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Characterization of Novel Ligands of ERα, Erβ, and PPARγ: The Case of Halogenated Bisphenol A and Their Conjugated Metabolites

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The capability of the flame retardants tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA) to activate peroxysome proliferator–activated receptors (PPARs) α, β, and γ and estrogen receptors (ERs) α and β has been recently investigated, but the activity of their biotransformation products and of their lower molecular weight analogues formed in the environment remains unexplored. The aim of this study was to investigate the relationship between the degree of halogenation of BPA analogues and their affinity and activity towards human PPARγ and ERs and to characterize active metabolites of major marketed halogenated bisphenols. The biological activity of all compounds was studied using reporter cell lines expressing these nuclear receptors (NRs). We used NR-based affinity columns to rapidly evaluate the binding affinity of halogenated bisphenols for PPARγ and ERs and to trap active metabolites of TBBPA and TCBPA formed in HepG2 cells. The agonistic potential of BPA analogs highly depends on their halogenation degree: the bulkier halogenated BPA analogs, the greater their capability to activate PPARγ. In addition, PPARγ-based affinity column, HGELN-PPARγ reporter cell line and crystallographic analysis clearly demonstrate that the sulfation pathway, usually considered as a detoxification process, leads for TBBPA and TCBPA, to the formation of sulfate conjugates which possess a residual PPARγ-binding activity. Our results highlight the effectiveness NR-based affinity columns to trap and characterize biologically active compounds from complex matrices. Polyhalogenated bisphenols, but also some of their metabolites, are potential disrupters of PPARγ activity.

Key Words: **halogenated BPA; affinity column; nuclear receptor; metabolism; obesity; endocrine disrupter.**

Over the past decades, growing concerns have been raised about human and wildlife exposure to endocrine disrupting chemicals (EDC). Bisphenol A (BPA), a major EDC used for the production of polycarbonates and epoxy resins, is among the highest volume chemicals produced worldwide (Jimenez-Diaz *et al.*, 2010). The occurrence of BPA in the environment, wildlife, and in humans has been demonstrated. In comparison, available data regarding BPA analogs is much scarcer. On the one hand, this group comprises bisphenols which differ at the level of the central part of the molecule bridging between the two phenols. All these bisphenols (BPF, BPC, BPS, etc.) are produced in much lower amounts than BPA and are weak estrogens (Perez *et al.*, 1998). On the other hand, analogs of BPA where the phenolic moieties are substituted with halogens (Br or Cl) deserve a broader attention because such substitution may significantly modify both their fate (Schauer *et al.*, 2006; Zalko *et al.*, 2006) and their capability to interact with nuclear receptors (NRs).

Tetrabromobisphenol A (TBBPA) is mainly used as a flame retardant to protect computer motherboards and other electronic equipment. Its production is currently estimated around 200,000 tons/year, and its presence has been reported in the environment (de Wit *et al.*, 2009) and in wildlife (Darnerud, 2003). In the environment, TBBPA can be debrominated into tri-, di-, and monoBBPA (Iasur-Kruh *et al.*, 2010). Its presence in human samples has only been monitored in a limited number of studies but was clearly demonstrated in maternal serum and mother milk (Cariou *et al.*, 2008; Johnson-Restrepo *et al.*, 2008).

Tetrachlorobisphenol A (TCBPA) has also been reported to be used as a flame retardant, but in much lower quantities than TBBPA (< 10,000 tons/year) (Chu *et al.*, 2005). The presence of TCBPA, as well as that of lower chlorinated analogs (monoCBPA, diCBPA, and triCBPA) in environmental samples has been unequivocally demonstrated (Fukazawa *et al.*, 2001; Gallart-Ayala *et al.*, 2010; Liu *et al.*, 2009). Chlorinated BPA analogs can be considered as an emerging group of contaminants, which is also supported by recent reports demonstrating their presence in human (Fernandez *et al.*, 2007; Jimenez-Diaz *et al.*, 2010). Contrary to TBBPA,

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and given the low levels of production of TCBPA, the origin of most chlorinated BPA in the environment is more likely the chlorination of BPA than the dechlorination of TCBPA. Indeed, BPA is readily chlorinated by reacting with sodium hypochlorite, which is commonly used as a bleaching agent in paper factories and also as a disinfectant in sewage treatments plants (Gallart-Ayala *et al.*, 2010; Yamamoto and Yasuhara, 2002). Like many other phenolic compounds (Deborde *et al.*, 2004), BPA easily chlorinates in aqueous media, and chlorinated BPA have also been found in seawater (Liu *et al.*, 2009).

Despite its weak estrogenicity compared with the natural ligand 17β-estradiol, BPA is a model EDC. Not all halogenated BPA analogs are estrogen receptor (ER) agonists. With an increasing number of bromine atoms, brominated BPAs are decreasingly potent ER agonists (Meerts *et al.*, 2001; Riu *et al.*, 2011). Conversely, the estrogenic activity of some chlorinated congeners exceeds that of BPA (Takemura *et al.*, 2005). Likewise, TBBPA could act as an antiandrogenic ligand (Christen *et al.*, 2010) and both TBBPA and TCBPA are candidate thyroid hormone disrupting chemicals (Kitamura *et al.*, 2002; Sun *et al.*, 2009). Recently, we demonstrated that brominated bisphenols (di-, triBBPA, and TBBPA) as well as TCBPA are potent peroxisome proliferator–activated receptor-(PPARγ) agonists (Riu *et al.*, 2011).

PPARγ is a member of the NR superfamily, and the disruption of regulation pathways under the control of these NR may be involved in the onset of diabetes, obesity, and reproductive disorders (Swedenborg *et al.*, 2009). BPA analogs with a lower degree of chlorination, but also the biotransformation product of halogenated BPA analogs, may also target PPARγ.

In this study, we first examined the effect of the degree of halogenation of chlorinated BPA on both their affinity and activity toward human ERs and PPARs, using ERα, ERβ, PPARα, PPARβ, and PPARγ reporter cell lines. Then, we took advantage of the difference observed between the ERα and PPARγ activities of BPA and its halogenated derivatives to explore the capability of ERα-based affinity columns (Riu *et al.*, 2008) and newly developed PPARγ-based affinity columns and to retain these different molecules. Affinity columns were further used in combination with high-performance liquid chromatography (HPLC) and mass spectrometry, to isolate and characterize the active metabolites of major halogenated BPA (TCBPA, TBBPA) formed *in vitro* after their biotransformation by human hepatoblastoma cells (HepG2).

MATERIALS AND METHODS

Chemicals. 17β-Estradiol, BPA, and TBBPA (2,2-Bis(3,5-dibromo-4-hydroxyphenyl)propane) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). TCBPA (2,2-Bis(3,5-dichloro-4-hydroxyphenyl)propane) was purchased from TCI Europe (Zwijndrecht, Belgium). CD4700, a PPARγ-selective agonist, either unlabelled or tritiated (specific activity: 1100 GBq/mmol), were gifts from Michel Rivier (Galderma Research, Sofia-Antipolis, France). ^{3}H-rosiglitazone (2000 GBq/mmol), ^{14}C-17β-estradiol (^{14}C-E2; 1.9 GBq/mmol) and ^{3}H-17β-estradiol (^{3}H-E2; 1,528 GBq/mmol), and ^{14}C-BPA (7.4 GBq/mmol) were purchased from PerkinElmer (Courtaboeuf, France), NEN Life Sciences Products (Paris, France), and Moravek Biochemicals (CA), respectively. All solvents (analytical grade) were purchased from Scharlau Chemie SA (Barcelona, Spain). Materials for cell culture were obtained from Invitrogen (Cergy-Pontoise, France) and luciferin from Promega (Charbonnieres, France).

Synthesis of halogenated BPA analogs. Radiolabeled TBBPA was synthesized from ring-^{14}C-BPA as previously described (Zalko *et al.*, 2006; Fig. 1). TBBPA radio-purity was checked by HPLC (> 99.8%). Its specific activity was 3.64 KBq/nmol, and its structure was confirmed by electrospray ionization-mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR).

Radiolabeled brominated BPA (3-monobromoBPA, 3,3′-dibromoBPA and 3,3′,5-tribromoBPA) were similarly synthesized from ring-^{14}C-BPA, using 2.2 equivalents of bromine, added to 1.48 MBq ^{14}C-BPA, and 2 mg unlabelled BPA in 80 µl methanol/water (1:1 vol/vol). They were individually isolated and purified using the HPLC system coupled to a Gilson 202 fraction collector (Gilson France, Villiers-Le-Bel, France). The radio-purity of all brominated analogs was checked by HPLC (> 99.8%). Their structures were confirmed by ESI-MS and NMR. Their specific activity was 168 Bq/nmol.

The synthesis of radiolabeled chlorinated BPA (3-monochloro-BPA, 3,3′-dichloro-BPA, 3,3′,5-trichloro-BPA and TCBPA) was adapted from Gallard *et al.* (2004). BPA, 1mM (3.7 MBq of ^{14}C-BPA) in buffered ultrapure water (phosphate buffer 10mM, pH 8) was chlorinated with NaOCl (2.5 µg/ml). After 10 min shaking at room temperature, reaction was quenched with 400 µl of $Na_2S_2O_4$ (0.1g/ml). Radiolabeled ^{14}C-chlorinated BPA analogs were individually isolated and purified by HPLC fractionation. Radiochemical purity of all chlorinated congeners was checked by radio-HPLC (> 99.8%). Their specific activity was 3.7 KBq/nmol, and their structures were confirmed by ESI-MS and NMR.

The monosulfate conjugate of TBBPA (TBBPA-sulf) was synthesized from TBBPA (adapted from Lindholst *et al.*, 2003). Chlorosulfonic acid (2.76 mmol), from Sigma-Aldrich, was added drop by drop to a constantly stirred ice-cooled solution of 1.84 mmol TBBPA dissolved in 3 ml pyridine. After 2 h at room temperature, the reaction was quenched with concentrated KOH (1:1). The solid portion was filtered off by suction and allowed to dry. Purification of TBBPA-sulf was obtained by solid-phase extraction (SPE) followed by HPLC. SPE:chromabond 1 g C_8 glass cartridges (Macherey Nagel, Hoerdt, France) were washed with 4 ml methanol and equilibrated with 4 ml of solution A (“A”):20mM ammonium acetate, pH 3.5/acetonitrile (95/5, vol/vol). The powder was dissolved in 5 ml “A” and loaded on cartridge. Elution was performed successively with 8 ml “A”, 4 ml “A”:acetonitrile (85:15, vol/vol), 4 ml “A”:acetonitrile (50:50, vol/vol), and 4 ml acetonitrile. TBBPA-sulf was eluted in the penultimate fraction. Purity and chemical structure were checked by HPLC-diode array detector (DAD), ESI-MS, and NMR.

Analytical procedure. HPLC analyses were performed on a Spectra system P1000 (Thermo Electron, les Ulis, France) equipped with a Rheodyne injector and an UV-DAD set at 254 nm (Spectra system UV6000LP; Thermo Electron), connected to a Flo-one/β A500 radioactivity detector (Radiomatic 610TR; PerkinElmer, Villebon-sur-Yvette, France). Radio-HPLC metabolic profiles of TBBPA and TCBPA incubated with HepG2 cells were carried out with an HPLC system based on a Nucleodur C_8 column (250 × 4 mm, 5 µm; Macherey Nagel) in the following conditions: mobile phases: A:“A”; B: 100% acetonitrile; gradient: 0–4 min, A: 100% isocratic; 4–6 min, linear gradient from A: 100% to A:B 70:30; 6–21 min, A:B 70:30 isocratic; 21–23 min, linear gradient from A:B 70:30 to A:B: 50:50; 23–33 min, A:B 50:50 isocratic; 33–34 min, linear gradient from A:B 50:50 to B: 100%; 34–50 min, B: 100% isocratic.

Cell incubation. HepG2 human hepatoblastoma cell (Aden *et al.*, 1979) (ATCC No. HB-8065) were cultured in minimum essential medium, 10% fetal calf serum, vol/vol, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a 5% CO_2 atmosphere at 37°C.

HepG2 cells (3.5×10^5 per well) were seeded in 12-well plates. After 16 h, the medium was replaced by fetal calf serum–free medium supplemented with 1µM ^{14}C-TBBPA (or ^{14}C-TCBPA) (3700 Bq/incubation).

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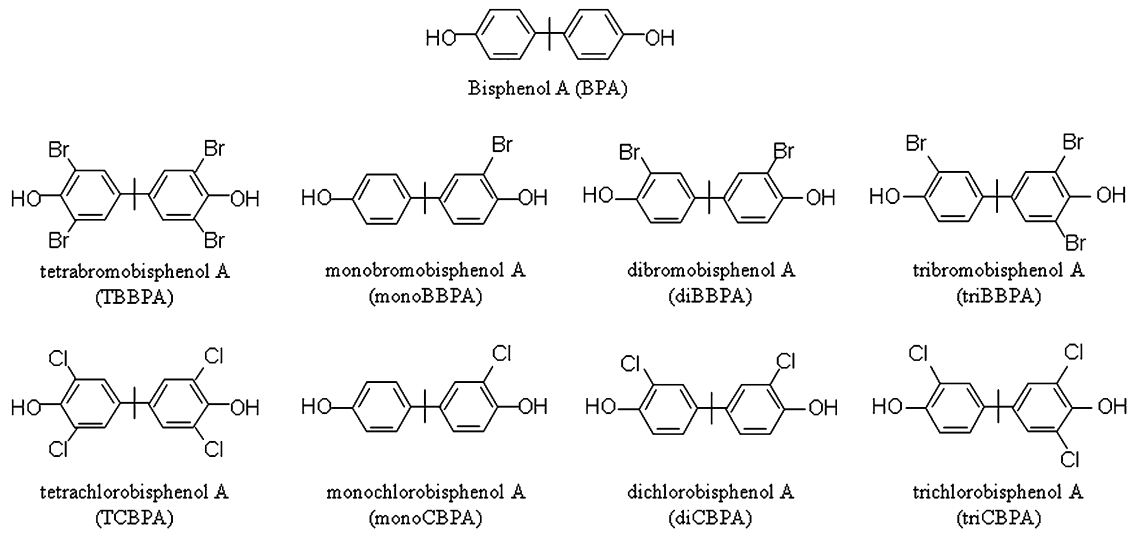


FIG. 1. Chemical structure of the halogenated BPA analogs used in this study.

Control incubations were carried out in the same conditions but without cells. At the end of the incubation period, media were removed and the cells were recovered by washing each well with 1 ml H_2O/acetonitrile (50:50, vol/vol). Supernatant and cells were processed, that is, (1) radioactivity was quantified by direct counting in a Packard scintillation counter (Model Tri-Carb 200CA; PerkinElmer) using Ultima Gold as the scintillation cocktail and (2) analyzed by radio-HPLC. For all vials, sample quenching was compensated by the use of quench curves and external standardization. All experiments were made in triplicate.

Reporter cell lines and stable gene expression assays. Generation of HGELN, HGELN-ERα, HGELN-ERβ, HGELN-GAL-PPARα, HGELN-GAL-PPARβ, and HGELN-GAL-PPARγ reporter cell lines was performed as described in Riu *et al.* (2011). Briefly, reporter cells were seeded at a density of 20,000 cells per well, in 96-well white opaque tissue culture plates, and maintained in Dulbecco's modified eagle medium (DMEM) without phenol red, supplemented with 5% dextran-coated, charcoal-treated FCS; 24 h later, culture medium was replaced by DMEM containing tested compounds. Assays were performed in absence of serum in order to avoid ligand capture by serum proteins; 16 h after exposure, media was replaced by media containing 0.3mM luciferin. Luminescence was measured in intact living cells for 2 s in a Microbeta Wallac luminometer.

Receptor production. The production of the human recombinant ERα-LBD (302-552, C381,417,530S) was performed in *Escherichia coli* as described in Riu *et al.* (2008). DNA encoding the human PPARγ-LBD (amino-acids Glu196-Tyr477) was amplified by PCR and cloned into the expression vector pET15b. The plasmid PPARγ (Glu196-Tyr477)-pET15b was transformed into *E. coli* BL21 (DE3) cells (Invitrogen), and PPARγ-LBD was expressed. ERα and PPARγ concentrations were determined by ligand-binding analysis with ^{3}H-E2 and ^{3}H-CD4700, respectively (the concentration was around 2.5μM and 25μM for ERα-LBD and PPARγ-LBD, respectively). NRs were aliquoted and stored at −80°C.

NR-based affinity columns. All experimental steps were realized at 4°C in order to circumvent protein denaturation. First, ERα-LBD or PPARγ-LBD were individually immobilized on Ni-NTA-agarose phase (purchased from Qiagen, France) and applied in an empty column. Affinity columns were performed as described in Riu *et al.* (2008).

Additional experiments were carried out to trap the metabolites formed in incubations of TCBPA or TBBPA with HepG2 cells that would behave as PPARγ ligands. An aliquot of each cell incubation (0.5 nmol of equivalent of the parent compound), diluted in 500 μl washing buffer (WB, final volume 1 ml, with 5% organic solvent) was directly applied on 2 nmol of immobilized PPARγ-LBD. WB and elution buffer (EB) volumes were 1 ml and 500 μl, respectively. Three volumes of methanol were added to the pooled eluted fractions in order to precipitate and denaturate the receptor. Supernatants, recovered after a centrifugation step at 5000 rpm (2 min), were evaporated to eliminate organic solvent and concentrated on C_{18} glass cartridges (Macherey Nagel) previously washed with 4 ml methanol and equilibrated with 1 ml EB. Elution was performed with 2 ml acetonitrile. Aliquots from eluted fractions were analyzed by radio-HPLC.

Crystallization. Prior to crystallization trials, the purified PPARγ-LBD was concentrated to 8.5 mg/ml in a buffer containing 20mM Tris-HCl, pH 8.5, 250mM NaCl, 5mM dithiothreitol, and 1mM ethylenediaminetetraacetic acid. Crystals with TBBPA-sulf were obtained by vapor diffusion in hanging drops at 293 K by mixing 1 μl of protein solution with 1 μl of well solution containing 1M trisodium citrate, 100mM HEPES, pH 7.5, 3.5% 1,2-propanediol, and TBBPA-sulf at 0.2mM in order to have a molar ratio protein:ligand of 1:2 in the drop. Crystals appeared after 1 day and grew to about 100 μm within few days. Crystals were transferred to a cryoprotectant comprised of the well solution containing 20% glycerol and the corresponding ligand at a concentration of 1mM and frozen in liquid nitrogen.

Crystallographic data collection, processing, and structure refinement. Diffraction data were collected using an ADSC Quantum Q315r detector at the ID14-4 beamline of the European Synchrotron Radiation facility (ESRF, Grenoble, France) at 2.30 Å. Diffraction data were processed using MOSFLM (Leslie, 1992) and scaled with SCALA from the CCP4 program suite (CCP4 1994). The structure was solved by using the previously reported structure 2ZVT from which the ligand was omitted. The initial $F_o - F_c$ difference map showed significant signal for the TBBPA-sulf, which could be fitted into the electron density. The structure was modeled with COOT (Emsley and Cowtan, 2004) and refined with phenix.refine from the PHENIX program suite (Afonine *et al.*, 2005).

RESULTS

Activity of Chlorinated BPAs on ERs and PPARs

The occurrence of chlorinated BPA derivatives in the ecosystem, likely originating from the chlorination of BPA,

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prompted us to evaluate the activities of these BPA analogs on ERs and PPARs, using stably transfected HGELN-ERα and -ERβ and HGELN-PPARα, -PPARδ, and -PPARγ cell lines. Dose-responses curves obtained with ERs and PPARγ cells (Fig. 2A) demonstrated the sensitivity of the assays for the natural ER ligand E2 (EC_{50} of 18.9 and 81.5pM for ERα and ERβ, respectively) and for pharmaceutical ligands of PPARγ, namely CD4700 and rosiglitazone (EC_{50} of 1.32nM and 29.6nM, respectively). All chlorinated BPAs were first tested for nonspecific modulation of luciferase expression on the HGELN parental cell line containing the same reporter gene as HGELN-ER or HGELN-PPAR cells but devoid of ERs or PPARs, then on HGELN-ERα and -ERβ and HGELN-PPARα, -PPARδ, and -PPARγ cell lines. All compounds except BPA were cytotoxic above 10μM, in the six HGELN cell lines. As shown in Figure 2B, BPA and all chlorinated BPA analogs displayed a full agonistic activity toward ERα with a 1,000 to 10,000-fold lower potency than E2. However, as observed for brominated BPAs (Riu *et al.*, 2011), the capability of these compounds to transactivate luciferase gene expression in HELN-ERβ cell lines was found to depend on their chlorination level (Fig. 2C). BPA and monoCBPA displayed the highest transactivation efficiency, followed by diCBPA and triCBPA, and finally TCBPA. Figure 2D shows that in contrast to HGELN-ERβ cells, the potency to transactivate luciferase gene expression in the HGELN-PPARγ cell line was reversed, the highest agonistic activity being observed for BPA analogs with the highest degree of halogenation (TCBPA and triCBPA). Compared with the reference agonist, rosiglitazone, these compounds were found to be 50- to 500-fold less potent toward PPARγ. Halogenated BPAs were also tested on HGELN-PPARα and HGELN-PPARβ cell lines, revealing no significant agonist activity on these PPAR subtypes. For each tested compounds, the EC_{50} values were calculated and reported in Table 1.

Establishment and Validation of NR-Based Affinity Columns

ERα-LBD affinity columns were previously demonstrated to efficiently trap estrogenic compounds from complex matrices (Riu *et al.*, 2008). We developed the same tool based on the human PPARγ-LBD, in order to characterize new compounds able to disrupt PPARγ activities. The functionality of ERα- and

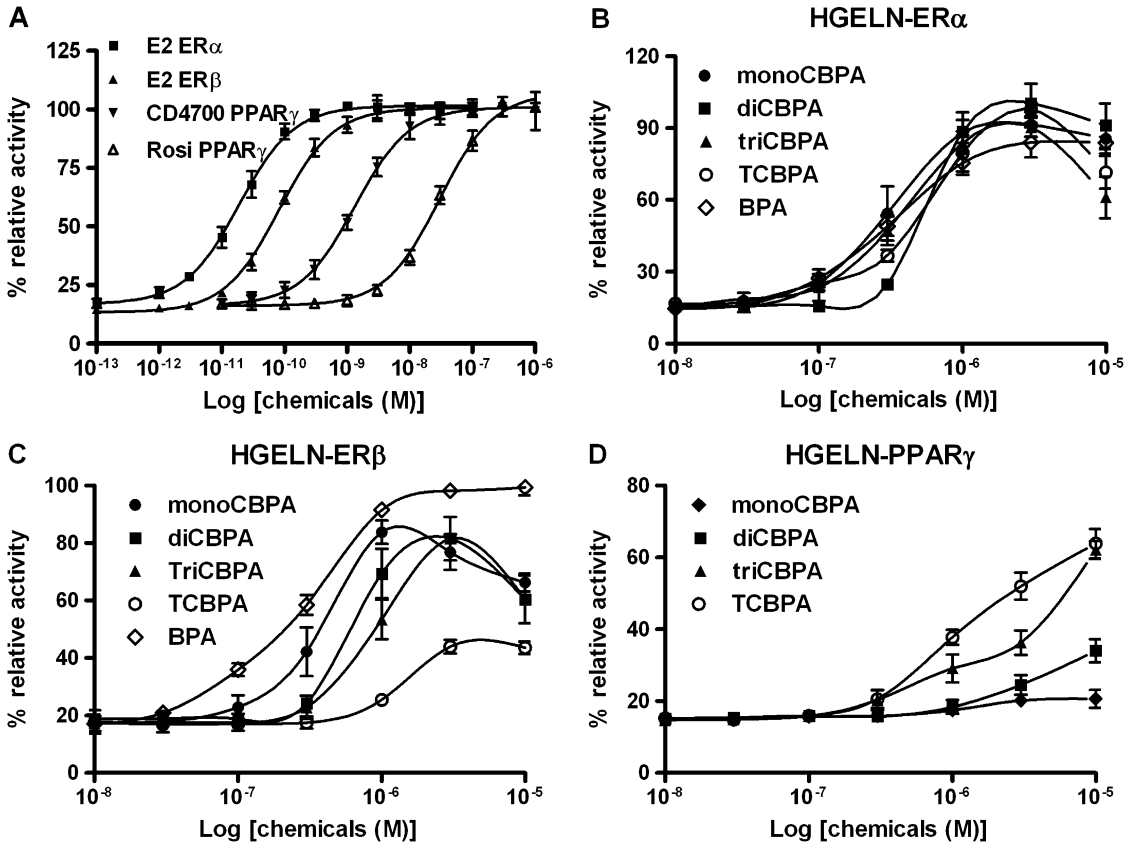


FIG. 2. Dose-response curves for reference high-affinity ligands, BPA, and its halogenated analogs in reporter cell lines. (A) HELN (ERα and ERβ) and HGELN-PPARγ luciferase assays of natural estrogen and pharmaceutical high-affinity ligands, (B) HELN-ERα luciferase assays of BPA and halogenated BPA, (C) HELN-ERβ luciferase assays of BPA and halogenated BPA, (D) HGELN-PPARγ luciferase assays of BPA and halogenated BPA. Results are expressed as a percentage of luciferase activity measured per well (mean ± SEM, $n = 4$). The values obtained in the presence of 10nM E2 and 100nM CD4700, respectively, were taken as 100.

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TABLE 1
Chlorinated BPA Potency to Activate ERs and PPARγ

Compound	HELN-ERa EC_{50} ± SE	HELN-Era EC_{50} ± SE	HGELN-PPARγ EC_{50} ± SE
E2	18.9pM ± 1.8	81.5pM ± 8.8	
BPA	0.29μM ± 0.03	0.28μM ± 0.05	
monoCBPA	0.28μM ± 0.04	0.35μM ± 0.12	ND
diCBPA	0.54μM ± 0.09	0.63μM ± 0.27	ND
triCBPA	0.34μM ± 0.17	0.88μM ± 0.36	2.34μM ± 0.86
TCBPA	0.53μM ± 0.25	1.15μM ± 0.51	1.28μM ± 0.16
Rosiglitazone			29.6nM ± 0.62
CD4700			1.32nM ± 0.13

Note. ND, not determined.

PPARγ-LBDs was verified by measuring their affinities for reference ligands as previously described (Pillon *et al*., 2005). The dissociation constant (K_d) determined by Scatchard analysis and using the human recombinant ERα-LBD was of 0.31nM for ^{3}H-E2, a value very similar to that obtained with the full-length receptor. Similarly, the binding affinities of the pharmaceutical ligands ^{3}H-rosiglitazone and ^{3}H-CD4700 for PPARγ-LBD were determined as 25 and 0.56nM, respectively. Having demonstrated the functionality of the bacterially expressed human receptors, we then examined the ability of NR-based affinity columns to retain high-affinity radiolabeled ligands (Fig. 3). With 91.6 ± 1.3% of the radioactivity loaded on immobilized ERα-LBD recovered in eluted fractions, the ERα-LBD column showed a high effectiveness to capture ^{14}C-E2. Likewise, as shown in Figure 3A, 89.9 ± 0.9% of the ^{3}H-CD4700 loaded on the PPARγ-LBD column was trapped, whereas no radioactivity was recovered in eluted fractions in the absence of PPARγ-LBD (data not shown). Together, these data validate affinity columns both as novel tools for the identification and characterization of ERα and PPARγ ligands.

Radiolabeled BPA and each of the halogenated analogs (1 nmol) were loaded separately on columns after the immobilization of 2 nmol of either ERα or PPARγ on the Ni-NTA phase. Using the ERα-based affinity column (Fig. 3B), most of the radioactivity was recovered in eluted fractions, for BPA (98.0 ± 1.9%), monoBBPA (94.6 ± 0.2%), diBBPA (96.2 ± 1.1%), and triBBPA (59.1 ± 7.3%). As observed in stably transfected cell line assays with which we showed that TBBPA was not able to activate ERα (Riu *et al*., 2011), TBBPA did not bind to the immobilized ERα-LBD, with most of the loaded radioactivity recovered in washing fractions. Conversely, using the PPARγ-LBD affinity column, most of the radioactivity was recovered in eluted fractions for TBBPA (97.5 ± 0.6%), triBBPA (94.9 ± 1.2%), and diBBPA (83.5 ± 2.1%). Neither monoBBPA nor BPA were trapped on the PPARγ-LBD affinity column. The same binding profile was observed for chlorinated BPA analogs (Fig. 3C). All of them were trapped on ERα-based affinity columns, with more than 90% of the radioactivity recovered in elution fractions for mono-, di-, and triCBPA, whereas only 63.1± 4.7% of the loaded radioactivity was recovered in elution fractions for TCBPA. In full agreement with cell-based assays (Fig. 2D), only the most chlorinated analogs were efficiently trapped on the PPARγ-LBD affinity column but to a lesser extent than the corresponding brominated BPA analogs: 27.7 ± 0.7%, 72.2 ± 0.4%, and 84.7 ± 0.5% of the radioactivity recovered in elution fractions for diCBPA, triCBPA, and TCBPA, respectively. As shown in Figure 3A, monoCBPA was not able to bind PPARγ-LBD immobilized on agarose columns, with almost all the radioactivity being recovered in the loading and washing fractions.

The nonspecific binding to the NR-LBD columns was evaluated for each tested compound using a large amount of non-radiolabeled high-affinity ligand (E2 or CD4700) trapped on the columns prior to the assays with the radiolabeled compound of interest. In additional controls carried out, none of the halogenated compounds was retained on the phase when no NR-LBD was immobilized on it. All together, these results demonstrate that NR-LBD affinity columns are able to capture both high- and low-affinity ligands and then could allow the identification and the characterization of unknown ligands from complex matrices.

Metabolites of Halogenated BPAs Are New PPARγ Ligands

In the HPLC system developed for this study, TCBPA and TBBPA were eluted at 36.5 and 37.5 min, respectively. After 24-h incubations of HepG2 cells with either ^{14}C-TBBPA or ^{14}C-TCBPA, more than 95% of the radioactivity was recovered in incubation media. As observed on the radio-chromatograms obtained from incubations with TCBPA (Fig. 4A) or TBBPA (Fig. 4B), both molecules were completely metabolized after 24 h into two metabolites, namely TC1/TC2 for TCBPA and TB1/TB2 for TBBPA. These metabolites accounted for 14.1 ± 5.6% (TC1), 85.9 ± 5.6% (TC2), 13.5 ± 2.9% (TB1), and 86.5 ± 2.9% (TB2) of the recovered radioactivity. ESI-MS analyses performed on incubation media extracts allowed to characterize TC1 and TB1 as glucuronic acid conjugates of TCBPA (Glc-TCBPA, R_T: 16.8 min) and TBBPA (Glc-TBBPA, R_T: 19.9 min), respectively, whereas TC2 and TB2 were identified as the sulfate conjugates of TCBPA (TCBPA-sulf; R_T: 25.8 min) and TBBPA (TBBPA-sulf; R_T: 27.4 min), respectively.

To further characterize these metabolites, TCBPA and TBBPA incubation media were separately loaded on the PPARγ-based column. As shown in Figures 4C and 4D, more than 80% of the radioactivity was retained on the column and recovered in elution fractions, which were subsequently processed and analyzed by radio-HPLC (Figs. 4E and 4F). A single peak was observed on each radiochromatogram with elution times of 25.8 min (TC2) and 27.4 min (TB2), for incubations carried out with TCBPA and TBBPA, respectively. These results clearly suggested that monosulfate conjugates of TBBPA and TCBPA would behave as PPARγ ligands. Conversely, none of the glucuronide conjugates was retained on the PPARγ column. Using the ERα-LBD column, neither

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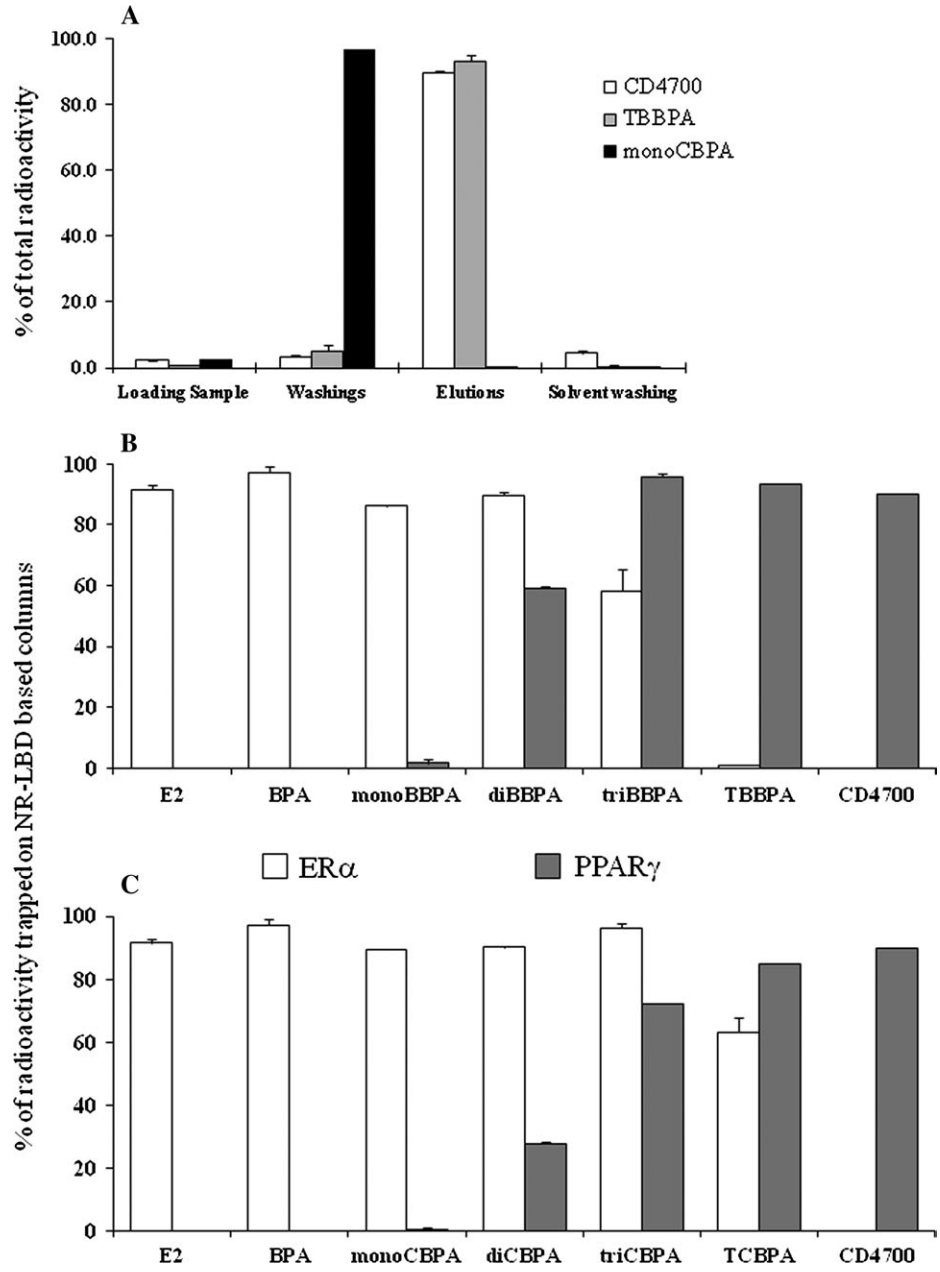


FIG. 3. Influence of the degree of halogenation of BPA analogs on their capture by NR-LBD–based affinity columns. Distribution of radioactivity: direct counting of ^{3}H-CD4700, ^{14}C-TBBPA, and ^{14}C-monoCBPA, respectively, loaded on PPARγ-LBD affinity columns (A). Determination of the capability of ERα-LBD (white bars) and PPARγ-LBD (gray bars) immobilized on agarose columns to capture pure radiolabeled brominated BPA (B) and chlorinated BPA (C) depending on the degree of halogenation of these bisphenols. Results are expressed in percentage of the radioactivity trapped on affinity columns by specific binding of the tested compound to the NR-LBD (mean ± SD, $n = 3$). Note: the radioactive fractions not captured by NR were recovered in the washing fractions.

glucuronide nor sulfate conjugates were trapped. To confirm and better characterize the agonistic activity of TCBPA-sulf and TBBPA-sulf, both ligands were chemically synthesized and purified. As shown in Figure 5, the two metabolites displayed residual agonistic activity on HGELN-PPARγ cell lines. Not only these sulfate-conjugated metabolites were able to bind PPARγ-LBD immobilized on affinity columns but they were also found to be able to activate this NR.

It is worth notifying that similar experiments performed with BPA provided monoglucuronide and monosulfate conjugates after 24-h incubation with HepG2 cells, none of them being trapped on ERα-LBD or PPARγ-LBD columns (data not shown).

Structural Analysis of PPARγ in Complex with TBBPA-sulf

In order to compare the binding mode of this metabolite with that of the parent compound TBBPA (Riu *et al.*, 2011), we

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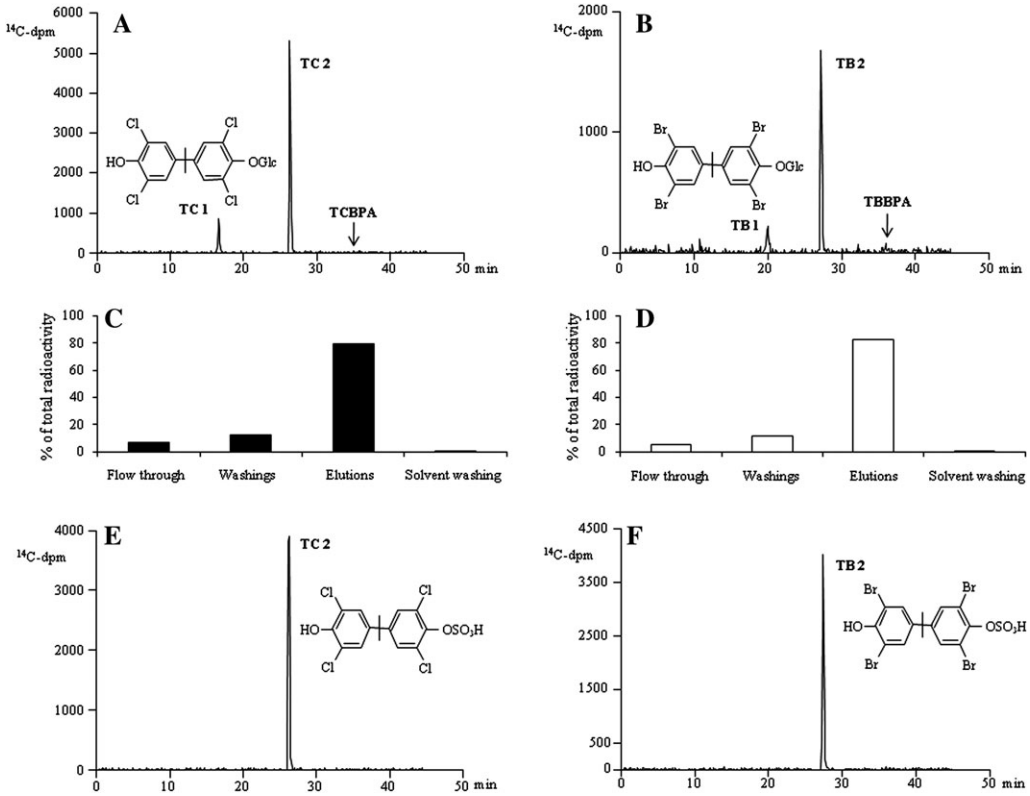


FIG. 4. Identification of new biologically active compounds using affinity columns. Typical radio-HPLC metabolic profiles obtained from incubations of ^{14}C-TCBPA, 1μM (A), and ^{14}C-TBBPA, 1μM (B), with HepG2 cells. Radioactivity distribution in affinity columns collected fractions (C and D) and radio-chromatograms corresponding to the eluted fractions (E and F) obtained from TCBPA and TBBPA incubation supernatant loaded on PPARγ-LBD columns, respectively (mean ± SD, $n = 3$).

solved the crystal structure of PPARγ-LBD bound to TBBPA-sulf (TB2) (Atomic coordinates and structure factors for the reported crystal structure has been deposited with the Protein Data Bank under accession code 3 pba.). Overall, the structure of PPARγ-LBD is identical when bound to TBBPA or TBBPA-sulf, with an RMSD value of 0.24 Å for superimposed alpha carbons (Fig. 6A). Contrary to TBBPA, TBBPA-sulf is not a symmetric molecule, and in principle, the sulfate group could have been positioned either on the H11/H12 side or near the β-sheet S1/S2.

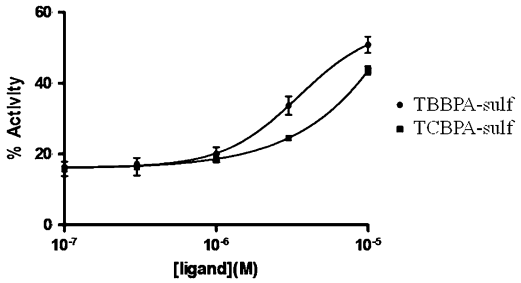


FIG. 5. Dose-response curves for sulfate-conjugated metabolites of TBBPA and of TCBPA in reporter cell lines. HGELN-PPARγ luciferase assays of monosulfate conjugate of TBBPA and monosulfate conjugate of TCBPA after their chemical synthesis and their purification. Results are expressed as a percentage of luciferase activity measured per well (mean ± SEM, $n = 4$). The value obtained in the presence of 100nM CD4700 was taken as 100.

Inspection of the omit $F_o - F_c$ difference map (Fig. 6B) revealed density for the ligand which could be placed in a position almost identical to that of TBBPA, the sulfate group pointing toward H11/H12 (Fig. 6A). Interestingly, accommodation of the sulfate group by PPARγ does not require any conformational change of the surrounding residue side chains. In this orientation, the sulfate moiety fills a portion of the ligand-binding pocket occupied by water molecules in the TBBPA structure and is potentially involved in hydrogen bonds with Tyr327, Lys367, His449, Gln286, and Ser289 (Fig. 6C). However, the electron density of the sulfate group appears as a blob, thus precluding the precise positioning of individual oxygen atoms. Finally, the presence of a conserved water molecule mediates the indirect interaction between TBBPA-sulf and PPARγ helix H12 (Fig. 6C). The rather poorly defined density of the sulfate moiety suggests a dynamic behavior of this chemical group. Overall, the crystallographic data validate the notion that this TBBPA metabolite is a PPARγ ligand with partial agonistic profile.

DISCUSSION

Most of the reported effects of EDC are attributed to their interference with hormonal signaling mediated by NR. Even

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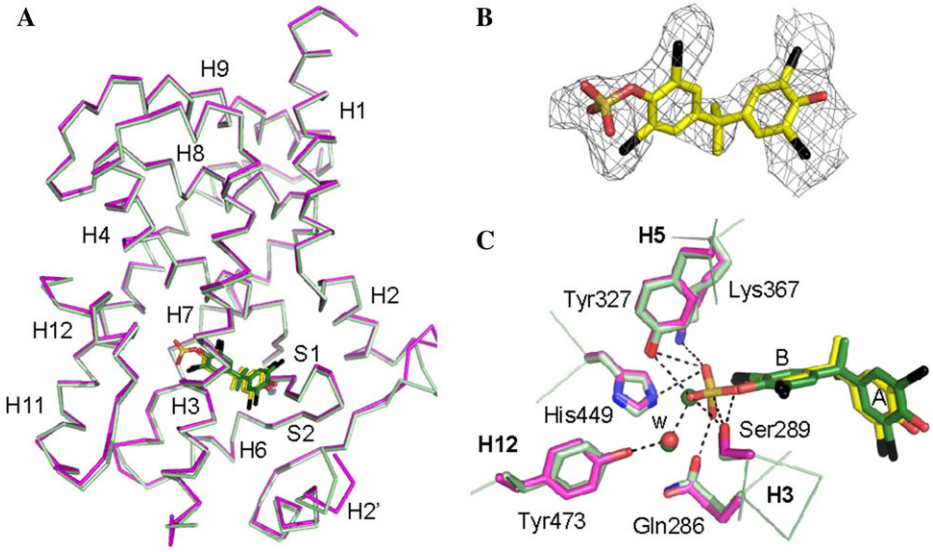


FIG. 6. Crystal structure of PPARγ-LBD in complex with TBBPA-sulf. (A) Superimposition of the co-crystal structure of PPARγ-LBD bound to TBBPA-sulf on the structure with TBBPA (PDB code 3OSW). Carbon atoms are shown in magenta in the TBBPA-sulf complex and in green in the TBBPA structure. TBBPA-sulf and TBBPA are shown in stick representation with each atom type colored: carbon, yellow and green, respectively; oxygen, red; bromine, black. (B) TBBPA-sulf in the $F_o - F_c$ omit map contoured at 1.5σ. (C) PPARγ residues (shown as magenta sticks) in contact with the sulfate group of TBBPA-sulf. Key interactions are highlighted as black dashed lines. Water molecules are shown as red spheres. Corresponding residues and water molecules in the TBBPA-bound PPARγ are shown as green sticks and green spheres, respectively.

low doses of contaminants can trigger biological effects when exposure occurs during critical windows of exposure (Newbold *et al.*, 2009; Vandenberg *et al.*, 2009). An extensive variety of man-made contaminants exist. Many of these chemicals undergo biotic or abiotic structural changes in the environment, and biotransformations at the level of target organisms can also modulate EDC's activity. These facts greatly complicate the picture and require the use of adequate approaches for the screening of environmental contaminants able to interfere with NR.

Environmental as well as human exposure data clearly indicate that halogenated bisphenols are emerging contaminants, which fate and cellular targets deserve to be better characterized (Cariou *et al.*, 2008; de Wit *et al.*, 2009; Fernandez *et al.*, 2007; Johnson-Restrepo *et al.*, 2008). The occurrence of brominated bisphenols mainly results from the extensive use of TBBPA as a flame retardant. Conversely, a growing body of evidence suggests that chlorinated BPA analogs arise from the abiotic chlorination of BPA residues (Fukazawa *et al.*, 2001; Gallart-Ayala *et al.*, 2010). In this study, we investigated both the ER and PPAR activities of BPA and related halogenated analogs and characterized their binding affinity using newly developed NR-LBD affinity columns, combined with radio-HPLC and MS analyses. The potential of these columns to be used as powerful tools to identify metabolites of interest was further validated using reference ligands, then applied to the study of TBBPA and TCBPA metabolites formed after 24-h incubation with HepG2 cells.

For chlorinated BPA analogs, the results obtained with HeLa cells stably transfected with ERα showed that these compounds are at least as potent agonists as BPA, consistent with a previous study (Takemura *et al.*, 2005). Contrary to brominated BPA (Riu *et al.*, 2011), the halogenation degree of chlorinated bisphenols did not decrease their ERα potency. Likely due to the lower ligand-binding pocket size of ERβ compared with ERα, chlorinated analogs with the lowest molecular weight (mono- and diCBPA) exhibited the highest agonistic activity in the HELN-ERβ cell line. As previously observed for brominated BPA analogs, in HGELN-PPARγ cells, the order of potency was reversed, with TCBPA > triCBPA > diCBPA, whereas monoCBPA as well as BPA showed no PPARγ activity at all. In binding affinity assays using NR-based affinity columns, we confirmed the ranking order observed with stably transfected cells for brominated BPA, as well as for chlorinated BPA, demonstrating a good correlation between binding affinities and agonistic activities. Taken together, these data demonstrate that the ERβ and PPARγ agonistic activities of BPA analogs highly depend on their halogenation degree, contrary to their ERα activities.

Up to now, very few environmental ligands of PPARγ have been characterized (Feige *et al.*, 2007; Grun and Blumberg, 2006; Takeuchi *et al.*, 2006). In fact, only mono(2-ethyl-hexyl) phthalate and tributyltin have been shown to significantly activate PPARγ, whereas organotins activate RXR/PPARγ, primarily by interacting with RXR (le Maire *et al.*, 2009). The use of PPARγ-LBD–based affinity columns clearly demonstrates a direct interaction of halogenated BPA derivatives with

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TABLE 2
Data Collection and Refinement Statistics

Complex	TBBPA-sulf
PDB code	3PBA
Space group	*C2*
Cell dimensions a, b, c (Å)	93.37, 61.56, 119.07
β (degree)	102.89
Resolution range (Å)	116.25–2.30 (2.42–2.30)[a]
No. of reflections	27,899
R_{sym}	0.107 (0.711)[a]
I/sI	9.4 (0.9)[a]
Completeness (%)	94.8 (97.7)[a]
Redundancy	2.6 (2.7)[a]
Refinement	
Resolution range (Å)	40.93–2.30
R (%)/R_{free} (%)	18.8/25.5
No. atoms	4192
No. protein atoms	3997
No. ligand atoms	25
No. water molecules	165
Average B-factor (Å^2)	
Protein B-factor (Å^2)	47.67
Ligand B-factor (Å^2)	89.45
Water B-factor (Å^2)	49.37
RMSD from ideality	
Bond lengths (Å)	0.004
Angles (°)	0.8
Ramachandran plot (%)	
Favored region	95.4
Additionally allowed regions	4.4
Generously allowed regions	0.2
Disallowed regions	0.0

[a]Values in parentheses are for highest resolution shell.

PPARγ. The fact that bisphenol analogs bearing at least two halogens are also ligands of PPARγ considerably strengthens the hypothesis that environmental ligands could be involved in the disruption of energy balance regulation. Like for other EDC, perinatal exposure could play a critical role. Indeed, a large body of data regarding EDCs nowadays underlines the importance of exposure during early stages of the development, which can later result in adverse effects (Newbold *et al.*, 2009). For BPA, this includes reproductive targets and obesity. As demonstrated by Somm *et al.* (2009), a perinatal exposure to BPA alters early adipogenesis in rat, a process known to be mediated by PPARγ. According to Cariou *et al.* (2008), significant levels of TBBPA are found in human cord blood (200 pg/g fresh weight) and maternal milk (0.1–37.4 ng/g lipid weight), demonstrating both prenatal and postnatal exposure in a large fraction of the tested population. Recently, a study conducted in Spain demonstrated the presence of mono-, di-, and triCBPA in human placenta (5.1–21.4 ng/g, 12.7–58.8 ng/g, and 4.0–31.2 ng/g fresh tissue, respectively), suggesting as well a perinatal exposure to chlorinated BPA (Jimenez-Diaz *et al.*, 2010).

We previously showed that ERα-LBD affinity columns are able to trap estrogenic compounds present in environment or in food samples (Pillon *et al.*, 2005; Riu *et al.*, 2008). In this study, the newly developed PPARγ-LBD columns were demonstrated to efficiently trap both parent compounds and compounds present in complex matrices, namely the metabolites formed in *in vitro* incubations. Human hepatoblastoma cells metabolized both TBBPA and TCBPA into the corresponding glucuronide and sulfate conjugates. BPA is known to be predominantly metabolized through conjugative pathways. Likewise, *in vivo*, TBBPA is mainly metabolized into TBBPA-monosulfate (Schauer *et al.*, 2006; Van der Ven *et al.*, 2008). The metabolic fate of TCBPA and other chlorinated BPA analogs *in vitro* and *in vivo* is still unknown, but conjugative metabolism is expected to be predominant, consistent with the results obtained in this study using incubations with HepG2 cells. Phase II metabolism, with the production of conjugated metabolites, is almost always a detoxification process. The occurrence of bioactive conjugates is not a common finding. It has previously been reported only for a very limited number of compounds, including morphine and estrogens (Glatt, 2000; Ritter, 2000). In this study, the development of PPARγ-LBD–based columns allowed to identify, for the first time, that the sulfation of halogenated BPA produces bioactive metabolites. Using bioluminescent cell lines assays expressing PPARγ and X-ray crystallography, we showed that the sulfate conjugates of TBBPA and TCBPA are able to bind PPARγ but also display an agonistic activity toward this NR. Chlorinated bisphenols have already been demonstrated to be present in human tissues. The occurrence of brominated bisphenols has been demonstrated in maternal blood, cord blood, and milk, suggesting direct fetal exposure during pregnancy (Cariou *et al.*, 2008), as well as a neonatal exposure to TBBPA residues (parent molecule and/or conjugates) through milk. The fact that halogenated analogs of BPA are selective PPARγ ligands highlights this NR as a new target for endocrine disruption. Taking into account their structure, their biotransformation process (biotic or abiotic), and their capability to activate different NR (ERs and PPARγ), these halogenated BPA analogs may contribute to the onset of metabolic disorders in human. Their additive effects could further increase such adverse outcomes and should therefore be examined as well.

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