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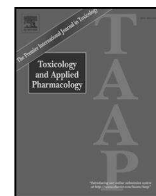

In vitro study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors

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ABSTRACT

Bisphenols are a group of chemicals structurally similar to bisphenol-A (BPA) in current use as the primary raw material in the production of polycarbonate and epoxy resins. Some bisphenols are intended to replace BPA in several industrial applications. This is the case of bisphenol-S (BPS), which has an excellent stability at high temperature and resistance to sunlight. Studies on the endocrine properties of BPS have focused on its interaction with human estrogen receptor alpha (hERα), but information on its interaction with other nuclear receptors is scarce. The aim of this study was to investigate interactions of BPS, BPF, BPA and its halogenated derivatives, tetrachlorobisphenol A (TCBPA), and tetrabromobisphenol A (TBBPA), with human estrogen receptors (hERα and hERβ), androgen receptor (hAR), and pregnane X receptor (hPXR), using a panel of in vitro bioassays based on competitive binding to nuclear receptors (NRs), reporter gene expression, and cell proliferation assessment. BPS, BPF, and BPA efficiently activated both ERs, while TCBPA behaved as weak hERα agonist. Unlike BPF and BPA, BPS was more active in the hERβ versus hERα assay. BPF and BPA were full hAR antagonists (BPA > BPF), whereas BPA and BPS were weak hAR agonists. Only BPA, TCBPA, and TBBPA, were hPXR agonists (TCBPA > TBBPA > BPA). These findings provide evidence that BPA congeners and derivatives disrupt multiple NRs and may therefore interfere with the endocrine system. Hence, further research is needed to evaluate the potential endocrine-disrupting activity of putative BPA substitutes.

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Introduction

Over the past few decades, concerns have grown about the possible health threat posed by endocrine-disrupting chemicals (EDCs), i.e., substances in our environment, food, and consumer products that interfere with hormone biosynthesis, metabolism, or activity and produce a deviation from normal homeostatic control or reproduction (Diamanti-Kandarakis et al., 2009). The direct interaction of chemicals, acting as receptor agonists or antagonists, with nuclear receptors (NRs), is a well-known mechanism of endocrine disruption. NRs are members of the steroid receptor superfamily, a large family of ligand-dependent transcriptional factors (Germain et al., 2006). Most research on EDCs has focused on their deleterious effects on sexual development and reproduction caused by interference with steroid signaling via human estrogen (hER) and androgen (hAR) receptors, because the outcome is readily identifiable and represents a sensitive health issue for a wide public (Henley and Korach, 2006). However, more recent reports have shown that several environmental chemicals can also affect hormone metabolism and synthesis by regulating their related enzymes, e.g., cytochrome P450, as activators of other NRs (Tabb and Blumberg, 2006), such as the human pregnane X receptor (hPXR). Indeed, activation of hPXR and up-regulation of their target genes by numerous compounds can increase the levels of endocrine-disrupting metabolites while at the same time altering the local bioavailability of endogenous androgens and estrogens. This provides a pathway for EDCs to alter steroid receptor activity without directly binding to steroid receptors. The problem posed by EDCs was addressed by European regulation (EU, 2006) on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), which set out the steps for authorizing their use and called for the development of safer alternatives. Subsequently, the European Commission (EC) published a new Directive (EU, 2011) that amended Directive 2002/72/EC to restrict the use of bisphenol-A (BPA) in plastic infant feeding bottles. Currently, a law banning the use of BPA in food packaging has passed its final stage in the French Senate and is set to be implemented in 2013 for packaging for children under the age of three and for all food packaging

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in France in 2015. The National Assembly of France has asked the government to submit a report on the potential toxicity of possible alternatives to BPA before 1 July 2014, six months before the ban takes effect.

BPA [2,2-bis(4-hydroxyphenyl)propane], one of the highest production volume chemicals worldwide (Vandenberg et al., 2010), is an industrial chemical used to make a hard clear plastic known as polycarbonate (Fiege et al., 2000), a component of numerous consumer products. BPA is also found in epoxy resins, which act as a protective lining on the inside of metal-based food and beverage cans. BPA has been detected in the environment (Kang et al., 2007) and in human fluids and tissues (Calafat et al., 2008; Jiménez-Díaz et al., 2010), and its toxicity has been intensively studied since the 1970s. Despite possessing only modest estrogenic activity in comparison to the natural estrogen 17β-estradiol (E_2), BPA has produced a range of adverse effects in laboratory animals, and major concerns have been raised about its impact on reproductive systems (Richter et al., 2007). Further receptor-mediated biological activities have been reported in different model systems, e.g., binding to the orphan estrogen-related receptor gamma (ERRγ) (Okada et al., 2008), thyroid hormone disruption (Moriyama et al., 2002), altered pancreatic β-cell function (Ropero et al., 2008), and obesity promotion (Newbold et al., 2008). However, although BPA is a well-known EDC, the effects of low doses remain controversial (Vandenberg et al., 2012).

Several chemicals that are structurally similar to BPA are utilized in the manufacture of resins and plastics. They consist of two phenolic rings joined by a bridging carbon or other chemical structures (Fig. 1) and are designated BPA analogs, congeners or bisphenols. Some of these are considered candidates for the partial replacement of BPA in the industrial applications, including bisphenol-S [bis(4-hydroxyphenyl)sulfone (BPS)], whose two phenolic rings are linked by a sulfur dioxide (SO_2) group. BPS is of interest for the preparation of high temperature resistant thermosetting thermoplastic polymers (Spitsbergen et al., 1971). BPS-based epoxy resins resist deformation by heat and thermal stability and offer shorter gelling gel times, the more rapid development of mechanical properties in cured systems, improved resistance to organic solvent attack, increased dimensional stability, and better wetting of glass reinforcements (Rwei et al., 2003). As well as in epoxy resins, BPS is widely used as a monomer in the production of cyclic carbonates (Kim et al., 2001) and sulfonated poly(ether ketone ether sulfone) (Changkhamchom and Sirivat, 2010), and is a chemical additive in pesticides, dyestuffs, color-fast agents, leather tanning agents, dye dispersants, and fiber improvers. BPS replaced BPA as a developer in dyes for thermal paper in Japan (Watanabe et al., 2004) and China (Liu, 2005) and has been detected in canned food (Viñas et al., 2010) and in paper products and currency bills (Liao et al., 2012a). In fact, widespread exposure of the general population to BPS has been demonstrated in various countries, with the detection of BPS levels ranging from 0.02 to 21 ng/ml (0.8–84 nM) in urine samples from people living in the U.S. and seven Asian countries (Liao et al., 2012b). BPS is much less biodegradable than BPA (Danzl et al., 2009; Ike et al., 2006) and, given its annually increasing production, it is expected to become as widespread as BPA (Liao et al, 2012c). There has been less research on BPS than on BPA, but preliminary studies have shown that it also possesses hormone-mimicking properties (Chen et al., 2002; Delfosse et al., 2012; Grignard et al., 2012; Hashimoto et al., 2001; Kitamura et al., 2005; Kuruto-Niwa et al., 2005). However, studies on BPS as an endocrine disrupter have focused on its interaction with human estrogen receptor alpha (hERα), and much less is known about its interaction with other NRs.

Bisphenol-F, [bis(4-hydroxyphenyl)methane, (BPF)], which has no substituent at the bridging carbon (except with H atoms), has a broad range of industrial applications. The BPF monomer is polymerized to prepare epoxy resins and polycarbonates for use in the manufacture of lacquer, varnishes, coatings, adhesive plastics, and other products (Jana et al., 2005). Although no information is available on human exposure, BPF has been detected in the environment (Fromme et al., 2002; Stachel et al., 2003) and has demonstrated an estrogenic effect in various in vivo (Yamasaki et al., 2002) and in vitro studies (Cabaton et al., 2009; Hashimoto and Nakamura, 2000; Hashimoto et al., 2001). BPF has also shown anti-androgenic activity in several human recombinant cell lines carrying hAR (Cabaton et al., 2009; Satoh et al., 2004).

Halogenated derivatives of BPA, such as tetrabromobisphenol-A [2,2-bis(4-hydroxy-3,5-dibromophenyl)propane, (TBBPA)] and tetrachlorobisphenol-A [2,2-bis(4-hydroxy-3,5-dichlorophenyl)propane, (TCBPA)] are both widely used as flame-retardants for building material, paints, and epoxy resin-containing plastic products such as electronic circuit boards, and other electronic equipment. Like BPA, both compounds are considered environmental contaminants (de Wit et al., 2009; Fukazawa et al., 2001) and have also been reported in human fluids and/or tissues (Cariou et al., 2008; Fernandez et al., 2007; Jimenez-Diaz et al., 2010; Johnson-Restrepo et al., 2008). Moreover, these compounds have been found to interact with and disrupt thyroid hormone receptor signaling (Kitamura et al. 2002). TBBPA and TCBPA are also potent peroxisome proliferator-activated receptor gamma (PPARγ) agonists (Riu et al., 2011a).

As noted, research has focused mainly on the endocrine disrupting activity of BPA, and much less attention has been paid to the toxicity of the other bisphenols proposed as substitutes, such as BPS. The present study was designed to develop a comprehensive NR interaction profile of five bisphenols in current use (BPS, BPF, BPA, TCBPA and TBBPA) in order to contribute additional information on their endocrine disruptive activity. For this purpose, we investigated the direct interaction of these compounds with hERα, hERβ, hAR,

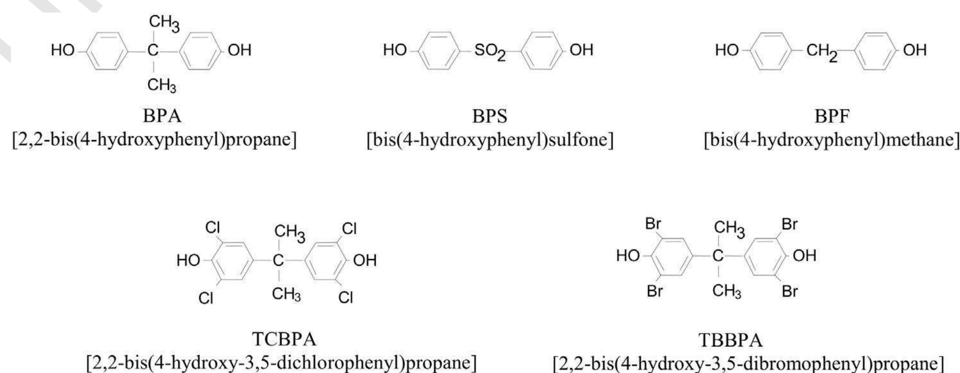

Fig. 1. Chemical structures of bisphenol-A (BPA) and derivatives.

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and hPXR, using a panel of steroid hormone receptor cell based assays to measure different endpoints at distinct levels of biological complexity.

Materials and methods

Chemicals and materials. Culture medium and fetal bovine serum (FBS) were obtained from Gibco (Invitrogen, Barcelona, Spain). E_2, EE_2, BPA, BPS, BPF, TBBPA, TCBPA, puromycin, geneticin, luciferin, methyl thiazolyl diphenyl tetrazolium bromide (MTT) and sulforhodamine B (SRB) were obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). $[^3H]$-E_2 (41.3 Ci/mmol specific activity), methyltrienolone (R1881) and tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate (SR12813) were purchased from NEN Life Science Products (Paris, France). Stock solutions (10 mM) of E_2 ($\geq$98% purity), EE_2 ($\geq$98% purity), R1881 ($\geq$98% purity), SR12813 ($\geq$98% purity), BPA ($\geq$99% purity), BPS (98% purity), BPF (98% purity), TBBPA (97% purity), and TCBPA (98% purity) were prepared in ethanol (>99.7% purity), and successive dilutions were performed in culture medium. Stock solutions were kept at −20 °C and dilution series were freshly prepared before each experiment. All other chemicals were of the highest quality available from commercial sources. All cell culture plastics were obtained from Falcon (VWR International Eurolab, Barcelona, Spain). An infinite M200 luminometer (Tecan, Barcelona, Spain) was used to detect luciferase activity in intact cells.

Charcoal–dextran treatment of serum to remove sex steroids. Sex steroids were removed from FBS by dextran-coated charcoal (DCC) stripping. Briefly, a suspension of 5% charcoal with 0.5% dextran T-70 was prepared. Aliquots of the DCC suspension of a volume similar to the serum aliquot to be processed were centrifuged at 1000 ×*g* for 10 min. Supernatants were aspirated, and serum aliquots were mixed with the charcoal pellets. This DCC-serum mixture was maintained in suspension by rolling (6 cycles/min) at 37 °C for 1 h. The suspension was centrifuged at 2000 ×*g* for 20 min, and the supernatant was then filtered through a 0.22 mm filter (Millipore). DCC-treated FBS (DCC-FBS) was stored at −20 °C until needed.

Plasmids. The plasmids used have been described elsewhere: pSG_5-ERα-puro (aa 1–595), pSG_5-ERβ-puro (aa 1–530), pSG_5-AR-puro (aa 1–919), and $pGAL_4$RE-ERE-βGlobin-Luc-SV-Neo (Balaguer et al., 1999; Paris et al., 2002) and $p(GAL_4RE)_5$-βGlob-Luc-SVNeo and pSG_5-GAL_4(DBD)-hPXR(LBD)-puro (Lemaire et al., 2006; Seimandi et al., 2005).

Generation of stable reporter cell lines and culture conditions. The stably transfected luciferase reporter MELN cell line was obtained as previously reported (Balaguer et al., 2001). Briefly, MELN cells were obtained by transfecting ERα-positive breast cancer MCF-7 cells with the estrogen-responsive gene ERE-βGlob-Luc-SV-Neo (Balaguer et al., 1999). MELN cells were cultured in Dulbecco's modified Eagle medium (DMEM) F12 with phenol red supplemented with 10% FBS, 1% antibiotic (penicillin/streptomycin), and 1 mg/ml G418. Basal luciferase activity in MELN cells was around 15% of maximal activity (100% for 10 nM E_2).

Generation of HELN-hERα and -hERβ reporter cell lines was performed in two steps (Balaguer et al., 1999; Escande et al., 2006). The estrogen responsive reporter gene was first stably transfected into HeLa cells, generating the HELN cell line and, in a second step, these HELN cells were transfected with -hERα or -hERβ plasmid constructs to obtain the HELN-hERα or -hERβ cell lines, respectively. HELN cells were cultured in DMEM supplemented with 5% FBS, 1% antibiotic, and 1 mg/ml G418. HELN-ER cells were cultured in DMEM F12 without phenol red supplemented with 6% DCC-FBS, 1% antibiotic, 1 mg/ml G418, and 0.5 μg/ml puromycin. Basal luciferase activity in HELN-hERα and HELN-hERβ cells was around 10% of maximal activity (100% for 10 nM E_2).

PALM cells were obtained as already described (Terouanne et al., 2000). Briefly, PC3 cells were co-transfected with an androgen responsive gene, MMTV-Luc-SV-Neo, and an androgen receptor expressing plasmid, pSG_5AR-puro. PALM cells were cultured in Ham's F12 supplemented with 10% FBS, 1 mg/ml G418, and 1 μg/ml puromycin. Basal luciferase in PALM cells was around 10% of maximal activity (100% for 10 nM R1881).

The HG_5LN-hPXR cell line was generated in two steps (Lemaire et al., 2006). In a first step, HeLa cells were stably transfected with a GAL_4RE_5-βGlob-Luc-SVNeo plasmid to produce the HG_5LN cell line, which expresses constitutively luciferase activity. Then, HG_5LN cells were stably transfected with the pSG_5-GAL_4(DBD)-hPXR(LBD)-puro plasmid to obtain the HG_5LN-hPXR cell line. HG_5LN and HG_5LN-hPXR cells were cultured in DMEM supplemented with 5% FBS, 1% antibiotic, and 1 mg/ml G418. Additionally, 0.5 μg/ml puromycin was added in HG_5LN-hPXR cell medium.

Because of the estrogenic activity of phenol red and FBS, experiments were performed in a test culture medium, i.e., DMEM F12 without phenol red supplemented with 6% DCC-FBS (for MELN, HELN-hERα, -hERβ, and HG_5LN-hPXR cells) or Ham's F12 supplemented with 6% DCC-FBS (for PALM cells) and 1% antibiotic in a 5% CO_2 humidified atmosphere at 37 °C. The test culture medium was used in transactivation and competitive binding assays.

Living cell luciferase assay. Reporter cells were seeded at a density of 5×10^4 cells per well in 96-well white opaque tissue culture plates in 150 μl test culture medium. Test compounds were prepared at 4× concentration in the same medium, and 50 μl was added per well 8 h after seeding. Cell lines were incubated for 16 h (except for PALM cells, which were incubated for 40 h) with the compounds at 37 °C. At the end of incubation, the medium containing test compounds was removed and replaced with test culture medium containing 0.3 mM luciferin. At this concentration, luciferin diffused into the cell and produced a stable luminescent signal 5 min later. This signal is approximately 10-fold less intense than the signal obtained after cell lysis but is perfectly stable for several hours. The 96-well plate was then introduced into a luminometer, and luminescence was measured in intact living cells for 2 s.

Agonist and antagonist assays. Agonistic activities of hERα, hERβ, hAR, and hPXR in HELN-derived, MELN, PALM, or HG_5LN-hPXR cells were tested in the presence of increasing concentrations (0.01–10 μM) of BPS, BPF, BPA, TCBPA, and TBBPA. Tests were performed in quadruplicate for each concentration. Results were expressed as a percentage of maximal luciferase activity. Maximal luciferase activity (100%) was obtained in the presence of 10 nM E_2, 10 nM R1881, and 3 μM SR12813 (for hERs, hAR, and hPXR, respectively). For each compound, the potency corresponding to the concentration yielding half-maximal luciferase activity (EC_{50} value) was calculated. The antagonistic activities of these compounds (tested at 0.01–10 μM) were determined by coincubation with E_2 (0.1 nM), R1881 (0.3 nM), and SR12813 (0.2 μM) agonists for hERs, hAR, and hPXR, respectively. At these concentrations, activities reach approximately 90, 60, 80 and 60% of maximal luciferase activity (for hERα, hERβ, hAR, and hPXR, respectively). Data were expressed as half-maximal inhibitory concentration (IC_{50} value) for each compound tested.

Whole-cell hERα and hERβ competitive binding assays. Briefly, HELN-hERα and -hERβ cells were seeded at a density of 2×10^5 cells per well in 24-well tissue culture plates and grown in test culture medium. After 24 h, HELN-hERα and -hERβ cells were labeled with 0.1 nM $[^3H]$-E_2 (41.3 Ci/mmol specific activity) at 37 °C for 3 h in the absence or presence of BPS, BPF, BPA (0.01–10 μM), or unlabelled E_2 (100 nM). The final incubation volume was 500 μl, and each well was

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tested in duplicate. After incubation, unbound material was aspirated and cells washed three times with 500 µl of cold PBS. Then, 250 µl lysis buffer (400 mM NaCl, 25 mM Tris phosphate pH 7.8, 2 mM DTT, 2 mM EDTA, 10% glycerol, 1% triton X-100) was added, and plates were shaken for 5 min. Total cell lysate (200 µl) was mixed with 4 ml of LSC-cocktail (Emulsifier-Safe, Packard BioScience), and [^{3}H] bound radioactivity was liquid scintillation-counted (LS-6000-SC, Beckman-Coulter, Roissy, France). Non-specific binding was determined in the presence of 100 nM unlabeled E_2. Specific binding was calculated by subtracting non-specific binding from total binding. Bound radioactivity values were expressed in disintegrations per minute (dpm). In the absence of a competitor, specific bound radioactivity was 750–1000 dpm.

Results were plotted as dpm versus concentration of tested compounds. IC_{50} values were defined as the compound concentration required to decrease maximal [^{3}H]-E_2 binding by 50%. Compound selectivity towards hERα or hERβ was evaluated using the relative binding affinity (RBA) to E_2. The RBA for each competitor was calculated as the ratio of E_2 to competitor concentration required to reduce specific radiolabeled binding by 50% (ratio of IC_{50} values). The RBA value for E_2 was arbitrarily set at 100.

MCF-7 cell lines. Human breast cancer MCF-7 cells were cultured for routine maintenance in DMEM with phenol red supplemented with 10% FBS. MCF-7 AR1 cells, which are stable transfectants of MCF-7 cells expressing the wild-type hAR, were obtained as already described (Szelei et al., 1997). MCF7-AR1 cells were cultured in DMEM with phenol red supplemented with 10% FBS and 0.6 mg/ml G418. Cell proliferation experiments were performed in test culture medium (DMEM without phenol red supplemented with 10% DCC-FBS) in a 5% CO_2 humidified atmosphere at 37 °C.

E-Screen bioassay. MCF-7 cells were used in the test of estrogenicity according to a technique slightly modified from that originally described by Soto (Soto et al., 1995). Briefly, MCF-7 cells were trypsinized and plated in 96-well culture plates at initial concentrations of 4×10^3 cells per well. Cells were allowed to attach for 24 h and the seeding medium was then removed and replaced with the test culture medium. A range of concentrations of the test compound was added to this medium in the sample wells. In each experiment, a dose–response curve (0.1 pM–1000 pM) for E_2 and a negative control (cell treated only with hormone-free medium) were included. The bioassay was ended on day 6 (late exponential phase) by removing the media from the wells, fixing the cells, and staining them with SRB. The cells were treated with cold 10% trichloracetic acid and incubated at 4 °C for 30 min, washed five times with tap water, and left to dry. Trichloroacetic-fixed cells were stained for 10 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Wells were rinsed with 1% acetic acid and air dried. Bound dye was solubilized with 10 mM Tris base (pH 10.5) in a shaker for 20 min. Finally, the absorbance was read in a Titertek Multiscan apparatus (Flow, Irvine, CA) at 492 nm. Linearity of the SRB assay with cell number was verified prior to cell growth experiments.

Agonistic assays were performed in the presence of increasing concentrations (0.01–10 µM) of the test compounds. For each compound, the ratio between the cell yield obtained and the proliferation of hormone-free control cells (negative control) was calculated. Tests were done in triplicate for each concentration. Results were expressed as proliferative effect (PE) [MCF-7 cell proliferation (fold-over control)]. The antagonistic activities of these compounds were determined by coincubation with the agonist E_2 at 100 pM.

A-Screen bioassay. A slightly modified version of the protocol described previously by Szelei (Szelei et al., 1997) was employed to evaluate the androgenic activity of the test compounds in the A-screen assay. Briefly, MCF-7 AR1 cells were trypsinized and plated in 96-well culture plates at initial concentrations of 5×10^3 cells per well and allowed to attach for 24 h. On the second day, the seeding medium was removed and replaced with the test culture medium. Assays were performed in the presence of increasing concentrations (0.01–10 µM) of the test compounds together with 100 pM E_2. In each experiment, a dose–response curve (0.1 pM–1000 pM) of R1881, a negative control (cell treated only with hormone-free medium), and a positive control (E_2 at 100 pM) were included. Cells were incubated for 5 days. The bioassay was terminated by removing the media from the wells, fixing the cells, and staining them with SRB. The fixation protocol and SRB colorimetric assay were done as described above for the E-screen assay. Results were also expressed as PE [MCF-7 cell proliferation (fold-over control)].

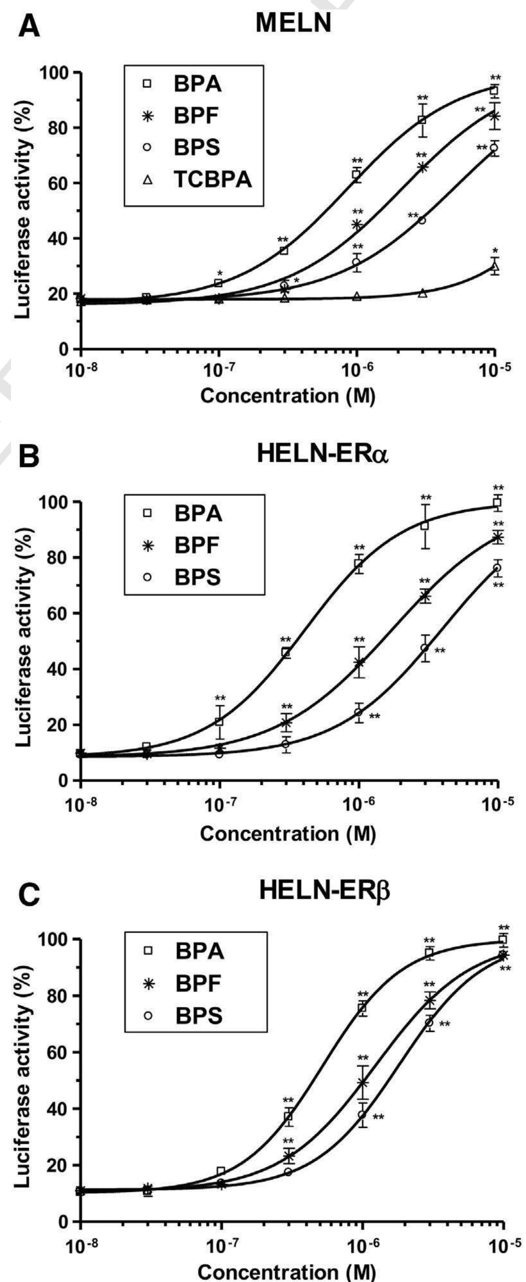

Fig. 2. Induction of luciferase activity in MELN, HELN-hERα, and -hERβ cells by BPA and derivatives. Cells were treated with BPA, BPF, BPS and TCBPA for 16 h at the indicated concentrations. Maximal luciferase activity (100%) was obtained with 10 nM E_2. Results are expressed as a percentage of maximal E_2 induction. Values were the mean ± SD from three separate experiments. *$p < 0.05$ and **$p < 0.01$ (versus 0.1% ethanol used as a control).

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Table 1
Effective and inhibitory concentrations for half-maximal luciferase activity (EC_{50} and IC_{50}) of BPA and derivatives on transcriptional activation through hERs, hAR and hPXR.

Compounds	MELN	HELN-ERα	HELN-ERβ	PALM	PALM	HG_5LN-PXR
	EC_{50} (μM)	EC_{50} (μM)	EC_{50} (μM)	EC_{50} (μM)	IC_{50} (μM)	EC_{50} (μM)
BPS	12.10 ± 0.92	3.96 ± 0.33	1.72 ± 0.27	70.54 ± 2.21	ne	ne
BPF	0.98 ± 0.05	1.73 ± 0.46	1.43 ± 0.19	ne	6.98 ± 0.15	ne
BPA	0.47 ± 0.03	0.41 ± 0.11	0.52 ± 0.09	55.38 ± 6.46	0.92 ± 0.02	17.72 ± 2.43
TCBPA	47.60 ± 5.26	nt	nt	ne	ne	8.49 ± 0.63
TBBPA	ne	nt	nt	ne	ne	11.97 ± 5.38

(ne) no effect.
(nt) no tested.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) toxicity assay. The effects of BPA, BPS, BPF, TBBPA, and TCBPA on cell viability were assessed with the MTT test, using Denizot and Lang's modified technique (1986). In short, cell lines (MCF-7, MCF-7 AR1, MELN, HELN-derived, PALM, and HG_5LN-hPXR) were seeded at a density of 5×10^4 cells per well in 96-well tissue culture-grade plate for 8 h, followed by treatment with different concentrations (0.01–10 μM) of each compound for a further 24 h. Cells were washed with PBS three times, and 100 μl of MTT solution (0.5 mg/ml) was then added to each well. After incubation (2 h), viable cells cleaved the MTT tetrazolium ring into a dark blue formazan reaction product, whereas dead cells remained colorless. The MTT-containing medium was gently removed, and DMSO was added to each well. After shaking, the plates were read in absorbance at 540 nm. Medium alone with no cells served as an additional control. Data were expressed as the mean of three wells.

Data analysis. For all assays, each compound was tested at various concentrations in at least three independent experiments, and data were expressed as mean ± SD. Individual dose–response curves, in the absence and presence of agonist, were fitted using the sigmoid dose–response function of a graphics and statistics software package (Graph-Pad Prism, version 4.0, 2003, Graph-Pad Software Inc., San Diego, CA, USA). Results are presented as EC_{50} and IC_{50} values. Data were analyzed for significant differences using one-way ANOVA followed by Dunnett's post-comparison test (vs. control). Differences were considered statistically significant at $p < 0.05$.

Results

Transcriptional activation of hERs by BPA congeners and derivatives

BPS, BPF, BPA, TCBPA, and TBBPA were tested on the MELN cell line, which stably expresses an estrogen-responsive luciferase reporter under the control of endogenous hERα. In this cell line, all compounds except the halogenated bisphenols, TCBPA and TBBPA, induced luciferase expression in a concentration-response manner (Fig. 2A) but with different potencies, in the order BPA > BPF > BPS, as indicated by their EC_{50} values (Table 1). TCBPA showed only 30% transactivation at 10 μM concentrations, whereas TBBPA, was found inactive in MELN cells.

We next explored whether the most hERα-active compounds, BPA, BPF, and BPS, could act as specific ER modulators. We used the stably transfected HELN-hERα and -hERβ cell lines, which allow characterization of ER selectivity (between subtypes) and activity (antagonistic, partial or full agonistic) within the same cellular context (Escande et al., 2006). As in previous studies (Molina-Molina et al., 2008), dose–response curves in these cells showed a slight difference in assay sensitivity for the natural estrogen E_2 and for the synthetic estrogen EE_2, with EC_{50} values of 0.019 and 0.007 nM for hERα and 0.067 and 0.24 nM for hERβ, respectively.

When BPS, BPF, and BPA were applied on HELN-hERα cells, the estrogenic responses were very similar to those obtained in MELN cells. BPA was the most effective agonist, exhibiting a full dose–response curve, followed by BPF and BPS, which induced 87 and 76% of maximal luciferase activity, respectively, at the highest concentration tested (Fig. 2B). BPS, BPF, and BPA behaved as full hERβ agonists, and BPA was again the most effective agonist (Fig. 2C), compared to other compounds. Interestingly, unlike BPS, which was more active in HELN-hERβ than in HELN-hERα cells, BPA and BPF were equally active in the hERβ than in hERα assay (Table 1). Finally, all three compounds were tested for non-specific modulation of luciferase expression on the HELN parental cell line, finding that luciferase expression was not induced at concentrations up to 10 μM (data not shown).

Effect of BPS, BPF and BPA on E_2 binding to hERα and hERβ

Whole-cell competitive binding assays were performed with HELN-hERα and -hERβ cells to determine whether the estrogenic effects observed in transactivation assays reflected the abilities of BPS, BPF and BPA (the most effective compounds) to bind to hERα and hERβ. Table 2 summarizes IC_{50} and RBA values for the two hERs. All three compounds showed subtype-selective differences in ligand binding to the two hER subtypes, with an approximately 2-fold greater affinity to hERβ than to hERα, the opposite effect to that of E_2. In fact, BPA was able to completely displace $[^3H]$-E_2 from hERα at 10 μM concentration, whereas BPF and BPS were less active, inhibiting $[^3H]$-E_2 binding by approximately 85 and 70%, respectively, at this concentration (Fig. 3A). In HELN-hERβ cells, BPF and BPS inhibited the binding of $[^3H]$-E_2 to this receptor in a concentration-dependent and competitive manner, although less effectively than BPA, which was also able to completely displace $[^3H]$-E_2 from hERβ at the highest concentration tested (Fig. 3B). These findings indicate that the ability of these compounds to act as hER agonists derives from receptor binding and the greater affinity of BPS for hERβ than for hERα correlated with the preferential agonism of hERβ activity in transactivation assays. However, BPA and BPF compete more effectively for binding to hERβ, but induce hERα and hERβ mediated gene expression with comparable efficacy.

Table 2
IC_{50} and relative binding affinity (RBA) values of BPA, BPS and BPF for hERα and hERβ.

Competitors	hERα		hERβ	
	IC_{50} (nM)	RBA (%)	IC_{50} (nM)	RBA (%)
E_2	0.12 ± 0.03	100	0.21 ± 0.01	100
BPS	6560 ± 530	0.001	3452 ± 878	0.006
BPF	2182 ± 87	0.005	1452 ± 261	0.014
BPA	839 ± 270	0.014	401 ± 126	0.052

(IC_{50}) Competitor concentration required to decrease maximal $[^3H]$-E_2 binding by 50%.
(RBA) Relative binding affinity of each competitor for hER subtypes. The RBA was calculated as the ratio of E_2 to competitor concentration required to reduce specific radiolabeled binding by 50% (ratio of IC_{50} values). The RBA value for E_2 was arbitrarily set at 100.

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Estrogenic effects of BPA congeners and derivatives in the E-Screen bioassay

The estrogenic potential of BPS, BPF, BPA, TCBPA, and TBBPA was further characterized by using the E-Screen bioassay to investigate their ability to stimulate cell proliferation in MCF-7 cells. In this cell line, the full ER agonist E_2 strongly induced significant proliferation in a dose-dependent manner, with an EC_{50} value of 0.018 nM. All tested compounds except TBBPA also increased cell proliferation (BPA > BPF > BPS > TCBPA), but their potency was very low in comparison to E_2 (Fig. 4). BPA and BPF showed full dose–response curves (EC_{50} = 0.47 and 1.01 μM, respectively), and a 6.8- and 6.3-fold increase in cell number, respectively, versus control-treated cells (hormone-free medium). BPS and TCBPA (both at 10 μM) also increased cell number by approximately 3.7- and 2.0-fold, respectively (EC_{50} = 12.1 and 45.8 μM, respectively). By contrast, TBBPA did not stimulate MCF-7 cell proliferation in the concentration range of 0.01–10 μM.

hAR in vitro activation by BPA congeners and derivatives

The potential androgenic and anti-androgenic activities of BPS, BPF, BPA, TCBPA, and TBBPA were examined by using PALM cells. The synthetic androgen R1881 was previously found to exert marked androgenic activity, with an EC_{50} value of 0.1 nM (Molina-Molina et al., 2006, 2008). BPA and BPS showed weak agonistic activity at 10 μM concentrations (20 and 15% of maximal activity, respectively) while BPF, TCBPA, and TBBPA did not (Fig. 5A). When the antagonistic activity of these compounds was tested, BPA and BPF at 10 μM concentrations proved to be potent hAR antagonists that strongly inhibited the luciferase activity induced by 0.2 nM of R1881 (Fig. 5B). Despite its weak agonistic activity, BPA was a better antagonist (IC_{50} = 0.92 μM) than BPF (IC_{50} = 6.98 μM), whereas BPS, TCBPA, and TBBPA showed no antagonistic activity towards hAR.

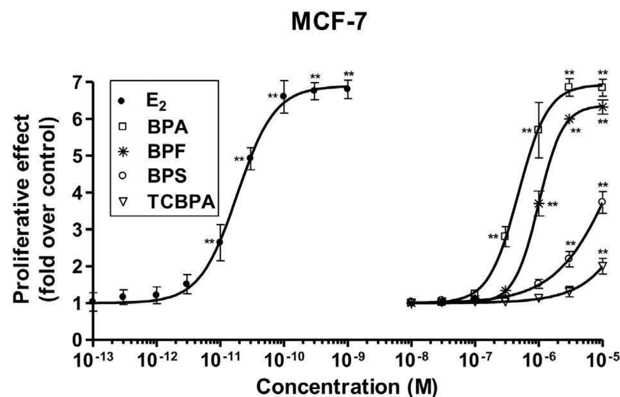

Fig. 4. Dose–proliferative response curves in MCF-7 cells. Cells were incubated for 144 h at 37 °C in the presence of E_2, BPA, BPF, BPS and TCBPA at the indicated concentrations. Results are expressed as proliferative effect (ratio between the highest cell yield obtained with the chemical and the proliferation of hormone-free control cells). Values were the mean ± SD from three separate experiments. *$p < 0.05$ and **$p < 0.01$ (versus hormone-free control).

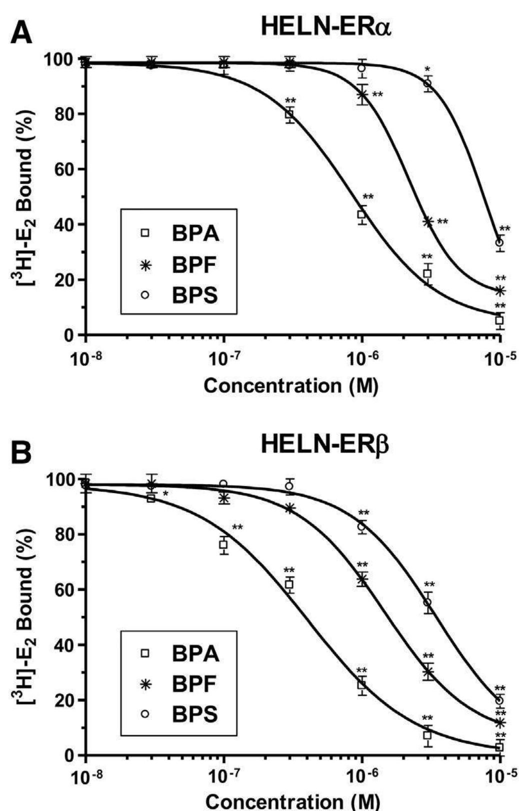

Fig. 3. Competitive inhibition of $[^3H]$-E_2 binding to hERα and hERβ by BPA, BPF and BPS. HELN-hERα and -hERβ cells were incubated with different concentrations (0.01–10 μM) of BPA, BPF and BPS in the presence of 0.1 nM $[^3H]$-E_2. Values were the mean ± SD from three separate experiments. *$p < 0.05$ and **$p < 0.01$ (versus 0.1 nM $[^3H]$-E_2).

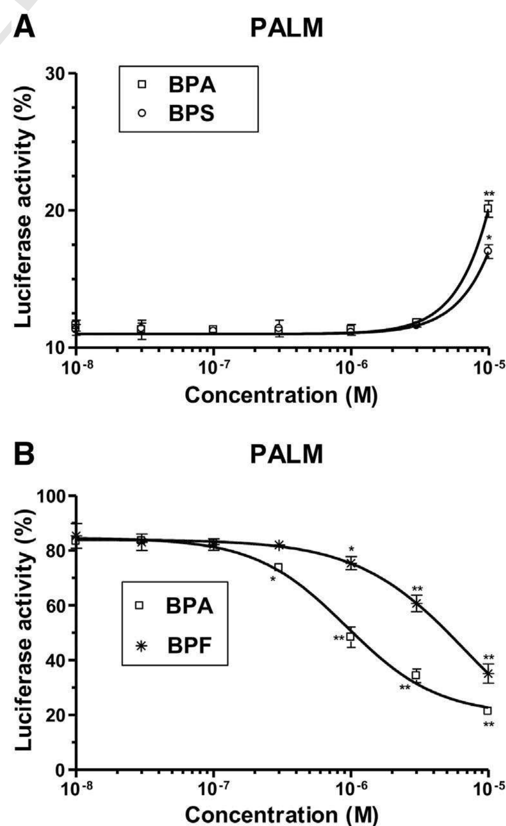

Fig. 5. Induction of luciferase activity in PALM cells by BPA and derivatives. Panel (A). PALM cells were treated with BPA and BPS for 40 h at the indicated concentrations. Maximal luciferase activity (100%) was obtained with 10 nM R1881. Values were the mean ± SD from three separate experiments. *$p < 0.05$ and **$p < 0.01$ (versus 0.1% ethanol). Panel (B). PALM cells were treated with 0.2 nM R1881 in the presence of increasing concentrations of BPA and BPF for 40 h. Maximal luciferase activity (100%) was obtained with 10 nM R1881. Values were the mean ± SD from three separate experiments. *$p < 0.05$ and **$p < 0.01$ (versus R1881 0.2 nM).

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Anti-proliferative potential of BPA congeners and derivatives on MCF-7 AR1 cells

The androgenic response of these compounds to hAR was further characterized by studying the effects of BPS, BPF, BPA, TCBPA, and TBBPA on MCF-7 AR1 cells, which are stable transfectants of MCF-7 cells that express wild-type hAR. Androgen agonists inhibit cell proliferation in MCF-7 AR1 cells, which serves as an end-point to assess the endogenous cell response to androgens (von Bueren et al., 2008). As expected, the full agonist R1881 strongly inhibited the proliferation induced by 100 pM E_2 in a dose-dependent manner in this cell line, with an IC_{50} value (concentration required for 50% of maximal inhibition of E_2-induced proliferation) of 44.5 pM. When the test compounds were applied to MCF-7 AR1 cells, BPA and BPS showed weak but significant inhibitory effects in these cells at 10 μM concentrations (Fig. 6). Consistent with their transactivation assay results, BPF, TCBPA, and TBBPA had no effect on this cell line, indicating that they are not androgenic.

Gene expression modulation via the human pregnane X receptor (hPXR)

We used two HeLa-derived reporter cell lines: the HG_5LN-hPXR line, to detect hPXR agonists or antagonists; and the parental HG_5LN cell line, which constitutively expresses luciferase activity, as a control for non-PXR-specific activities. As previously reported (Creusot et al., 2010), the cholesterol-lowering drug SR12813 exhibited marked hPXR agonistic activity in HG_5LN-hPXR cells, with an EC_{50} value of 69 nM. When BPS, BPF, BPA, TCBPA, and TBBPA were tested for their ability to activate hPXR, only BPA and its halogenated derivatives, TCBPA and TBBPA, were found to be weak-to-moderate hPXR activators (Fig. 7). TCBPA was the most potent of these compounds, activating hPXR with an EC_{50} of 8.49 μM. BPA and TBBPA also induced significant activation of luciferase activity (40 and 50%, respectively). By contrast, BPS and BPF were unable to activate hPXR after 16 h of exposure at concentrations up to 10 μM (Table 1). None of the compounds tested were able to activate luciferase expression in HG_5LN cells, demonstrating that the activity observed in HG_5LN-hPXR cells was hPXR-specific. Finally, prompted by a report by Dring et al. (2010), the test compounds were tested for their ability to antagonize hPXR in our cell model. However, no antagonistic activity was detected (data not shown).

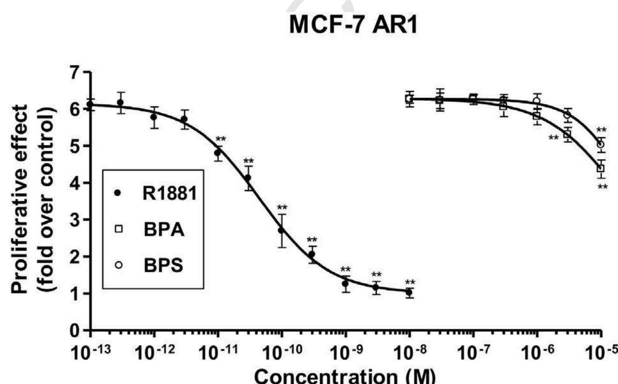

Fig. 6. Anti-proliferative response curves in MCF-7 AR1 cells. Cells were treated with 100 pM E_2 in the presence of increasing concentrations of R1881, BPA and BPS for 5 days at 37 °C. Results are expressed as proliferative effect (ratio between the highest cell yield obtained with the chemical and the proliferation of hormone-free control cells). Values were the mean ± SD from three separate experiments. *p < 0.05 and **p < 0.01 (versus hormone-free control).

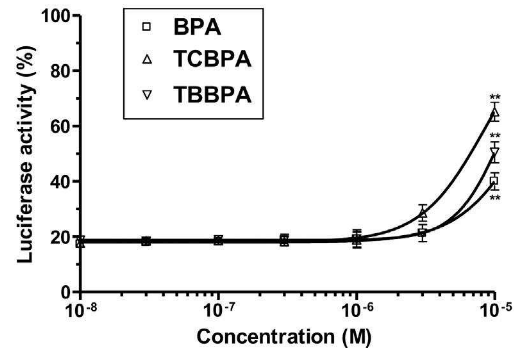

Fig. 7. Induction of luciferase activity in HG_5LN-hPXR cells by BPA and derivatives. HG_5LN-hPXR cells were treated with BPA, TCBPA and TBBPA for 16 h at the indicated concentrations. Maximal luciferase activity (100%) was obtained with 3 μM SR12813. Results are expressed as percentage of maximal SR12813 induction. Values were the mean ± SD from three separate experiments. *p < 0.05 and **p < 0.01 (versus 0.1% ethanol).

Cell viability

The MTT test was used to assess the cytotoxicity of BPA, BPS, BPF, TBBPA, and TCBPA was assessed for the six cell lines used in this study. In all assays, the tested compounds were devoid of any cytotoxicity (cell survival ranging from 95 to 100%) in the 0.01–10 μM range (data not shown).

Discussion

This study contributes evidence that BPS and BPF disrupt the function of several NRs and may therefore interfere with the endocrine system in humans. It was found that BPS, BPF, and BPA all compete with natural ligands for binding to NRs, trigger the expression of cell type-specific genes, and promote cell proliferation in in vitro bioassays. This interference in NR signaling has been considered crucial for assessing the toxicology of BPA and is of no less importance for BPS and BPF, which have been proposed to replace BPA in many of its multiple applications. Given the disrupting effects of BPS observed in different in vitro test systems and its higher resistance to environmental degradation in comparison to BPA or BPF, the proposal to utilize BPS instead of BPA in various products (e.g., baby bottles, food and beverage cans, thermal paper, currency bills) should be viewed with caution (Danzl et al., 2009; Ike et al., 2006). Most of the reported effects of EDCs are attributed to their interference with hormonal signaling mediated by NRs; hence, before any decision is taken on appropriate BPA alternatives, the complete characterization of their NR-mediated effects is essential.

In the present study, the agonistic and antagonistic activities of BPA congeners and derivatives were assessed by using a panel of in vitro bioassays for the detection of different steroid receptor-mediated activities (hERα, hERβ, hAR and HPXR). The estrogenic responses of BPA congeners and derivatives in HELN-ERα cells were highly similar to those obtained in MELN cells, as previously reported for other ERα ligands (Molina-Molina et al., 2008). All of the bisphenols tested except for TBBPA exhibited a marked estrogenic activity (BPA > BPF > BPS > TCBPA), with EC_{50} values in the micromolar range, showing a 25,000- to 250,000-fold less potent transactivation activity, compared to E_2 or the potent pharmaceutical estrogen EE_2. Although fewer data are available on BPS than on BPA and other bisphenols, various in vitro assays have confirmed its estrogenic activity via hERα (Chen et al., 2002; Hashimoto et al., 2001; Kitamura et al., 2005; Kuruto-Niwa et al., 2005). Our results indicate that the ability of BPS to act as hERα agonist derives from its receptor binding affinity. We also studied the effects of the tested

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bisphenols on cell proliferation using the E-screen bioassay to further characterize the estrogenic response of these compounds towards hERα. The ranking order of estrogenic potency in MCF-7 cells was BPA > BPF > BPS > TCBPA, whereas TBBPA showed no estrogenic activity in this cell line, confirming the results observed with ours stably transfected cells. In this context, when a wide range of bisphenols were ranked by proliferative potency in MCF-7 cells (Perez et al., 1998), experimental data suggested that not only the distance between *para* hydroxyl groups but also the nature of the bridging carbon substituent determined their estrogenicity. In this regard, bis(4-hydroxyphenyl)ketone, which is more polar than the other bisphenols because of its bridging carbonyl group, has shown the poorest proliferative effect (Perez et al., 1998) and weakest cell-type protein induction in MCF-7 cells (Rivas et al., 2002). This implies that a higher polarity, as in BPS, reduces the estrogenicity. This is consistent with the earlier report by Dodds and Lawson (1936), who used an uterotrophic assay and found bis(4-hydroxyphenyl)ketone to be the least active estrogenic compound out of a series of bisphenol derivatives. The bridging carbon in BPA is replaced with a SO_2 group in BPS, conferring a higher polarity and consequently a lower estrogenicity in this hERα-driven effects model.

Previous reports on halogenated derivatives of BPA (Meerts et al., 2001) indicated that the inclusion of chlorine or bromine atoms in the *meta* position of the aromatic ring of bisphenols had no significant effect on the estrogenic potency. However, the introduction of two atoms in two *meta* positions of one aromatic ring drastically decreased the estrogenic potency (Riu et al., 2011a; Rivas et al., 2001). In agreement with these findings, the results of our reporter gene and MCF-7 cell proliferation bioassays showed a tendency for TCBPA to exert a weak hERα agonistic activity.

Although some attention has been paid to relationship between the structure/function of bisphenols and ERα activation, very little information is available on the binding or activation of ERβ. We have found that BPS can efficiently stimulate hERβ-mediated gene expression and show a higher affinity for binding to hERβ than to hERα. The binding affinity of BPS for hERβ was consistent with the estrogenic activity in the reporter gene assay, demonstrating a good correlation between binding affinity and agonistic activity. The ligand polarity may again explain these results. In a study of selective ligands for α and β ER isoforms, Hillisch et al. (2004) suggested that bulky substitutions below the so-called D-ring in the E_2 molecule lead to ERα agonists, whereas substitutions above the so-called B and C rings preferentially yield ERβ agonists, suggesting that ligand polarity modifies affinity to ER isoforms. Moreover, Nilsson and Gustafsson (2011) reported that each class of ER ligands induces a unique ER conformation that promotes specific co-regulator protein interactions and associations of ER N- and C-terminal activation functions (AF-1 and AF-2, respectively). In this regard, when BPS was tested in HELN cells stably transfected with AF-1 deleted hERs, deletion of the A/B domain in hERβ markedly altered its transactivation potency, indicating that BPS is dependent on AF-1 (Delfosse et al., 2012). In summary, the response of a given cell or tissue to E_2 and to synthetic agonists and antagonists is influenced not only by the relative levels of ERα and ERβ but also by their ligand polarity, which modifies their affinity for ER isoforms.

Beside BPS, other bisphenols such BPF, bisphenol B (BPB), and bisphenol E (BPE) are being considered as putative BPA substitutes and have been investigated in food stimulants and materials in contact with food (Gallart-Ayala et al., 2011). BPF diglycidyl ether (BFDGE) and BPA diglycidyl ether (BADGE) are the building blocks of epoxy resins that coat food and beverage cans and are additives in organosol resins. When in contact with aqueous solution and acidic food, both BADGE and BFDGE are partly converted to BADGE.2HCl and BFDGE.2HCl and to BADGE.$2H_2O$ and BFDGE.$2H_20$, among other compounds. The migration of BADGE and BFDGE from food contact materials was recently investigated (Coulier et al., 2010), and the European commission has set a limit of 1 mg/kg for BADGE and its hydrolytic and chlorinated derivatives (EU, 2005). BADGE and its hydrolysis products are common contaminants in indoor dust (L. Wang et al., 2012; Y. M. Wang et al., 2012). Their inhalation, alongside the consumption of canned food, contributes to the exposure of children and adults to bisphenol derivatives. Concerns raised by the presence of BPF residues in canned food are related not only to its estrogenic effects but also its anti-androgenic effects, attributable to its ability to bind to the AR. In 2004, the anti-androgenic activity of BPF was observed in AR-luciferase reporter gene assays using Chinese hamster ovary cells (Satoh et al., 2004) and human breast cancer cells (Stroheker et al., 2004). Furthermore, anti-androgenic effects were also shown by some BPF derivatives (BFDGE and hydrolytic byproducts) (Satoh et al., 2004). In the present study, the potential activity of BPS, BPF, BPA, TCBPA, and TBBPA via hAR was investigated by using PALM cells. As expected, BPA and BPF showed potent anti-androgenic activity (BPA > BPF), whereas only BPA and BPS out of the above compounds evidenced weak agonistic activity at the highest concentration tested. The findings for BPA were consistent with reports of the mixed agonistic/antagonistic activity of some compounds, e.g., cyproterone acetate, chlormadinone acetate, and hydroxyflutamide (Kemppainen and Wilson, 1996; Wilson et al., 2002; Wong et al., 1995). At high concentrations, these ligands appear to induce a receptor conformation that is compatible with AR DNA binding and transcriptional activation.

Given that androgen agonists inhibit cell proliferation in MCF-7 AR1 cells (von Bueren et al., 2007), we assessed the ability of BPA congeners and derivatives to inhibit cell proliferation in this cell line. The cells were stably transfected with a full hAR (Szelei et al., 1997) and expressed approximately 5-fold more hAR in comparison to wild-type MCF-7 cells. Although MCF-7 AR1 cells retain the capacity to proliferate in response to estrogen treatment, androgens inhibit estrogen-induced proliferation and the cells arrest in G0/G1 phase. Only BPA and BPS inhibited cell proliferation at 10 μM concentrations in this cell line, indicating that both compounds are weakly androgenic and confirming the results of our transactivation assays.

It is now well-known, that the metabolic inactivation and excretion of xenobiotics are promoted by the activation of multiple signaling pathways to trigger hepatic biotransformation, biliary excretion, and renal elimination (L. Wang et al., 2012; Y. M. Wang et al., 2012). Some of these clearance mechanisms are coordinated by NRs such as PXR. PXR is an important transcription factor controlling xenobiotic detoxification and is strongly expressed in the liver and the intestine, the primarily exposed organs (Lamba et al., 2004). The ligand-binding pocket of PXR accommodates a wide range of structurally unrelated endogenous and exogenous ligands (Di Masi et al., 2009). For instance, hPXR is activated by: endogenous ligands, e.g., bile acids and steroid hormones (Timsit and Negishi, 2007); xenobiotics, e.g., pharmaceuticals (Berthier et al., 2012); endocrine disrupters, e.g., BPA and phthalates (DeKeyser et al., 2001; Mnif et al., 2007); and natural plant compounds, e.g., zearalenone (Ayed-Boussema et al., 2001). Hence, we investigated whether they could be PXR activators. The ability of BPA and derivatives to activate transcription via hPXR was examined using HG_5LN-hPXR cells. We found that only BPA and its halogenated derivatives TCBPA and TBBPA, but not BPS or BPF, were able to activate the hPXR (TCBPA > TBBPA > BPA). In a cell-based reporter assay using HepG2 cells, Sui et al. (2012) found that BPA exhibited agonistic activity for hPXR and that BPF and BPS were inactive, as observed in the present study. They reported that a key structural requirement for the hPXR-mediated activity of BPA and its derivatives is the presence of at least one *para* phenolic group. Moreover, the number and position of methyl groups in the bridge between the two phenolic rings appeared to play a significant role in hPXR activity. In fact, its agonistic activity was abolished by the loss of both methyl groups, as in BPF, or by their replacement with a SO_2 group, as in BPS. However, unlike in the present study, BPA halogenated derivatives TCBPA and TBBPA were unable to

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activate the hPXR in HepG2 cells, which may be explained by the ability of human hepatoblastoma cells to metabolize TCBPA and TBBPA, as demonstrated by Riu et al. (2011b).

The present study demonstrates the ability of BPA congeners and derivatives to act at different levels of the NR signal transduction pathway, modulating reporter gene expression, competitive binding, and cell proliferation. The combination of multiple assays offers a rational and informative approach for assessing the disruption capacity of these compounds. Although these bisphenols are active at micromolar concentrations (concentrations unlikely to be leached from bisphenol-containing products or reported in human fluids and tissues), some studies have confirmed that lower concentrations of these compounds appear to affect non-genomic signaling in estrogen-responsive cells, with potential consequences for cell function. For example, BPA binds to both ERs, triggering non-classical estrogenic effects at nanomolar concentrations and altering the function of key cell types involved in human metabolism, such as pancreatic β cells and adipocytes (Soriano et al., 2012; vom Saal et al., 2010). Moreover, BPS is active via non-genomic signaling pathways in pituitary cells (Viñas and Watson, 2013a) at low-dose ranges likely to be present in food items and human fluids. Furthermore, in the real world, environmental and even occupational exposures are rarely due to a single chemical but rather involve complex chemical mixtures. Therefore, it cannot be ruled out that synergistic effects exerted via different NRs bound to bisphenols alone or to other EDCs may mediate the putative in vivo endocrine disrupting effect of these compounds (Viñas and Watson, 2013b). These findings call into question the advisability of replacing BPA with other bisphenols and underscore the need for further investigation of putative BPA substitutes.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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