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Bisphenol A at Low Nanomolar Doses Confers Chemoresistance in Estrogen Receptor Alpha Positive and Negative Breast Cancer Cells

Elizabeth W. LaPensee, Traci R. Tuttle, Sejal R. Fox, and Nira Ben-Jonathan

Department of Cancer and Cell Biology, University of Cincinnati, Ohio, USA

Address correspondence to Nira Ben-Jonathan, Ph.D, Department of Cell and Cancer Biology, University of Cincinnati, 3125 Eden Ave, Cincinnati, OH 45267-0521 USA. Telephone: (513) 558-4821. Fax: (513) 558-4823. E-mail: Nira.Ben-Jonathan@uc.edu

Running Title: Chemoresistance by Bisphenol A

Key Words: bisphenol A, breast cancer cells, chemotherapeutic agents, cytotoxicity, estrogen receptors

Abbreviations:

BPA- bisphenol A

CSS - charcoal-stripped serum

DES- diethylstilbestrol

E2- estradiol

ERα/β- estrogen receptor α or β

ERR- estrogen-related receptor

GPR30 - G protein-coupled receptor 30

MTT- 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo [1,5-a]pyrimidin-3-yl]phenol

PHTPP - 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo [1,5-a] pyrimidin-3-yl]phenol

Article Descriptor: Breast Cancer

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Outline

Abstract

Background. Resistance to chemotherapy is a major problem facing breast cancer patients, and identifying potential contributors to chemoresistance is a critical area of research. Bisphenol A (BPA) has long been suspected to promote carcinogenesis, but the high doses of BPA used in many studies generated conflicting results. In addition, the mechanism by which BPA exerts its biological actions is unclear. While estrogen has been shown to antagonize anti-cancer drugs, the role of BPA in chemoresistance has not been examined.

Objective. The objective was to determine whether BPA at low nanomolar concentrations opposes the action of doxorubicin, cisplatin and vinblastine in the ERα positive T47D and the ERα negative MDA-MB-468 breast cancer cells.

Methods. The responsiveness of cells to anti-cancer drugs and BPA was determined by the MTT cytotoxicity assay. Specific ERα and ERβ inhibitors and real-time PCR were used to identify potential receptor(s) that mediate the actions of BPA. Expression of anti-apoptotic proteins was assessed by Western blotting.

Results. BPA antagonizes the cytotoxicity of multiple chemotherapeutic agents in both ERα positive and negative breast cancer cells independent of the classical ERs. Both cell types express alternative ER receptors, including GRP30 and members of the estrogen related receptor (ERR) family. Increased expression of anti-apoptotic proteins is a potential mechanism by which BPA exerts its anti-cytotoxic effects.

Conclusions. BPA at environmentally relevant doses reduces the efficacy of chemotherapeutic agents. These data provide considerable support to the accumulating evidence that BPA is hazardous to human health.

Introduction

Bisphenol A (BPA) is a monomer of polycarbonate plastics which is used in numerous consumer products, including food and water containers, baby bottles, lining of metal food and beverage cans, medical tubing, epoxy resins and dental fillings (Welshons et al. 2006). Small amounts of BPA can be liberated from incompletely polymerized polycarbonates or via partial hydrolysis, especially upon heating (Le et al. 2008). Decades of continuous release of free BPA into food, beverages, and the environment have resulted in a widespread human exposure to this chemical. Many studies in the USA, Europe and Japan have documented BPA levels ranging from 0.2 to 10 ng/ml (~0.5-40 nM) in adult and fetal human serum (Welshons et al. 2006) as well as in breast milk (Kuruto-Niwa et al. 2007). Being lipophilic, BPA can also accumulate in fat, with detectable levels of BPA found in half of breast adipose tissue samples examined (Fernandez et al. 2007).

Given the structural similarity of BPA to the potent estrogenic compound diethylstilbestrol (DES), its ability to promote carcinogenesis has long been suspected (Keri et al. 2007). Studies with rodents have revealed that early life exposure to BPA causes increased susceptibility to mammary and prostate tumorigenesis (Prins et al. 2007; Soto et al. 2008), however there is less evidence for carcinogenic activity of BPA when administered to adult animals. Studies with human breast cancer cells have yielded inconsistent data with respect to the mitogenic, apoptotic or transcriptional properties of BPA (Dairkee et al. 2008; Diel et al. 2002; Singleton et al. 2006; Soto et al. 1995). This inconsistency is attributed to the wide variations in BPA doses used, some of which are at the micromolar levels. BPA often exhibits a 'U' or an inverted 'U' shaped dose-dependent curve. Consequently, extrapolation from an action, or lack of action, of BPA at high doses to its presumed bioactivity at low doses is unwarranted. Thus, to support the argument that BPA poses risks to human health, it is necessary to establish its effectiveness at environmentally relevant concentrations (the low nanomolar range).

The mechanism by which BPA exerts it biological actions is enigmatic. The binding affinity of BPA to estrogen receptor (ER) α or ERβ is 10,000 and 1000 fold lower than that of estradiol (E2), respectively (Kuiper et al. 1998). This suggests that BPA should mimic or compete with

endogenous estrogens only at the μM range. Yet, BPA at nM doses often displays activities that are similar to those of E2 (Watson et al. 2005; Welshons et al. 2006). To reconcile this dilemma, several speculations have been proposed. One view is that BPA binds differently within the ligand binding domain of ERα or ERβ and recruits a dissimilar set of co-regulators (Safe et al. 2002). Other investigators maintain that BPA elicits its responses via non-classical estrogen receptors, including membrane-anchored ERs (Watson et al. 2005), G-protein-coupled receptor 30 (GPR30; (Thomas and Dong 2006)), or members of the estrogen-related receptors (ERR) such as ERRγ, which has a high binding affinity to BPA (Okada et al. 2008).

While most studies to date have examined whether BPA stimulates breast cancer cell proliferation, its potential effects on chemotherapeutic efficacy have received little attention. Chemotherapy, alone or in combination with hormonal or targeted therapy, remains the mainstay treatment in metastatic breast disease. A wide variety of anti-cancer drugs are available, including doxorubicin, cisplatin and vinblastine. Most regimens combine agents that act by different mechanisms to improve efficacy. Although treatment of breast cancer patients with these anti-cancer drugs has shown good success, tumor resistance remains a major obstacle. Some tumors are intrinsically resistant to certain drugs while others can acquire resistance following treatment. Although the effects of environmental pollutants on drug transporters as well as on metabolic and detoxifying enzymes have been explored to some extent (Brockmoller et al. 2000; Chen et al. 1998; Han and Zhang 2004), there is no information on whether endocrine disruptors can modulate the responsiveness of breast cancer cells to anti-cancer drugs.

The objectives of this study were to: 1) compare the effects of low doses of BPA on cisplatin, doxorubicin and vinblastine cytotoxicity in the estrogen-responsive T47D breast cancer cells, 2) examine whether BPA exerts similar effects on the estrogen-insensitive MDA-MB-468 cells, 3) compare expression of classical (ERα and ERβ) and non-classical (GPR30, ERRα, ERRβ and ERRγ) estrogen receptors in the two cell lines, 4) determine the effects of an ERα antagonist (ICI182,780; ICI), and an ERβ-specific antagonist (PHTPP) on the ability of BPA to antagonize the cytotoxic effects of doxorubicin, and 5) examine whether the chemoresistant effects of BPA are mediated by altered expression of anti/pro-apoptotic proteins of the Bcl-2 and survivin families.

Materials and Methods

Drugs and inhibitors. Doxorubicin (Sigma, St Louis, MO), cisplatin (Sigma) and vinblastine (Biomol, Plymouth Meeting, PA) were dissolved in water at stock concentrations of 1 mg/ml (doxorubicin and cisplatin) or 0.1 mg/ml. ICI182780 (100 mM; Tocris Bioscience, Ellisville, MO) and PHTPP (50 mM; Tocris) were dissolved in DMSO or ethanol, respectively. Drugs and inhibitors were diluted in culture medium immediately before treatment.

Cell lines and culture conditions. T47D and MDA-MB-468 cells were obtained from the American Type Culture Collection (Manassas, VA). T47D cells were maintained in RPMI (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Hyclone), 5μg/ml bovine insulin, 10 mM HEPES, 1 mM sodium pyruvate and 50 μg/ml normocin (Invivogen, San Diego, CA). 468 cells were cultured in low glucose DMEM (Hyclone) supplemented with 10% FBS and 50 μg/ml normocin. For all experiments, T47D cells were plated in phenol red-free RPMI with 5% charcoal stripped serum (CSS) and ITS+ supplement **(**1:200; BD biosciences, Bedford, MA**)** and treated in RPMI with 3% CSS and ITS+. MDA-MB-468 cells were plated in phenol red-free DMEM supplemented with 3% CSS and treated in DMEM with 1% CSS.

Cytotoxicity assay. Cells were plated at a density of 6000 or 8000 cells/well in 96 well plates in plating medium. The following day, cells were incubated with BPA for 24 hrs in treatment medium. In the case of inhibitors, ICI and PHTPP were added to the cells 1 hr before BPA. After BPA treatment for 24 hrs, the various drugs were added for an additional 1 to 4 days in the continuous presence of BPA. Cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) methods. MTT was added at a final concentration of 0.5 mg/ml for 2 hrs. Following medium aspiration, the formazan dye was extracted with DMSO and absorbance was read at 570 nm using a plate reader (Bio-Tek, Winooski, VT).

Western blotting. Following treatment, cells were homogenized in buffer (10nM Tris-HCl, 5mM EDTA, 50nM NaCl, 50mM sodium fluoride, 30mM sodium pyrophosphate, 1% Triton-X, 200μM sodium orthovanadate, 1mM phenylmethylsulfonyl, 1μg/ml pepstatin, μg/ml leupeptin, 5μg/ml aprotinin). Protein concentration was determined using the Pierce BCA protein assay.

Cell lysates (40μg proteins) were electrophoresed onto 12% or 15% SDS-PAGE gels. After transfer to PVDF membranes, samples were blocked in 5% dry milk and incubated overnight with the following primary antibodies: Bcl-2, Bcl-xL, survivin (1:1000 each; Cell Signaling, Danvers, MA), ERα (1:400, Santa Cruz Biotechnology, Santa Cruz, CA), ERβ (1:3000, Upstate, Danvers, MA) or β-actin (1:10,000; Sigma). After incubation with horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ), products were developed on film using SuperSignal chemiluminescence reagents (Pierce, Rockford, IL).

Real-Time PCR. Total RNA was isolated using Tri-Reagent (MRC, Cincinnati, OH) and cDNA was synthesized as previously described (Hugo et al. 2006). PCR was performed on 200 ng cDNA using intron-spanning primers for ERα, ERβ, GRP30, ERRα, ERRβ and ERRγ; β2-microglobulin (β2M) was used as a reference gene. Primer sequences are listed in Table 1. Quantitative real-time PCR was performed using Immolase heat-activated Taq DNA polymerase (Bioline, Taunton, MA). SYBR Green I (Invitrogen, Carlsbad, CA) was used for fluorometric product detection using a SmartCycler I (Cepheid, Sunnyvale, CA). Cycle parameters were 96ºC for 15 min for polymerase activation, followed by 40 cycles of 95ºC for 15 sec, 57ºC for 15 sec and 72ºC for 30 sec with an optical read stage at 83.5ºC for 6 sec. Product purity was confirmed by DNA melting curve analysis. After correction for β2M, fold changes in gene expression were calculated from the cycle threshold measurements as described (Pfaffl et al. 2002).

Data analysis. Statistical differences were determined by one-way ANOVA followed by Newman-Keuls post hoc analysis. p values <0.05 were considered significant. All experiments were performed at least three times.

Results

BPA protects T47D cells from chemotherapeutic-induced cytotoxicity. We first examined the sensitivity of the estrogen-responsive T47D cells to selected anti-cancer drugs, and whether BPA protects the cells from drug-induced cytotoxicity. As shown in Figure 1, doxorubicin induced a dose dependent decrease in cell viability that was either completely, or partially, antagonized by a 24 hr pretreatment with a low dose of BPA (1 nM). The cells were less sensitive to cisplatin,

with the highest tested dose (400 ng/ml) decreasing viability by approximately 40%. BPA prevented drug-induced cytotoxicity at all tested cisplatin doses. The cytotoxic effects of vinblastine on T47D cells resembled that of doxorubicin. Pre-treatment with BPA was highly effective only against the lowest dose of vinblastine (1 ng/ml). In all cases, BPA alone increased cell viability.

BPA antagonizes chemotherapeutic agents in MDA-MB-468 cells. We next examined whether BPA protected the estrogen-unresponsive MDA-MB-468 cells from the same anti-cancer drugs (Fig 2). Similar to T47D cells, doxorubicin treatment resulted in a dose dependent decrease in 468 cell viability. BPA completely, or partially, protected the cells from all doses of doxorubicin. The MDA-MB-468 cells were significantly more sensitive to cisplatin than T47D cells, with the 400ng/ml dose of cisplatin inhibiting cell viability by more than 80%. All doses of cisplatin were antagonized by a pretreatment with BPA. BPA protected MDA-MB-468 cells only from the lowest dose of vinblastine. Unlike T47D cells, BPA alone has no effect on cell viability.

BPA, at low nM concentrations, protects cells from doxorubicin-induced cytotoxicity. The next experiment evaluated the ability of increasing, environmentally relevant doses of BPA to antagonize the cytotoxic effect of one dose of doxorubicin. Fig 3 shows that BPA alone (1 nM or 10 nM) significantly increased cell viability in the T47D, but not the MDA-MB-468 cells. In both cell types, doxorubicin treatment induced an approximately 35% decrease in cell viability. A 24 hr pretreatment with BPA at all doses examined completely protected the cells from dox-induced cytotoxicity.

The protective effects of BPA are not mediated via classical estrogen receptors. To determine if the protective effects of BPA involve ERα or ERβ we used ICI, an antagonist of both receptors, as well as PHTPP, a specific ERβ antagonist. As shown in Fig 4, upper panels, neither ICI nor PHTPP has any effect on their own on T47D or MDA-MB-468 cell viability. Furthermore, the ability of BPA to antagonize doxorubicin-induced cytotoxicity in either cell line was not altered in the presence of ICI or PHTPP. Using Western blotting, we next probed for both ERα and ERβ in T47D and MDA-MB-468 cells treated for 1, 4 or 48 hrs with the above inhibitors. Fig 4, lower panels, demonstrate that T47D cells, but not 468 cells, express ERα, while both cell types

express ERβ. Treatment with ICI caused a time-dependent decrease in ERα expression in T47D cells, reducing it to an undetectable level by 48 hrs. On the other hand, ERβ expression in MDA-MB-468 cells increased at 4hrs and decreased after 48hrs in response to ICI treatment. PHTPP had no effect on ERα, increased the expression of ERβ in T47D cells, and had no effect on ERβ in MDA-MB-468 cells.

Relative receptor expression in T47D and MDA-MB-468 cells. Using real-time PCR, the next set of experiments compared the expression of several putative estrogen receptors in the two cell lines. Data were presented as percent of ERα expression in T47D cells. Fig 5 shows that the expression of ERβ is similar in the two cells lines, being less than 1% that of ERα. ERRα is the most highly expressed of the alternative receptors in both cell lines, nearing 10% of ERα in T47D cells. The expression levels of GRP30 and ERRγ are similar in T47D cells, with ERRγ being slightly higher than GRP30 in MDA-MB-468 cells. ERRβ was undetectable in both cell lines.

BPA may promote chemoresistance by altering anti-apoptotic proteins. We next explored the effects of BPA and doxorubicin on the expression of several pro-survival proteins. As shown in Fig 6, treatment of T47D cell with BPA for 24 hrs increased both Bcl-2 and Bcl-xL expression. BPA and doxorubicin alone increased expression of survivin, but their combination had no further effect. Both doses of doxorubicin caused a small decrease in Bcl-2 expression, which was partially prevented when the cells were pre-treated with BPA. In 468 cells, Bcl-2 expression was higher when cells were exposed to 75 ng/ml dox and BPA than in 75 ng/ml doxorubicin alone. BPA alone did not increase the expression of Bcl-xL in 468 cells. In both cell lines, Bcl-xL expression was higher in cells treated with 150 mg/ml doxorubicin and BPA as compared to 150 ng/ml doxorubicin alone. Survivin expression was increased in both cell types in response to BPA or doxorubicin alone but was not further augmented by their combination.

Discussion

This is the first report that BPA antagonizes chemotherapeutic agents in both ERα positive and negative breast cancer cells. Importantly, unlike some previous studies which have used

micromolar concentrations of BPA, our data were obtained using low nanomolar concentrations, which are relevant to human exposure levels. BPA confers chemoresistance to several anti-cancer drugs, including doxorubicin, cisplatin and vinblastine, that act by different mechanisms. As judged by specific ERα/ERβ antagonists, BPA does not appear to mediate its effects through either ERα or ERβ. Given that both cell lines express non-classical estrogen receptors such as GPR30 and members of the ERR family, these could serve as putative BPA receptors. The ability of BPA to alter the expression of Bcl-2 and Bcl-xL suggests a potential mechanism by which it confers chemoresistance in the two breast cancer cell lines.

We postulated that BPA might play a role in chemoresistance following the reports that estradiol antagonizes anti-cancer drugs. For example, taxol-induced cytotoxicity in MCF-7 cells was abrogated by 0.1μM estradiol (Huang et al. 1997). This was confirmed in a later study implicating JNK activation in the modulation of apoptosis and estradiol protection (Razandi et al. 2000). In addition, estradiol antagonizes dox-induced cytotoxicity in MCF-7 cells (Teixeira et al. 1995). Our data show that BPA protects T47D cells from several anti-cancer drugs. More unexpected was the effect of BPA on the estrogen unresponsive MDA-MB-468 cells, raising the prospect that ERα does not mediate the chemoresistant effects of BPA.

The few reports on the effects of BPA on mitogenesis have used the ER positive MCF-7 cells. Olsen et al., observed increased MCF-7 cell proliferation in response to BPA, with the relative proliferative potential being 60,000 times lower than that of estradiol (Olsen et al. 2003). Such effects of BPA were further confirmed by Samuelsen et al., whose MCF-7 data are a prime example of an inverted U shaped curve which is often observed when treating cells with increasing doses of BPA. In this study, cell proliferation was unchanged in the presence of 10 nM BPA, increasing more than 40% with 100 nM BPA, peaking with 1 μM BPA and declining at higher doses (Samuelsen et al. 2001). These studies are in agreement with our data which show an approximate 25% increase in cell viability in T47D cells in response to BPA. Despite the lack of a mitogenic effect of BPA in the MDA-MB-468 cells, its ability to antagonize the anti-cancer drugs was observed with as little as 0.01 nM BPA.

Of particular interest is the ability of BPA to antagonize the cytotoxic effects of three chemotherapeutic agents that induce cell death by different mechanisms. Doxorubicin causes DNA damage by chelating metal ions, generating free radicals and inhibiting topoisomerase, thereby blocking transcription (Aubel-Sadron and Londos-Gagliardi 1984). Cisplatin, a platinum based compound, causes DNA intrastrand crosslinking and inhibits replication (Stewart 2007). Vinblastine acts by interfering with microtubule dynamics, resulting in mitotic arrest and cell death (Toso et al. 1993). As mentioned above, estradiol protects against a microtubule altering, as well as a DNA damaging drug (Huang et al. 1997; Teixeira et al. 1995). Thus, drugs with different intracellular targets may have a common mechanism for inducing cell death. Future studies should examine whether BPA protects cells from death ligands which induce apoptosis by binding to pro-apoptotic death receptors.

BPA weakly competes with 17β-estradiol in binding to the estrogen receptor. Using a cell based transcription assay with a reporter gene, Hiroi et al reported that BPA exhibits agonistic activity when signaling through ERβ but has both agonistic and antagonistic activity when interacting with ERα (Hiroi et al. 1999). Whereas T47D cells express both ERα and ERβ, MDA-MB-468 cells have long been used as a model for ER negative breast cancer. We show that MDA-MB-468 cells express ERβ protein, whose levels can be modulated by treatment with ICI or PHTPP. Like others (Fan et al. 2003; Long and Nephew 2006), we show that ICI rapidly and dramatically degraded the ERα protein, suggesting that the use of ICI is comparable to targeting the receptor with siRNA. The finding that BPA exerted its anti-cytotoxic effects when ERα or ERβ were inhibited suggests that BPA activates a non-classical ER(s).

Non-classical ERs include the G-protein coupled receptor GPR30 and members of the estrogen related receptor family: ERRα, ERRβ and ERRγ. BPA binds to GPR30 with an IC_{50} of 630 nM, as compared to estradiol with an IC_{50} of 17.8 nM (Thomas and Dong 2006). Interestingly, ICI binds to GPR30 and acts as an agonist (Prossnitz et al. 2007). Although 17-β estradiol does not bind to members of the ERR family, ERRs can bind to functional EREs in ER target genes (Huppunen and Aarnisalo 2004). Among the ERR's, BPA binds strongly to ERRγ, with a K_D of 5.5 nM , a much more environmentally relevant dose than that needed to bind to ERα or ERβ (Matsushima et al. 2007). This makes ERRγ the most likely candidate for mediating the

protective effects of BPA. Importantly, ERRγ mRNA is expressed in 75% of breast tumors as compared to normal mammary epithelial cells (Ariazi and Jordan 2006). We show that both T47D and MDA-MB-468 cells express GPR30, ERRα and ERRγ, while ERRβ was undetectable. These data identified potential receptors that should be pursued using approaches such as siRNA to determine which receptor(s) mediate the chemoprotective effects of BPA.

The mechanisms underlying chemoresistance include altered expression of pro/anti-apoptotic proteins, increased activity of membrane transporters such as P-glycoprotein, the status of tumor suppressors and the efficiency of DNA repair processes. The anti-apoptotic Bcl-2 and Bcl-xL proteins and the pro-survival Inhibitor of Apoptosis protein survivin, are major players in tumor growth and resistance to cytotoxic insults. Estrogen increases Bcl-2 protein expression in MCF-7 cells, with cells transfected with Bcl-2 antisense twice as sensitive to doxorubicin treatment in the presence of estrogen than controls (Teixeira et al. 1995). Another study suggested that increased Bcl-2 in response to estrogen protects cells from taxol-induced cytotoxicity (Huang et al. 1997). Our data indicate that upregulation of Bcl-2 and Bcl-xL is a plausible mechanism by which BPA confers resistance to doxorubicin and possibly other anti-cancer drugs. The survivin data agree with another study that found increased expression of this protein following doxorubicin treatment (Tirro et al. 2006). However, the contributions of survivin are less critical when proteins such as Bcl-2 and Bcl-xL, which are upstream of survivin, mediate survival.

In conclusion, we have shown that low doses of BPA confer chemoresistance to multiple anti-cancer drugs, possibly by increasing expression of anti-apoptotic Bcl-2 proteins. Importantly, the effects of BPA are observed in a cell line lacking ERα, indicating that BPA acts via non-classical receptors. These data highlight a previously unrecognized function of BPA in cancer management, thereby adding a strong support to the growing recognition of the adverse effects of BPA on human health.

References

Ariazi EA and Jordan VC. 2006. Estrogen-related receptors as emerging targets in cancer and metabolic disorders. Curr Top Med Chem 6:203-215.

Aubel-Sadron G and Londos-Gagliardi D. 1984. Daunorubicin and doxorubicin, anthracycline antibiotics, a physicochemical and biological review. Biochimie 66:333-352.

Brockmoller J, Cascorbi I, Henning S, Meisel C, Roots I. 2000. Molecular genetics of cancer susceptibility. Pharmacology 61:212-227.

Chen X, Carystinos GD, Batist G. 1998. Potential for selective modulation of glutathione in cancer chemotherapy. Chem Biol Interact 111-112:263-275.

Dairkee SH, Seok J, Champion S, Sayeed A, Mindrinos M, Xiao W et al. 2008. Bisphenol A induces a profile of tumor aggressiveness in high-risk cells from breast cancer patients. Cancer Res 68:2076-2080.

Diel P, Olff S, Schmidt S, Michna H. 2002. Effects of the environmental estrogens bisphenol A, o,p'-DDT, p-tert-octylphenol and coumestrol on apoptosis induction, cell proliferation and the expression of estrogen sensitive molecular parameters in the human breast cancer cell line MCF-7. J Steroid Biochem Mol Biol 80:61-70.

Fan M, Bigsby RM, Nephew KP. 2003. The NEDD8 pathway is required for proteasome-mediated degradation of human estrogen receptor (ER)-alpha and essential for the antiproliferative activity of ICI 182,780 in ERalpha-positive breast cancer cells. Mol Endocrinol 17:356-365.

Fernandez MF, Arrebola JP, Taoufiki J, Navalon A, Ballesteros O, Pulgar R et al. 2007. Bisphenol-A and chlorinated derivatives in adipose tissue of women. Reprod Toxicol.

Han B and Zhang JT. 2004. Multidrug resistance in cancer chemotherapy and xenobiotic protection mediated by the half ATP-binding cassette transporter ABCG2. Curr Med Chem Anticancer Agents 4:31-42.

Hiroi H, Tsutsumi O, Momoeda M, Takai Y, Osuga Y, Taketani Y. 1999. Differential interactions of bisphenol A and 17beta-estradiol with estrogen receptor alpha (ERalpha) and ERbeta. Endocr J 46:773-778.

Huang Y, Ray S, Reed JC, Ibrado AM, Tang C, Nawabi A et al. 1997. Estrogen increases intracellular p26Bcl-2 to p21Bax ratios and inhibits taxol-induced apoptosis of human breast cancer MCF-7 cells. Breast Cancer Res Treat 42:73-81.

Hugo ER, Brandebourg TD, Comstock CE, Gersin KS, Sussman JJ, Ben-Jonathan N. 2006. LS14: a novel human adipocyte cell line that produces prolactin. Endocrinology 147:306-313.

Huppunen J and Aarnisalo P. 2004. Dimerization modulates the activity of the orphan nuclear receptor ERRgamma. Biochem Biophys Res Commun 314:964-970.

Keri RA, Ho SM, Hunt PA, Knudsen KE, Soto AM, Prins GS. 2007. An evaluation of evidence for the carcinogenic activity of bisphenol A. Reprod Toxicol 24:240-252.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT et al. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252-4263.

Kuruto-Niwa R, Tateoka Y, Usuki Y, Nozawa R. 2007. Measurement of bisphenol A concentrations in human colostrum. Chemosphere 66:1160-1164.

Le HH, Carlson EM, Chua JP, Belcher SM. 2008. Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing cerebellar neurons. Toxicol Lett 176:149-156.

Long X and Nephew KP. 2006. Fulvestrant (ICI 182,780)-dependent interacting proteins mediate immobilization and degradation of estrogen receptor-alpha. J Biol Chem 281:9607-9615.

Matsushima A, Kakuta Y, Teramoto T, Koshiba T, Liu X, Okada H et al. 2007. Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERR gamma. J Biochem 142:517-524.

Okada H, Tokunaga T, Liu X, Takayanagi S, Matsushima A, Shimohigashi Y. 2008. Direct Evidence Revealing Structural Elements Essential for the High Binding Ability of Bisphenol A to Human Estrogen-Related Receptor-gamma. Environ Health Perspect 116:32-38.

Olsen CM, Meussen-Elholm ET, Samuelsen M, Holme JA, Hongslo JK. 2003. Effects of the environmental oestrogens bisphenol A, tetrachlorobisphenol A, tetrabromobisphenol A, 4-hydroxybiphenyl and 4,4'-dihydroxybiphenyl on oestrogen receptor binding, cell proliferation and regulation of oestrogen sensitive proteins in the human breast cancer cell line MCF-7. Pharmacol Toxicol 92:180-188.

Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36.

Prins GS, Birch L, Tang WY, Ho SM. 2007. Developmental estrogen exposures predispose to prostate carcinogenesis with aging. Reprod Toxicol 23:374-382.

Prossnitz ER, Arterburn JB, Sklar LA. 2007. GPR30: A G protein-coupled receptor for estrogen. Mol Cell Endocrinol 265-266:138-142.

Razandi M, Pedram A, Levin ER. 2000. Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. Mol Endocrinol 14:1434-1447.

Safe SH, Pallaroni L, Yoon K, Gaido K, Ross S, McDonnell D. 2002. Problems for risk assessment of endocrine-active estrogenic compounds. Environ Health Perspect 110 Suppl 6:925-929.

Samuelsen M, Olsen C, Holme JA, Meussen-Elholm E, Bergmann A, Hongslo JK. 2001. Estrogen-like properties of brominated analogs of bisphenol A in the MCF-7 human breast cancer cell line. Cell Biol Toxicol 17:139-151.

Singleton DW, Feng Y, Yang J, Puga A, Lee AV, Khan SA. 2006. Gene expression profiling reveals novel regulation by bisphenol-A in estrogen receptor-alpha-positive human cells. Environ Res 100:86-92.

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 103 Suppl 7:113-122.

Soto AM, Vandenberg LN, Maffini MV, Sonnenschein C. 2008. Does breast cancer start in the womb? Basic Clin Pharmacol Toxicol 102:125-133.

Stewart DJ. 2007. Mechanisms of resistance to cisplatin and carboplatin. Crit Rev Oncol Hematol 63:12-31.

Teixeira C, Reed JC, Pratt MA. 1995. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. Cancer Res 55:3902-3907.

Thomas P and Dong J. 2006. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. J Steroid Biochem Mol Biol 102:175-179.

Tirro E, Consoli ML, Massimino M, Manzella L, Frasca F, Sciacca L et al. 2006. Altered expression of c-IAP1, survivin, and Smac contributes to chemotherapy resistance in thyroid cancer cells. Cancer Res 66:4263-4272.

Toso RJ, Jordan MA, Farrell KW, Matsumoto B, Wilson L. 1993. Kinetic stabilization of microtubule dynamic instability in vitro by vinblastine. Biochemistry 32:1285-1293.

Watson CS, Bulayeva NN, Wozniak AL, Finnerty CC. 2005. Signaling from the membrane via membrane estrogen receptor-alpha: estrogens, xenoestrogens, and phytoestrogens. Steroids 70:364-371.

Welshons WV, Nagel SC, Vom Saal FS. 2006. Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology 147:S56-S69.

Table 1. Human gene-specific primers for quantitative real-time RT-PCR.

Gene	Accession Number	Forward primer (5'→3')	Reverse Primer (5'→3')	Product Size (bp)
ESR1	NM_000125	CAGGCACATGAGTAACAAAGG	CAAGGAATGCGATGAAGTAGAG	195
ESR2	NM_001437	CAGTTATCACATCTGTATGCGG	ACTCCATAGTGATATCCCGA	208
ESRRA	NM_004451	ACTGCAGGATGAGCTGG	TGCACAGAGTCTGAATTGG	185
ESRRB	NM_004452	CTGGTGTACGCTGAGGA	TACATGGAATCGGAGTTGG	172
ESRRG	NM_001438	CATATTCCAGGCTTCTCCA	GACAAGTTCATCCTCAAACGA	122
GPR30	NM_001039966	ACGAGACTGTGAAATCCGCAACCA	ATCAGGCTGGAGGTGCACTTGGAA	153
B2M	NM_004048	GGCATTCCTGAAGCTGAC	GAATCTTTGGAGTACGCTGG	114

Primer pairs were designed using the program PerlPrimer and are all intron-spanning pairs. ESR1- estrogen receptor alpha, ESR2 - estrogen receptor beta, ESRRA - estrogen-related receptor alpha, ESRRB - estrogen-related receptor beta, ESSRG - estrogen-related receptor gamma (all 3 transcripts), GPR30 – G protein-coupled receptor 30, B2M - beta-2-microglobulin.

Figure Legends

Figure 1 BPA protects T47D cells from several chemotherapeutic agents. Cells were treated with BPA for 24 hrs followed by increasing concentrations of doxorubicin, cisplatin or vinblastine for an additional 96 hrs. Cytotoxicity was determined by the MTT assay. Each value is a mean ± SEM of six replicates of a single experiment, repeated 3 times with similar results. * designates significant differences ($p < .05$) compared to control. ** designates significant differences compared to the corresponding drug dose.

Figure 2 BPA antagonizes anti-cancer drugs in MDA-MB-468 cells. Cells were treated with BPA for 24 hrs followed by increasing concentrations of doxorubicin, cisplatin or vinblastine for an additional 96 hrs. Cytotoxicity was determined by the MTT assay. Each value is a mean ± SEM of six replicates of a single experiment, repeated 3 times with similar results. * designates significant differences ($p < .05$) compared to control. ** designates significant differences compared to corresponding drug dose.

Figure 3 Low doses of BPA protect T47D and MDA-MB-468 cells from doxorubicin treatment. Cells were treated with increasing doses of BPA for 24 hrs followed by doxorubicin for an additional 24 hrs. Cytotoxicity was determined by the MTT assay. Each value is a mean ± SEM of six replicates of a single experiment, repeated 3 times with similar results. * designates significant differences ($p < .05$) compared to control. ** designates significant differences compared to doxorubicin.

Figure 4 BPA mediates its protective effects independent of the classical estrogen receptors. *Upper Panels:* T47D or MDA-MB-468 cells were treated with 100nM ICI or PHTPP one hr before BPA (10nM). Following 24hr pretreatment with BPA, cells were exposed to doxorubicin (25 ng/ml) for an additional 24 hrs. Cytotoxicity was determined by the MTT assay. Each value is a mean ± SEM of six replicates of a single experiment, repeated 3 times with similar results. * designates significant differences ($p < .05$) compared to control. ** designates significant differences compared to doxorubicin. *Lower Panels:* T47D and MDA-MB-468 cells were treated

with 100nM ICI or PHTPP for 1, 4 or 48 hrs. Western blots were probed for ERα or ERβ; β-actin served as a loading control. Shown are representative blots, repeated at least three times.

Figure 5 T47D and MDA-MB-468 cells express several types of estrogen receptors, as determined by real-time PCR. Both cell lines express ERβ, as well as non-classical estrogen receptors such as GRP30, ERRα and ERRγ. Data are expressed as percent of ERα in T47D cells after corrections for β2M. Each value is mean ± SEM of five separate experiments.

Figure 6 BPA may mediate chemoresistance by altering the expression of pro-survival proteins. Cells were pretreated with 10nM BPA for 24 hrs followed by exposure to doxorubicin (75-150 ng/ml) for an additional 24 hrs. Western blots were probed for Bcl-2, Bcl-xL and survivin with β-actin serving as a loading control. Shown are representative blots, repeated at least three times.

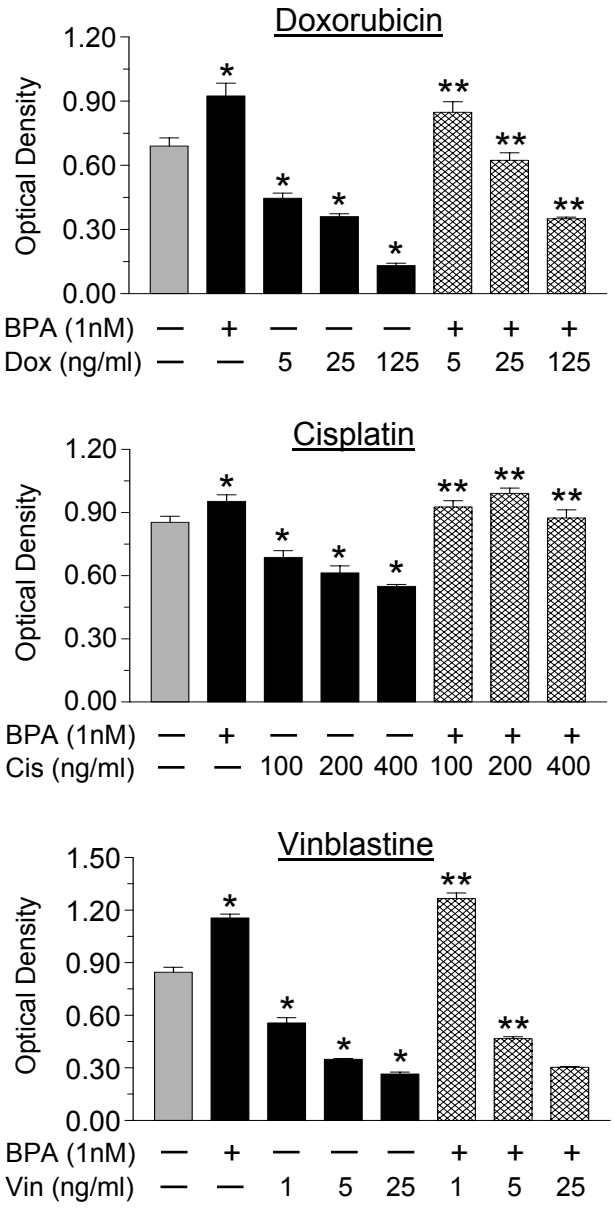
Doxorubicin
Optical Density
1.20
0.90
0.60
0.30
0.00
BPA (1nM) — + — — — + + +
Dox (ng/ml) — — 5 25 125 5 25 125
Cisplatin
Optical Density
1.20
0.90
0.60
0.30
0.00
BPA (1nM) — + — — — + + +
Cis (ng/ml) — — 100 200 400 100 200 400
Vinblastine
Optical Density
1.50
1.20
0.90
0.60
0.30
0.00
BPA (1nM) — + — — — + + +
Vin (ng/ml) — — 1 5 25 1 5 25

Figure 1

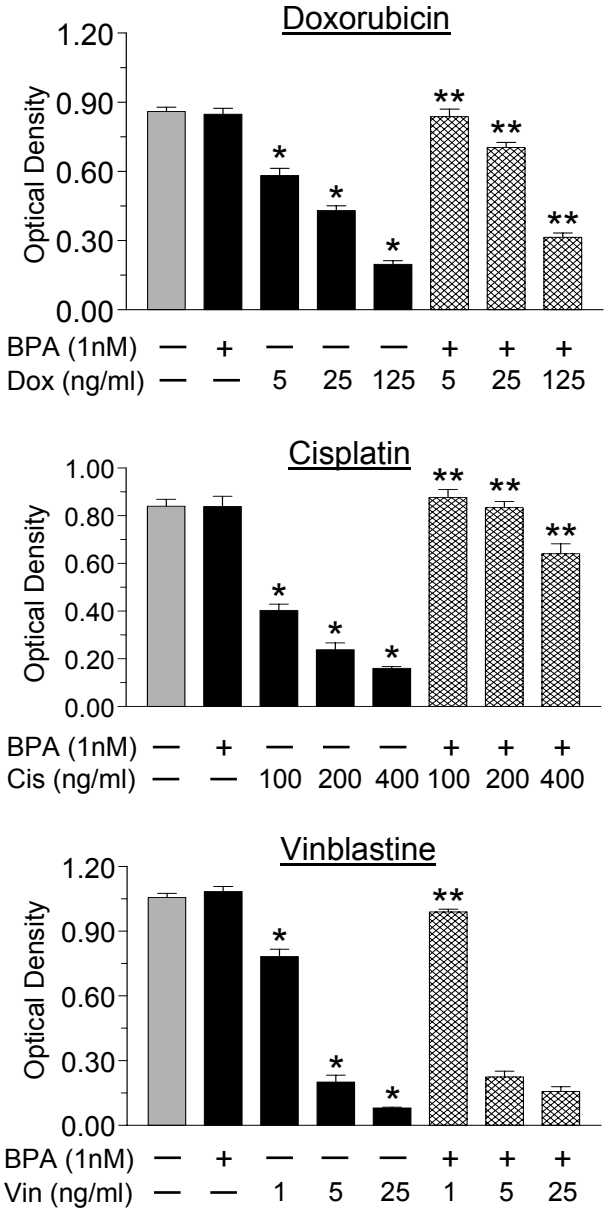
Doxorubicin
Optical Density
1.20
0.90
0.60
0.30
0.00
BPA (1nM) — + — — — + + +
Dox (ng/ml) — — 5 25 125 5 25 125
Cisplatin
Optical Density
1.00
0.80
0.60
0.40
0.20
0.00
BPA (1nM) — + — — — + + +
Cis (ng/ml) — — 100 200 400 100 200 400
Vinblastine
Optical Density
1.20
0.90
0.60
0.30
0.00
BPA (1nM) — + — — — + + +
Vin (ng/ml) — — 1 5 25 1 5 25

Figure 2

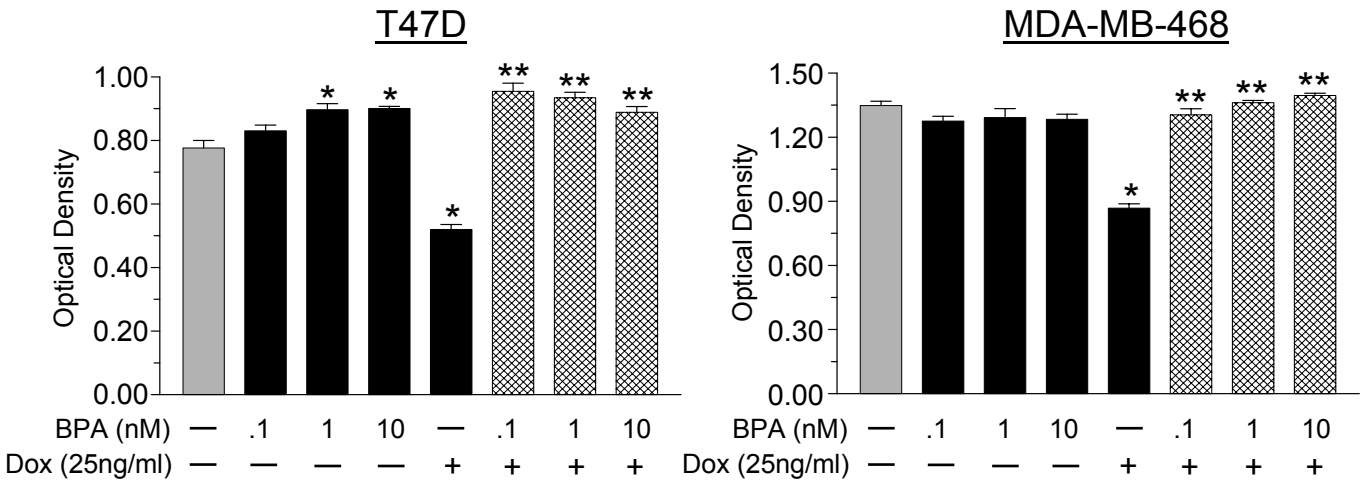
T47D
MDA-MB-468
Optical Density
1.00
0.80
0.60
0.40
0.20
0.00
1.50
1.20
0.90
0.60
0.30
0.00
BPA (nM) — .1 1 10 — .1 1 10
Dox (25ng/ml) — — — — + + + +
*
**

Figure 3

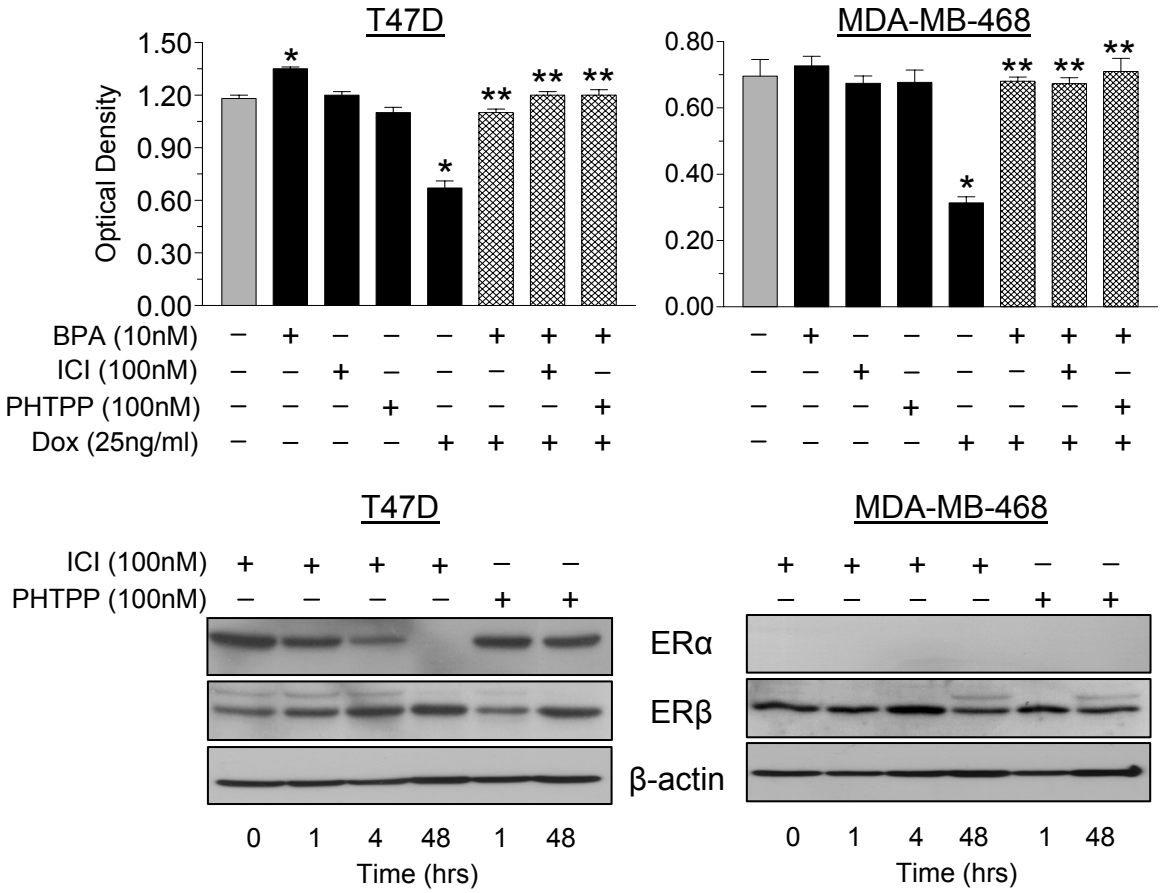
T47D
MDA-MB-468
Optical Density
1.50
1.20
0.90
0.60
0.30
0.00
0.80
0.60
0.40
0.20
0.00
BPA (10nM)
ICI (100nM)
PHTPP (100nM)
Dox (25ng/ml)
ERα
ERβ
β-actin
0 1 4 48 1 48
Time (hrs)

Figure 4

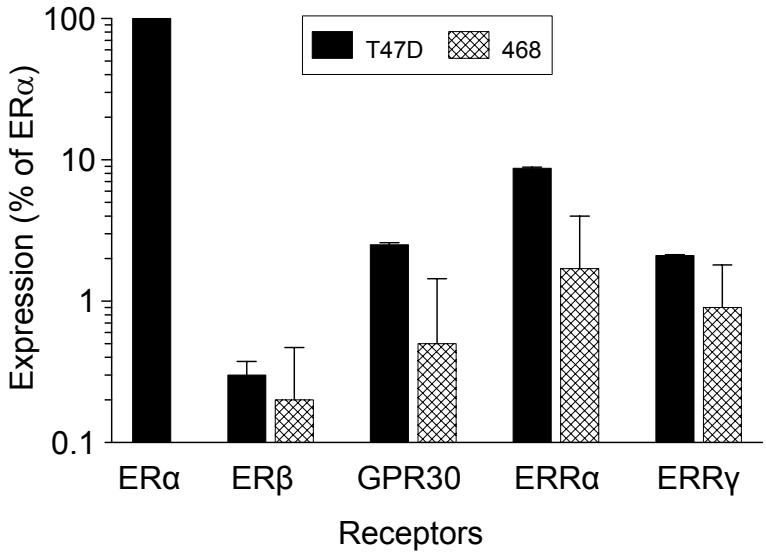
100
10
1
0.1
Expression (% of ERα)
T47D
468
ERα
ERβ
GPR30
ERRα
ERRγ
Receptors

Figure 5

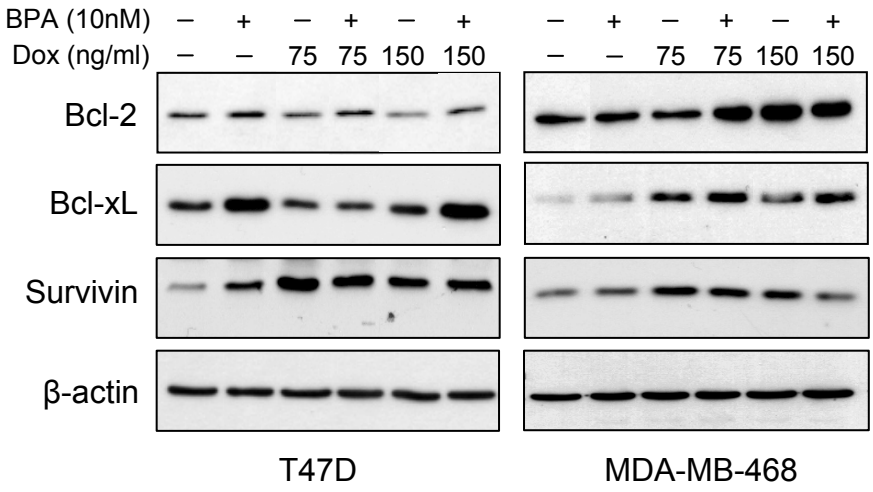
BPA (10nM) – + – + – + – + – + – +
Dox (ng/ml) – – 75 75 150 150 – – 75 75 150 150
Bcl-2
Bcl-xL
Survivin
β-actin
T47D
MDA-MB-468

Figure 6