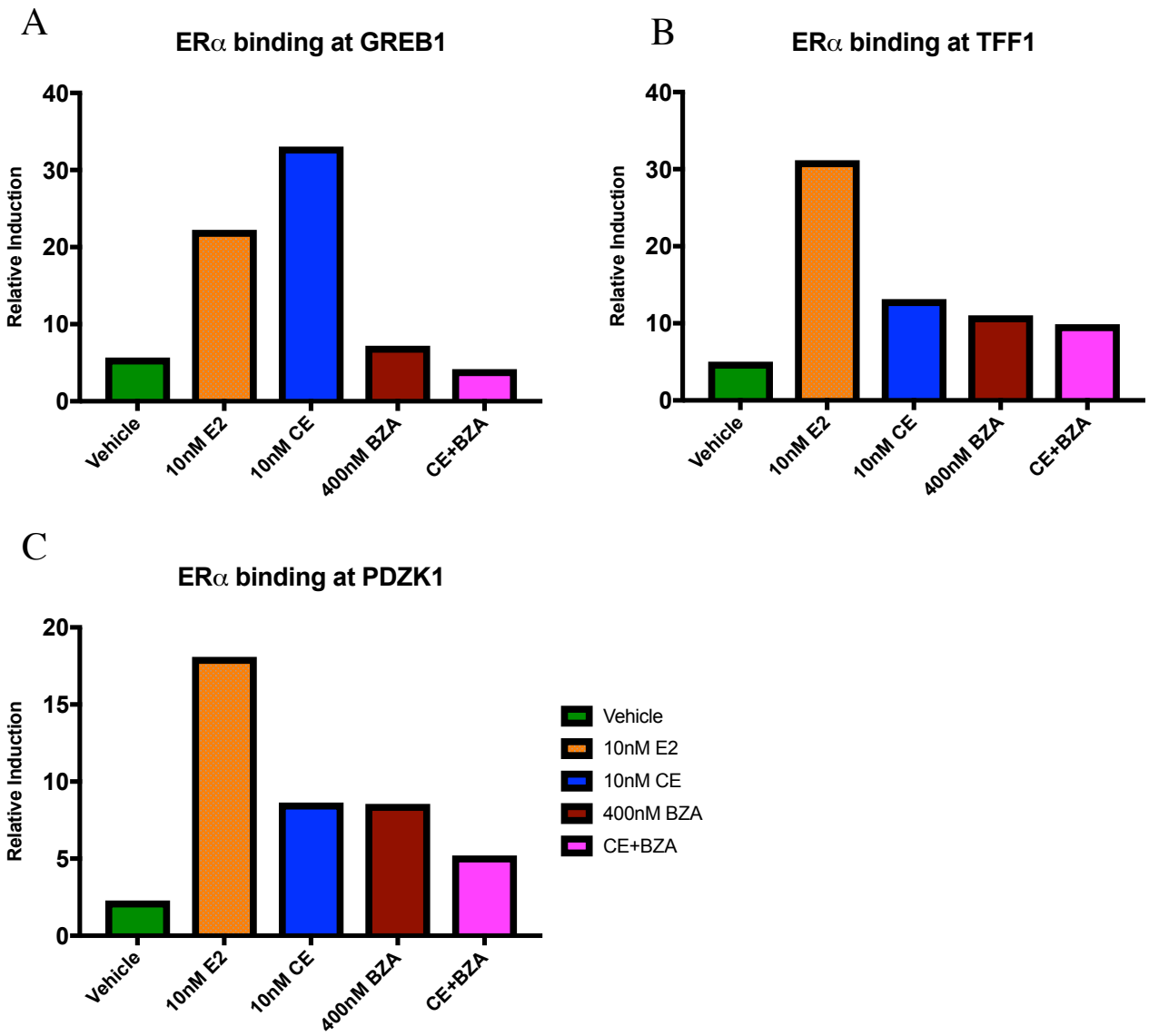


Figure 2.8 ERα occupancy at target gene loci in MCF7 cells. 10nM E2 and 10nM CE increased ERα binding at loci of GREB1, TFF1 and PDZK1 genes. 400nM BZA and CE+BZA decreased ERα binding at gene loci. Results from one representative experiment shown.

separate differentially expressed genes (DEGs) into six subgroups based on mRNA expression level changes. These groups included genes that were upregulated in all treatment groups, genes that were downregulated in all treatments, genes that were upregulated in the E2 and CE groups and downregulated in the BZA and CE+BZA groups, etc. (Figure 2.9). ChIP-seq showed that overall, the number of ER binding sites was higher in E2 and CE groups, compared to vehicle, however a majority of those sites were lost in the BZA and CE+BZA treatment groups (Figure 2.9 and 2.10). The overwhelming majority of genes showed the same pattern of regulation for the E2 and CE groups. This was also true for the BZA and CE+BZA groups. There were, however, uniquely upregulated or downregulated genes in each treatment, as well as unique binding sites (Figure 2.9, Table 2). Figure 2.9 represents combined data from RNA-seq and ChIP-seq: out of 1408 total differentially expressed genes, 137 were found to also have ERα binding at either proximal (+/-5 kilobases (kb) to transcription start site) and/or distal (+/-50kb to transcription start site) sites in at least one treatment condition. From Ingenuity Pathway Analysis (IPA), it was determined that combination therapy decreased pathways related to Estrogen-mediated S-phase entry and cell cycle regulation, while increasing pathways related to cell cycle checkpoint regulation and DNA damage checkpoint regulation (Table 3). When IPA analysis was used to evaluate diseases and biological functions, pathways related to cell proliferation, survival and viability were most significant, and were activated by E2 and CE treatment and repressed by BZA and CE+BZA treatment. On the other hand, cell death and apoptosis pathways were upregulated by BZA and CE+BZA, while downregulated by E2 and CE (Table 4). Because IPA analysis identified aryl hydrocarbon receptor (AHR) (Table 3) signaling as the top canonical pathway, I investigated this pathway further.

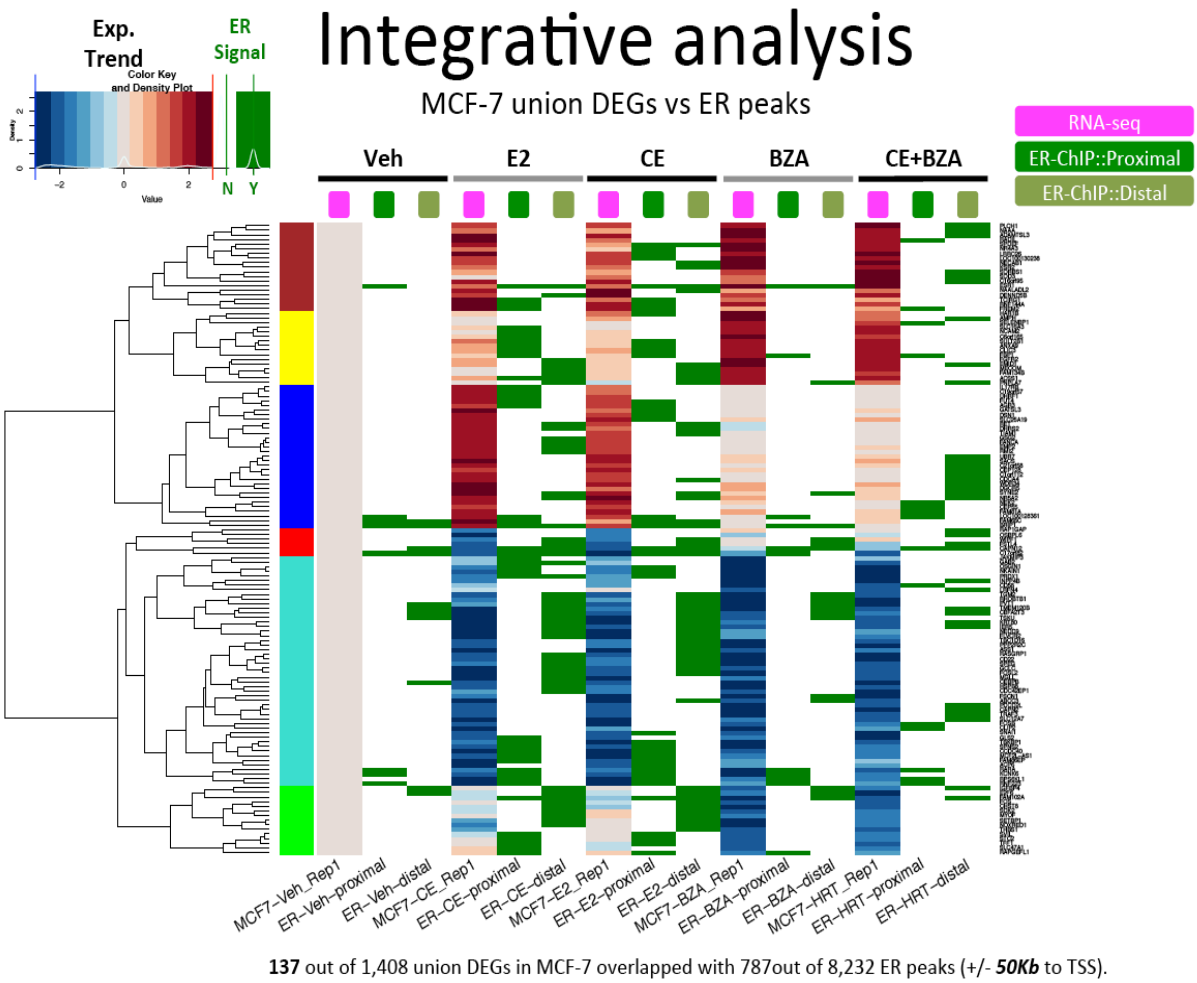


Figure 2.9 Integrative analysis of RNA-seq and ChIP-seq studies. RNA-seq and ChIP-seq analysis performed from triplicate samples from MCF7 cells. Heatmap of DEGs shown (red – increased expression; blue – decreased expression; figure legend, top left). DEGs were divided into six groups based on expression. DNA binding peaks from ChIP-seq (green bars) were found at proximal (+/-5kb) and/or distal (+/-50kb) sites. Taken together, 137 DEGs (out of 1,408) showed overlap with ERα binding peaks in at least one treatment condition.

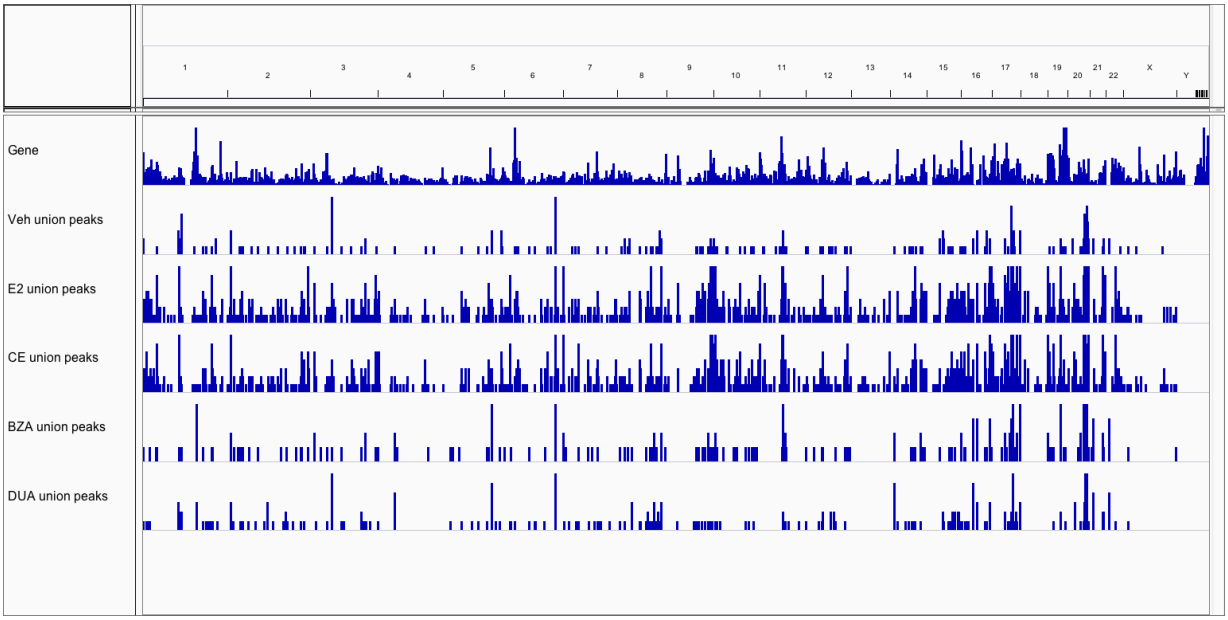


Figure 2.10 Distribution of ERα binding along genome in MCF7 cells. ChIP-seq peak visualization from Integrative Genomics Viewer showing ERα binding across chromosomes in comparison to RefSeq gene track (Gene). E2 and CE treatment resulted in highest ERα binding. BZA and CE+BZA (DUA) showed fewer binding peaks thank E2 or CE.

Treatment	Varying DEGs	Unique binding sites
E2 (vs CE)	19 genes up in E2, down in CE	366 (0 corresponding to DEGs)
CE (vs E2)	73 genes up in CE, down in E2	588 (4 corresponding to DEGs)
BZA (vs CE+BZA)	28 genes up in BZA, down in CE+BZA	167 (0 corresponding to DEGs)
CE+BZA (vs BZA)	38 genes up in CE+BZA, down in BZA	1 (0 corresponding to DEGs)

Table 2. Uniquely upregulated or downregulated genes and correspondence to binding sites.

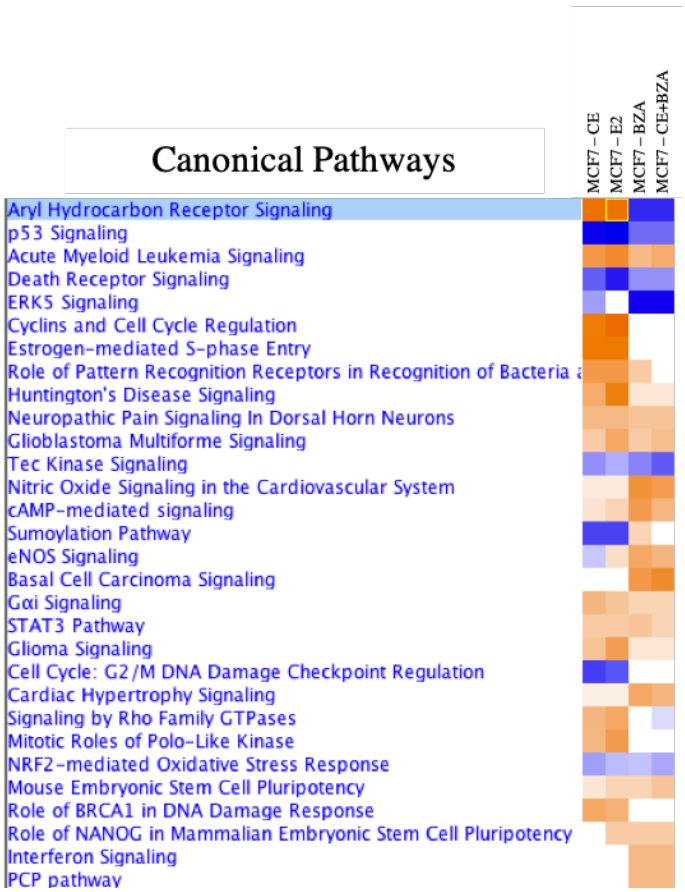


Table 3. Top canonical pathways from IPA analysis of RNA-seq data.

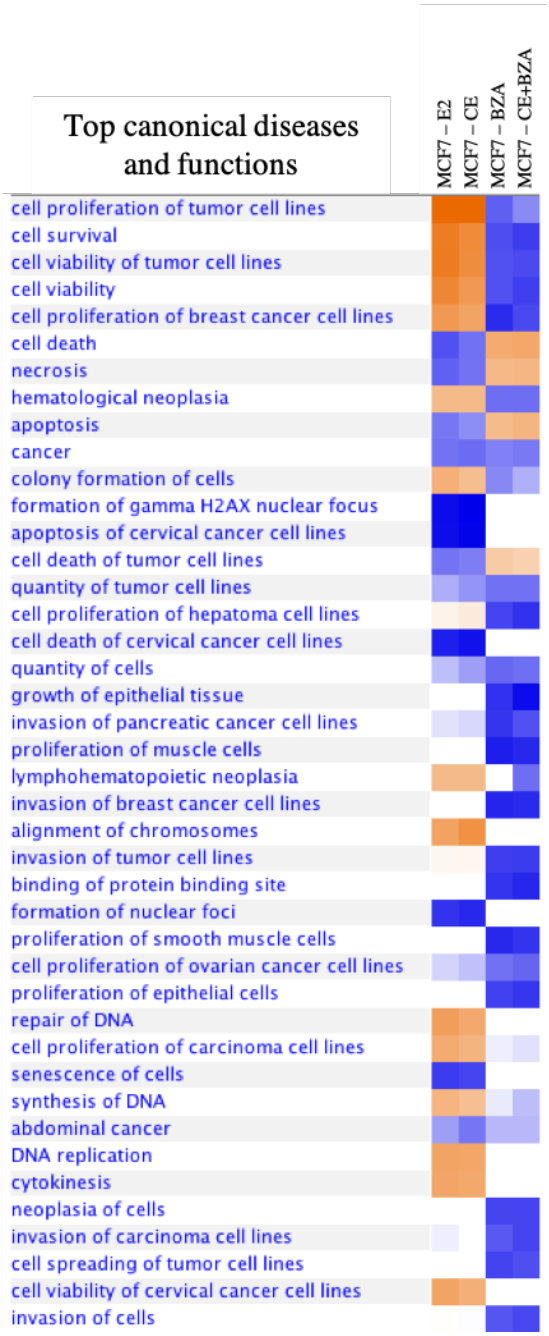


Table 4. Top canonical diseases and functions from IPA analysis from RNA-seq

AHR has been known to play several roles in cancer. AHR is a transcription factor activated by various polycyclic aromatic hydrocarbons, such as dioxin (TCDD) and 3-methylcholanthrene (3MC).[65] AHR heterodimerizes with AHR nuclear translocator at various response elements on DNA and is able to induce gene transcription. Upregulated genes include AHR receptor repressor (AHRR, which has been reported as a tumor suppressor), and the cytochrome P450 enzymes CYP1A1 and CYP1B1, which are important for the metabolism of carcinogens. The role of AHR in cancer is mixed: AHR can either be pro- or anti-tumorigenic, depending on the context. In breast cancer, studies suggest that AHR may play an anti-estrogenic role.[66–68] Data from patient cohorts suggest that ERα-positive breast cancer patients with high AHR expression have a better prognosis than those with low AHR expression (Figure 2.11A).[69] However, this is not true for all breast cancer patients (Figure 2.11B). Studies have also shown that tamoxifen activates AHR-responsive genes in MCF7 cells as well as in ERα-negative cell lines.[70] Raloxifene, another SERM, has been shown to induce cell death in an AHR-dependent manner in ERα-negative cells.[71] The effect of BZA on AHR signaling has not yet been evaluated.

As previously mentioned, IPA analysis identified the AHR pathway as the top canonical pathway based on our sequencing data. Based on previous studies performed with tamoxifen and raloxifene, we expected that BZA, as well as CE+BZA, would stimulate the AHR pathway and increase expression of its downstream targets. When we looked at the genes involved in the AHR pathway as described by IPA, it became clear why there was an opposite effect of what we anticipated: E2F1, a gene that is increased by E2 and CE, and decreased by BZA and CE+BZA, is included in the larger AHR network. Due to its role as a transcription factor, E2F1 is also upstream of many genes included in the larger AHR network and therefore we were seeing an effect opposite of what we had expected on the AHR pathway as a whole. When we look at

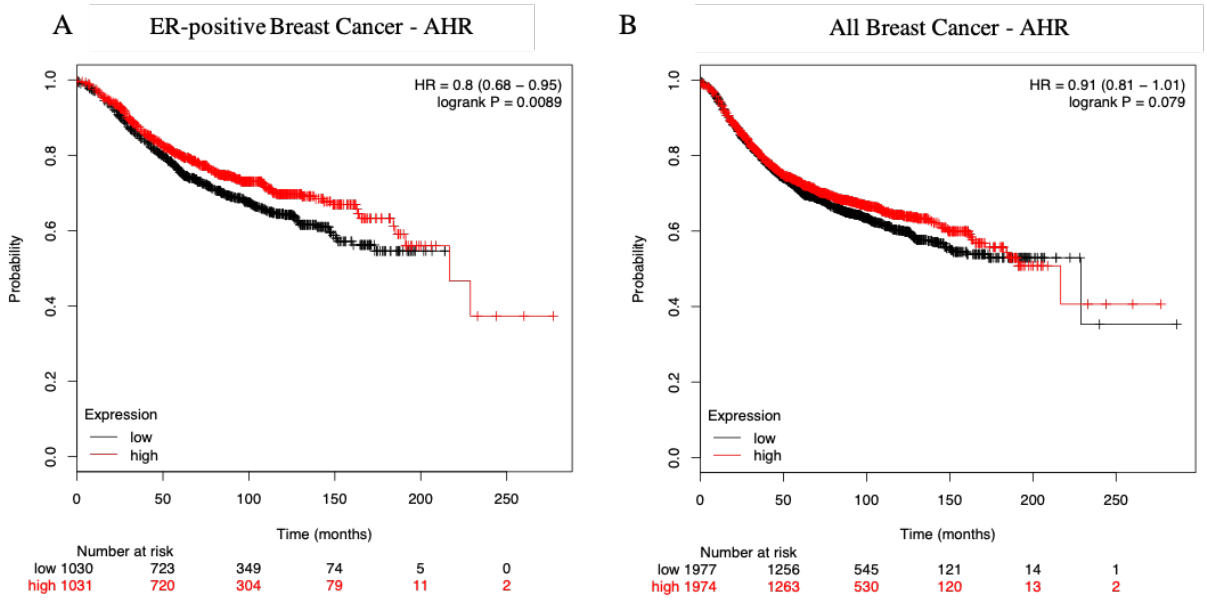


Figure 2.11 AHR expression corresponds with better prognosis in ER-positive breast cancer. Kaplan Meier plot of ER-positive breast cancer patients showing higher probability of survival in patients with higher AHR expression (p=0.0089) (A). Kaplan Meier plot of all breast cancer patients suggests that AHR is not a predictor of prognosis (p=0.079) (B).

targets directly downstream of AHR, our sequencing data shows that E2 and CE decrease expression of AHRR and CYP1A1, whereas BZA and CE+BZA increase AHRR. We decided to further evaluate the effects of BZA on the AHR pathway in cell lines.

In our studies, BZA affected the AHR pathway in both ERα-positive and ERα-negative cell lines. MCF7 cells were first tested for AHR and AHRR expression after E2, CE, and/or BZA treatment, with 3MC used as control. It was determined that AHR expression does not vary significantly in response to treatment (Figure 2.12A). However, BZA and CE+BZA increased expression of AHRR in MCF7 cells (Figure 2.12B). Further, in MCF7 cells BZA increased expression of two AHR-responsive genes, AHRR and CYP1A1, after 24 hours of treatment (Figure 2.13A-C). 4-Hydroxy tamoxifen (4OHT) was used as a positive control, as it had previously been shown to increase expression of AHRR, CYP1A1 and CYP1B1 in ERα-positive and ERα-negative cell lines.[70] Similar results were seen in MDA-MB-231 cells (Figure 2.13D-F). In ERα-negative C4-12 cells, BZA was able to increase the expression of AHRR, CYP1A1 and CYP1B1 (Figure 2.13G-I). T47D cells did not show a difference in gene expression when treated with BZA or the control 4OHT (data not shown); however, this could be due to their low AHR protein levels (Figure 2.14). Studies suggest that lower AHR levels correspond to earlier stages of malignancy, which could be why T47D cells express lower amounts of AHR protein as compared to MCF7, C4-12 and MDA-MB-231 cells.[72] siRNA knockdown of AHR was performed to determine whether AHR is required for BZA- or 4OHT-activated increase of AHRR, CYP1A1 and CYP1B1; however, the results were inconclusive (data not shown). Overall, results from these experiments suggest that BZA, similar to tamoxifen and raloxifene, acts as an AHR agonist and highlights one potential mechanism through which BZA exerts its inhibitory action on breast cancer cells.

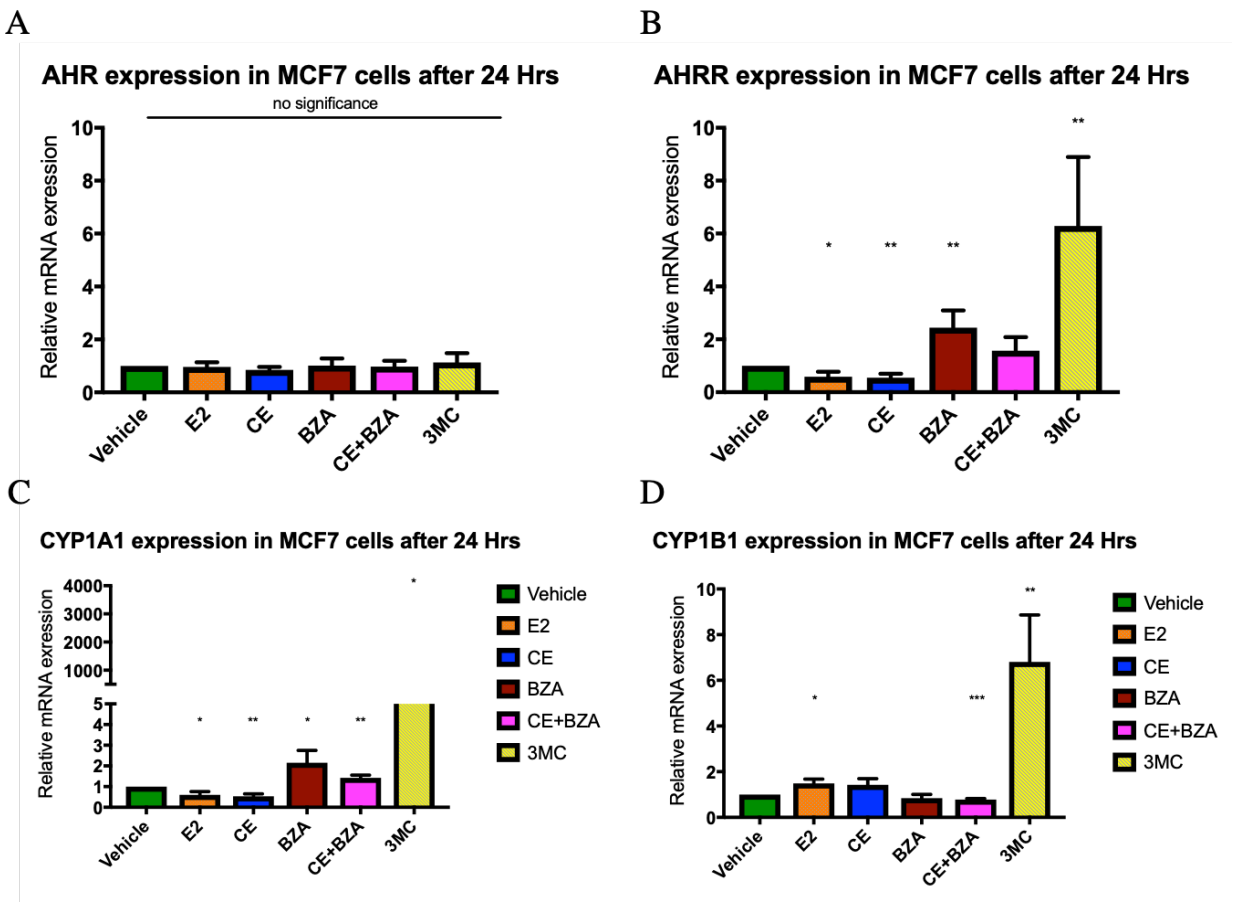


Figure 2.12 BZA and CE+BZA upregulate expression of AHRR in MCF7 cells. AHR expression in MCF7 cells was not altered based on 24-hour treatment with E2, CE, BZA, CE+BZA or the positive control 3MC (A). AHRR expression in MCF7 cells was decreased by E2 and CE, was increased by BZA and CE+BZA, as well as with the positive control, 3MC, after 24 hours (B). CYP1A1 expression was decreased by E2 and CE, and increased by BZA, CE+BZA and 3MC after 24 hours (C). CYP1B1 was increased by E2 and 3MC, decreased by CE+BZA after 24 hours of treatment (D).

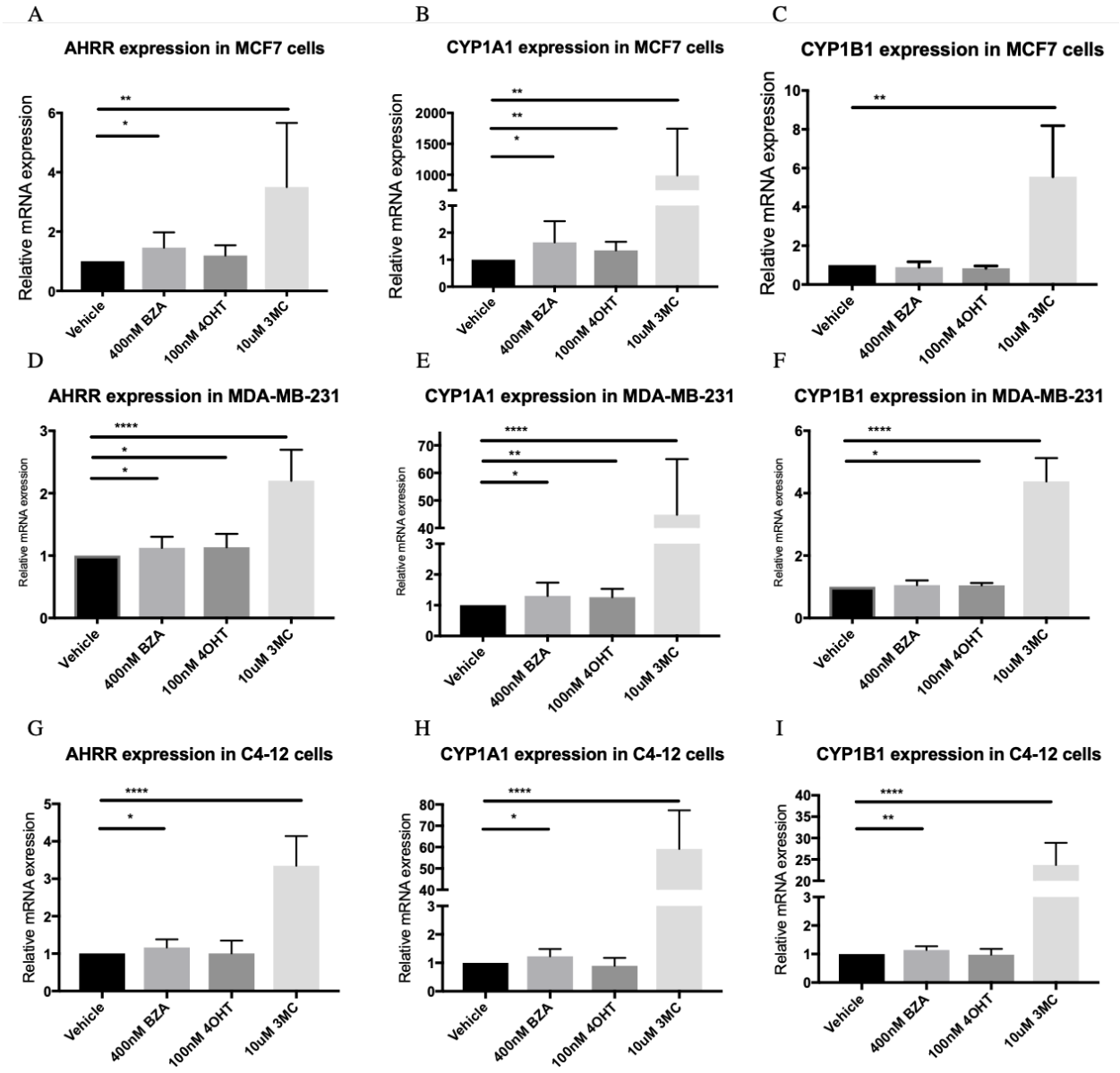


Figure 2.13 BZA upregulates expression of AHR pathway genes in ER-positive and ER-negative cell lines. ER-positive MCF7 cells treated for 24 hours with 400nM BZA showed increased expression of AHRR and CYP1A1, but not CYP1B1 (A-C). ER-negative MDA-MB-231 cells treated for 24 hours with 400nM BZA showed increased AHRR, CYP1A1, but not CYP1B1 (D-F). ER-negative C4-12 cells treated for 24 hours with 400nM BZA showed increased AHRR, CYP1A1 and CYP1B1 (G-I). 4OHT and 3MC were used as positive controls. Data are represented as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, by Student's *t* test.

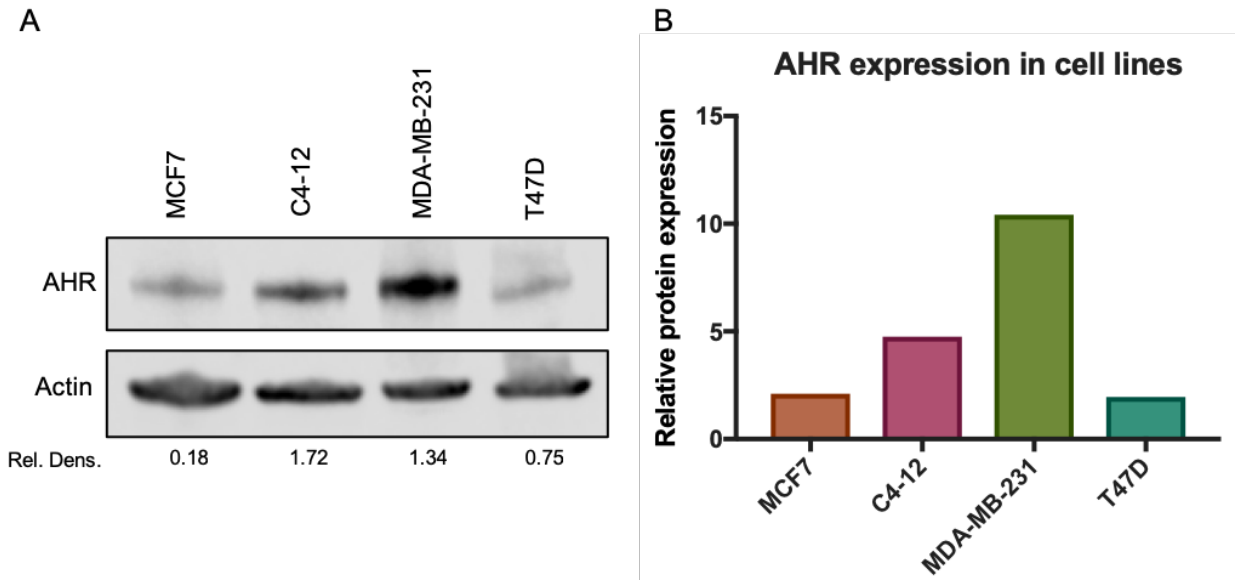


Figure 2.14 AHR expression in breast cancer cell lines. Western blot for AHR and actin in MCF7, C4-12, MDA-MB-231 and T47D cells with relative density (Rel. Dens.) of AHR below (A). Graph showing relative protein expression of AHR in cell lines (B).

Discussion

Cell-based studies were performed to better understand the mechanism of action of CE+BZA. It was determined that when tested individually, CE effects most closely resemble those of E2, whereas the CE+BZA combination was most similar to BZA alone. However, there were notable differences among the different treatments. BZA, as a single agent and in combination with CE, was found to decrease growth rate, gene expression and ERα binding in ERα-positive cell lines. At a higher level, BZA also increased activation of pathways related to cell death and decreased activation of pathways related to cell proliferation, survival and viability.

BZA also targets the AHR pathway, both in ERα-positive and -negative cell lines, and thus may be exerting its effect, at least in part, through the AHR pathway. These experiments show that CE+BZA treatment alters ERα binding to DNA, thus exerting a unique transcription profile and therefore decreasing cell growth and proliferation.

This study, for the first time, shows the effects of CE+BZA treatment on a global level in MCF7 cells. Both transcriptome and cistrome profiles were presented for CE, BZA and the CE+BZA combination. Previous studies have shown genes that are regulated by CE and/or BZA, but it is important to understand their functions on a global level to be able to make conclusions as well as define novel methods of action.

This study is the first to establish a link between BZA and the AHR pathway. Previous work on the AHR pathway has not defined a clear role of AHR in breast cancer: studies show that it has both pro- and anti-tumorigenic properties. The data presented here shows that BZA is able to activate the AHR pathway, as measured by increase of AHR-responsive genes AHRR, CYP1A1 and CYP1B1. AHRR has been reported to act as a tumor suppressor in breast cancer.[67]

Based on patient cohort data, increased AHR levels correspond with a better prognosis in ER-positive breast cancer. Therefore, activation of AHR by BZA is a benefit to women taking BZA and CE+BZA combination therapy. Moreover, our study is in line with previous studies on the effects of SERMs on the AHR pathway. Most similar, was a study by DuSell *et al* in which 4OHT, a metabolite of tamoxifen, was seen to activate the AHR pathway.[70] Based on their results, 4OHT was used as one control in the experiments presented here. In my study, 4OHT was not able to induce relative mRNA expression to the same level as in the DuSell study. Therefore, since I determined that BZA was able to induce similar effects of gene expression as 4OHT, and DuSell reported that 4OHT induces AHR pathway genes at a high rate, there may be an even more significant role for BZA than is reported in this study.

This study has shown that CE+BZA treatment decreases cell proliferation due to a decrease in ERα protein levels, decrease in ERα binding to gene loci and decreased gene expression. BZA also targets the AHR pathway to exert its effects. Based on the results observed in cell lines, I continued this study with animal models in order to determine whether similar effects of decreased cell proliferation exists and therefore leads to an overall decrease in tumor growth.

CHAPTER III

CE+BZA TREATMENT DELAYS TUMOR ONSET AND DECREASES THE TOTAL VOLUME OF TUMORS IN THE PYMT TRANSGENIC MOUSE MODEL, THEREBY EXTENDING ANIMAL SURVIVAL

Background

The role of CE+BZA treatment in breast cancer development or progression is not well understood. The short duration of the SMART trials did not allow conclusions about breast cancer risk to be made.[51–54] Previous mouse models shed some light on the effect of CE, BZA and other SERMs on the mammary gland and on mammary tumors. In one study, tamoxifen, a SERM, was found to have a number of effects on mammary tumors in an ovariectomized C3(1)/SV40 Large T-antigen transgenic mouse model. Tamoxifen decreased the number of mammary intraepithelial neoplasia lesions, decreased the total number of tumors per mouse, decreased overall tumor volume and improved the overall survival of mice.[73] In another study, the effect of various SERMs was evaluated alone or together with CE in C57BL/6 ovariectomized mice. In this study, Peano and colleagues found that BZA reduced CE stimulation of uterine weight, total number of branch points in the mammary ductal tree, and overall gene expression.[44] Further, a study to test the effects of CE+BZA combination on tumors was performed using ovariectomized nude mice with MCF7 xenografts. This study determined that BZA blocked CE stimulation of Ki67 and gene expression, and inhibition of apoptosis.[48] Overall, there is evidence to support a role for CE+BZA in reducing breast cancer outgrowth, but the studies have so far been limited. Therefore, I decided to use the transgenic PyMT mouse model to investigate the effects of CE+BZA on breast cancer growth.

The PyMT mouse model was used for these studies because their mammary tumors closely resemble the human condition. These mice express the polyoma virus middle T antigen under control of the mouse mammary tumor virus long terminal repeat, thus restricting it to the mammary gland. PyMT is oncogenic due to the PyMT protein disrupting the Src family, Ras, and PI3 kinase pathways, which are often altered in human breast cancer. PyMT mammary gland tumors start out ERα-positive and lose positivity over time. These tumors can be classified into four stages: hyperplasia, adenoma, early carcinoma and late carcinoma. At the age of four to six weeks, 50 to 60 percent of mice develop hyperplasia, which is 30-40 percent ERα-positive. At eight to nine weeks, the majority of mice have adenoma, or mammary intraepithelial hyperplasia, a premalignant lesion. These premalignant lesions are 50-80 percent ERα-positive. Mice aged 8-12 weeks have early carcinoma, which is most similar to human ductal carcinoma in situ (DCIS), and the lesions are mosaic for ERα. Finally, at ten weeks of age, about half the mice have advanced or late carcinoma, which is most similar to human invasive ductal carcinoma. This late stage is 90 percent ERα-negative, although small clusters of ERα-positive cells remain in 80 percent of the mice. Pulmonary metastases appear in a majority of the mice at fourteen weeks of age.[74,75]

Due to their similarity to the human condition, as well as their expression of ERα, these mice are an excellent model to test the effects of CE+BZA on tumor onset and progression. These mice also have an intact immune system, giving them some advantages over cell line xenograft models in immune compromised mice. We therefore initially decided to use this model to test the effects of CE+BZA on tumor growth in a whole organism. In later studies, we used a patient derived xenograft model to test the effects of CE+BZA in tumor tissue of human origin (Chapter IV).

Results

To better understand the effects of CE+BZA *in vivo*, the PyMT mouse model was my primary model. PyMT mice were bred in-house for all experiments. Mice were genotyped to identify presence of transgene and heterozygous female mice were used for all experiments. For the first set of experiments, we ovariectomized the mice at four to five weeks of age to create an environment free of hormones, resembling a post-menopausal woman. Following ovariectomy, mice were given a daily oral gavage of vehicle, CE (3mg/kg), BZA (2mg/kg) or CE+BZA. Although the molar ratio of CE to BZA in prescribed Duavee HRT therapy is approximately one to forty, we chose to use a three to two ratio to more closely mimic published animal studies.[44,48] E2 injection (5µg/kg) was used as a control, in addition to an E2+BZA group. Mice were monitored weekly for tumor formation and size and were sacrificed when their tumor reached maximum allowed size (Figure 3.1). Each treatment group had at least eight mice in the study. CE+BZA, as well as BZA alone and E2+BZA, delayed tumor onset compared to E2 and CE alone (Figure 3.2). Mice treated with BZA, CE+BZA and E2+BZA survived longer than those treated with E2 or CE alone (Figure 3.2).

The same study was repeated with intact mice to rule out possible effects of ovariectomizing the mice. Mice started treatments at week four to five and were given a daily oral gavage of vehicle, CE, BZA or CE+BZA. E2 was not used as a control because the mice had normal circulating hormones. Mice were monitored for tumor formation and growth weekly and were sacrificed when tumor burden became too great. Similar results were observed in intact mice compared to ovariectomized mice: BZA and CE+BZA treatment delayed tumor onset and prolonged survival compared to CE alone (Figure 3.3). This result was not anticipated, considering intact mice have circulating E2 that can bind to ER in the mouse mammary gland.

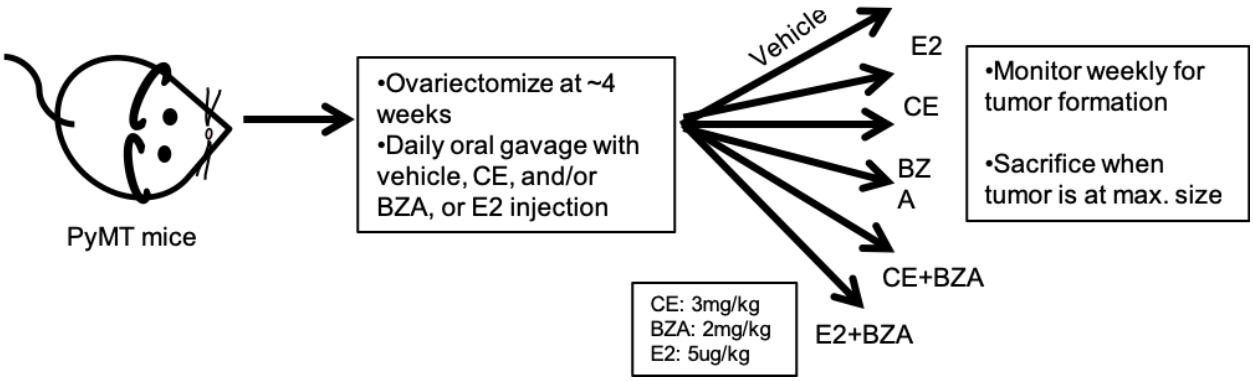


Figure 3.1 Schematic of ovariectomized mouse study. PyMT mice were ovariectomized at approximately 4 weeks of age. Once healed, mice were randomized and entered the study. Mice received a daily oral gavage of vehicle, CE, BZA, CE+BZA or an injection of E2. Mice were monitored weekly for tumor formation and size. Mice were sacrificed when tumors reached maximum allowed size.

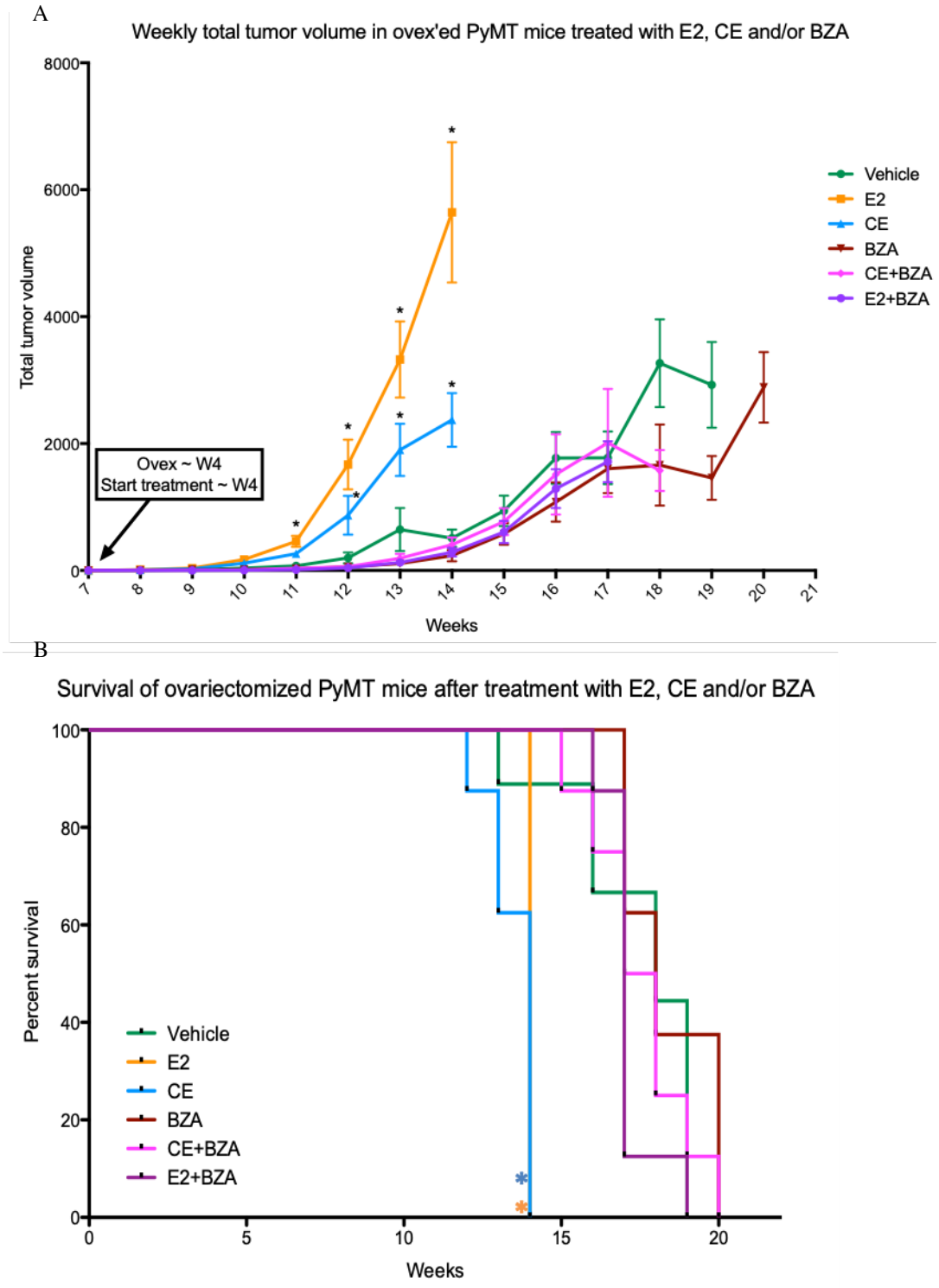


Figure 3.2 CE+BZA decreases tumor growth and increases survival of ovariectomized PyMT mice. Total tumor volume in ovariectomized PyMT mice treated with vehicle, E2, CE, and/or BZA over time (weeks) (A). Kaplan Meier curve showing survival

Figure 3.2, continued

of ovariectomized PyMT mice treated with vehicle, E2, CE, and/or BZA (B). Data are represented as mean ± SEM; *p<0.05, by Student's *t* test.

Week	8	9	10	11	12	13	14	15	16	17	18	19	20
Mice in vehicle group	9	9	9	9	9	9	8	8	8	6	6	3	
Mice in E2 group	8	8	8	8	8	8							
Mice in CE group	8	8	8	8	8	7	5						
Mice in BZA group	8	8	8	8	8	8	8	8	8	8	5	3	3
Mice in CE+BZA group	8	8	8	8	8	8	8	8	7	6	4		
Mice in E2+BZA group	8	8	8	8	8	8	8	8	8	7			

Table 5. Number of mice in ovariectomized mouse study per week.

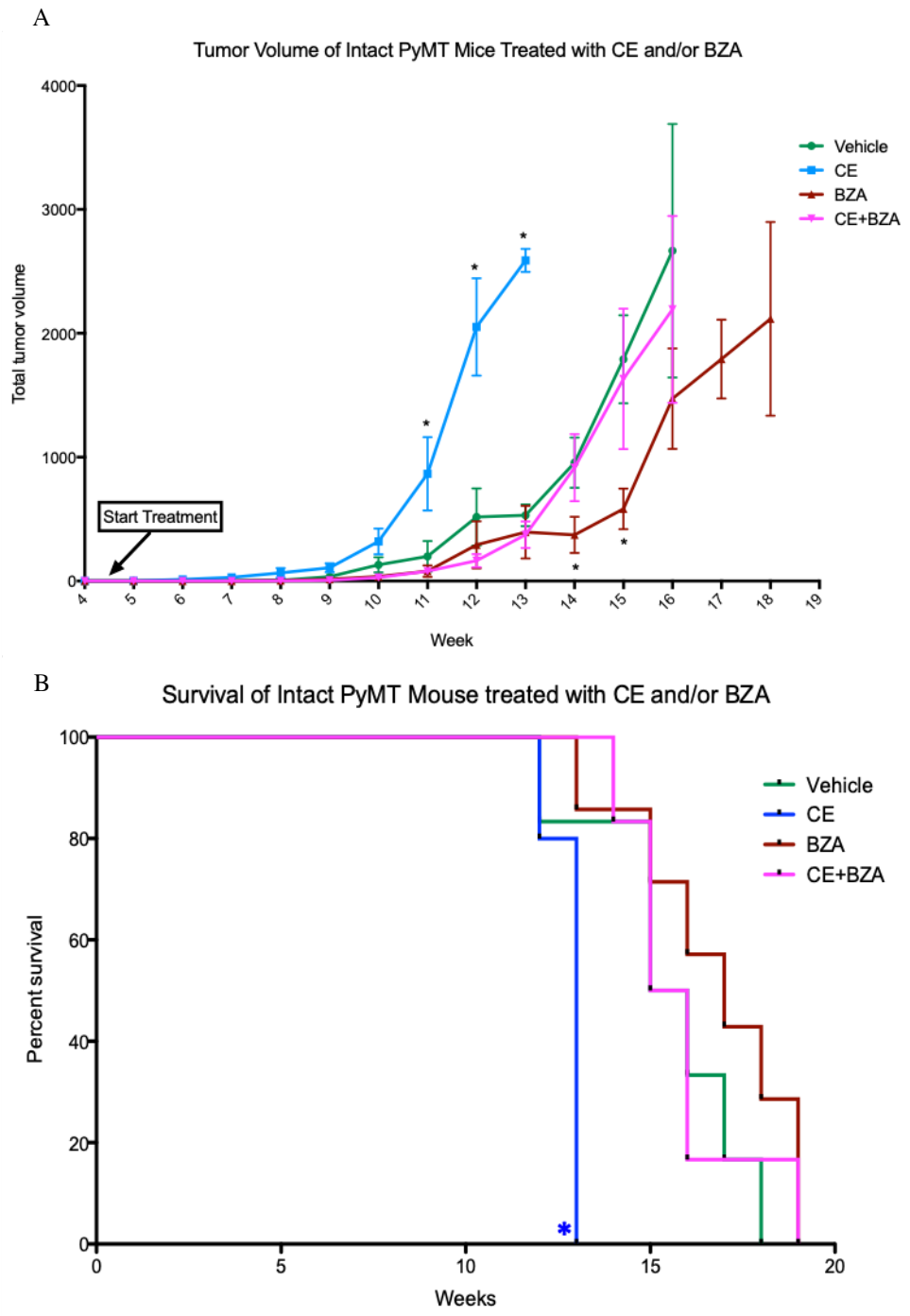


Figure 3.3 CE+BZA decreases tumor growth and increases survival of intact PyMT mice. Total tumor volume in intact PyMT mice treated with vehicle, CE, and/or BZA over time (weeks) (A). Kaplan Meier curve showing survival of intact PyMT mice treated with vehicle, CE, and/or BZA (B). Data are represented as mean ± SEM; *p<0.05, by Student's *t* test.

Week	8	9	10	11	12	13	14	15	16	17	18
Mice in vehicle group	7	7	7	7	7	6	6	6	3		
Mice in CE group	5	5	5	5	5	4					
Mice in BZA group	7	7	7	7	7	7	6	6	5	4	3
Mice in CE+BZA group	6	6	6	6	6	6	6	5	3		

Table 6. Number of mice in intact mouse study per week

However, the amount of E2 circulating in mice is very low, about 13.5 pg/mL during follicular phase, whereas a normal woman's levels are 27 pg/mL during follicular phase.[76,25] During menopause, the levels of E2 decrease in women to below 30pg/mL.[77] The onset of tumors for all mice in the ovex group (except those supplemented with E2) was somewhat delayed, compared to the intact group. Overall, however, the pattern of tumor growth based on CE, BZA and CE+BZA treatment was similar in the ovex and intact groups, due to a relatively low level of circulating E2, one comparable to post-menopausal women. Lung metastasis and ERα expression were evaluated in both the intact and ovariectomized mice at endpoint. However, no obvious differences were observed between groups, possibly due to the fact that tumors looked very similar at their endpoint. Although the age of the mice was different, because mice treated with E2 or CE were sacrificed earlier and those treated with BZA and CE+BZA were sacrificed later, tumors had reached such a large size that they were most likely very similar to each other. Therefore, a time point analysis was performed.

Mice in the time point study were treated similar to those in the intact studies. Intact mice were split into four treatment groups (vehicle, CE, BZA, CE+BZA) and were sacrificed at three time points: eight, ten and twelve weeks (Figure 3.4). When comparing the number of lung metastases, CE-treated mice had more metastases than those treated with vehicle, BZA or CE+BZA (Figure 3.5). The area of the metastases, when taken as a percentage of total lung area in cross sections, was statistically higher in CE-treated mice at 12 weeks (Figure 3.5). Although the area of lung metastasis in BZA- and CE+BZA-treated mice was smaller than in vehicle-treated mice, the difference was not statistically significant (Figure 3.5). I also observed that ERα mRNA expression in intact mice treated with BZA and CE+BZA was higher than in vehicle-treated or CE-treated mice (Figure 3.6).

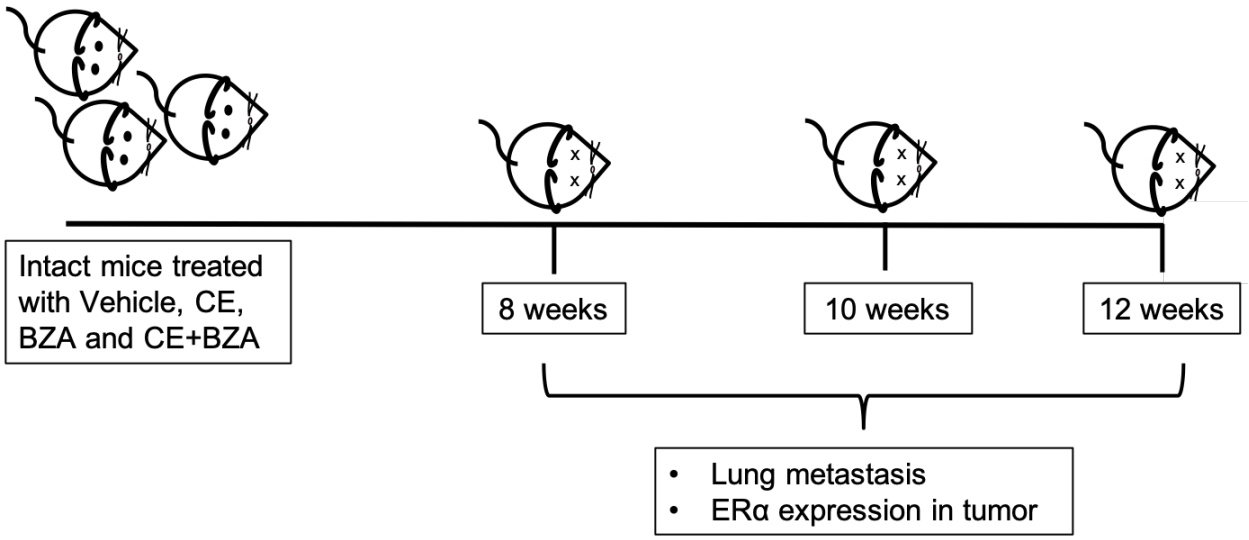
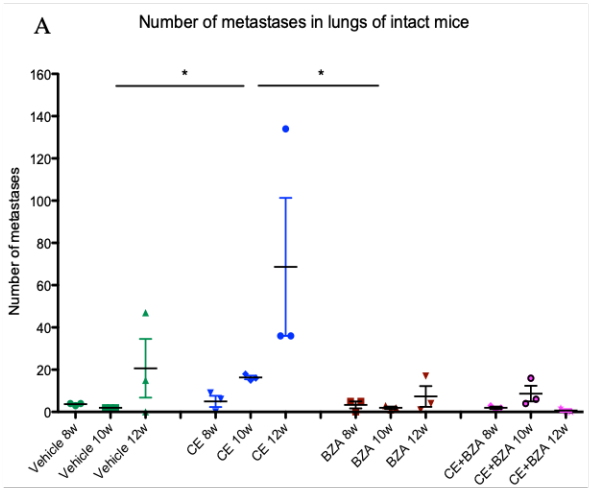
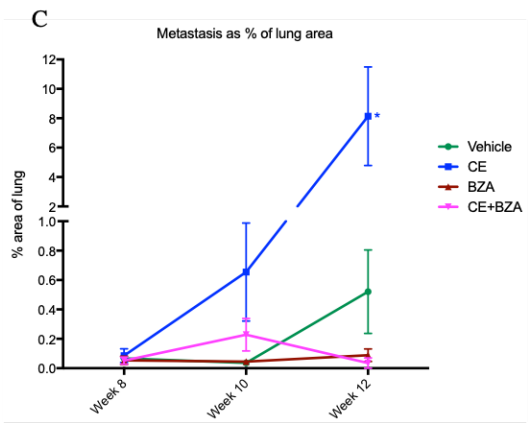


Figure 3.4 Schematic of timepoint intact mouse study. PyMT mice were randomized into groups to receive a daily oral gavage with vehicle, CE, BZA or CE+BZA. Mice were sacrificed at 8, 10 or 12 weeks for evaluation of lung metastases and tumor ERα expression.



B

Number of lung metastases per mouse				
	Vehicle	**CE**	**BZA**	**CE+BZA**
8 w	3, 4, 4	0, 6, 9	0, 5, 5	1, 2, 3
10 w	2, 2, 2	15, 16, 18	1, 2, 3	4, 6, 16
12 w	0, 15, 47	36, 36, 134	1, 4, 17	0, 2, 0



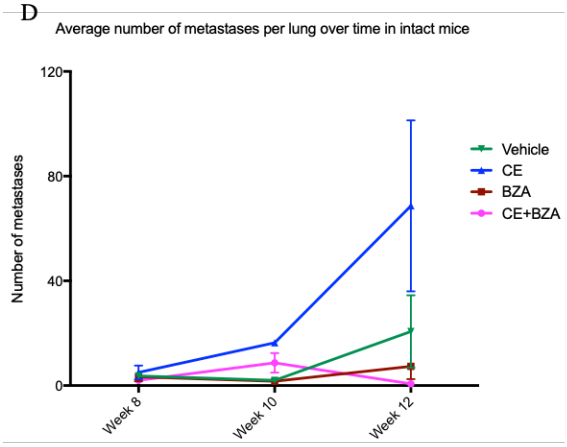


Figure 3.5 CE+BZA decreases lung metastases in intact PyMT mice. Metastases in lungs of intact mice at 8, 10 and 12 weeks of age with vehicle, CE, BZA or CE+BZA treatment (A). Table showing the number of lung metastases of intact mice at 8, 10 and 12 weeks of age with vehicle, CE, BZA or CE+BZA treatment (B). Metastases area as a percentage of total lung area in intact mice at 8, 10 and 12 weeks of age with vehicle, CE, BZA or CE+BZA treatment (C). Average number of metastases per lung over time in intact mice at 8, 10 and 12 weeks of age with vehicle, CE, BZA or CE+BZA treatment (D). Data are represented as mean ± SEM; *p<0.05, by Student's *t* test.

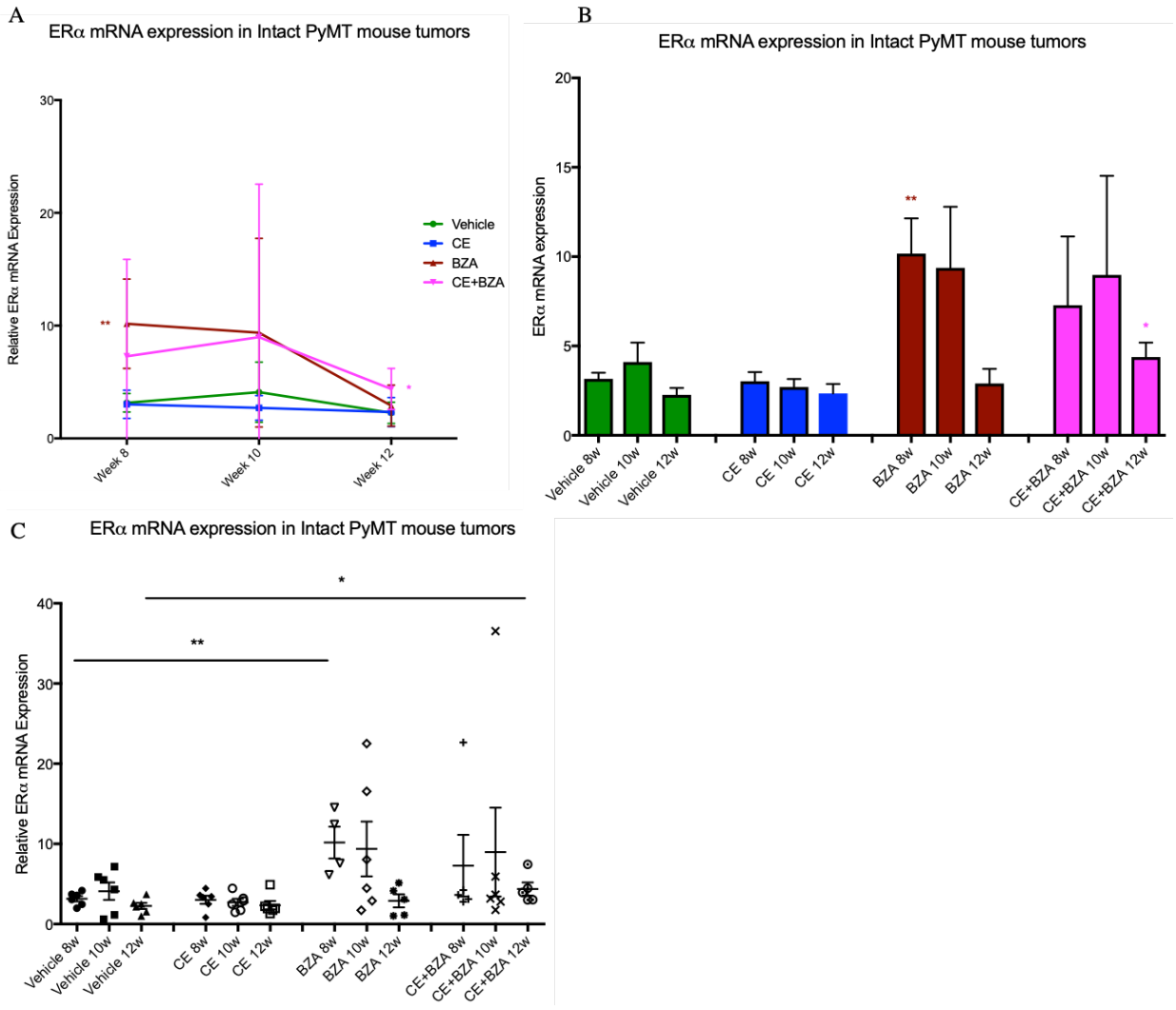


Figure 3.6 CE+BZA increases ERα expression in intact PyMT mouse tumors. ERα expression in tumors of intact mice treated with vehicle, CE, BZA or CE+BZA over time (weeks) (A-C). Data are represented as mean ± SEM; *p<0.05, **p<0.01 by Student's *t* test.

In cell-based studies, I observed that BZA was activating the AHR pathway (Chapter II), therefore I decided to evaluate AHR activation in tumors of timepoint mice. AHR and AHRR expression was highest in tumors of intact PyMT mice treated with CE or CE+BZA (Figure 3.7 A, B). Mice treated with BZA also showed higher expression of CYP1A1 and CYP1B1 at certain time points (Figure 3.7 C, D). These results provide further evidence that BZA acts as an AHR agonist and highlights one potential mechanism through which BZA exerts its actions on breast cancer cells.

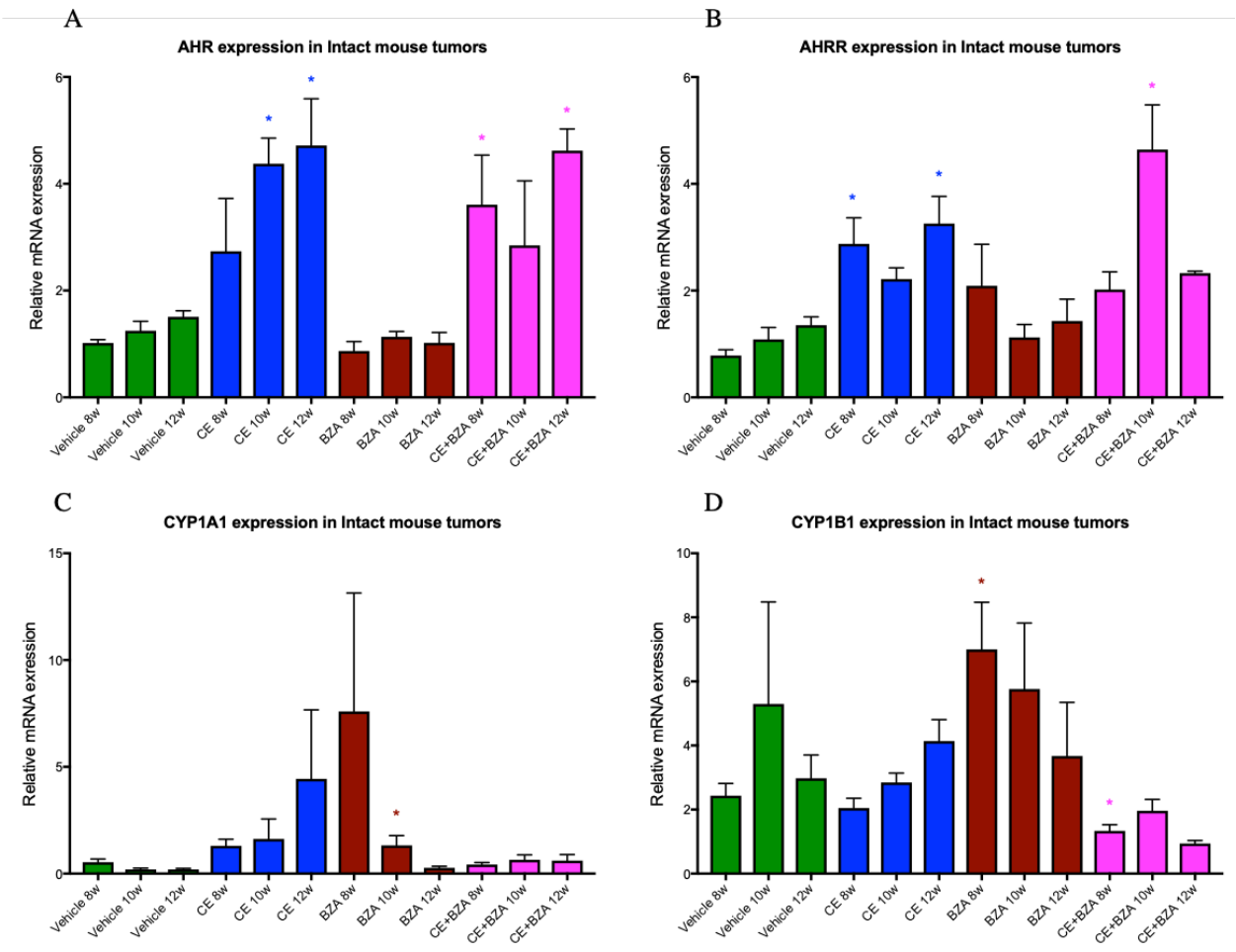


Figure 3.7 CE+BZA increases expression of AHR pathway members in intact mouse tumors. AHR expression in intact mouse tumors was increased by CE+BZA and CE treatment (A), but not BZA alone treatment. AHRR expression in intact mouse tumors was increased by CE and CE+BZA treatment, but not BZA alone (B). CYP1A1 expression in intact mouse tumors was increased by BZA treatment, but not CE or CE+BZA (C). CYP1B1 expression in intact mouse tumors was increased by BZA treatment and decreased by CE+BZA treatment (D). Data are represented as mean ± SEM; *p<0.05, by Student's *t* test.

Discussion

In the PyMT mouse model, CE+BZA treatment was able to delay tumor onset, decrease total tumor volume and extend survival. CE+BZA had an effect in both ovariectomized and intact mice. Mice treated with CE alone had a higher number of metastases in the lungs compared to both vehicle and BZA alone. ERα expression was higher in tumors of BZA- and CE+BZA-treated mice. AHRR expression was increased in CE- and CE+BZA-treated mouse tumors. BZA increased expression of CYP1A1 in mouse tumors. Overall, these data show that CE+BZA treatment is able to delay tumor onset and prolong survival in mice.

Results in the PyMT mouse model closely resemble those from cell-based studies. In both cells and mouse tumors, BZA and CE+BZA treatment led to a decrease in proliferation of tumor cells, resulting in a smaller tumor burden in mice. It is possible that BZA and CE+BZA can also act on immune cells and other cells in the tumor microenvironment, thereby having an effect on tumor growth in the mice. Although the net result of that is seen in the PyMT mice, which have an intact immune system, further studies are required to determine whether cells other than those of the mammary gland are being affected and thereby having an effect on tumor growth.

In mice treated with CE alone, there were more lung metastases as compared to those treated with vehicle or BZA alone. Lung metastases in the PyMT model are ERα-negative, therefore it is likely that BZA or CE+BZA is not inhibiting their growth inside the lung. However, since BZA and CE+BZA treatment decreases cell growth and proliferation, there are fewer cells that are available to metastasize from mammary gland into the lung. Most likely,

BZA and CE+BZA causes a delay in metastasis and eventually all mice in this aggressive model will develop lung metastases, regardless of treatment.

This study is the first to evaluate the effects of BZA on the AHR pathway, and more specifically the effects in a mouse model. In this study, we saw that BZA is able to activate the AHR pathway not only in cell lines, but also in a mouse model. The role of AHR is not fully understood in breast cancer, however data suggests that higher levels of AHR correspond with a better prognosis in patients (Figure 2.11). In the PyMT mice, AHR expression was increased by CE and CE+BZA treatment, AHRR expression was increased by CE and CE+BZA treatment, and expression of CYP1A1 and CYP1B1 both were increased by BZA treatment. Although these results are not exactly the same as we saw from cell-line studies, this may be due to the fact that the LBD of AHR has only about 85% homology in mice and humans. Additionally, the AHR trans-activation domain of AHR in mice and humans only has a 58% homology. Therefore, the mouse studies could be underestimating the potential effect of BZA on AHR in humans.

Further, this study is the first to show the effects of CE+BZA in the PyMT mouse model. Previous studies have used breast cancer cell line xenografts in order to evaluate the effect of CE+BZA or have evaluated the effects of the combination on non-malignant mouse mammary gland.[44,48,78] Although there is no perfect model to study breast cancer, the benefit of the PyMT mouse is that it has an intact immune system, and this study is the first to evaluate the effect of CE+BZA on tumor growth in such a model. The PyMT model is also very aggressive with tumors growing quickly. Other models may show an even greater effect of CE+BZA on tumor volume and overall survival.

Chapter IV

CE+BZA TREATMENT DECREASES TUMOR GROWTH IN A PDX MOUSE MODEL

Background

The PyMT mouse is an excellent model to study the effects of compounds on the mammary gland in immune competent mice. However, the tissue is not of human origin. To better understand the human condition, a patient derived xenograft (PDX) model was used. In such models, a piece of primary or metastatic tissue from a patient is transplanted into a mouse. This tissue is often heterogeneous and therefore closely resembles the tumor of origin. Many PDX models are able to maintain their original heterogeneity and are often indistinguishable from their human tissue of origin.[79,80] The PDX model used in this study was purchased from Jackson labs. The model was derived from a stage IV ERα-positive breast adenocarcinoma collected from a lung metastasis of a 70-year-old white female patient. This model is useful for testing the effect of CE+BZA on human tissue.

Results

In this study, an ERα-positive breast cancer PDX mouse was used. Pieces of a human ERα-positive tumor were implanted orthotopically into SCID mice. Once tumor size reached approximately 125 mm^3, mice were randomized and were treated daily with an oral gavage of vehicle, CE, BZA or CE+BZA (Figure 4.1). Tumor size was monitored weekly. Similar to the PyMT mouse experiments, CE and BZA were used at a dose of 3mg/kg and 2mg/kg, respectively. The mice were not ovariectomized and therefore no E2 control was included.

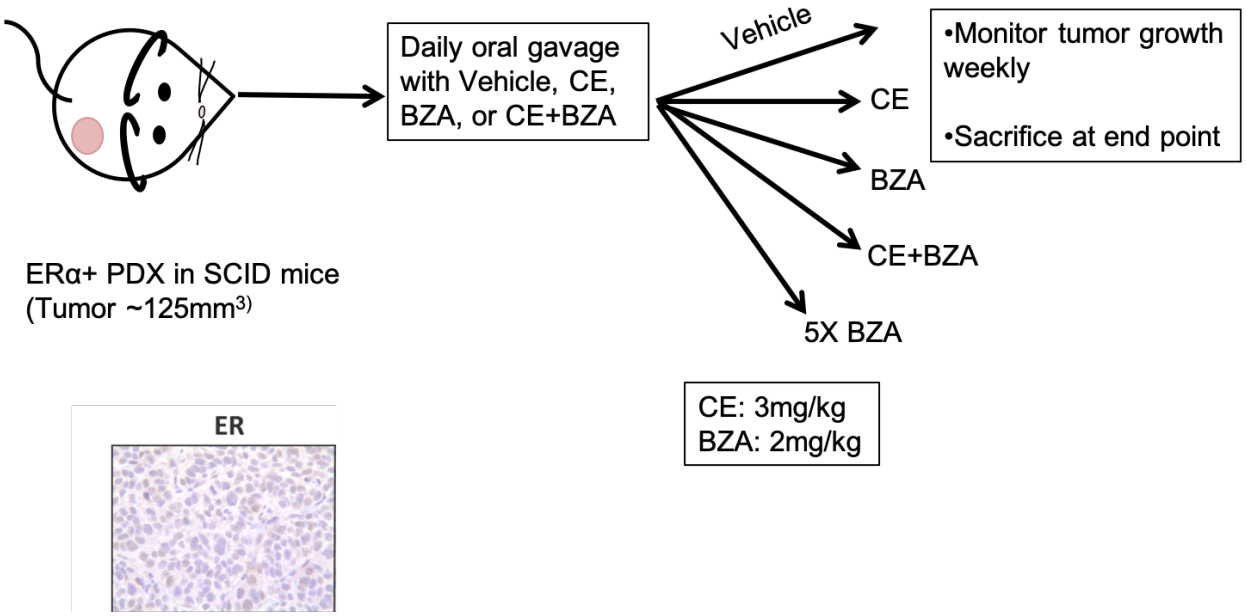


Figure 4.1 Schematic of PDX mouse study. ERα-positive PDX sample pieces were implanted into SCID mice and allowed to grow to a size of approximately 125mm^3, after which the mice were randomized and began treatment with vehicle, CE, BZA and CE+BZA. A BZA dose of 5X (10mg/kg) was used for comparison. Mice were monitored weekly for tumor growth and were sacrificed once tumors reached maximum allowed size. ERα expression of PDX sample prior to mouse implantation is shown at the bottom left.

A 5X BZA group was also added to show the effect of 10mg/kg of drug, which is a concentration more similar to Duavee drug-treatment in women, compared to the treatment we used. The average tumor volume for all groups (CE, BZA, CE+BZA and 5X BZA) was below vehicle (Figure 4.2). A statistically significant decrease in tumor size was observed for BZA, CE+BZA and 5X BZA groups at various time points in the study. Mice treated with BZA and 5X BZA survived longer than those in the vehicle-treated group (Figure 4.2). When tumor weight was measured there was not a statistically significant difference among treatments, however the tumor weights from mice treated with CE+BZA approached statistical significance at the endpoint (Figure 4.2). Overall, CE+BZA treatment decreased tumor volume and increased overall survival in the PDX mouse model.

Discussion

An ERα-positive PDX model was used to better understand the effect of CE+BZA on human tumor tissue. Overall, CE+BZA treatment was able to decrease tumor volume and decrease tumor weight in the PDX model. BZA alone was able to prolong the survival of PDX mice. PDX models are great tools to use in the lab since they better represent actual patient tumors and the tissue is a heterogeneous mixture from the patient. However, some problems may arise. The mice used in this study were infected with Epstein Barr Virus (EBV) and thus contracted T-cell lymphoma. The EBV most likely came from the patient tumor, as similar results have been reported in the literature.[81,82] Although the T-cell lymphoma presented an additional variable in the PDX model, CE+BZA was able to exert its effect and result in an overall smaller tumor volume compared to vehicle.

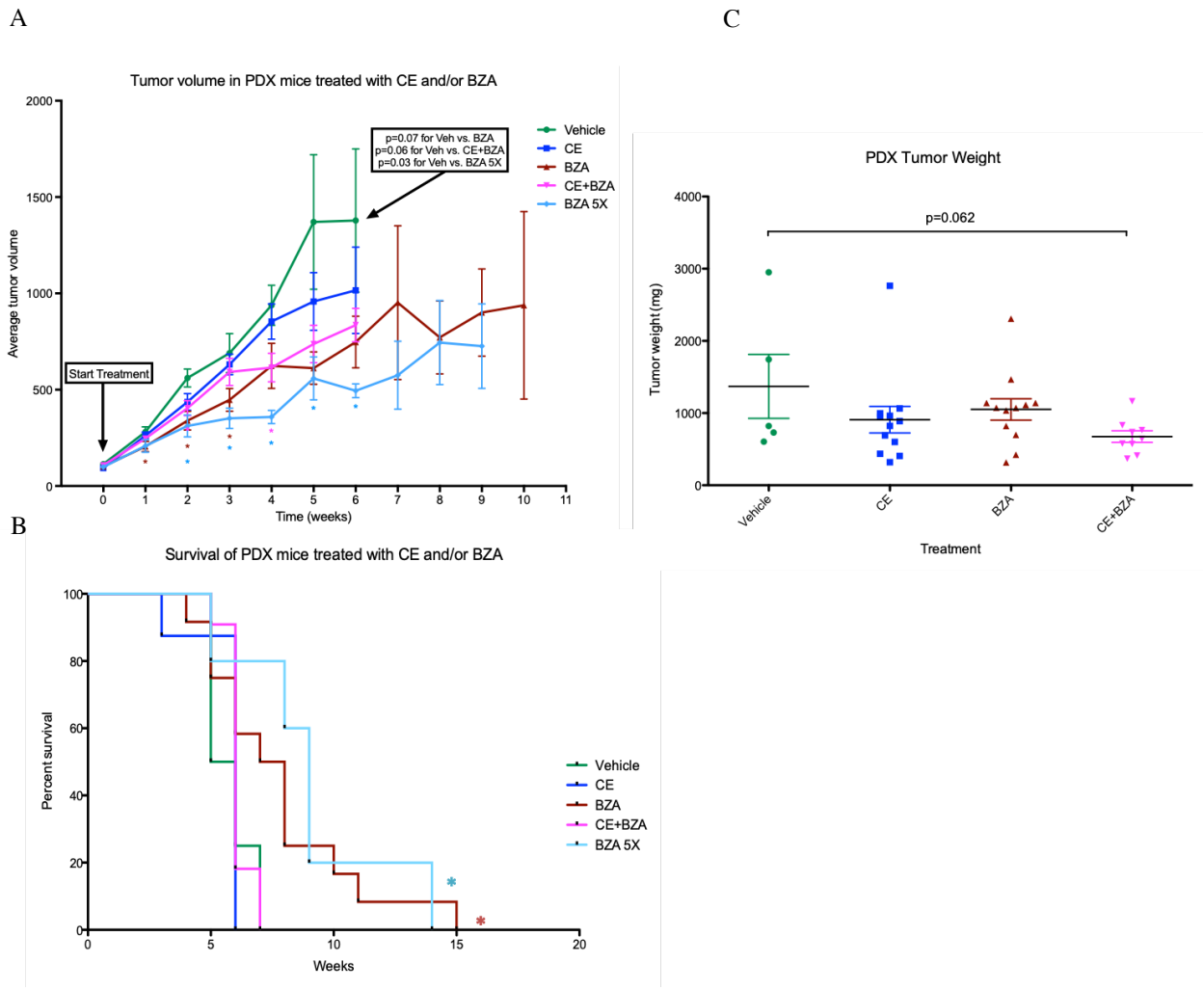


Figure 4.2 CE+BZA decreases tumor growth and increases survival but does not alter tumor weight in PDX mice. Average tumor volume in PDX mice over time upon treatment with vehicle, CE, BZA, CE+BZA or 5X BZA (A). Kaplan Meier survival curve of PDX mice over time (weeks) (B). Tumor weight (mg) of PDX mice at endpoint (C). Data are represented as mean ± SEM; *p<0.05, by Student's *t* test.

Week	0	1	2	3	4	5	6	7	8	9	10
Mice in vehicle group	8	8	8	8	8	8	4				
Mice in CE group	11	11	11	11	10	10	10				
Mice in BZA group	12	12	12	12	12	11	9	7	6	5	3
Mice in CE+BZA group	11	11	11	11	11	11	10				
Mice in 5X BZA group	6	6	6	6	6	6	5	5	5	3	

Table 7. Number of mice in PDX study per week.

This study is one of the few studies evaluating the effect of BZA on a PDX model. One such example was a study evaluating the effect of 10mg/kg BZA alone or with palbociclib, a CDK4/6 inhibitor, on tumor growth in a PDX model that had a mutant ERα.[83] In their study, Wardell *et al* found that BZA alone was effective at decreasing tumor volume in PDX mice. The results are similar to my study, however the models themselves are different. To date, however, mine is the first study evaluating the role of BZA at a dose of 2mg/kg on tumor growth. To our knowledge, this is also the first study evaluating the role of CE on tumor growth in a PDX model. In order to understand the effect of HRT in the form of CE+BZA on human breast cancer outgrowth, it is essential to perform studies with the best models available. Therefore, this model sheds some light on what the effect of CE+BZA may be on human tumor outgrowth. The results presented here should alleviate some perceived risks of this particular hormone replacement therapy for patients and providers.

CHAPTER V

SUMMARY AND CONCLUSIONS; ONGOING WORK AND FUTURE DIRECTIONS; DISCUSSION

Summary and conclusions

This study has shown that due to its action on ERα, CE+BZA is able to decrease target gene expression and cell growth, thereby decreasing overall tumor volume in mouse models. CE+BZA decreases ERα-positive cell growth and proliferation and does not alter growth of ERα-negative cell lines. CE+BZA decreased ERα binding at target gene loci and also decreased expression of ERα-regulated genes in ERα-positive cell lines. When siRNA against ERα was used, CE+BZA-treated cells exhibited higher gene expression compared to control, suggesting that ERα is required for regulating gene expression upon treatment. CE+BZA also decreased ERα protein expression over time in MCF7 cells. RNA sequencing studies showed that CE+BZA decreased pathways related to Estrogen-mediated S-phase entry and cell cycle regulation, while increasing pathways related to cell cycle checkpoint regulation and DNA damage regulation. IPA analysis from RNA-seq also showed that CE+BZA treatment downregulated pathways related to cell proliferation, survival and viability. CE+BZA upregulated cell death and apoptosis pathways; AHR signaling was the top canonical pathway identified by IPA. Upon further investigation, it was determined that BZA acted as an AHR agonist in both ERα-positive and ERα-negative cell lines. BZA was able to increase expression of AHR-responsive genes: AHRR, CYP1A1, CYP1B1. Due to these results, we decided to move to animal studies.

Results from the two mouse models of breast cancer we used in this study, the PyMT transgenic mouse model and an ERα-positive PDX mouse model, both correlated to the results observed in cell-based studies in an overall decrease of tumor cell proliferation. In PyMT mice, CE+BZA treatment decreased the overall growth of tumors. CE+BZA delayed tumor onset in ovariectomized and intact PyMT mice. Mice treated with CE+BZA also survived longer. Lung metastases in CE+BZA-treated mice were of a smaller size. ERα expression was higher in tumors of CE+BZA-treated mice. CE+BZA treatment led to a higher expression of AHR and AHRR in mouse tumors. In an ERα-positive PDX mouse model, CE+BZA treatment decreased the average tumor volume and prolonged survival of mice. Tumor weight of CE+BZA-treated mice was lower than of those treated with vehicle. Overall, this study has shown that BZA in combination with CE is able to counteract CE's stimulatory effects, thereby decreasing gene expression, cell proliferation and tumor growth. This work suggests that similar effects may be seen in postmenopausal women taking CE+BZA as a HRT drug: CE+BZA will not stimulate the growth of new tumors and may actually decrease or stop the growth of undetectable pre-existing lesions, therefore decreasing overall breast cancer cases.

Ongoing work and future directions

As previously mentioned, our lab is participating in the Promise Study, a "window of opportunity" clinical trial (Figure 5.1). In this trial headed by Dr. Swati Kulkarni at Northwestern University, women diagnosed with DCIS are randomized to receive placebo or CE+BZA for about four weeks prior to their lumpectomy or mastectomy surgery. The primary objective of this study is to assess whether CE+BZA treatment for 28±7 days is able to reduce proliferation as measured by Ki67 protein expression. Our lab is evaluating one of the secondary objectives: to assess changes in global gene expression profiling in women with DCIS treated with

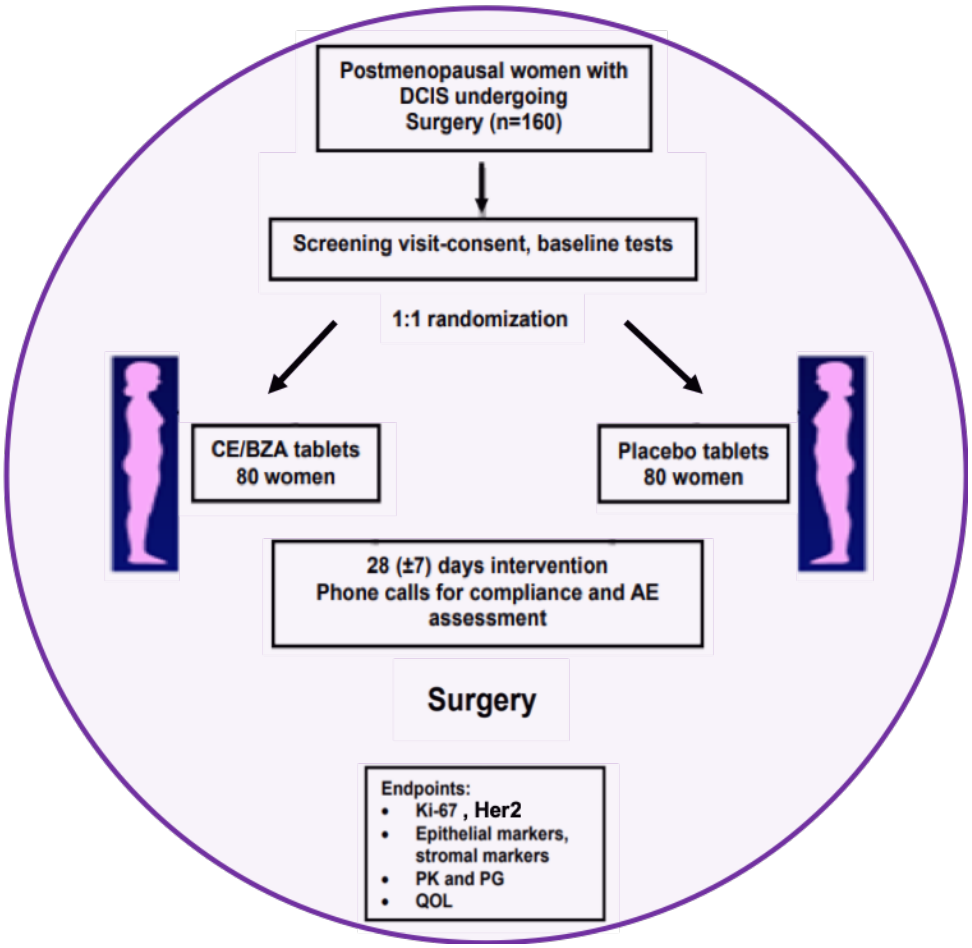


Figure 5.1 Schematic of the Promise study, a window of opportunity clinical trial. Postmenopausal women with DCIS will be randomized at diagnosis to receive either placebo or CE+BZA for 28±7 days prior to surgery. Biopsy and surgery samples will be analyzed by our lab by RNA-seq analysis to determine the effect of CE+BZA treatment.

CE+BZA compared to placebo. Patient accrual has already begun for this study and we have so far collected biopsy and surgery samples from a number of patients. These samples are stained and evaluated by Dr. Jeremy Segal, a pathologist at the University of Chicago. After evaluation, I use a laser microdissection microscope to cut out areas of DCIS from the sample slides for RNA extraction, and finally make RNA-Seq libraries.

Although some samples have gone through the entire process including sequencing, we have yet to draw any conclusions from the data due to the current small sample number. We are waiting for more patients to enroll in the study, and because this is a multi-center trial, we hope that we will soon have enough patient samples to draw at least preliminary conclusions. Samples from patients will also undergo multiplex staining for biomarkers in the Tlsty lab at UCSF. Dr. Tlsty's group previously determined that DCIS cases that are triple positive for p16, Cox-2 and Ki67 are associated with increased risk of invasive cancer[84]. Therefore, this biomarker testing may help determine whether CE+BZA treatment is decreasing the likelihood that a DCIS case becomes invasive. We anticipate that the results from this window of opportunity trial will show that CE+BZA treatment lowers Ki67 protein expression and, similar to results in the study presented here, upregulates pathways associated with apoptosis and cell death while downregulating pathways associated with cell proliferation, survival and viability. We anticipate that this study will also show an activation of the AHR pathway in patient tumors and will help clarify the role of the AHR pathway in breast cancer.

The results of this study have opened up several other avenues of work that is yet to be done. Since CE is a mixture of many compounds, it will be beneficial to understand the contribution of each on the growth of breast cancer in both cell lines and mouse models. It is likely that CE exerts its effect due to the blend of contribution from each compound in the

mixture; however, the effect of each compound should be investigated fully. Another direction of research is understanding the role of the AHR pathway in breast cancer. The role of AHR pathway in breast cancer is not fully understood and may be dependent on the stage of the tumor. More work is needed to fully tease out the role of AHR as well as AHRR, CYP1A1 and CYP1B1. The PyMT mouse model can also be used to study the effect of CE+BZA on immune cells. E2 has been shown to have an effect on both T cells and B cells since ERα is expressed in both cell types.[85] Therefore, it is possible that the CE+BZA combination may be exerting an effect on the immune system in patients receiving HRT. Likewise, the effect of CE+BZA can be studied on the tissue microenvironment in the PyMT mouse model. In the Promise study, we are also planning on evaluating the effect of CE+BZA on the DCIS microenvironment. Also, it will be interesting to see the effects of CE+BZA in more PDX studies. Since PDX samples reflect one patient at a time, it will be beneficial to evaluate the effect of the combination therapy on several PDX models to gain a better understanding of its effects.

Discussion

CE+BZA treatment has unique properties: clinical trials have shown that it is able to alleviate vasomotor symptoms and this study has determined that it decreases cancer cell growth and proliferation. Our study suggests that the CE+BZA combination therapy is safe for women who are considering hormone replacement therapy. The WHI trial results tremendously affected the patterns of prescribing and use of hormone replacement therapy. The WHI trial was stopped early "due to increased breast cancer risk and lack of overall benefit."[86] Based on the initial trial results, there was a dramatic decrease in the number of women taking HRT that still persists. Studies show that the number of women aged 45-64 taking PremPro declined from about 9% prior to the WHI to 2.7% after the results were published.[87] However, some results of the WHI

are often overlooked or not mentioned. The CE only arm of the study in fact showed favorable results: there was a significant decrease in total breast cancer and invasive breast cancer in women who had a hysterectomy and were taking CE alone.[37,86] Of course, not everyone will, or should, get a hysterectomy. Since the initial publication of WHI findings, it has been determined that women who started HRT closer to the time of menopause tended to have a reduced risk of coronary heart disease, compared to the risk seen in women who started HRT farther away from menopause.[88] Moreover, studies suggest that the cancer cases presented in the CE+MPA arm of the WHI were largely due to an outgrowth of pre-existing, undetectable lesions, not a stimulation of new tumor formation.[42,43]

In a survey of women taken after the first results of the WHI were published, 100% reported having at least heard of the WHI study, and more than half reported that it had affected their use of HRT. The main effects for those who were affected by the WHI study were decreasing the use and stopping the use of HRT all together. Among past users of HRT in the study, 36% reported discontinuing HRT with the most commonly cited reasons being fear of cancer or media advice against using HRT. One surprising result was that those women taking CE alone also changed their therapy after hearing the results of the WHI. This led researchers to conclude that there was a misunderstanding of the WHI study results: at the time the survey was taken, the WHI trial was halted only for those women taking PremPro, the combination of CE+MPA. One contributing factor to this misunderstanding was the verbiage used in the media: "hormones" and "hormone replacement therapy" instead of more specific terminology like "estrogen plus progestin" or "combination therapy."[89]

Partially due to the negative reaction after the WHI results were published, some women have favored the use of compounded hormone therapy (CHT) to treat menopausal symptoms.

The hormones included in the CHT formulation can include E2, progesterone, testosterone, as well as others.[90] Although a prescription is often needed for CHT, the FDA does not oversee the drug compounding in the same manner and there have been cases of both over-and under-dosing, at times with deleterious effects. One FDA study found that of 29 compounded drugs obtained from 12 different compounding pharmacies, 34% failed at least one quality test.[91] This FDA study found that nine products had a dose of progesterone lower than required. A 2007 study described three women who developed endometrial cancer after using CHT due to potential under-dosing of progesterone.[92] Progesterone creams are also often used, yet it is not always possible to accurately test the progesterone levels in tissues and blood levels do not give accurate results because they do not increase after progesterone creams are used.[93] Testosterone levels in some women taking CHT were found to be substantially higher than normal, which can lead to negative consequences if used for a prolonged period of time.[94] The FDA regards terms such as "bioidentical hormone" used by CHT proponents as a marketing ploy, implying a benefit for a drug without a medical or scientific basis.[90] Proponents of CHT often suggest that it is safer than commercially available HRT and that the CHT provides better symptom relief, yet there is no scientific basis for these remarks.[95] Unfortunately, the volume of CHT use is approaching the volume of FDA-approved HRT use, and CHT use is predicted to grow in the future.[96] Therefore, it would benefit women and providers to have an alternative to the feared PremPro. Based on previous cell and animal work, clinical trials, and work presented here, we believe that CE+BZA will provide women relief from menopausal symptoms in a safe and effective manner.

This study has the potential to impact the lives of many. Based on census data and menopause statistics, it is estimated that in 2019, there are approximately 29 million menopausal and postmenopausal women. Of those, approximately 70%, or 20 million, will suffer from hot

flashes. When we compare to all breast cancer cases, the affected population is about 100-fold larger. We hope that this study will lead to a reduction of the stigma that all HRT is "bad" and that there will be a reduction in the use of potentially harmful bioidentical hormones. Finally, we hope that the results from this study will decrease the number of women suffering from menopausal symptoms.

LIST OF ABBREVIATIONS

3MC	3-methylcholanthrene
4OHT	4-hydroxytamoxifen
AHR	Aryl hydrocarbon receptor
AHRR	Aryl hydrocarbon receptor repressor
BZA	Bazedoxifene
CE	Conjugated equine estrogens
ChIP	Chromatin immunoprecipitation
CHT	Compounded hormone therapy
DCIS	Ductal carcinoma in situ
DEG	Differentially expressed gene
Duavee	Trade name for CE+BZA
E2	17-beta estradiol
EBV	Epstein-Barr virus
ER	Estrogen receptor (ERα and ERβ)
GFP	Green fluorescent protein
HRT	Hormone replacement therapy
IPA	Ingenuity pathway analysis
MPA	Medroxyprogesterone acetate
PDX	Patient-derived xenograft
Premarin	Trade name for CE
PremPro	Trade name for combination of CE+MPA
SCR	Scrambled control for siRNA studies
SERD	Selective estrogen receptor degrader
SERM	Selective estrogen receptor modulator
SMART	Selective estrogens, Menopause And Response to Therapy clinical trials
TCDD	Tetracholorodibenzo-p-dioxin (simply, dioxin)
TSEC	Tissue selective estrogen complex
WHI	Women's Health Initiative clinical trials

METHODS

Tissue Culture

MCF7, C4-12, MDA-MB-231 cells were grown in DMEM medium (Corning) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals), 1% penicillin streptomycin (PenStrep; Gibco) and 2mM L-Glutamine (Gibco). T47D and ZR-75 cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% FBS and 1% PenStrep. For treatment, cells were culture for 48 hours in steroid deprived media: phenol red free DMEM or RPMI supplemented with 10% charcoal-stripped FBS and 1% PenStrep. Doses specified in the text were used for various compounds in this study: estradiol (Sigma-Aldrich), 4OHT (Sigma-Aldrich), 3MC (Sigma-Aldrich), CE and BZA (both, a gift from Pfizer).

Cell proliferation experiments

Cell growth was evaluated using the IncuCyte live cell imager (Essen BioScience). MCF7 and MDA-MB-231 cell lines had nuclear-GFP tag, therefore total cell number could be calculated. Cell confluence was used for C4-12 cells, which did not have a nuclear GFP tag. Cell number and percent confluence was determined using the IncuCyte integrated software.

Western blotting

Cells were grown to approximately 70% confluence and lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher). The resulting total cell lysate was run on a SDS-PAGE gel (BioRad), transferred onto nitrocellulose membrane (BioRad), blocked with 1% nonfat dry milk (LabScientific) and immunoblotted using antibodies for proteins of interest: anti-ER (HC-20 or F-10 from Santa Cruz), anti-actin (AC-15 from Santa Cruz or 20536-1-AP from

Proteintech), anti-AHR (D5S6H from Cell Signaling), anti-AHRR (ab85666 from abcam). Protein expression was normalized to beta-actin loading control. Alternatively, proteins were visualized using the Wes Simple Western (ProteinSimple) according to the manufacturers protocol.

qRT-PCR and RNA-seq studies

Cells were grown to a confluence of approximately 70% and were treated for 24 hours or as indicated. Cells were scraped, spun down and snap frozen. Total RNA was extracted using the Qiagen RNeasy kit, and RNA was converted to cDNA using qScript (QuantaBio). Transcript levels were analyzed by real time PCR using Taqman reagents (Life technologies) and probes from IDT in the StepOnePlus Real-Time PCR System (Applied Biosystems). RNA-seq libraries were prepared using 500ng total purified RNA with the KAPA Stranded mRNA-Seq kit (KR0960) and AMPure XP beads (Beckman Coulter). Amplification of libraries was performed with 12 PCR cycles and TruSeq primers (IDT) were used.

ChIP and ChIP-seq studies

Cells were grown to a confluence of approximately 70% and were treated for 45 minutes with drug concentrations as indicated. Cells were fixed with 1% formaldehyde for 10 minutes and crosslinking was quenched with 0.125M glycine for 5 minutes. Fixed cells were lysed with ChIP lysis buffer (1mL 1M Tris pH 8.0; 200μL 5M NaCl; 1mL 0.5M EDTA; 1mL NP-40; 1g SDS; 0.5g deoxycholate; water to 100mL) and sonicated using the Diagenode Biorupter for 30 minutes (30 second on, 30 seconds off cycles). Chromatin was diluted 1:10 in ChIP dilution buffer (1.7mL 1M Tris pH 8.0; 3.3mL 5M NaCl; 5mL 10% NP-40; 200μL 10% SDS; water to 100mL). 10% of sheared chromatin was removed and frozen as input. 4μg antibody was added,

and samples were incubated in a rotator at 4°C overnight, followed by 1-hour incubation with 30μL magnetic Protein G Mag Sepharose beads (GE Healthcare). Anti-ER HC-20 antibody (Santa Cruz) was used with IgG (Santa Cruz) as control. Immunoprecipitated chromatin was washed with cold ChIP wash buffer I, (2mL 1M Tris pH 8.0; 3mL 5M NaCl; 400μL 0.5M EDTA; 10mL 10% NP-40; 1mL 10% SDS; water to 100 mL), followed by cold ChIP wash buffer II (2mL 1M Tris pH 8.0; 10mL 5M NaCl; 400μL 0.5M EDTA; 10mL 10% NP-40; 1mL 10% SDS; water to 100mL), cold ChIP wash buffer III (1mL 1M Tris pH 8.0; 5mL of 5M LiCl; 200μL 0.5M EDTA; 10mL 10% NP-40; 10mL 10% deoxycholate; water to 100mL) and finally cold TE (pH 8.0). Elution from beads was performed twice for 15 minutes by incubation at 65°C with 100μL Elution buffer (1% SDS, 0.1M $NaHCO_3$). De-crosslinking was performed overnight at 65°C in elution buffer with 200mM NaCl. Next, samples were treated with RNAse A (Life Technologies) at 37°C for 30 minutes, followed by proteinase K (Life Technologies) for 45 minutes at 45°C. Finally, DNA fragments were purified using Qiagen PCR purification kit and resuspended in 40μL nuclease-free water. Real time qPCR was performed using SYBR green reagents (Life Technologies). For ChIP-seq, libraries were prepared using Kapa Biosystems LTP library preparation kit (KK8232) according to the manufacturers protocol.

RNA- and ChIP-Seq analysis

As mentioned previously, Wen-Ching Chan at the University of Chicago Center for Research Informatics performed sequencing analysis. A brief description of his work is summarized in this methods section. Quality of raw sequencing data was assessed using FastQC v0.11.4.[97] RNA reads were mapped to the UCSC human reference genome hg19 using TopHat v2.1.0 using default parameters.[98] DNA reads were aligned to hg19 using BWA MEM v0.7.12-

r1039 with default parameters.[99] Alignment results went through sample reconfiguration using Picard 1.70.

Individual RNA-seq alignments were evaluated for transcriptional expression using Cufflinks v2.2.1 and Rsubread::featureCounts.[100,101] Several methods of differential expression analysis were employed to discover DEGs between pair-wise groups based on expression estimation of individual mRNAs: Cuffdiff v2.2.1, DESeq v1.24.0, DESeq2 v1.12.4, edgeR v3.14.0 and limma v3.28.17.[100,102–105] To obtain groups with similar expression trends based on identified DEGs, in-house scripts were implemented using R and Perl languages.

Binding enrichment for ChIP-seq analysis was evaluated by comparing IP versus input samples using MACS v2.1.0, MeDUSA v1.22.0 and Q v1.2.0.[106–108] Enrichment regions were visualized using Integrative Genomics Viewer (IGV v2.3).

siRNA studies

Cells were grown to approximately 50% confluence in full DMEM media. On the day of siRNA treatment, siRNA (siGENOME SMARTpool, Dharmacon) and Dharmafect transfection reagent (Dharmacon) was diluted in Opti-MEM medium (Gibco), and finally added to cells with steroid deprived DMEM. siRNA treatment lasted 48 hours, after which drug treatment was performed as stated.

PyMT mouse studies

PyMT mice were bred in-house for all experiments: wild type females were bred with males heterozygous for the PyMT transgene. Mice were genotyped by PCR to identify presence of transgene (method below) and heterozygous female mice were used for all experiments. For

the first set of experiments, mice were ovariectomized at four to five weeks of age. Mice were first anesthetized with ketamine/xylazine (both Henry Schein) solution to prevent movement and allow for ease of manipulation. Once asleep, mice were shaved on the area of surgery, and an incision was made first in the skin, and then in the muscle layer. The ovary was gently pulled out of the body with tweezers and the uterine horns were tied off just below the ovary. Next, the ovary was cut, and the horn placed back into the body. The wounds were closed by suture first in the muscle layer and finally in the skin. The procedure was repeated on the contralateral side. Mice were given an injection of buprenorphine (Reckitt Benckiser Healthcare) as an analgesic after the surgery. The mice were then placed on a heated (25-27°C) surface until they woke and began to move without difficulty. After approximately 48 hours, when the wounds were healed, mice began receiving a daily oral gavage of vehicle, CE (3mg/kg), BZA (2mg/kg) or CE+BZA. E2 injection (5µg/kg) was used as a control, in addition to an E2+BZA group. Mice were monitored weekly for tumor formation and size and were sacrificed at an endpoint when the tumor reached maximum size. For the second set of experiments, mice were not ovariectomized, however received treatment and were monitored for tumor size in the same manner. Tumor size was measured with calipers and volume was determined by the equation $Tumor\ volume = \frac{1}{2}(length \times width^2)$, where length was the greatest diameter, and width was the smallest diameter.

Mouse genotyping

Mouse tail tissue was used for genotyping experiments. Mouse tails were snipped at an age of approximately three weeks. Tail snips were digested with Extracta reagent (QuantaBio) according to manufacturer's guidelines. Extracted DNA was run on a thermal cycler (Bio-Rad) using AccuStart II PCR SuperMix (QuantaBio) according to manufacturer's guidelines. PCR

products were run on a 2% agarose (DOT scientific) gel with approximately 0.2µg/mL ethidium bromide (Sigma) and visualized under UV light.

Patient-derived xenograft experiments

SCID mice were purchased from Taconic at an age of approximately 6 weeks. A PDX tumor from a donor mouse (Model TM00284) was dissected into pieces of approximately $2mm^3$. Recipient mice were anesthetized with isoflurane. When asleep, a shallow incision was made in the mouse and two tumor pieces were implanted subcutaneously on each side of the mouse abdomen. The incisions were closed with suture and the mice were placed on a heated (25-27°C) surface until they woke and began to move without difficulty. After the tumor had grown to the size of $125mm^3$, mice were randomized and were treated with an oral gavage of Vehicle, CE, BZA or CE+BZA. Tumor size was monitored weekly. Similar to the PyMT mouse experiments, CE and BZA were used at a dose of 3mg/kg and 2mg/kg, respectively. A 5X BZA group was also added (10mg/kg BZA).

qRT-PCR for mouse tumors

Mouse tumors were resected upon autopsy. Tumors were snap frozen in liquid nitrogen and crushed manually on dry ice. RNA was extracted from crushed tumors using the Quiagen RNeasy kit and qRT-PCR was performed as described above.

Tissue slide preparation

Mouse tumors and lungs were resected upon autopsy. Tissues were put into plastic cassettes and were fixed with 4% paraformaldehyde for a minimum of 24 hours. Next, tissues were delivered to the University of Chicago Human Tissue Resource Center where they were

embedded into paraffin, sectioned and mounted on slides. Slides were stained with hematoxylin and eosin or with Anti-ER antibody (MC-20, Santa Cruz). Tumor slides were scanned using the Leica ScanScope XT slide scanner and the Aperio software was used to quantify the amount of ER in the slides. Lung slides were scanned using Panoramic CRI scanner at 40X magnification and were analyzed for metastases using FIJI software.

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