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Repeated exposure to 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP), an active metabolite of bisphenol A, aggressively stimulates breast cancer cell growth in an estrogen receptor β (ERβ)-dependent manner

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Running title: MBP stimulation of breast cancer cell growth through ERβ

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Abbreviations

BPA, bisphenol A; DCC-FBS, dextran-coated charcoal-treated FBS; DMSO, dimethyl sulfoxide; E2, 17β-estradiol; ERα/β, estrogen receptor α/β; ERE, estrogen-responsive element; ERRγ, estrogen-related receptor γ; FBS, fetal bovine serum; GPR30, G protein-coupled receptor-30; HEK293, human embryonic kidney 293; MBP, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene; MEMα, minimum essential medium α; PBS, phosphate-buffered saline; PHTPP, 4-[2-phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]-pyrimidin-3-yl]phenol; PI, propidium iodide; RT-PCR, reverse transcription-polymerase chain reaction; TGF-β1, transforming growth factor-β1.

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Abstract

Bisphenol A (BPA), recognized as an endocrine disruptor, is thought to exert its activity through a mechanism involving the activation of estrogen receptors (ERα/β). However, a major problem is that very high concentrations of BPA are required (*i.e*., those in excess of environmental levels) for effective activation of ERα/β-mediated transcriptional activities *in vitro*, despite the BPA-induced estrogenic effects observed *in vivo*. To elucidate the causal reasons, we successfully identified a BPA metabolite, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP), which exhibits highly potent estrogenic activity both *in vivo* and *in vitro*. We have focused on the biological relationship between breast tumor promotion and MBP/BPA, because BPA is considered to be a human carcinogen owing to its breast tumor-promoting properties. In general, humans are exposed to many endocrine disruptors, including BPA. In the present study, we used the ERα/β-positive human breast cancer cell line, MCF-7, as an experimental model to investigate the effects of repeated exposure to BPA/MBP at concentrations found in the environment on the expression of ERα/β and to determine the particular ER subtype involved. We demonstrated that repeated exposure to MBP, but not to BPA, significantly downregulated ERα protein expression and stimulated the proliferation of MCF-7 cells through the activation of ERβ-mediated signaling.

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Introduction

Ordinarily, humans are exposed to many chemicals present in the environment. Many of them, such as bisphenol A (BPA), can be used to produce polycarbonate and epoxy resins; these are used in a variety of consumer products, including baby bottles, food can lining, and dental sealant (Brotons et al., 1995; Olea et al., 1996; Yamamoto and Yasuhara, 1999). Thus, we may be exposed to BPA monomers that leach out from those materials. According to one study, BPA has been detected in >90% of the human urine samples tested in the United States (Calafat et al., 2008), which suggests the widespread nature of exposure to BPA. An accumulation of experimental evidence suggests the adverse health effects considered to be attributable to BPA include disorders of reproductive function, obesity, and cancer. In addition, there is reasonable evidence, such as breast/prostate tumor-promoting properties, to suggest that BPA is a human carcinogen (Seachrist et al., 2016).

It is generally accepted that BPA is an endocrine disruptor that can exert estrogenic effects via estrogen receptors (ERα and ERβ) *in vivo* (Steinmetz et al., 1997; Welshons et al., 2006; Richter et al., 2007; Kundakovic et al., 2013), although it exhibits only very weak estrogenic properties in *in vitro* assays relative to 17β-estradiol (E2), a physiological ligand for ERs (Krishnan et al., 1993; Kuiper et al., 1997). To explain the inconsistency between the *in vivo* and the *in vitro* effects of BPA, it was proposed that BPA was susceptible to modification by certain drug-metabolizing enzyme-mediated metabolic processes that resulted in the formation of more active compound(s) in the *in vivo* environment. We originally identified 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP), now recognized as an active metabolite of BPA, which can be obtained by co-incubation of BPA and liver S9 fraction including human samples, in the presence of an NADPH-generating system. In the literature, MBP was demonstrated to be a much stronger activator of ERs than is the parent compound, BPA (Yoshihara et al., 2001; Yoshihara et al., 2004). Further, we found that the estrogenic activity

of MBP was approximately 500-fold more potent than that of BPA in *in vivo* experiments performed in ovariectomized Wistar rats (Okuda et al., 2010). In addition, potent estrogenic effects were observed in experiments on medaka (*Oryzias latipes*) and zebrafish, in which MBP exhibited approximately 250-fold and 1000-fold stronger estrogenic activity than did BPA (Ishibashi et al., 2005; Moreman et al., 2018). As mentioned above, MBP is thought to be a key candidate for resolving the difference of *in vitro* and *in vivo* effects of BPA. We reported that MBP exhibited comparable EC_{50} values for the activation of human ERα and ERβ transiently expressed in NIH/3T3 cells (Yoshihara et al., 2004). Under physiological conditions, the expression of ERs is regulated by their ligand, E2 (Nirmala and Thampan, 1995; Nawaz et al., 1999; Duong et al., 2006); this phenomenon suggested that prolonged, but not transient, exposure to MBP may lead to a change in the expression of ERα/β, which may enhance the malignancy of cancer cells.

Ligand-mediated ER degradation has been demonstrated in different cell types, including human breast cancer MCF-7 cells, which are known to be ERα/β-positive (Nirmala and Thampan, 1995; Nawaz et al., 1999; Speirs et al., 1999a; Duong et al., 2006). The proliferation of MCF-7 cells is stimulated by E2, which results from the activation of the E2/ERα signal transduction pathways (Foster et al., 2001). Unlike ERα, the physiological role of ERβ is not fully understood; however, the β-type ER is recognized as a functional repressor of ERα owing to its ability to heterodimerize with ERα when they are co-expressed (Cowley et al., 1997; Ogawa et al., 1998; Pettersson et al., 2000; Powell and Xu, 2008). Furthermore, it has been suggested that ERβ may be a tumor suppressor owing to its reduced expression during cancer development (Iwao et al., 2000). However, the accumulated evidence suggests that ERβ also acts as a mediator of estrogen action in breast cancer cells (Speirs et al., 1999a; Hamilton et al., 2015; Ma et al., 2017). Thus, in the present study, we investigated the effects of repeated exposure to BPA, together with MBP, at concentrations

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similar to environmental levels on the expression of ERα/β, and investigated the ER subtype that was involved in these effects. Our results indicated that repeated exposure to MBP, but not BPA, downregulated ERα protein expression and stimulated the proliferation of MCF-7 cells through the activation of ERβ.

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Materials and Methods

Reagents.

ICI 182,780 (fulvestrant) (purity: ≥98%) (CAS No. 129453-61-8) and 4-[2-phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]-pyrimidin-3-yl]phenol (PHTPP) (purity: ≥98%) (CAS No. 805239-56-9) were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. BPA (4,4'-(propane-2,2-diyl)diphenol) (purity: ≥99%) (CAS No. 80-05-7) was purchased from Nacalai Tesque (Kyoto, Japan). The chemical synthesis of MBP (purity: ≥98%) (CAS No. 13464-24-9) was performed in accordance with previously reported methods (Yoshihara et al., 2004; Okuda et al., 2010). E2 (purity: ≥98%) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Cell cultures and chemical treatments.

The cell culture conditions and methods used were based on previously described procedures (Takeda et al., 2013; Takeda et al., 2016; Suzuki et al., 2017a). Briefly, the human breast cancer cell lines, MCF-7 and MDA-MB-231, were routinely grown in phenol red-containing minimum essential medium α (MEMα) (FUJIFILM Wako Pure Chemical Corporation), supplemented with 10 mM HEPES, 5% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL), in a humidified incubator in an atmosphere of 5% CO_2 at 37ºC. Twenty-four hours prior to the treatment of cells with chemicals, the culture medium was changed to phenol red-free MEMα (FUJIFILM Wako Pure Chemical Corporation), supplemented with 10 mM HEPES, 5% dextran-coated charcoal-treated FBS (DCC-FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). ICI 182,780, PHTPP, BPA, and MBP were prepared in cell culture-grade dimethyl sulfoxide (DMSO). In the experiments in Figure 1B and 1C, the cells were treated with ICI 182,780 and PHTPP for 24 h after cDNA transfection (see *Transfection and luciferase analysis*), and then collected for

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the dual-luciferase assay. The details of the chemical treatment method, except for the experiment of Figure 1, are shown in Figure 2B. The first treatment (treatment 1) comprised vehicle (for the control) or ligands (BPA or MBP, for the booster (–/+)). After 24 h of the first treatment, the culture medium was changed to fresh phenol red-free MEMα, and the cells were further treated with the vehicle (for control or booster (–)) or with ligands (for booster (+); treatment 2). The results shown in Figure 8 are from experiments performed after MBP treatment in the presence or absence PHTPP. The results shown in Figure 9 are from experiments in which the cells were treated with 2 nM MBP for 48 h without alteration of the culture medium (indicated as 2 nM MBP), to compare the conditions between 2 nM MBP and booster (+) (*see* Fig. 9A). After a total treatment time of 48 h, the cell samples were collected for the dual-luciferase assay, cell proliferation analysis, cell cycle analysis, western blotting analysis, and real-time reverse transcription-polymerase chain reaction (RT-PCR).

Transfection and luciferase analysis (dual-luciferase assay).

The experiments were performed as described previously, with minor modifications (Takeda et al., 2013; Okazaki et al., 2018). In brief, 24 h prior to transfection, MCF-7 and MDA-MB-231 cells (5×10^4 cells/well) were seeded in 24-well plates containing MEMα. Each expression plasmid was transfected by using Lipofectamine® LTX with PLUS™ reagent (Thermo Fisher Scientific, Waltham, MA, USA). DNA mixtures containing 300 ng estrogen-responsive element $(ERE)_3$-Luc plasmid were co-transfected with 2 ng *Renilla* luciferase reporter plasmid (pRL-CMV) in 24-well plates. At 24 h after transfection, the cells were washed with phosphate-buffered saline (PBS), the culture medium was exchanged for phenol red-free MEMα-supplemented with 5% DCC-FBS, and chemical treatments were applied in accordance with the methods described in *Cell cultures and chemical treatments*. After 24 h or 48 h of treatment, cell extracts were prepared in 100 μL passive lysis buffer (Promega, Madison, WI, USA), from which 20 μL was extracted to perform the firefly

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luciferase and *Renilla* luciferase assays (Dual-Luciferase Reporter Assay System, Promega). The ratio of firefly luciferase activity to *Renilla* luciferase activity was presented as the relative luciferase activity.

Cell proliferation analysis (MTS assay).

The MTS assay was performed as described previously (Takeda et al., 2016; Suzuki et al., 2017b). Briefly, the cells were seeded into 96-well plates at a density of ~5×10^3 cells/well in the cell proliferation study. After chemical treatments or incubation, the degree of cell proliferation was analyzed by using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS reagent; Promega).

Cell cycle analysis.

The chemically treated cells were harvested (2×10^5 cells/sample) and fixed in 70% ethanol at 4ºC overnight. The fixed cells were harvested and re-suspended in 450 μL PBS containing 50 μg/mL propidium iodide (PI) (Nacalai Tesque) and 100 μg/mL RNaseA (Qiagen, Inc., Hilden, Germany) and incubated at 26ºC for 30 min in the dark. Finally, the stained cells were analyzed by using a FACSCalibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A minimum of 1×10^4 cells was counted per sample. The obtained results were analyzed by using ModFit LT™ v3.0 (Verity Software House, Topsham, ME, USA).

Antibodies and western blotting analysis.

Antibodies specific for ERα (ab79413; Abcam, Cambridge, MA, USA), ERβ (ab3576; Abcam), and β-actin (sc-47778; Santa Cruz Biotechnology) were used. Whole cell extracts were prepared by using lysis buffer (50 mM HEPES-NaOH at pH 7.5, 25 mM NaCl, 1% NP-40, and 1 mM phenylmethylsulfonyl fluoride) supplemented with cOmplete™ Mini Protease Inhibitor Cocktail (Sigma Co., St. Louis, MO, USA). The cell extracts that were transfected with human ERα or ERβ cDNA were used as the positive control (indicated as Std.; Figs. 6 and 9B). SDS-PAGE/western immunoblotting was performed based on previously described

procedures (Suzuki et al., 2017b). The antibodies for ERα (1:2000 dilution), ERβ (1:2000 dilution), and β-actin (1:4000 dilution) were used as the primary antibodies for the detection of ERα, ERβ, and β-actin, respectively. Anti-rabbit IgG (1:10000 dilution) and anti-mouse IgG (1:10000 dilution) antibodies conjugated with peroxidase were used as the secondary antibodies. Luminata™ Forte Western HRP Substrate (Millipore, Billerica, MA) was used for the detection of blotted bands. The quantification of band intensities was performed by using ImageJ 1.46r software (http://imagej.nih.gov/ij/) and the obtained values were normalized to β-actin, an endogenous control.

Preparation of total RNA and real-time RT-PCR

Real-time RT-PCR was as performed as described previously (Okazaki et al., 2018). Briefly, total RNA was prepared from MCF-7 cells by using TRIzol RNA Isolation Reagent (Thermo Fisher Scientific). cDNA was synthesized from 600 ng of total RNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time RT-PCR was performed using an Applied Biosystems StepOne Real-Time PCR System (Thermo Fisher Scientific) with Fast SYBR Green Master Mix (Thermo Fisher Scientific). The following primers used: ERα (sense), 3'-GAA AGG TGG GAT ACG AAA AGA CC-5'; ERα (antisense), 3'-GCT GTT CTT CTT AGA GCG TTT GA-5'; 18S-ribosomal RNA (sense), 3'-CGG CTA CCA CAT CCA AGG A-5'; 18S-ribosomal RNA (antisense), 3'-GCT GGA ATT ACC GCG GCT-5'. The mRNA expression of *ERα* in each sample was normalized to the corresponding mRNA expression of *18S-ribosomal RNA*.

Data analysis.

The EC_{50} values of BPA and MBP were determined, and the concentrations of the compounds that elicited ERE-mediated transcriptional activities equivalent to 10% of the value of the positive control (1 nM E2) were defined as PC_{10} (EPA, 2011). Dose-response curves were fitted by using SigmaPlot 11® software (Systat Software, Inc., San Jose, CA,

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USA). Differences were considered significant for *P*-values of less than 0.05. The statistical significance of differences between the two groups was calculated by using Student's *t* test. Other statistical analyses performed were Dunnett's or Tukey-Kramer's tests, as post-hoc tests following the analysis of variance (ANOVA) (details are indicated in Figure legends). These calculations were performed by using Statview 5.0 J software (SAS Institute Inc., Cary, NC, USA).

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Results

ERβ as a negative regulator of ERα-mediated transcriptional activity. It has been reported that estrogen signaling can be modulated by several methods; for example, when ERα and ERβ are simultaneously expressed in cells, E2/ERα-mediated transcriptional activity can be suppressed by the β-type ER in MCF-7 and other cell lines, including in human embryonic kidney 293 (HEK293) cells co-transfected with ERα/ERβ expression plasmids (see the model described in Fig. 1A) (Pettersson et al., 2000; Takeda et al., 2013; Takeda, 2014). It is generally considered, especially in *in vitro* conditions, that the expression of the individual ERs is one of the key determinants for the output of E2 signaling. ERα-positive MCF-7 cells produce endogenous E2, but at very low levels, to stimulate their own growth via E2/ERα and express the ERβ subtype (Miki et al., 2007). Therefore, in this study, we selected the MCF-7 cell line as a model and investigated the effects of two established antagonists, ICI 182,780 and PHTPP, which are specific for ERα and ERβ, respectively, in the absence of exogenous E2. As shown in Figure 1B and C, ER/ERE-mediated transcriptional activity in MCF-7 cells was inhibited by ICI 182,780 in a concentration-dependent manner up to 1 μM, whereas PHTPP stimulated the transcriptional activity in a concentration-dependent manner up to 10 μM. These data strongly suggested that E2/ER-mediated estrogen signaling was triggered mainly by the ERα subtype and that ERβ exerted a negative impact on ERα in the conditions in which cells co-expressed both subtypes (Fig. 1A).

MBP as a positive stimulator of ER-positive breast cancer cell proliferation in a repeated exposure-dependent manner. Humans are routinely exposed to a variety of environmental chemicals, including BPA, which can act as ligands and stimulate ERs. It is important to investigate whether repeated exposure of BPA to cells that express both ER subtypes may result in the perturbation of ERα/ERβ expression, which may be coupled with

the dysregulation of estrogen receptor signaling. Therefore, we first investigated the effects of BPA and its metabolite MBP (structures described in Fig. 2A) on the proliferation of MCF-7 cells. MCF-7 cells were treated with individual bisphenols in accordance with the experimental scheme described in Figure 2B [*i.e.*, booster (–) vs. booster (+)]. As shown in Figure 3A, there were no observable effects of repeated exposure of BPA on the proliferation of MCF-7 cells, although BPA stimulated cell proliferation in a concentration-dependent manner up to 10 μM. In contrast, when the effect MBP was examined, cell proliferation was positively stimulated by the molecule in a repeated exposure-dependent manner up to 25 nM [*i.e.*, booster (+)]; conversely, MCF-7 cell growth was suppressed by MBP concentrations above 2.5 μM (Fig. 3B). To conduct further study of whether the MBP-mediated "booster effects" were dependent on the expression status of ERs in cells, we utilized the human breast cancer cell line MDA-MB-231, which is ER negative (Takeda et al., 2013; Weigel and deConinck, 1993). MBP did not exert a stimulatory effect on the proliferation of MDA-MB-231 cells (data not shown). To confirm the phenomenon observed in Figure 3B, cell cycle analysis was performed by using flow cytometry. In support of the MBP-mediated upregulation of MCF-7 cell growth, the percentage of cells in the S phase (*i.e.*, DNA replication), which is a preliminary step in cell division, was clearly increased in an MBP-treatment-dependent manner [*i.e.*, 25.4% for control, 36.9% for booster (–), and 45.7% for booster (+)] (Fig. 4A and B). These results indicated that MBP may positively modulate the proliferation of ER-positive MCF-7 cells through the mitogenic activity of cell cycle acceleration. Thus, questions arose on the nature of the driving force for the MBP-mediated stimulation of MCF-7 cell proliferation associated with the stimulation of cell cycle progression. To address this issue, we studied the effects of BPA and MBP on ER/ERE-mediated transcriptional activity in the presence or absence of repeated exposure (see Fig. 2B). The panels in Figure 5 show the line graphs representing dose-response profiles for the

estrogenic activities of BPA and MBP, together with the data of 1 nM E2, which was used as the positive control. As BPA and MBP displayed lower estrogenic potency in booster (–) than in booster (+) conditions, accurate EC_{50} values were not obtained (indicated as N.D.); thus, we also determined the value of PC_{10}. The comparison of the EC_{50} values for the activation of ERs by BPA and MBP revealed that remarkable boosting effects were observed after the treatment of both BPA and MBP (BPA, PC_{10} values of 665 nM [booster (–)] vs. 138 nM [booster (+)]; and MBP, PC_{10} values of 4.8 nM [booster (–)] vs. 0.6 nM [booster (+)] (panels A–D). In addition, when focusing on the EC_{50} values between BPA and MBP in booster (+) conditions, much lower EC_{50} values were found for MBP exposure, 519 nM vs. 2.8 nM (panels B and D).

MBP induced downregulation of ERα mRNA/protein in a repeated exposure-dependent manner. It has been reported that the ERα protein may be subject to ligand-mediated degradation, preferentially in the presence of E2 (*i.e.*, >1 nM) (Nirmala and Thampan, 1995; Nawaz et al., 1999). Given that MBP behaves as an ERα ligand to induce similar biological effects to E2, the protein expression of ERα may be downregulated after repeated exposure to MBP. Thus, we analyzed the protein expression of ERα, together with ERβ, in MCF-7 cells. To use BPA concentrations that reflected those found in biological samples (Schönfelder et al., 2002; Welshon et al., 2006; Vandenberg et al., 2010), we treated MCF-7 cells with or without repeated exposure of BPA and MBP at 1 nM. When the membranes were blotted for BPA, no modulatory effects of 1 nM BPA on ERα/β expression were found relative to the control in either condition (booster +/–); however, the BPA metabolite MBP did reduce the expression of ERα in a treatment-dependent manner without significant alteration of the protein expression of ERβ, although there was a tendency for ERβ to be upregulated (Fig. 6A and B). To determine whether MBP also affected *ERα* mRNA expression after repeated exposure, we performed real-time RT-PCR analysis of

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samples derived from both conditions (booster +/–). Similarly, as shown in Figure 7, 1 nM MBP, but not 1 nM BPA, downregulated the mRNA expression of *ERα* in a repeated exposure-dependent way. Collectively, these data suggested that the protein expression ERα can also be downmodulated by MBP via a reduction in *ERα* mRNA.

Involvement of ERβ in MBP estrogenic action in a repeated exposure-dependent manner. We further investigated whether the MBP-mediated booster effects were dependent on the basal expression status of ERs (ERα and ERβ); for this, the MDA-MB-231 cell line, which is ERα negative and has a very low expression of ERβ, was employed (Weigel and deConinck, 1993; Takeda et al., 2013). As expected, it was clearly shown that neither the MBP-mediated stimulation of ER/ERE nor the MBP booster effect were detected in MDA-MB-231 cells (data not shown). To determine whether functional ERβ was indeed involved in the MBP-induced transcriptional activation of ERβ/ERE in MCF-7 cells after repeated exposure [*i.e.*, booster (+) conditions)], we performed an ERE-Luc analysis of samples that were treated with MBP in combination with four different concentrations of PHTPP (*i.e.*, 0.1, 0.25, 0.5, and 1 μM), a selective antagonist of the ERβ subtype. When compared with the MBP-only treatment group at concentrations between 0.25 and 100 nM, the MBP-activated transcriptional activities were clearly attenuated by PHTPP in a concentration-dependent manner (Fig. 8A). Furthermore, we analyzed whether the introduction of PHTPP abrogated the MBP-enhanced cell proliferation and found that MCF-7 cell proliferation stimulated by MBP was dose-dependently dampened by PHTPP (Fig. 8B). The effects of MCF-7 cells exposed to 1 nM MBP for a total of 48 h with repeated exposure [*i.e.*, booster (+): 1 nM MBP + 1 nM MBP] or without [*i.e.*, booster (–): 1 nM MBP + vehicle instead of MBP] are shown in Figures 4, 6B, 7, and 8B; however, at 24 h after the first exposure to MBP, the culture medium, including MBP, was replaced with fresh medium together with MBP or with vehicle. Therefore, in this experimental regimen (*see* Fig. 2B), one possible criticism might be that the

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apparent effects of repeated-exposure to MBP, booster (+) versus booster (–), can be simply explained by the difference in the "total" exposure of cells to MBP. Thus, we analyzed the effects of 2 nM MBP on cells treated for 48 h without the booster by a comparison of the effects of cells repeatedly exposed to 1 nM MBP [booster (+)] (see experimental regimen described in Fig. 9A). The obtained results of western blotting indicated that in comparison with the booster + results with 1 nM MBP, the expression of both ERα and ERβ proteins was significantly upregulated by 2 nM MBP treatment (Fig. 9B). Furthermore, it was unexpectedly revealed that an inactive effect of PHTPP (0.5 μM) on the cell viability after treatment at 2 nM MBP for 48 h without its booster was observed (Fig. 9C), implicating the involvement of the MBP-mediated ERα activation.

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Discussion

An accumulation of evidence suggests that BPA has a carcinogenic activity in the human breast because of its tumor-promoting properties (Seachrist et al., 2016). We are continually exposed to environmental BPA. In the current study, we investigated the effects of repeated exposure of BPA, together with MBP, at concentrations similar to environmental levels on the human breast cancer MCF-7 cell line, and demonstrated that i) "repeated" exposure to MBP, but not parent BPA, stimulated the proliferation of MCF-7 cells through the mitogenic activity of cell cycle acceleration, and ii) the bisphenol BPA utilized the ERβ subtype to induce mitogenic action.

It has been reported that BPA can be detected at concentrations between 0.3 nM and 40 nM in human biological samples from fetal serum and maternal plasma (Schönfelder et al., 2002; Welshon et al., 2006; Vandenberg et al., 2010). The combination of the data in Figure 3 and 5, indicated the following: i) although BPA can act as a positive stimulator of MCF-7 cell proliferation, as well as ER/ERE-mediated transcriptional activity, the former effect by this bisphenol at concentrations below 40 nM (Fig. 3A) may not be fully indicated by the data in Figure 5A/B, because reasonably high concentration of BPA are required to induce the full activation of ER/ERE-mediated transcriptional activity (>500 nM) and, importantly, ii) it was revealed that in good accordance with the MBP-mediated stimulation of MCF-7 cell proliferation, which was observed at concentrations below 40 nM (*i.e.*, physiological concentrations), the MBP-mediated activation of ER/ERE-mediated transcriptional activity in MCF-7 cells was also seen at concentrations below 40 nM, which implied the occurrence of a key interaction between the MBP activation of ERs and MCF-7 cell proliferation. Although the detailed regulation machinery of the ERα/β protein associated with MBP exposure in MCF-7 cells is unknown at present, after consideration of these observations, it was suggested that MBP can utilize ERβ (for which expression was unaffected) to induce its

biological effects (*e.g.*, the stimulation of breast cancer cell proliferation) in ERα/β-positive cells after repeated exposure. Through the construction of the three-dimensional analysis models of MBP with human ERα/β, it was suggested that MBP, but not BPA, interacted with amino acid residues in ERα/β that were important for the binding of E2 in ERs (Baker and Chandsawangbhuwana, 2012). We have reported that the EC_{50} values for ERα and ERβ activation by MBP were 0.68 nM and 0.46 nM, respectively, in NIH/3T3 cells transfected with expression plasmids encoding human ERα or ERβ (Yoshihara et al., 2004). Although we were unable to obtain the EC_{50} values for the individual subtypes of ERα and ERβ after the activation by MBP in MCF-7 cells, it was suggested that MBP may behave as an equipotent activator of human ERα/β.

In this study, it was also revealed that BPA itself can stimulate MCF-7 cell proliferation at concentrations below 40 nM; as recently reported, this effect of BPA might be mediated by nuclear ER-independent signaling pathways: i) Shimohigashi and Matsushima's research group reported that BPA had a high affinity (K_d=5.50 nM) for orphan nuclear receptor estrogen-related receptor γ (ERRγ) and that the interaction between BPA and ERRγ may explain the biological effects of low-dose BPA (Takayanagi et al., 2006; Okada et al., 2008); and ii) BPA targets G protein-coupled receptor-30 (GPR30)/EGFR expressed in the cell membrane to cause non-genomic estrogenic signaling (Dong et al., 2011; Ge et al., 2014). Exposure to environmental chemicals may be associated with many undesirable health outcomes; however, the negative effects of these environmental chemicals are generally weakened by drug-metabolizing enzymes (phase I and II reactions) after exposure. In the case of BPA, it has been reported that under *in vivo* conditions, BPA is readily converted to BPA-glucuronide (via phase II reaction); however, BPA-glucuronide has itself also been proven to be a possible active metabolite that induces adipogenesis in human and murine preadipocytes (Boucher et al., 2015). Although it has been suggested that ERβ has a possible

protective role against breast tumor progression, the accumulated evidence suggests that ERβ also acts as a mediator of estrogen action, coupled with tumorigenesis, in breast cancer cells, and that its expression is remarkably upregulated in tamoxifen-resistant breast cancers (Dotzlaw et al., 1999; Speirs et al., 1999a, 1999b; Power and Thompson, 2003; Hamilton et al., 2015; Ma et al., 2017). Collectively, including our findings here, it is suggested that the negative biological effects of BPA are exerted through several methods, such as through BPA itself and BPA metabolites, including BPA-glucuronide and MBP, and that the MBP-mediated estrogenic effects on breast cancer cells, which were possibly amplified by repeated exposure, were mediated by ERβ (Fig. 10).

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Authorship Contributions

Participated in research design: Takeda.

Conducted experiments: Hirao-Suzuki, Okuda.

Performed data analysis: Hirao-Suzuki, Takeda.

Wrote or contributed to the writing of the manuscript: Hirao-Suzuki, Takeda, Okuda, Takiguchi, Yoshihara.

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References

Baker ME and Chandsawangbhuwana C (2012) 3D models of MBP, a biologically active metabolite of bisphenol A, in human estrogen receptor α and estrogen receptor β. *PLoS One* **7:** e46078.

Boucher JG, Boudreau A, Ahmed S, and Atlas E (2015) *In Vitro* Effects of Bisphenol A β-D-Glucuronide (BPA-G) on Adipogenesis in Human and Murine Preadipocytes. *Environ Health Perspect* **123:** 1287–1293.

Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, and Olea N (1995) Xenoestrogens released from lacquer coatings in food cans. *Environ Health Perspect* **103:** 608–612.

Calafat AM, Ye X, Wong LY, Reidy JA, and Needham LL (2008) Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. *Environ Health Perspect* **116:** 39-44.

Cowley SM, Hoare S, Mosselman S, and Parker MG (1997) Estrogen receptors α and β form heterodimers on DNA. *J Biol Chem* **272:** 19858–19862.

Dong S, Terasaka S, and Kiyama R (2011) Bisphenol A induces a rapid activation of Erk1/2 through GPR30 in human breast cancer cells. *Environ Pollut* **159:** 212–218.

Dotzlaw H, Leygue E, Watson PH, and Murphy LC (1999) Estrogen receptor-β messenger RNA expression in human breast tumor biopsies: relationship to steroid receptor status and regulation by progestins. *Cancer Res* **59:** 529–532.

Duong V, Licznar A, Margueron R, Boulle N, Busson M, Lacroix M, Katzenellenbogen BS, Cavaillès V, and Lazennec G (2006) ERα and ERβ expression and transcriptional activity are differentially regulated by HDAC inhibitors. *Oncogene* **25:** 1799–1806.

EPA, (2011) Estrogen Receptor Transcriptional Activation (Human Cell Line – HeLa- 9903). *U.S. Environmental Protection Agency, Washington, DC 20460*, pp. 1–10.

Foster JS, Henley DC, Bukovsky A, Seth P, and Wimalasena J (2001) Multifaceted regulation of cell cycle progression by estrogen: regulation of Cdk inhibitors and Cdc25A independent of cyclin D1-Cdk4 function. *Mol Cell Biol* **21:** 794–810.

Ge LC, Chen ZJ, Liu HY, Zhang KS, Liu H, Huang HB, Zhang G, Wong CK, Giesy JP, Du J, and Wang HS (2014) Involvement of activating ERK1/2 through G protein coupled receptor 30 and estrogen receptor α/β in low doses of bisphenol A promoting growth of Sertoli TM4 cells. *Toxicol Lett* **226:** 81–89.

Hamilton N, Marquez-Garban D, Mah VH, Elshimali Y, Elashoff D, Garon EB, Vadgama J, and Pietras R (2015) Estrogen Receptor-β and the Insulin-Like Growth Factor Axis as Potential Therapeutic Targets for Triple-Negative Breast *Cancer Crit Rev Oncog* **20:** 373–390.

Ishibashi H, Watanabe N, Matsumura N, Hirano M, Nagao Y, Shiratsuchi H, Kohra S, Yoshihara S, and Arizono K (2005) Toxicity to early life stages and an estrogenic effect of a bisphenol A metabolite, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene on the medaka (*Oryzias latipes*). *Life Sci* **77:** 2643-2655.

Iwao K, Miyoshi Y, Egawa C, Ikeda N, and Noguchi S (2000) Quantitative analysis of estrogen receptor-β mRNA and its variants in human breast cancers. *Int J Cancer* **88:** 733–736.

Krishnan AV, Stathis P, Permuth SF, Tokes L, and Feldman D (1993) Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132:** 2279–2286.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, and Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138:** 863–870.

Kundakovic M, Gudsnuk K, Franks B, Madrid J, Miller RL, Perera FP, and Champagne FA (2013) Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. *Proc Natl Acad Sci USA* **110:** 9956–99561.

Ma R, Karthik GM, Lövrot J, Haglund F, Rosin G, Katchy A, Zhang X, Viberg L, Frisell J, Williams C, Linder S, Fredriksson I, and Hartman J (2017) Estrogen Receptor β as a Therapeutic Target in Breast Cancer Stem Cells. *J Natl Cancer Inst* **109:** 1–14.

Miki Y, Suzuki T, Tazawa C, Yamaguchi Y, Kitada K, Honma S, Moriya T, Hirakawa H, Evans DB, Hayashi S, Ohuchi N, and Sasano H (2007) Aromatase localization in human breast cancer tissues: possible interactions between intratumoral stromal and parenchymal cells. *Cancer Res* **67:** 3945–3954.

Moreman J, Takesono A, Trznadel M, Winter MJ, Perry A, Wood ME, Rogers NJ, Kudoh T, and Tyler CR (2018) Estrogenic Mechanisms and Cardiac Responses Following Early Life Exposure to Bisphenol A (BPA) and Its Metabolite 4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (MBP) in Zebrafish. *Environ Sci Technol* **52:** 6656–6665.

Nawaz Z1, Lonard DM, Dennis AP, Smith CL, and O'Malley BW (1999) Proteasome-dependent degradation of the human estrogen receptor. *Proc Natl Acad Sci USA* **96:** 1858–1862.

Nirmala PB, and Thampan RV (1995) Ubiquitination of the rat uterine estrogen receptor: dependence on estradiol. *Biochem Biophys Res Commun* **213:** 24–31.

Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y, and Muramatsu M (1998) Molecular cloning and characterization of human estrogen receptor βcx: a potential inhibitor of estrogen action in human. *Nucleic Acids Res* **26:** 3505–3512.

Okada H, Tokunaga T, Liu X, Takayanagi S, Matsushima A, and Shimohigashi Y (2008) Direct evidence revealing structural elements essential for the high binding ability of bisphenol A to human estrogen-related receptor-γ. *Environ Health Perspect* **116:** 32–38.

Okazaki H, Hirao-Suzuki M, Takeda S, Takemoto Y, Mizunoe R, Haraguchi K, Watanabe K, Takiguchi M, and Aramaki H (2018) Bisphenol AF as an activator of human estrogen receptor β1 (ERβ1) in breast cancer cell lines. *J Toxicol Sci* **43:** 321–327.

Okuda K, Takiguchi M, and Yoshihara S (2010) *In vivo* estrogenic potential of 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene, an active metabolite of bisphenol A, in uterus of ovariectomized rat. *Toxicol Lett* **197:** 7–11.

Olea N, Pulgar R, Pérez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V, Soto AM, and Sonnenschein C (1996) Estrogenicity of resin-based composites and sealants used in dentistry. *Environ Health Perspect* **104:** 298–305.

Pettersson K, Delaunay F, and Gustafsson JA (2000) Estrogen receptor β acts as a dominant regulator of estrogen signaling. *Oncogene* **19:** 4970–4978.

Powell E and Xu W (2008) Intermolecular interactions identify ligand-selective activity of estrogen receptor α/β dimers. *Proc Natl Acad Sci USA* **105:** 19012–19017.

Power KA and Thompson LU (2003) Ligand-induced regulation of ERα and ERβ is indicative of human breast cancer cell proliferation. *Breast Cancer Res Treat* **81:** 209–221.

Richter CA, Taylor JA, Ruhlen RL, Welshons WV, and Vom Saal FS (2007) Estradiol and Bisphenol A stimulate androgen receptor and estrogen receptor gene expression in fetal mouse prostate mesenchyme cells. *Environ Health Perspect* **115:** 902–908.

Schönfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, and Chahoud I (2002) Parent bisphenol A accumulation in the human maternal-fetal-placental unit. *Environ Health Perspect* **110:** A703–A707.

Seachrist DD, Bonk KW, Ho SM, Prins GS, Soto AM, and Keri RA (2016) A review of the carcinogenic potential of bisphenol A. *Reprod Toxicol* **59:** 167–182.

Speirs V, Malone C, Walton DS, Kerin MJ, and Atkin SL (1999a) Increased expression of estrogen receptor β mRNA in tamoxifen-resistant breast cancer patients. *Cancer Res* **59:** 5421–5424.

Speirs V, Parkes AT, Kerin MJ, Walton DS, Carleton PJ, Fox JN, and Atkin SL (1999b) Coexpression of estrogen receptor α and β: poor prognostic factors in human breast cancer? *Cancer Res* **59:** 525–528.

Steinmetz R, Brown NG, Allen DL, Bigsby RM, and Ben-Jonathan N (1997) The environmental estrogen bisphenol A stimulates prolactin release *in vitro* and *in vivo*. *Endocrinology* **138:** 1780–1786.

Suzuki M, Takeda S, Okazaki H, Watanabe K, Takiguchi M, and Aramaki H (2017a) Cannabidiolic acid-mediated interference with AP-1 transcriptional activity in MDA-MB-231 breast cancer cells. *Nat Prod Commun* **12:** 759–761.

Suzuki M, Takeda S, Teraoka-Nishitani N, Yamagata A, Tanaka T, Sasaki M, Yasuda N, Oda M, Okano T, Yamahira K, Nakamura Y, Kobayashi T, Kino K, Miyazawa H, Waalkes MP, and Takiguchi M (2017b) Cadmium-induced malignant transformation of rat liver cells: Potential key role and regulatory mechanism of altered apolipoprotein E expression in enhanced invasiveness. *Toxicology* **382:** 16–23.

Takayanagi S, Tokunaga T, Liu X, Okada H, Matsushima A, and Shimohigashi Y (2006) Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor γ (ERRγ) with high constitutive activity. *Toxicol Lett* **167:** 95–105.

Takeda S (2014) Δ^9-Tetrahydrocannabinol targeting estrogen receptor signaling: the possible mechanism of action coupled with endocrine disruption. *Biol Pharm Bull* **37:** 1435–1438.

Takeda S, Okazaki H, Kudo T, Kakizoe K, Himeno T, Matsumoto K, Shindo M, and Aramaki H (2016) Bongkrekic acid as a Warburg effect modulator in long-term estradiol-deprived MCF-7 breast cancer cells. *Anticancer Res* **36:** 5171–5182.

Takeda S, Yoshida K, Nishimura H, Harada M, Okajima S, Miyoshi H, Okamoto Y, Amamoto T, Watanabe K, Omiecinski CJ, and Aramaki H (2013) Δ^9-Tetrahydrocannabinol disrupts estrogen-signaling through up-regulation of estrogen receptor β (ERβ). *Chem Res Toxicol* **26:** 1073–1079.

Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, and Schoenfelder G (2010) Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. *Environ Health Perspect* **118:** 1055–1070.

Weigel RJ and deConinck EC (1993) Transcriptional control of estrogen receptor in estrogen receptor-negative breast carcinoma. *Cancer Res* **53:** 3472–3474.

Welshons WV, Nagel SC, and 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.

Welshons WV, Nagel SC, and 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.

Yamamoto T and Yasuhara A (1999) Quantities of bisphenol a leached from plastic waste samples. *Chemosphere* **38:** 2569–2576.

Yoshihara S, Makishima M, Suzuki N, and Ohta S (2001) Metabolic activation of bisphenol A by rat liver S9 fraction. *Toxicol Sci* **62:** 221–227.

Yoshihara S, Mizutare T, Makishima M, Suzuki N, Fujimoto N, Igarashi K, and Ohta S (2004) Potent estrogenic metabolites of bisphenol A and bisphenol B formed by rat liver S9 fraction: their structures and estrogenic potency. *Toxicol Sci* **78:** 50–59.

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Footnotes

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Figure Legends

Figure 1. ERβ inhibition of transcriptional activity mediated by ERs in MCF-7 cells. (A) A model of ERα signaling abrogation by ERβ. (B, C) MCF-7 cells were transiently transfected with an ERE-luciferase reporter plasmid. After transfection, the cells were treated with ICI 182,780 (1 pM–1 μM) (B) or PHTPP (10 pM–10 μM) (C). The control sample was incubation with vehicle (indicated as Ctl.). After 24 h, the cells were harvested and assayed for luciferase activity, with all transfections efficiencies were normalized by using the internal *Renilla* control plasmid. The data are presented as the mean ± S.D. (n = 3) fold-induction from the vehicle-treated control (indicated as 1). Significant differences [for (B) and (C) one-way ANOVA, followed by Dunnett's post hoc test] compared with control are marked with asterisks (*$P < 0.05$).

Figure 2. Structures and experimental scheme for the treatment with BPA or MBP. (A) The structures of BPA and MBP. (B) The experimental scheme for treatment with BPA or MBP. In treatment (1), the cells were treated with vehicle for control or ligands (BPA or MBP) for booster (–) and booster (+). In treatment (2), the culture medium was changed after 24 h of treatment (1), and the cells were then treated with vehicle for control/booster (–), or ligands for booster (+). The details of the experimental scheme for the treatments are described in the Materials and Methods. After chemical treatment for a total of 48 h, samples of the treated cells were collected for the analysis of gene expression, luciferase activity, cell proliferation, and cell cycle.

Figure 3. Effects of repeated exposure of BPA and MBP on the proliferation of MCF-7 cells. MCF-7 cells were exposed to BPA (0.1 nM–10 μM) (A) or MBP (0.1 nM–10 μM) (B) and then cell proliferation was measured. The chemical treatments and cell proliferation analysis

were performed in accordance with the methods described in Materials and Methods. The control sample (indicated as Ctl.) comprised incubation with vehicle alone. The open and closed circles represent booster (–) and booster (+), respectively. The data are presented as the mean ± S.D. (n = 6) percentage relative to the control. Significant differences [for (B) two-way ANOVA, followed by repeated (related) measures with Tukey-Kramer's post hoc test] are marked with asterisks (*P < 0.05). P values were for the line graph obtained when MBP was treated at the concentrations up to 25 nM, compared with the booster (–) line graph.

Figure 4. Repeated exposure of MBP stimulated cell cycle progression in MCF-7 cells. The MCF-7 cells were treated with 1 nM MBP for booster (–) and booster (+) and then stained with PI. The distribution of cells in each phase of the cell cycle was determined by flow cytometric analysis of PI staining of the DNA. (A) Representative histograms are shown. (B) The expected phase of the cell cycle and the percentage of cells in each phase are indicated. The data are presented as the mean ± S.D. (n = 3). Significant differences [for (B) two-way ANOVA, followed by Dunnett's post hoc test] compared with control are marked with asterisks (*P < 0.05).

Figure 5. Effects of repeated exposure of BPA and MBP on transcriptional activity mediated by ERs in ERα/β-positive MCF-7 cells. MCF-7 cells were transiently transfected with an ERE-luciferase reporter plasmid. After transfection, the cells were treated with BPA (25 nM–10 μM) (A, B) or MBP (0.25–100 nM) (C, D) for booster (–) (left panel) and booster (+) (right panel). The control sample (indicated as Ctl.) was treated with vehicle. After chemical treatment for a total of 48 h, the cells were harvested and assayed for luciferase activity; all transfections efficiencies were normalized by using the internal *Renilla* control plasmid. (A)–(D) EC_{50} and PC_{10} values were determined. Details are described in Materials and Methods

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section. The data are presented as the mean ± S.D. (n = 3) of the fold-induction from the vehicle-treated control (indicated as 1). N.D., not determined (because of low estrogenic activities not displaying a saturation curve profile).

Figure 6. Effects of repeated exposure to BPA and MBP on the protein expression of ERα and ERβ in MCF-7 cells. (A, B, upper panel) Western blotting of ERα and ERβ. MCF-7 cells were treated with 1 nM BPA (A) or MBP (B) for booster (–) and booster (+) or vehicle (control). After chemical treatment for a total of 48 h, total cell lysates were prepared, and western blotting was performed by using antibodies specific for ERα, ERβ, and β-actin, respectively; β-actin was used as an internal loading control. The cell lysates prepared with human ERα or ERβ cDNA-transfected cells were used as the positive control (indicated as Std.) (A, B, lower panel). The band intensity of ERα (left panel) and ERβ (right panel) was quantified by using ImageJ 1.46r software, and the values were normalized to those of β-actin. The data are presented as the mean ± S.D. (n = 3) of the fold change from the vehicle-treated control (indicated as 1). Significant differences (two-way ANOVA, followed by Dunnett's post hoc test) compared with control are marked with asterisks (*P < 0.05).

Figure 7. Effects of repeated exposure of BPA, MBP, and BPA/MBP on the mRNA expression of *ERα* in MCF-7 cells. Real-time RT-PCR analysis of ERα. (A: booster (–), B: booster (+) MCF-7 cells were treated with 1 nM BPA, 1 nM MBP, or their combination (BPA/MBP) for booster (–) and booster (+) or vehicle (indicated as Ctl.) for 48 h. The data are presented as the mean ± S.D. (n = 6) of the fold-induction from the vehicle-treated control. Significant differences (two-way ANOVA, followed by Dunnett's post hoc test) compared with control are marked with asterisks (*P < 0.05).

Figure 8. Effects of the ERβ antagonist, PHTPP, on MBP-stimulated transcriptional activity and cell proliferation. (A) MCF-7 cells were transiently transfected with an ERE-luciferase reporter plasmid. After transfection, the cells were treated with MBP (0.25–100 nM) for booster (+) in the absence (MBP only) or presence of 0.1, 0.25, 0.5, or 1 μM PHTPP. The control sample was treated with vehicle (indicated as Ctl.). After chemical treatment for a total of 48 h, the cells were harvested and assayed for luciferase activity; all transfections efficiencies were normalized by using the internal *Renilla* control plasmid. The data are presented as the mean ± S.D. (n = 3) of the fold-induction from the vehicle-treated control (indicated as 1). (B) MCF-7 cells were exposed to 1 nM MBP for booster (+) in the absence or presence of 0.1, 0.25, 0.5, 1 μM PHTPP, and then cell proliferation was measured. A control incubation was treated with vehicle alone (indicated as Ctl.). The data are presented as the mean ± S.D. (n = 6) percentage of the control. Significant differences (two-way ANOVA, followed by Dunnett's post hoc test) compared with control are marked with asterisks (*P < 0.05).

Figure 9. Comparison of the effects of repeated exposure of 1 nM MBP (booster (+)) and 2 nM MBP exposure on ERα/β protein expression and cell proliferation in MCF-7 cells. (A) The experimental scheme for the treatment with 1 nM MBP for booster (+) or 2 nM MBP for up to 48 h. The treatment of 1 nM MBP for booster (+) followed the same method as booster (+) in Figure 2B. In contrast, the experimental scheme for 2 nM MBP treatment involved no culture medium change and additional exposure. (B, left panel) Western blotting of ERα and ERβ. MCF-7 cells were treated with 1 nM MBP for booster (+) or 2 nM MBP. After chemical treatment for a total of 48 h, total cell lysates were prepared, and western blotting of antibodies specific for ERα, ERβ, and β-actin was performed; β-actin was used as an internal loading control. Cell lysates prepared with human ERα or ERβ cDNA-transfected cells were

used as the positive control (indicated as Std.) (B, right panel) The band intensity of ERα (left panel) and ERβ (right panel) was quantified by using ImageJ 1.46r software, and the values were normalized to β-actin. The data are expressed as the mean ± S.D. (n = 3) of fold change from booster (+) cells (indicated as 1). (C) MCF-7 cells were exposed to 2 nM MBP in the presence or absence of 0.5 μM PHTPP. After incubation for 48 h, the cell proliferation was measured. The control sample was treated with vehicle alone (indicated as Ctl.). The data are presented as the mean ± S.D. (n = 6) as a percentage of the control. Significant differences [Student's t test for (B, right panel); one-way ANOVA, followed by repeated (related) measures with Tukey-Kramer's post hoc test for (C)] as compared with booster (+) (B) or control (C) are marked with asterisks (*P < 0.05). N.S., not significant.

Figure 10. Summary of the effects of repeated exposure to MBP, an endocrine-disrupting chemical, on human breast cancer cells. ERα signaling tends to be abrogated by co-existing ERβ in human breast cancers with no exposure to MBP. In this study, it was revealed that repeated exposure of MBP stimulated ERE-driven transcriptional activity and cell proliferation, even though repeated exposure of MBP induced the downregulation of ERα. Mechanistically, the MBP-mediated stimulation of transcriptional activity/cell proliferation occurred in an ERβ-dependent manner. These effects were selectively observed with MBP, but not with the parent compound, BPA.

Figure 1

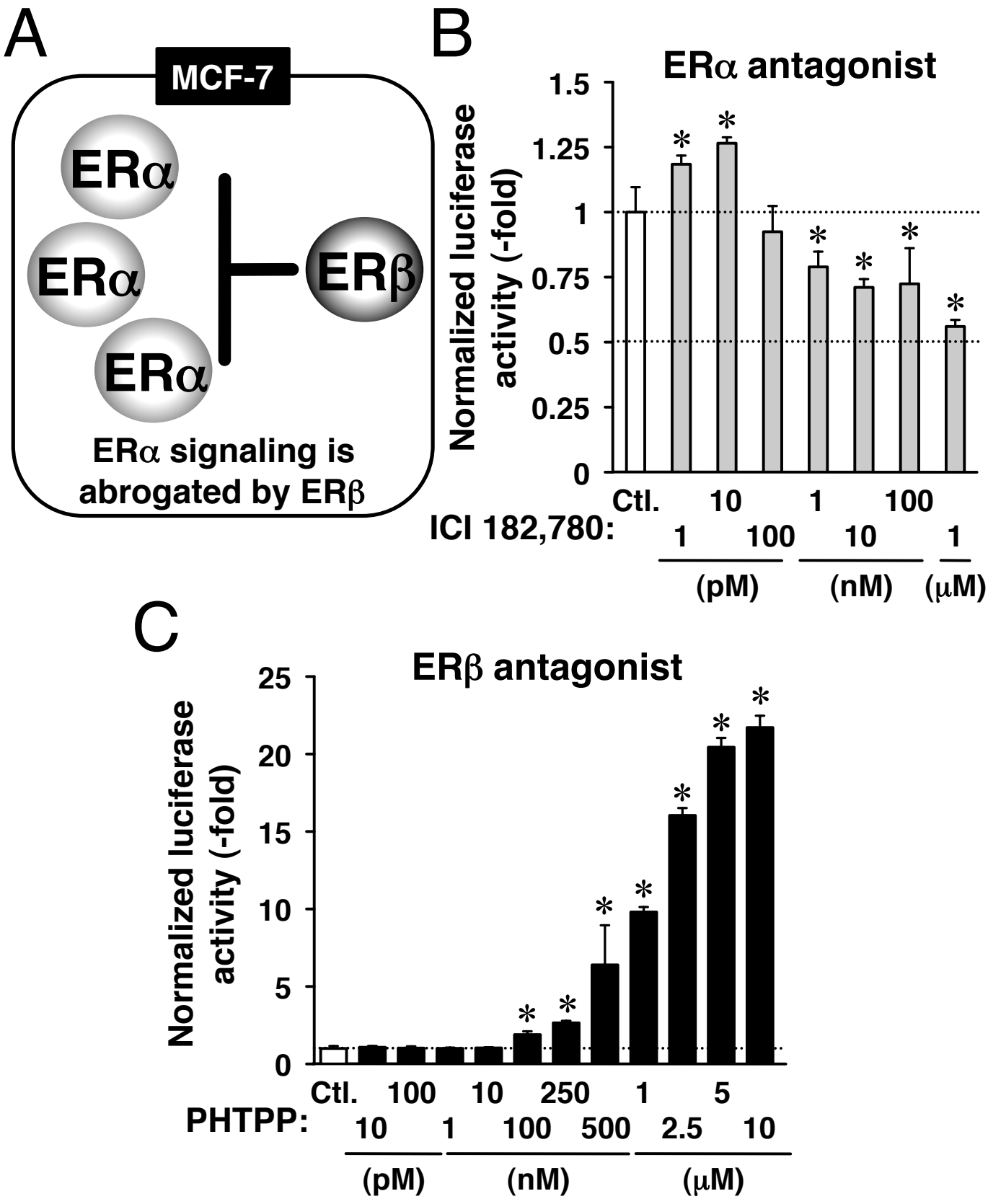
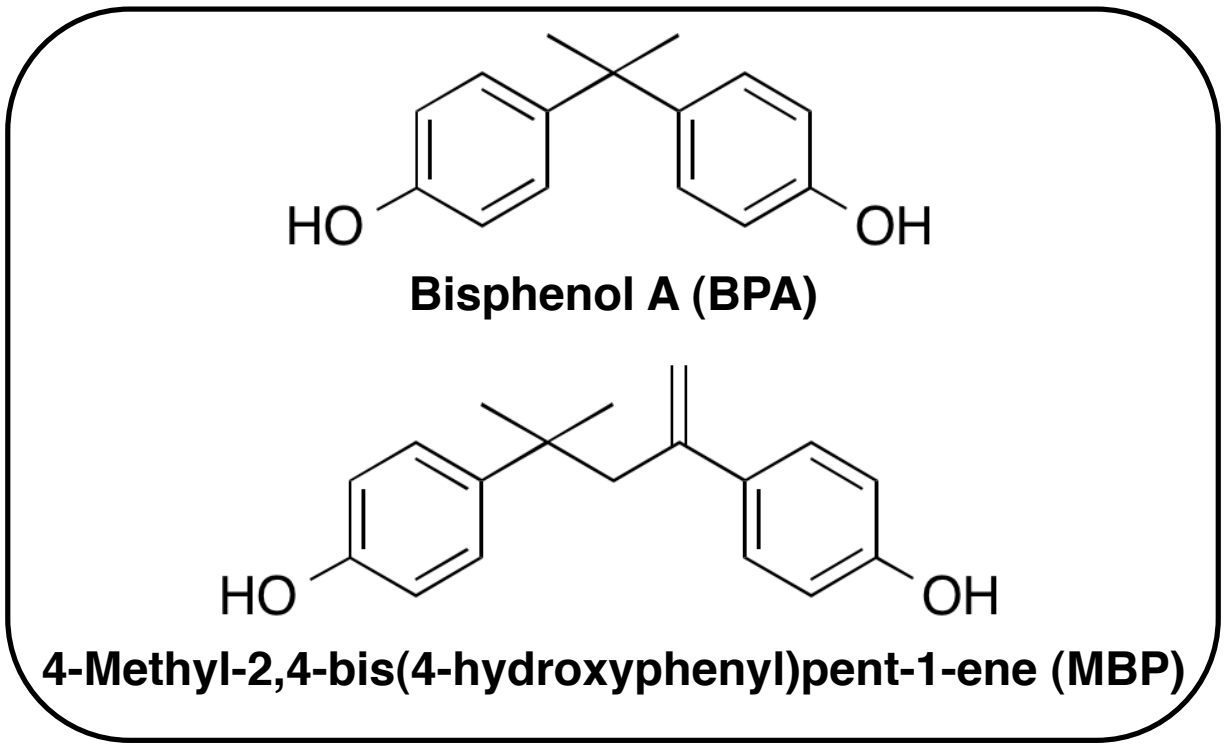

A
MCF-7
ERα
ERα
ERα
ERβ
ERα signaling is abrogated by ERβ
B
ERα antagonist
Normalized luciferase activity (-fold)
1.5
1.25
1
0.75
0.5
0.25
0
Ctl.
10
1
100
ICI 182,780:
1
100
10
1
(pM)
(nM)
(μM)
C
ERβ antagonist
Normalized luciferase activity (-fold)
25
20
15
10
5
0
Ctl.
100
10
250
1
5
PHTPP:
10
1
100
500
2.5
10
(pM)
(nM)
(μM)

Figure 2

A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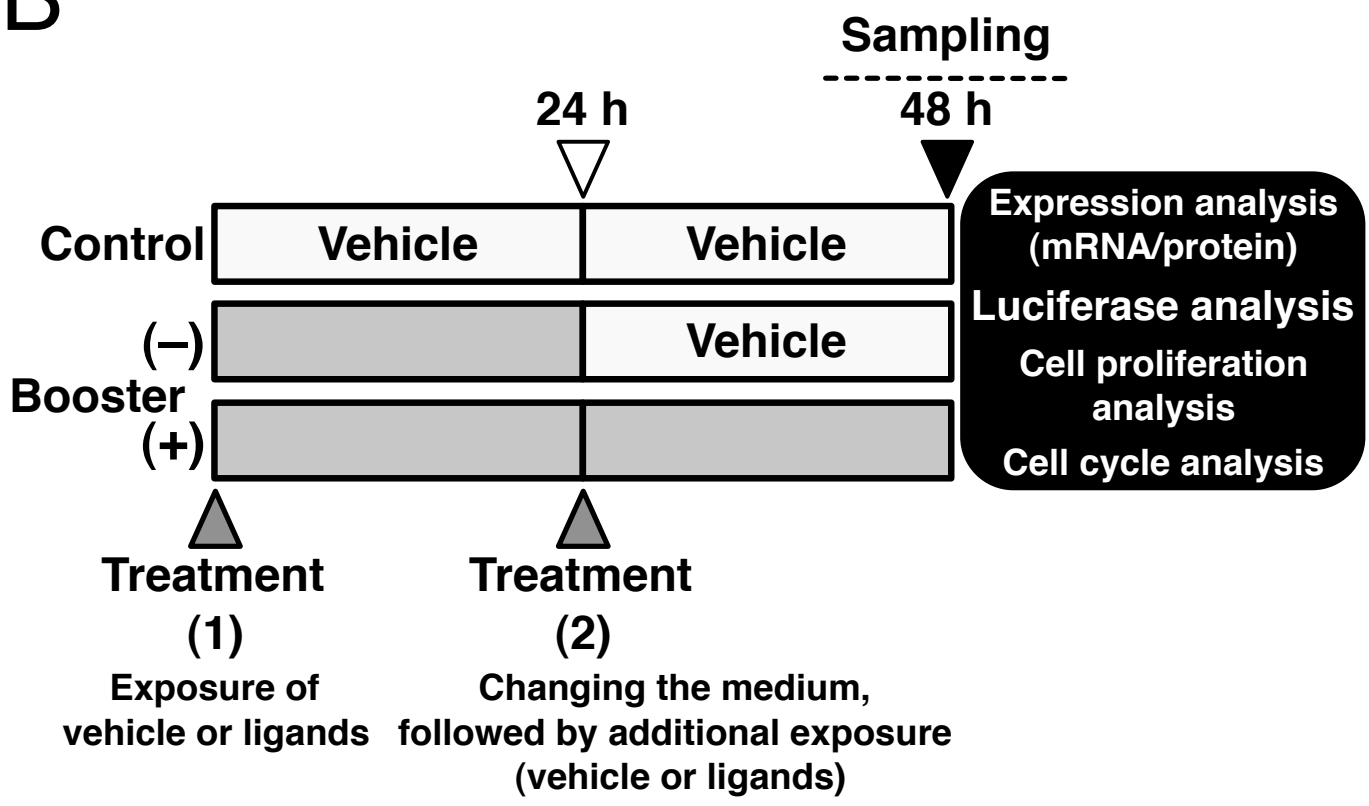
OH
Bisphenol A (BPA)
HO
OH
4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP)

B


Sampling
24 h
48 h
Control
Vehicle
Vehicle
(–)
Booster
(+)
Vehicle
Expression analysis (mRNA/protein)
Luciferase analysis
Cell proliferation analysis
Cell cycle analysis
Treatment
(1)
Exposure of vehicle or ligands
Treatment
(2)
Changing the medium, followed by additional exposure (vehicle or ligands)

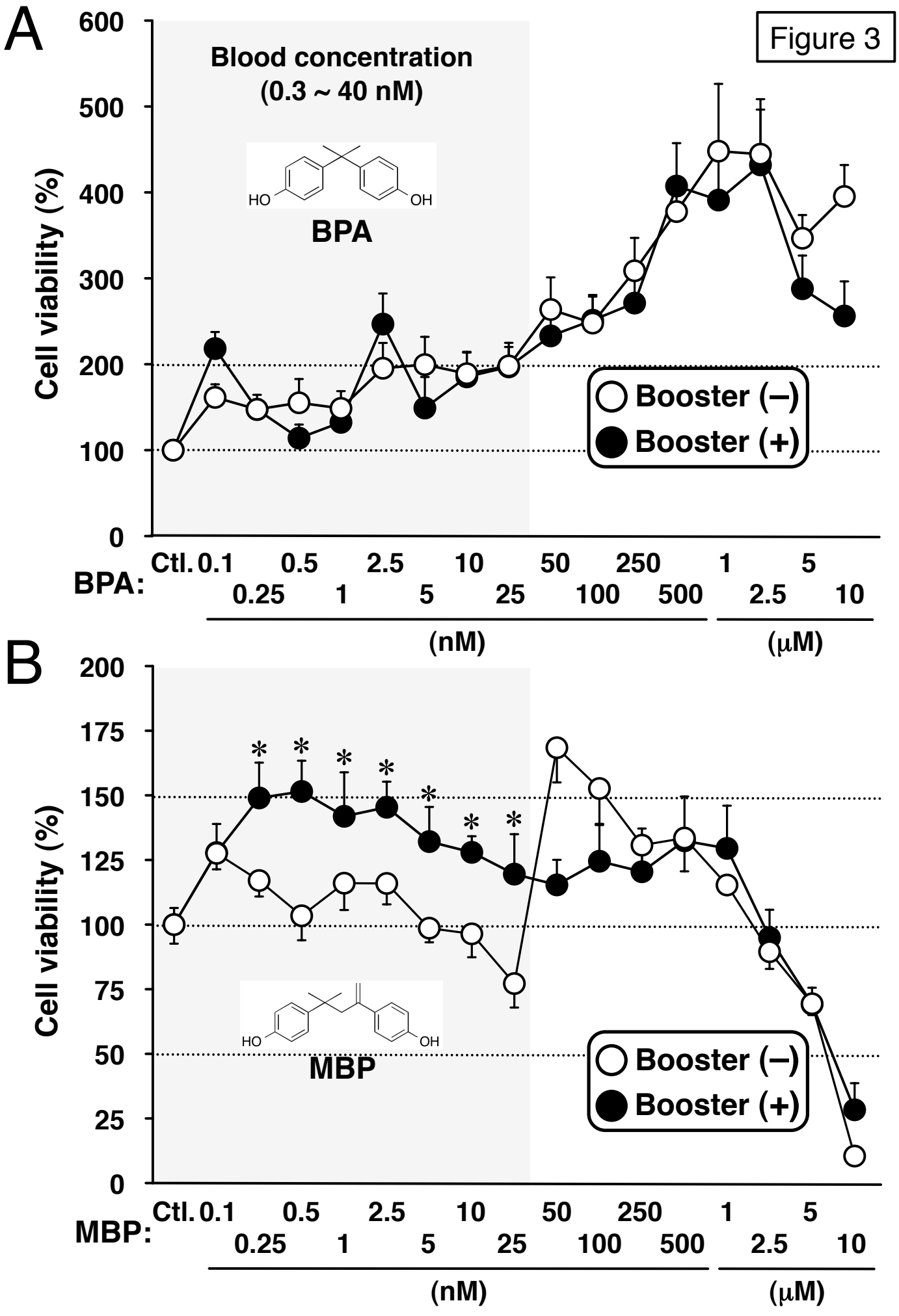
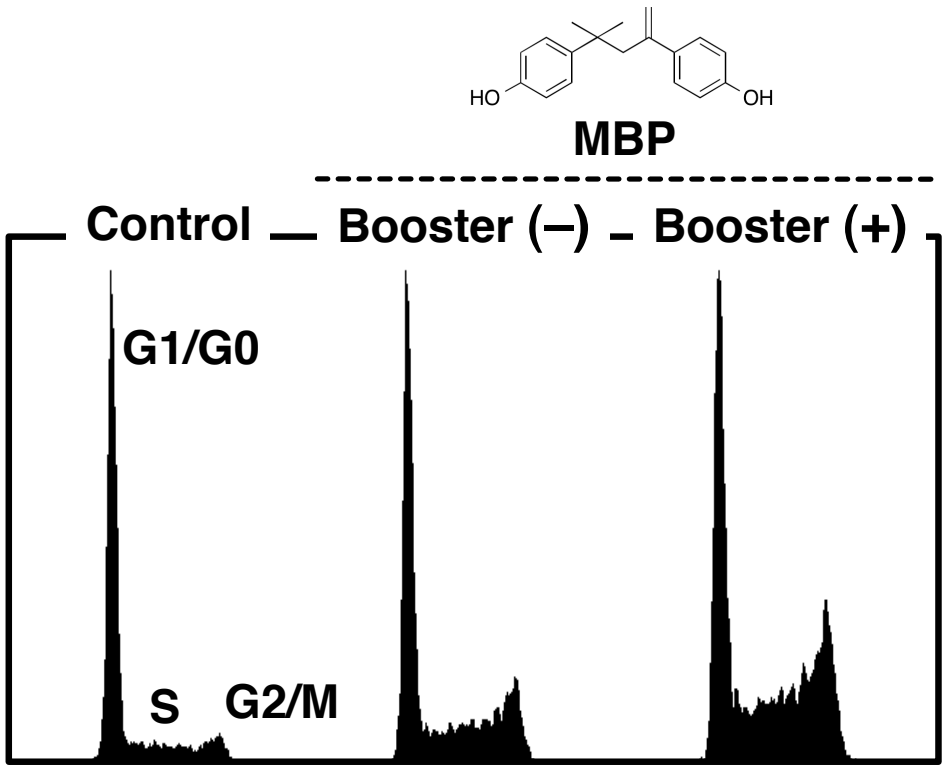

A
Figure 3
Blood concentration
(0.3 ~ 40 nM)
HO
OH
BPA
Cell viability (%)
600
500
400
300
200
100
0
Booster (–)
Booster (+)
Ctl. 0.1 0.5 2.5 10 50 250 1 5
BPA: 0.25 1 5 25 100 500 2.5 10
(nM) (μM)
B
200
175
150
125
100
75
50
25
0
HO
OH
MBP
Cell viability (%)
Booster (–)
Booster (+)
Ctl. 0.1 0.5 2.5 10 50 250 1 5
MBP: 0.25 1 5 25 100 500 2.5 10
(nM) (μM)

Figure 4

A


HO
OH
MBP
Control
Booster (–)
Booster (+)
G1/G0
S
G2/M

B

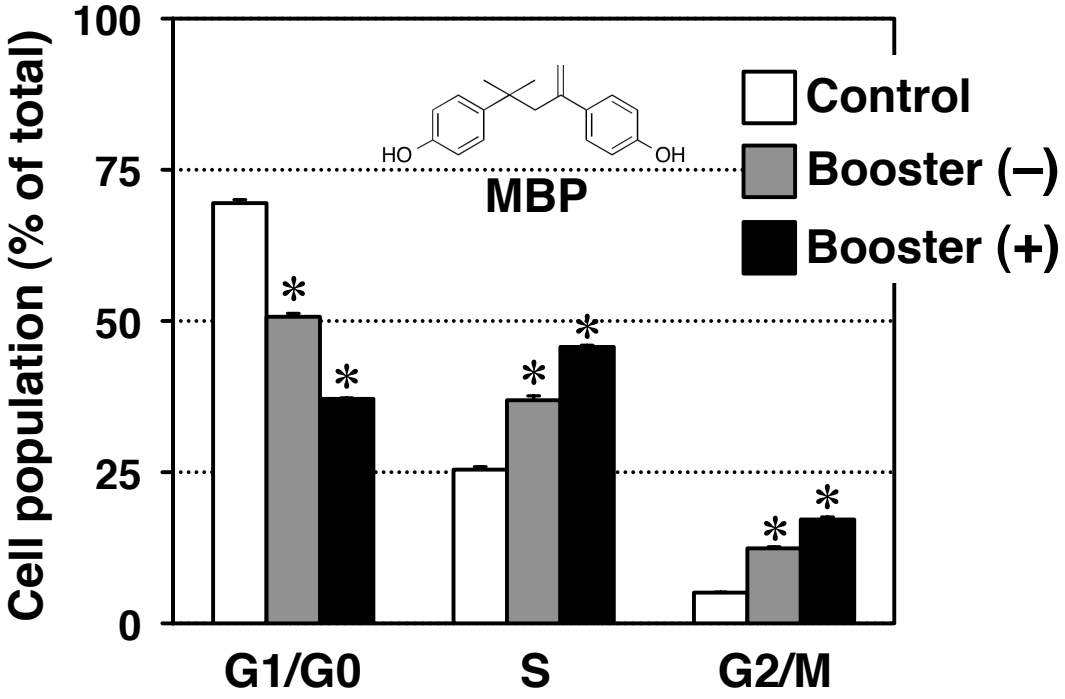

Cell population (% of total)
100
75
50
25
0
HO
OH
MBP
Control
Booster (–)
Booster (+)
*
G1/G0
S
G2/M

Figure 5

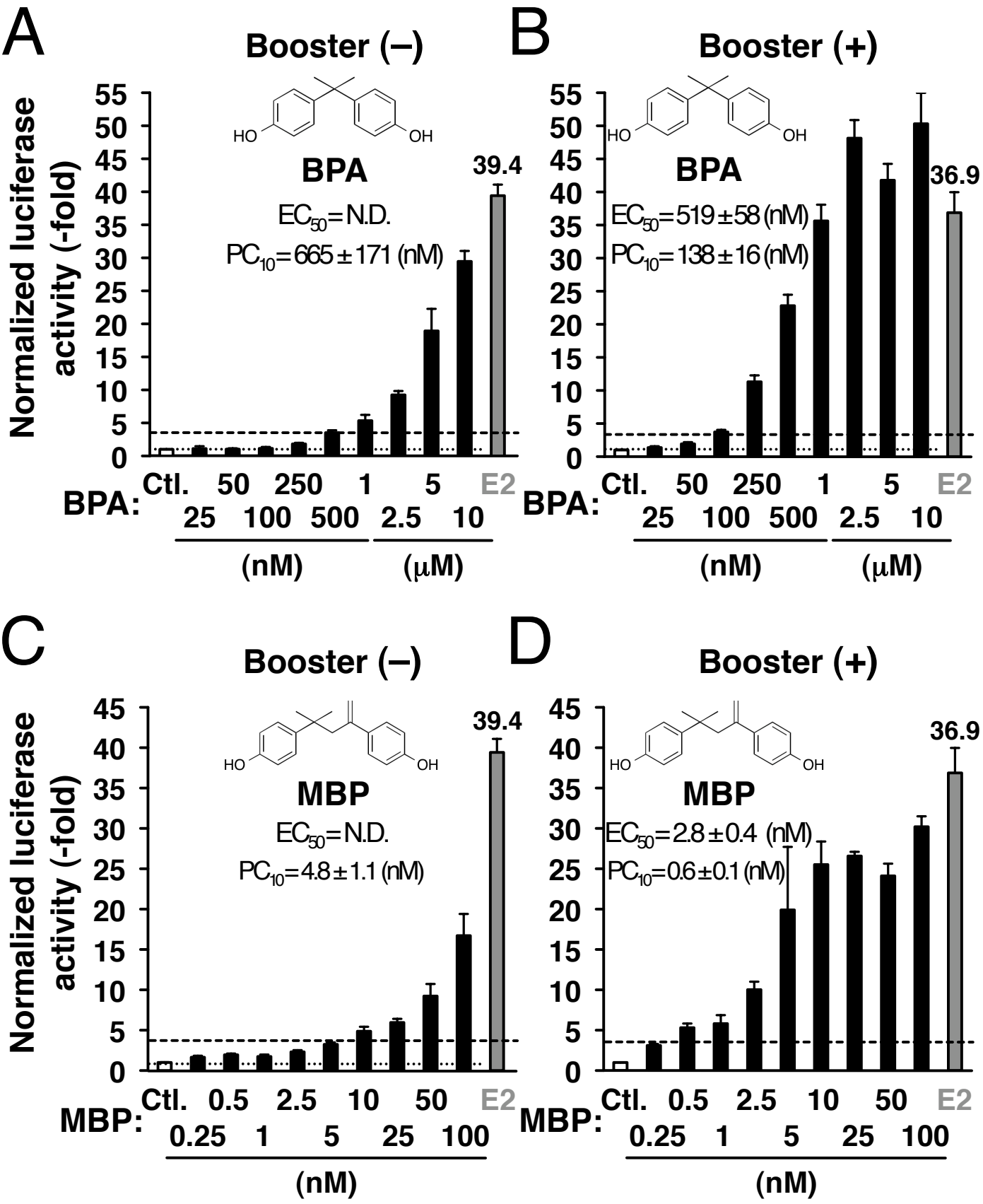
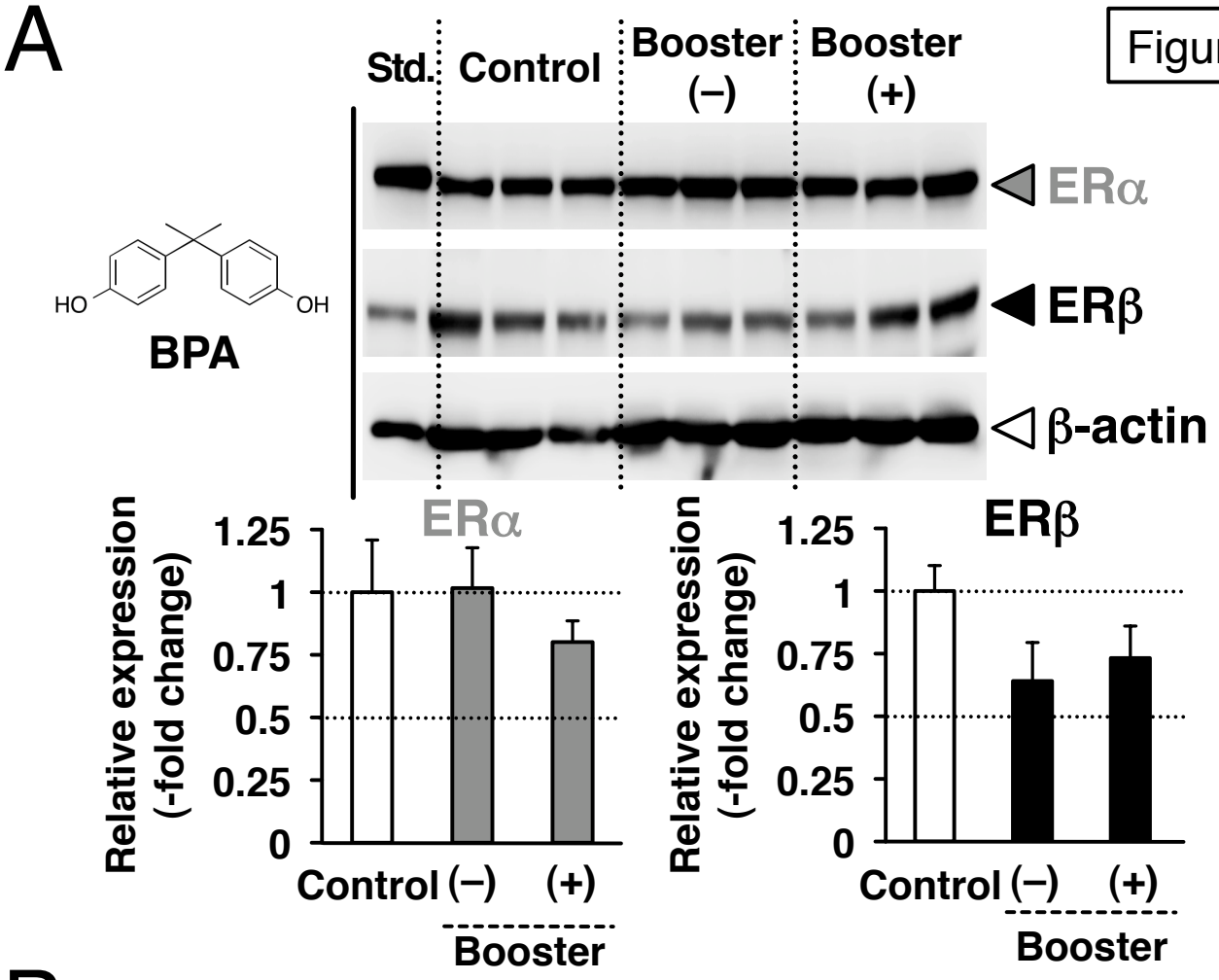

A
Booster (–)
Normalized luciferase activity (-fold)
BPA
EC50=N.D.
PC10=665±171 (nM)
39.4
BPA:
Ctl. 50 250 1 5 E2
25 100 500 2.5 10
(nM) (μM)
B
Booster (+)
BPA
EC50=519±58 (nM)
PC10=138±16 (nM)
36.9
C
Booster (–)
MBP
EC50=N.D.
PC10=4.8±1.1 (nM)
39.4
MBP:
Ctl. 0.5 2.5 10 50 E2
0.25 1 5 25 100
(nM)
D
Booster (+)
MBP
EC50=2.8±0.4 (nM)
PC10=0.6±0.1 (nM)
36.9

Figure 6


A
Std.
Control
Booster (–)
Booster (+)
ERα
ERβ
β-actin
HO
OH
BPA
Relative expression (-fold change)
1.25
1
0.75
0.5
0.25
0
Control
(–)
(+)
Booster

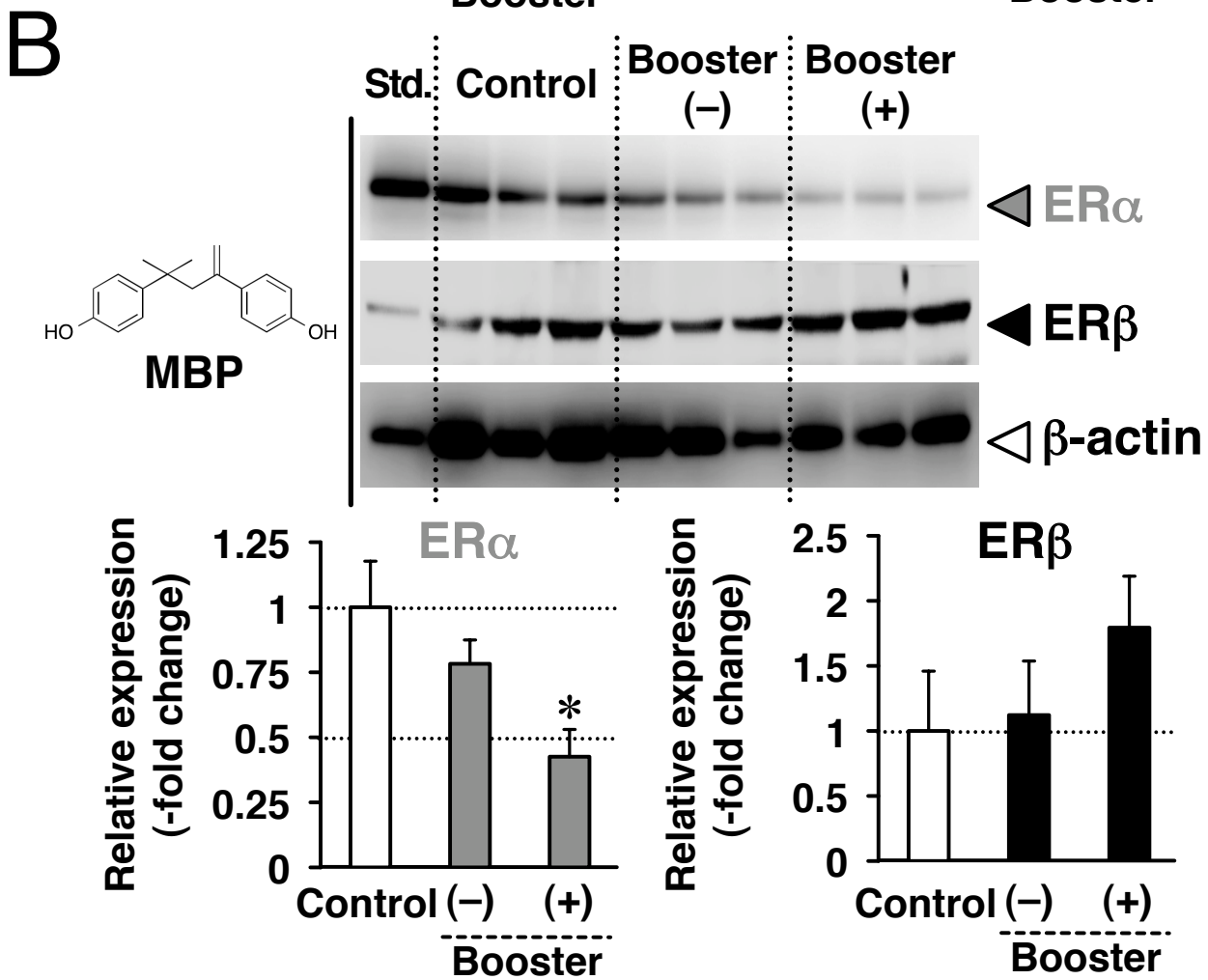

B
Std.
Control
Booster (–)
Booster (+)
ERα
ERβ
β-actin
HO
OH
MBP
Relative expression (-fold change)
1.25
1
0.75
0.5
0.25
0
*
2.5
2
1.5
1
0.5
0
Control
(–)
(+)
Booster

Figure 7

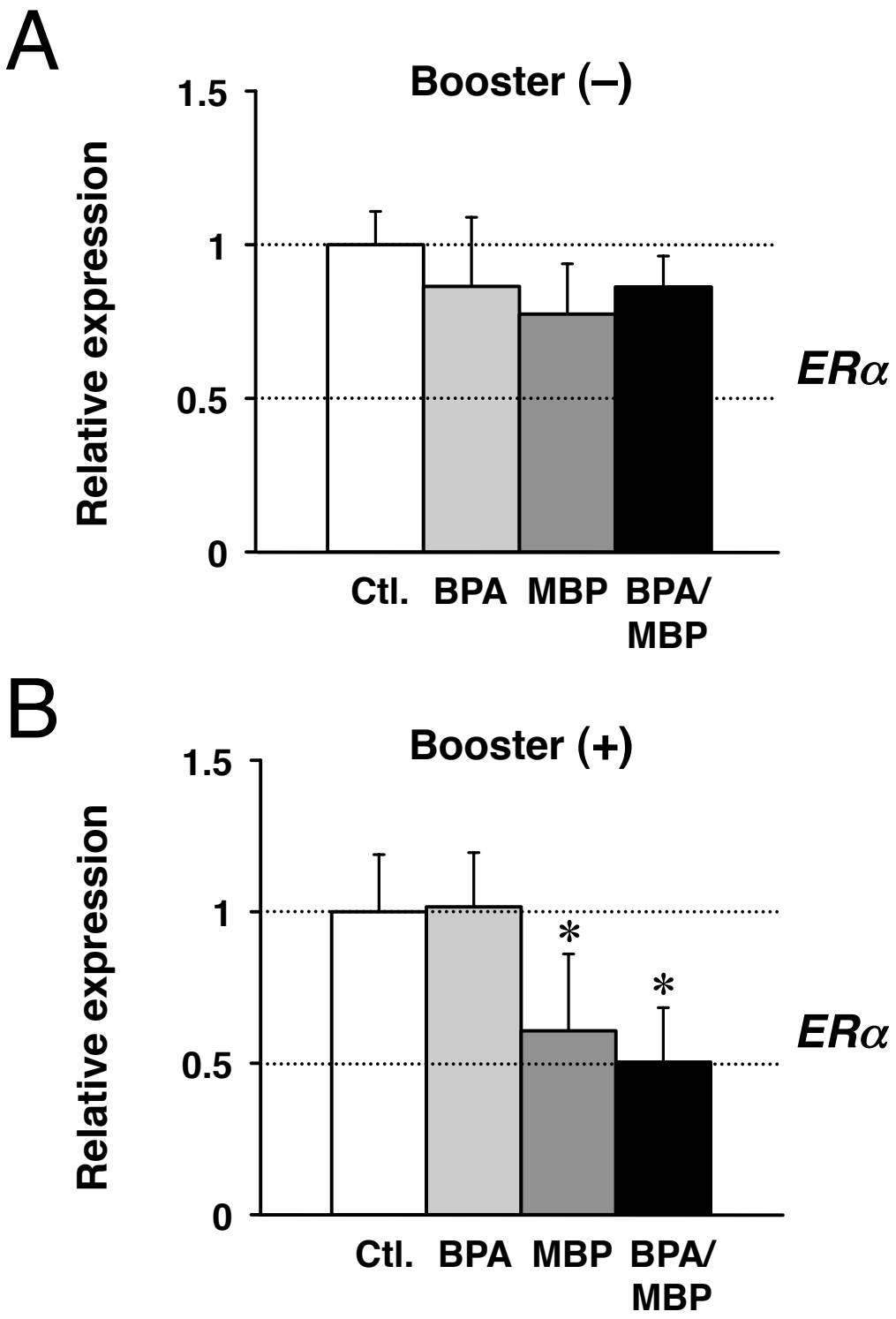

A
Booster (–)
Relative expression
1.5
1
0.5
0
ERα
Ctl.
BPA
MBP
BPA/
MBP
B
Booster (+)
Relative expression
1.5
1
0.5
0
*
*
ERα
Ctl.
BPA
MBP
BPA/
MBP

Figure 8

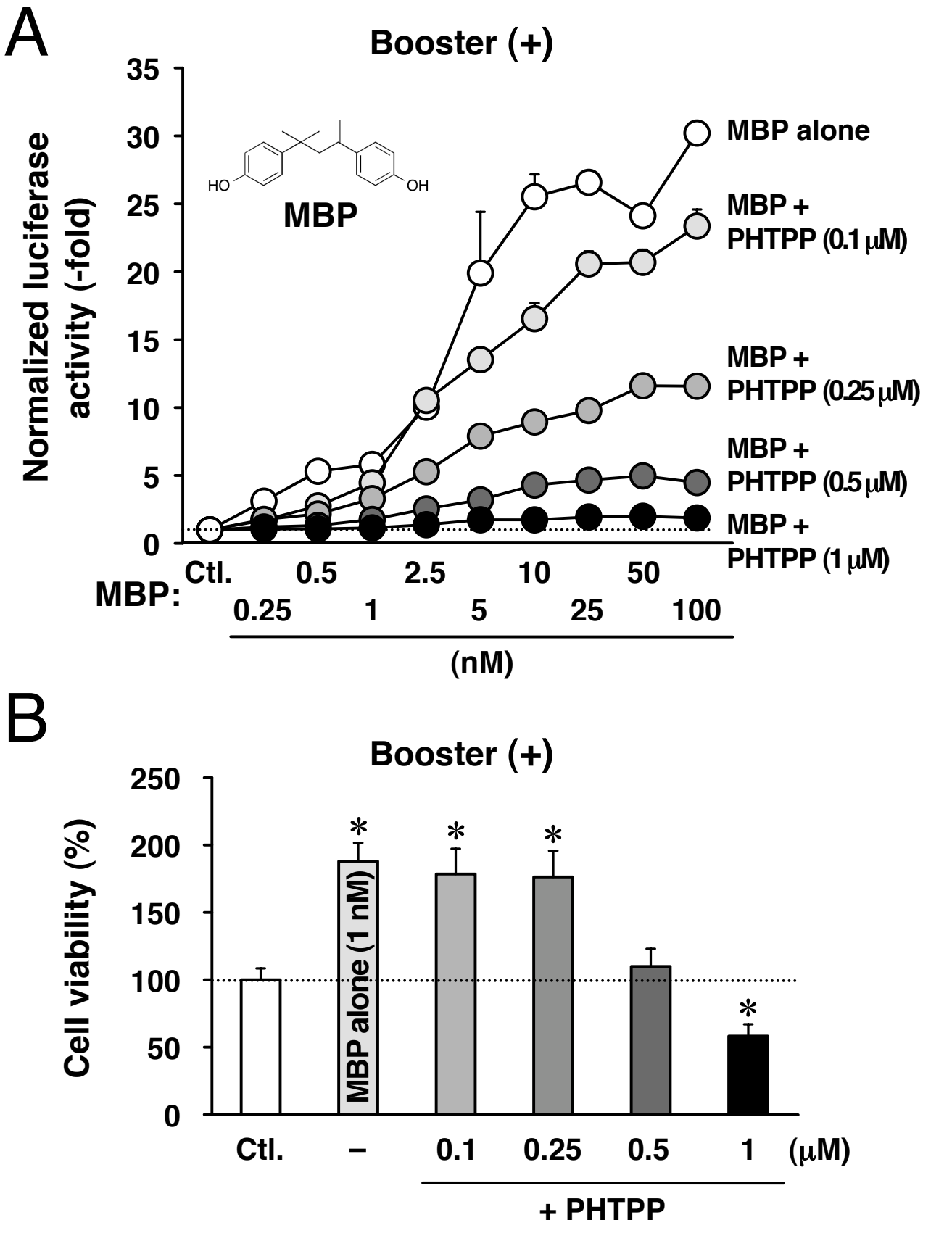

A
Booster (+)
Normalized luciferase activity (-fold)
35
30
25
20
15
10
5
0
HO
OH
MBP
MBP alone
MBP + PHTPP (0.1 μM)
MBP + PHTPP (0.25 μM)
MBP + PHTPP (0.5 μM)
MBP + PHTPP (1 μM)
MBP:
Ctl.
0.5
2.5
10
50
0.25
1
5
25
100
(nM)
B
Booster (+)
Cell viability (%)
250
200
150
100
50
0
MBP alone (1 nM)
*
Ctl.
–
0.1
0.25
0.5
1
(μM)
+ PHTPP

Figure 9

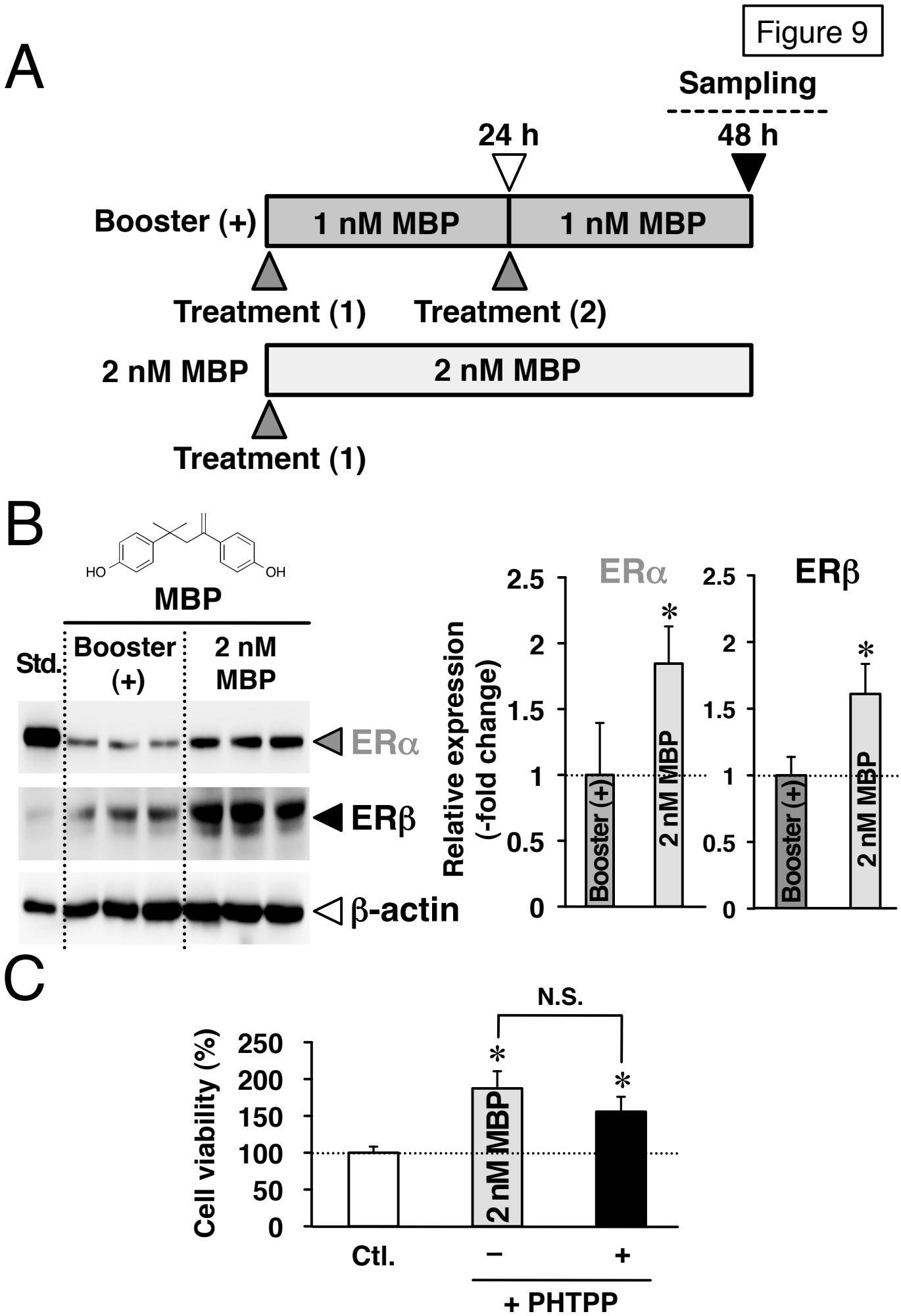
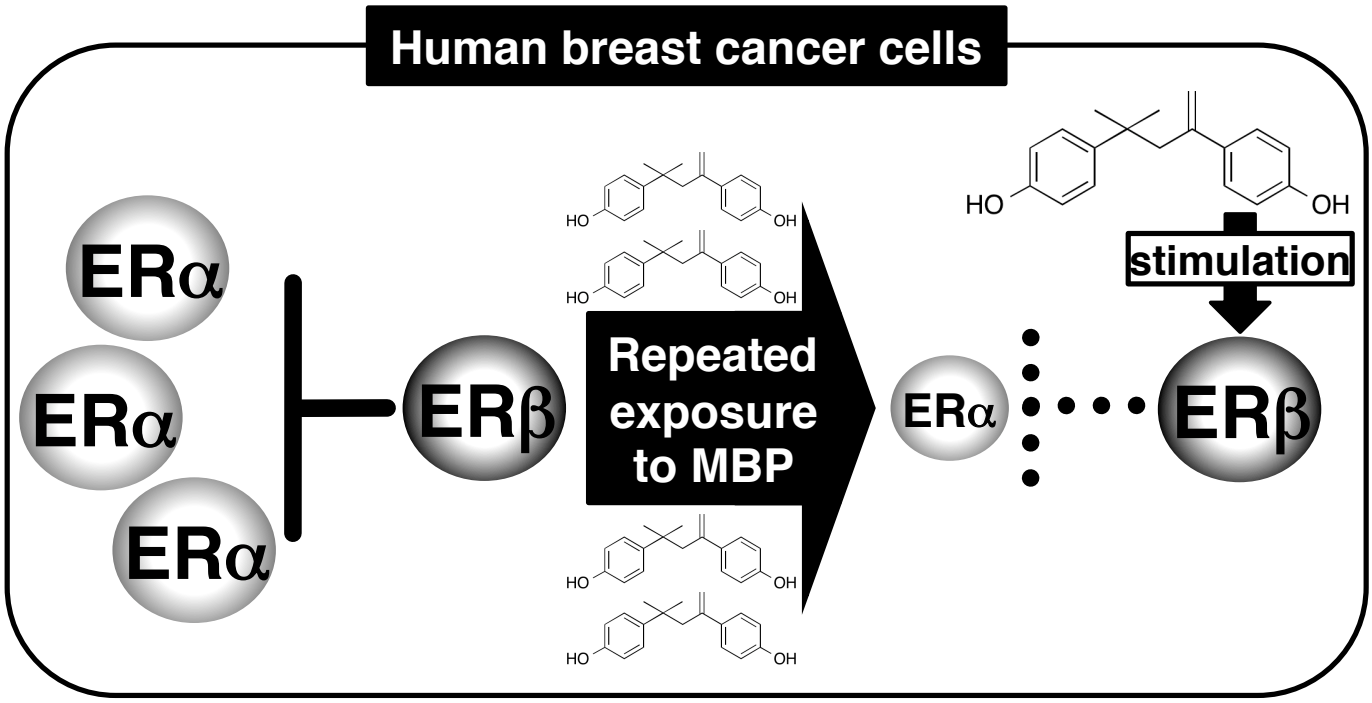

A
Sampling
24 h
48 h
Booster (+)
1 nM MBP
1 nM MBP
Treatment (1)
Treatment (2)
2 nM MBP
2 nM MBP
Treatment (1)
B
HO
OH
MBP
Std.
Booster (+)
2 nM MBP
ERα
ERβ
β-actin
Relative expression (-fold change)
ERα
ERβ
2.5
2
1.5
1
0.5
0
Booster (+)
2 nM MBP
C
Cell viability (%)
250
200
150
100
50
0
N.S.
Ctl.
–
+
2 nM MBP
+ PHTPP

Figure 10


Human breast cancer cells
ERα
ERα
ERα
ERβ
HO
OH
HO
OH
Repeated exposure to MBP
HO
OH
HO
OH
HO
OH
stimulation
ERα
ERβ