Effects of primary metabolites of organophosphate flame retardants on transcriptional activity via human nuclear receptors

Reference:
Kojima Hiroyuki, Takeuchi Shinji, Van den Eede Nele, Covaci Adrian.- Effects of primary metabolites of organophosphate flame retardants on transcriptional activity via human nuclear receptors
Toxicology letters - ISSN 0378-4274 - 245(2016), p. 31-39
Full text (Publishers DOI): http://dx.doi.org/doi:10.1016/J.TOXLET.2016.01.004
To cite this reference: http://hdl.handle.net/10067/1322260151162165141
Effects of primary metabolites of organophosphate flame retardants on transcriptional activity via human nuclear receptors

Hiroyuki Kojima\textsuperscript{a,}\textsuperscript{*}, Shinji Takeuchi\textsuperscript{a}, Nele Van den Eede\textsuperscript{b}, Adrian Covaci\textsuperscript{b}

\textsuperscript{a}Hokkaido Institute of Public Health, Kita-19, Nishi-12, Kita-ku, Sapporo 060-0819, Japan;

\textsuperscript{b}Toxicological Center, Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Antwerp, Belgium

\textsuperscript{*}Corresponding author. Tel.: +81-11-747-2733; fax: +81-11-736-9476.

E-mail address: kojima@iph.pref.hokkaido.jp (H. Kojima).
Abstract

Organophosphate flame retardants (OPFRs) have been used in a wide variety of applications and detected in several environmental matrices, including indoor air and dust. Continuous human exposure to these chemicals is of growing concern. In this study, the agonistic and/or antagonistic activities of 12 primary OPFR-metabolites against ten human nuclear receptors were examined using cell-based transcriptional assays, and compared to those of their parent compounds. As a result, 3-hydroxylphenyl diphenyl phosphate and 4-hydroxylphenyl diphenyl phosphate showed more potent estrogen receptor α (ERα) and ERβ agonistic activity than did their parent, triphenyl phosphate (TPHP). In addition, these hydroxylated TPHP-metabolites also showed ERβ antagonistic activity at higher concentrations and exhibited pregnane X receptor (PXR) agonistic activity as well as androgen receptor (AR) and glucocorticoid receptor (GR) antagonistic activities at similar levels to those of TPHP. Bis(2-butoxyethyl) 3’-hydroxy-2-butoxyethyl phosphate and 2-hydroxyethyl bis(2-butoxyethyl) phosphate act as PXR agonists at similar levels to their parent, tris(2-butoxyethyl) phosphate. On the other hand, seven diester OPFR-metabolites and 1-hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate did not show any receptor activity. Taken together, these results suggest that hydroxylated TPHP-metabolites show increased estrogenicity compared to the parent compound, whereas the diester OPFR-metabolites may have limited nuclear receptor activity compared to their parent triester OPFRs.

Key words: flame retardants, human, nuclear receptor, organophosphate, reporter gene assay, urinary metabolite.
1. Introduction

The phasing-out of polybrominated diphenyl ethers (PBDEs) saw an increase in the use of organophosphate flame retardants (OPFRs) as additives to flame retardants and plasticizers in a variety of applications, such as building materials, textiles and electric appliances, resulting in their widespread environmental dispersion (Reemtsma et al., 2008; van der Veen and de Boer, 2012). Many studies have reported that various OPFRs are widely distributed in both indoor and outdoor environments (Hartmann et al., 2004; Marklund et al., 2003; Takigami et al. 2009; Stapleton et al., 2009). In particular, the presence of OPFRs in indoor dust has been worldwide reported, with several OPFRs, such as tris(2-butoxyethyl) phosphate (TBOEP), tris(1-chloro-2-propyl) phosphate (TCIPP), triphenyl phosphate (TPHP), tris(2-chloroethyl) phosphate (TCEP), tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), and tri-n-butyl phosphate (TNBP), predominately found in indoor dust from residential houses and offices (Tajima et al., 2014; Van den Eede et al., 2011). The concentrations of OPFRs detected in indoor dust in recent years have been higher than those of PBDEs (Ali et al., 2012; Stapleton et al., 2012). As TCEP, TDCIPP, TCIPP and TBOEP have been shown to be carcinogenic or possibly carcinogenic in animal studies (WHO 1998; 2000), continuous human exposure to these OPFRs is of growing concern.

Two previous studies using rodents showed that TDCIPP is quickly metabolized to bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), which is excreted primarily in the urine (Lynn et al., 1981; Nomeir et al., 1981). Recent human biomonitoring studies have also reported that OPFRs are readily metabolized, via hydrolysis or hydroxylation, and excreted in
the urine as their dialkyl and diaryl compounds, which function as biomarkers for OPFR exposure (Butt et al., 2014; Cooper et al., 2011; Dodson et al., 2014; Van den Eede et al., 2013b). Van den Eede et al. (2015b) reported that 1-hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate (BCIPHIPP), diphenyl phosphate (DPHP), and BDCIPP were detected in >95% of human urine samples and bis(2-butoxyethyl) 2-hydroxyethyl phosphate (BBOEHEP) was found in >80% of those. In particular, DPHP, a primary metabolite of TPHP, was frequently detected at ppb levels in human urine (Cooper et al., 2011; Meeker et al., 2013; Reemtsma et al., 2011; Schindler et al., 2009). Thus, although various OPFR-metabolites are frequently detected in human urine and are probably quickly eliminated from the human body, it remains unclear whether these metabolites have adverse effects on human health during their residence time in the body.

The nuclear receptor superfamily is comprised of ligand-inducible transcription factors that specifically regulate the expression of target genes involved in metabolism, development, and reproduction (McKenna et al., 1999). Their primary function is to mediate the transcriptional response in target cells to hormones, such as sex steroids, adrenal steroids, vitamin D3, and thyroid and retinoid hormones, in addition to a variety of other metabolic ligands. Recent in vitro studies have suggested that several OPFRs act as endocrine disruptors via nuclear receptors, including hormone receptors (Kojima et al., 2013; Suzuki et al., 2013). In particular, our previous study using cell-based transactivation assays revealed that several OPFRs have agonistic and/or antagonistic activity against estrogen receptors (ERs), androgen receptor (AR), glucocorticoid receptor (GR) and pregnane X receptor (PXR) (Kojima et al.,
2013). In addition, other studies have shown that TPHP could act as an activator of the peroxisome proliferators-activated receptor (PPAR)γ to induce adipogenesis (Belcher et al., 2014; Pillai et al., 2014). In the present study, we characterized the agonistic and antagonistic activity of twelve metabolites of TBOEP, TCIPP, TPHP, TCEP, TDCIPP, and TNBP, which are predominately found in indoor dust (Tajima et al., 2014; Van den Eede et al., 2011), against the following human nuclear receptors: ERα/β, AR, GR, TRα1, retinoic acid receptor (RAR)α, retinoic X receptor (RXR)α, PPARα/γ and PXR. In this article, we provide the first evidence that hydroxylated TPHP-metabolites might possess multiple effects on transcriptional activity via human nuclear receptors, whereas dealkylated OPFR-metabolites do not show these activities.

2. Materials and methods

2.1. Chemicals, biochemicals and cells

Di-n-butyl phosphate (DNBP; >97% pure), diphenyl phosphate (DPHP; >99% pure) and TPHP (>99% pure) were purchased from Sigma-Aldrich (Bornem, Belgium). TDCIPP, TCIPP, TBOEP, TNBP, and TCEP (each >99% pure) were purchased from Wako Pure Chemical Industries, Ltd. (Wako; Osaka, Japan). Bis(2-chloro-1-methylethyl) phosphate (BCIPP; >91% pure), bis(2-chloroethyl) phosphate (BCEP; >97.5% pure), bis(2-butoxyethyl) phosphate (BBOEP; >97.5% pure), and BDCIPP (>97.5% pure) were synthesized in the Max Planck Institute (Göttingen, Germany). 4-Hydroxylphenyl diphenyl phosphate (HO-p-TPHP; >90% pure), 4-hydroxylphenyl phenyl phosphate (HO-DPHP; >90% pure), bis(2-butoxyethyl)
3'-hydroxy-2-butoxyethyl phosphate (HO-TBOEP; >90% pure), BBOEHEP (>95% pure), and BCIPHIPP (>90% pure) were also synthesized in the Max Planck Institute. 3-Hydroxylphenyl diphenyl phosphate (HO-m-TPHP; >90% pure) was kindly provided from Dr. Heather Stapleton (Duke University, USA). The chemical structures of the 12 OPFR-metabolites and their parent compounds are shown in Fig. 1.

17β-Estradiol (E2; >97% pure), 5α-dihydrotestosterone (DHT; 95% pure), hydrocortisone (HC; >98% pure), 9-cis retinoic acid (9-cis RA; 98% pure), and hydroxyflutamide (HF; >99% pure) were purchased from Wako. 3,3',5-triiodo-L-thyronine (T3; 99% pure), all trans-retinoic acid (at-RA; >98% pure), rifampicin (RIF; >97% pure), ciprofibrate (>99% pure), rosiglitazone (>99% pure), and mifepristone (RU-486; 98% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was used as a vehicle and purchased from Wako, and all compounds used were dissolved in DMSO at a concentration of 1 × 10⁻² M.

Dulbecco’s modified Eagle’s medium (D-MEM), and D-MEM plus Ham’s F-12 nutrient mixture (D-MEM/F-12) were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) and charcoal/dextran-treated FBS (CD-FBS) were obtained from Hyclone (Logan, UT, USA). The penicillin-streptomycin solution (antibiotics) was obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Bovine serum albumin (BSA) and 4-methylumbelliferyl-β-D-galactoside (4-MUG) were obtained from Sigma-Aldrich.

CHO-K1 cells and simian kidney COS-7 cells were obtained from the American Type Culture Collection. CHO-K1 cells were maintained in D-MEM/F-12 supplemented with 5%
FBS and antibiotics, and COS-7 cells were maintained in D-MEM supplemented with 10% FBS and antibiotics.

2.2. Plasmids

The expression plasmids, pcDNAERα, pcDNAERβ, pZeoSV2AR, pSG5-GR, pZeo-TRα1, pcDNA-RXRα, pcDNA-RARα, pSG5-hPXR, pcDNA-PPARα, and pTransExpress-PPARγ1, encoding full-length receptor proteins, as well as the reporter plasmids, pGL3-tkERE, pIND-ARE, pGRE-tk-Luc, pIND-TREpal, pXREM-3A4-Luc, and pGL4-10xAOXluc2, were prepared as previously described (Kojima et al., 2009; 2011; 2013).

The internal control plasmid, pCMVβ-Gal, was purchased from Clontech (Palo Alto, CA, USA).

2.3. Reporter gene assays

For detection of human ERα, ERβ, AR, GR, TRα1, RARα, and RXRα activity, we plated the CHO-K1 cells in 96-well microtiter plates (Nalge, Nunc, Denmark) at a density of 8,400 cells per well in phenol red-free D-MEM/F-12 containing 5% CD-FBS (complete medium) one day before transfection. COS-7 cells (8,400 cells/well) in D-MEM containing 10% CD-FBS were used as host cells for the detection of human PXR, PPARα, and PPARγ1 activity. These cells were transiently transfected with the receptor expression plasmid, the reporter plasmid, and the internal control plasmid (pCMVβ-Gal) using the FuGENE 6 Transfection Reagent (Promega, Madison, WI, USA) (Kojima et al., 2009; 2011; 2013). After a 3-hr transfection period, cells were dosed with various concentrations of the test compounds or with 0.3% DMSO (vehicle control) in complete medium. To avoid any cytotoxic effects
associated with the test compounds, assays were performed for test compounds at concentrations of less than $3 \times 10^{-5}$ M. After an incubation period of 24 hr, cells were rinsed with phosphate-buffered saline (pH 7.4) and lysed with passive lysis buffer (50 µl/well; Promega).

We measured firefly luciferase activity using a MiniLumat LB 9506 luminometer (Berthold, Wildbad, Germany) in one reaction tube with a 5-µl aliquot of the cell lysate using the Luciferase Assay System (Promega), according to the manufacturer’s instructions. Luciferase activity was normalized against the β-galactosidase activity for each treatment. Results are expressed as means ± SD from at least three independent experiments performed in triplicate.

2.4. β-Galactosidase activity assay

The measurement of β-galactosidase activity was performed using a fluorescence method as described in our previous paper (Takeuchi et al., 2005).

2.5. Evaluation of agonistic and antagonistic activities

To estimate the potency of the receptor agonistic activities of the tested compounds, the luminescence intensity of the assay was represented in a dose-response curve. The concentration of the compound equal to 20% of the maximal response of the positive control was obtained from the dose-response curve of the luminescence intensity and expressed as an REC20 value (20% relative effective concentration). The results for the receptor antagonistic activities of each compound were expressed as RIC20 values (20% relative inhibitory concentration), which is the concentration of the test compounds showing 20% inhibition of
the activities induced by the endogenous hormones. These values can be compared to those of
the positive controls and other compounds described in previous our reports (Kojima et al.

2.6. Statistical Analysis

An analysis of variance (ANOVA) followed by Bonferroni correction was used to
evaluate the differences in transcriptional levels between the control group and each of the
chemical groups in the ERβ, AR and GR antagonist assays. The level of significance was set
at $p<0.05$. Data were presented as the mean± S.D. of three triplicate experiments.

3. Results

3.1. Agonistic activities of the OPFR-metabolites against ERα/β, AR, GR, and TRα1

We found that HO-m-TPHP and HO-p-TPHP among the 12 OPFR-metabolites tested
induced ERα- and ERβ-mediated estrogenic activity of greater than 20% that of the
E2–induced maximal activity. However, none of the 12 OPFR-metabolites showed AR, GR or
TRα1 agonistic activity in these assays (data not shown).

Figure 2A shows the dose-response curves of ERα agonistic activities for TPHP and four
TPHP-metabolites. HO-m-TPHP and HO-p-TPHP showed more potent ERα agonistic activity
than did their precursor TPHP, whereas DPHP and HO-DPHP did not show any ERα
agonistic activity. The order of the relative ERα agonistic activity of these compounds was
HO-p-TPHP > HO-m-TPHP > TPHP > DPHP = HO-DPHP. From a comparison of REC20
values, the estrogenic activity via ERα of HO-p-TPHP, the most potent ERα agonist in this
study, was about 100,000-fold lower than that of E$_2$, but over 10-fold higher than that of TPHP (Table 1).

Figure 2B shows the dose-response curves of ER$\beta$ agonistic activities for TPHP and four TPHP-metabolites. These results indicated that HO-$m$-TPHP, HO-$p$-TPHP, and TPHP could act as ER$\beta$ agonists while DPHP and HO-HPHP did not show any ER$\beta$ agonistic activity, as also seen in above results for the ER$\alpha$ agonistic assay. A comparison of REC$_{20}$ values revealed that the estrogenic activity via ER$\beta$ of HO-$p$-TPHP was about 57,000-fold lower than that of E$_2$, but HO-$m$-TPHP and HO-$p$-TPHP induced about 2- and 20-fold higher responses, respectively, than did TPHP (Table 1).

3.2. Antagonistic activities of the OPFR-metabolites against ER$\alpha$/$\beta$, AR, GR, and TR$\alpha_1$

We found that HO-$m$-TPHP and HO-$p$-TPHP among the 12 OPFR-metabolites tested had antagonistic activity against ER$\beta$, AR and GR. However, none of the 12 OPFR-metabolites showed antagonistic activity against ER$\alpha$ or TR$\alpha_1$ (data not shown).

Figure 3a shows the dose-response curves of ER$\beta$ antagonistic activities for HO-$m$-TPHP, HO-$p$-TPHP, TPHP and TAM, a well-known ER antagonist. HO-$m$-TPHP and HO-$p$-TPHP inhibited ER$\beta$-mediated estrogenic activity of greater than 20% that of the activity induced by $1 \times 10^{-10}$ M E$_2$, while TPHP showed slight ER$\beta$ antagonistic activity of less than 20% that of the activity induced by $1 \times 10^{-10}$ M E$_2$. Although HO-$m$-TPHP and HO-$p$-TPHP were ER$\beta$ agonists, they could also act as ER$\beta$ antagonists at higher concentrations. As shown in Table 1, the RIC$_{20}$ value for HO-$p$-TPHP, the most potent ER$\beta$ antagonist in this study, was $5.4 \times 10^{-6}$
M and its antiestrogenic activity via ERβ was 900-fold lower than that of TAM (RIC20: 6.0 × 10⁻⁹ M).

Figure 3b shows the AR antagonistic effects of HO-m-TPHP and HO-p-TPHP at high concentrations as well as that of TPHP. A comparison of RIC20 values showed that the anti-androgenic activities of the hydroxylated TPHP-metabolites and TPHP were about 650-fold lower than that of HF, a known AR antagonist (Table 1).

Figure 3c shows that HO-m-TPHP and HO-p-TPHP as well as TPHP had weak inhibitory effects on the GR-mediated transcriptional activity induced by 3 × 10⁻⁸ M of HC. A comparison of RIC20 values revealed that the GR antagonistic activity of HO-p-TPHP, the most potent GR antagonist in this study, was an estimated 75-fold lower than that of RU-486, a known GR antagonist (Table 1).

Based on their β-galactocidase activity, except for TPHP, HO-m-TPHP and HO-p-TPHP, which were slightly cytotoxic at a concentration of 3 × 10⁻⁵ M, none of the six OPFRs or 12 OPFR-metabolites tested in this study showed any cytotoxic effects against CHO-K1 cells at the doses tested (≤ 3 × 10⁻⁵ M).

3.3. Agonistic activities of the OPFR-metabolites against RARα, RXRα, PXR, and PPARα/γ1

We found that HO-TBOEP, BBOEHEP, HO-m-TPHP and HO-p-TPHP among the 12 OPFR-metabolites possess PXR agonistic activity. However, none of the 12 OPFR-metabolites showed RARα, RXRα, PPARα or PPARγ1 agonistic activity in these
assays (data not shown).

Figure 4 shows the dose-response curves of HO-TBOEP, BBOEHEP, HO-\textit{m}-TPHP and HO-\textit{p}-TPHP showing PXR agonistic activity greater than 20\% that of the maximum activity of RIF. The \textit{REC}_{20} values of the 12 OPFR-metabolites and their precursors for PXR agonistic activity are given in Table 1. The relative potencies of their PXR agonistic activities were as follows: TDCIPP > TBOEP > HO-\textit{m}-TPHP > TPHP > HO-\textit{p}-TPHP > TNBP > HO-TBOEP > BBOEHEP. Comparisons of their \textit{REC}_{20} values suggested that their agonistic activities against PXR were between 4.5- and 90.3-fold lower than that of RIF (Table 1).

None of the 12 OPFR-metabolites induced PPAR\textgamma{}1 agonistic activity greater than 20\% that of the maximum activity of rosiglitazone. Figure 5 shows the dose-response curves of TPHP, four TPHP-metabolites and rosiglitazone in the PPAR\textgamma{}1 assay. Although TPHP showed weak PPAR\textgamma{}1 agonistic activity of about 15\% that of the maximum response of rosiglitazone, the activities of the two hydroxylated TPHP metabolites (HO-\textit{m}-TPHP and HO-\textit{p}-TPHP) were less potent than that of TPHP. On the other hand, the TPHP-derived diaryl compounds, DPHP and HO-DPHP, did not show any PPAR\textgamma{}1 agonistic activity.

Based on their \textbeta{}-galactosidase activity, except for HO-\textit{m}-TPHP and HO-\textit{p}-TPHP, which were slightly cytotoxic at a concentration of \(3 \times 10^{-5} \) M, none of the twelve OPFR-metabolites in this study showed any cytotoxic effects against COS-7 cells at the doses tested (\(\leq 3 \times 10^{-5} \) M).

\textbf{4. Discussion}
Recently, environmental pollution by OPFRs has been of increasing concern in terms of public health and the need for risk assessment (van der Veen and de Boer, 2012). Our previous study provided evidence that several OPFRs show potential endocrine disruption activity via human nuclear receptors (Kojima et al., 2013). In this study, to further elucidate the endocrine-disrupting properties of OPFRs in humans, we characterized activities of 12 primary metabolites (Fig. 1) derived from TBOEP, TCIPP, TPHP, TCEP, TDCIPP, and TNBP against 10 nuclear receptors using cell-based reporter gene assays with CHO-K1 and COS-7 cells. The results summarized in Table 1 reveal that two hydroxylated TPHP-metabolites among the 12 compounds tested exhibited ERα/β and PXR agonistic activity as well as ERβ, AR, and GR antagonistic activity, while two TBOEP-metabolites showed only PXR agonistic activity. However, it was also found that none of the 12 OPFR-metabolites, together with the 11 OPFRs reported in our previous paper (Kojima et al., 2013), possess TRα1, RARα, RXRα, or PPARα/γ activity.

The metabolization of TPHP by human liver microsomes has been reported to produce several primary metabolites, HO-\textit{p}-TPHP, DPHP and HO-DPHP, through the hydroxylation and \textit{O}-dealkylation of TPHP (Fig. 1) (Sasaki et al., 1984; Van den Eede et al., 2013a). Recent studies have shown that \textit{in vitro} metabolism of TPHP using chicken embryonic hepatocytes induced the formation of HO-\textit{m}-TPHP, together with DPHP and HO-\textit{p}-TPHP (Su et al., 2014; 2015), although HO-\textit{m}-TPHP has not been confirmed as a metabolite formed by human liver enzymes. However, as HO-\textit{m}-TPHP is present as an impurity/hydrolysis product of the flame retardant resorcinol bis(diphenylphosphate) (RDP), humans could be directly exposed to this
compound (Ballesteros-Gomes et al., 2015). Several analytical studies have shown that DPHP was frequently detected as a major metabolite of TPHP at levels in order of a few ng/ml in human urine samples (Cooper et al., 2011; Meeker et al., 2013; Reemtsma et al., 2011; Schindler et al., 2009). In the assays of the 12 OPFR-metabolites against nuclear receptors, HO-m-TPHP and HO-p-TPHP showed multiple ERα, ERβ, and PXR agonistic activities, as well as ERβ, AR and GR antagonistic activity. In particular, HO-p-TPHP activated ERα and ERβ at lower concentrations than the parent, TPHP, and HO-m-TPHP (Figs. 3a and b). This suggests that a para-substituted OH group connected to a benzene ring may be important for the induction of high levels of estrogen activity, a notion also supported by several studies reporting that hydroxylated polychlorinated biphenyls (HO-PCBs) with para-substituted HO-groups possess the most potent estrogenic effects via ERα, followed by meta- and ortho-substituted HO-PCBs (Arulmozhiraja et al., 2005; Kitamura et al., 2005). Additionally, this result implies that the metabolism of TPHP to HO-p-TPHP and HO-m-TPHP by oxidation enzymes enhances their endocrine-disrupting effects, such as estrogenic activity, suggesting metabolic activation against ERs. Interestingly, we found that HO-p-TPHP and HO-m-TPHP induce ERα- and ERβ-mediated transcriptional activities, but simultaneously inhibit ERβ-, AR- and GR-mediated transcriptional activities (Table 1). Such an activity pattern in same the assay systems using CHO-K1 cells is similar to those of 2’,3’,4’,5’-tetrachlorobiphenyl-2-ol (2’-HO-CB61) and 2’,3,3’,4’,5’-pentachlorobiphenyl-4-ol (4’-HO-CB106) (Takeuchi et al., 2011) and those of butylbenzyl phthalate (plasticizer) and the methoxychlor (insecticide) metabolite, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) (Takeuchi et al., 2005).
These findings, together with those of the present study, indicate that chemicals showing such pleiotropic effects may unexpectedly exist in the environment. Furthermore, hydroxylated TPHP-metabolites also showed PXR agonistic activity similar to that of TPHP (Fig. 6). Thus, HO-\textit{p}-TPHP and HO-\textit{m}-TPHP are thought to act as modulators against various nuclear receptors. On the other hand, DPHP and HO-DPHP did not exhibit any receptor activity. These results suggest that TPHP and hydroxylated TPHP-metabolites (triaryl phosphates) have multiple and pleiotropic effects against nuclear receptors, whereas DPHP and HO-DPHP (diaryl phosphates) were inactive against nuclear receptors. This difference might reflect the fact that diaryl phosphates show increased hydrophilicity, an important factor in their interactions with nuclear receptor, compared to triaryl phosphates.

TBOEP is an OPFR produced in large volumes and used in a broad range of applications, and is detectable at high concentrations in indoor dust (Tajima et al., 2014; Van den Eede et al., 2011). TBOEP-metabolizing studies using human liver preparations revealed the presence of several primary metabolites, such as BBOEP, HO-TBOEP and BBOEHEP (Fig. 1) (Van den Eede et al, 2013a; 2015a). BBOEHEP, in particular, is frequently detected in human urine (Van den Eede et al., 2015b). Although our previous study showed that the parent TBOEP has only PXR agonistic activity among various nuclear receptor assays (Kojima et al., 2013), we found that HO-TBOEP and BBOEHEP exhibited weaker PXR agonistic activity than did TBOEP, while an \textit{O}-dealkylated metabolite, BBOEP, did not show any activity (Fig. 5). This implies that TBOEP-OH and BBOEHEP are structurally similar to TBOEP, allowing them to act as PXR activators, whereas the structural properties of BBOEP might lead to reduction in
its binding to PXR (Fig. 1).

Recent studies have reported that TDCIPP was detected in indoor dust in homes and work environments (Meeker and Stapleton, 2010; Van den Eede et al., 2011). In addition to an in vivo study using rats (Lynn et al., 1981), in vitro metabolic studies using human liver microsomes or hepatocytes reported the formation of BDCIPP as a major metabolite of TDCIPP through its O-dealkylation (Abdallah et al., 2015; Van den Eede et al., 2013a). Several human monitoring studies have reported that BDCIPP is frequently detected in human urine samples (Carignan et al., 2013; Cooper et al., 2011; Hoffman et al., 2014). Although TDCIPP itself has AR and GR antagonistic activities as well as PXR agonistic activity (Kojima et al., 2013), the present study indicated that BDCIPP did not possess any receptor activity, including AR antagonistic activity (Table 1). This suggests that BDCIPP, formed through O-dealkylation of TDCIPP, does not affect the nuclear receptor activity, as in the case of DPHP and BBOEP.

The in vitro biotransformation of TCIPP, TCEP and TNBP by human liver preparations showed that these compounds are predominately metabolized to BCIPP, BCEP and DNBP, respectively (Van den Eede et al., 2013a). In this study, we also examined the nuclear receptor activity of the dechlorinated and hydroxylated TCIPP-metabolite, BCIPHIPP. The results revealed that these four metabolites did not exhibit any nuclear receptor activity, although TCIPP is a PXR agonist, and TNBP is a PXR agonist as well as an AR and GR antagonist (Kojima et al., 2013). These results suggest that the diester phosphates BCIPP, BCEP and DNBP lose their activity against nuclear receptors as also seen in DPHP, BBOEP and BDCIPP.
These decreases in receptor activity commonly observed in the diester phosphates appear to resemble those with regard to PXR agonistic activity observed in several oxon-forms of phosphorus insecticides (Kojima et al., 2011). Thus, the metabolization of a triester phosphate (R₃-P=O) to a diester phosphate (R₂-P(=O)-OH) by liver esterase increases the hydrophilicity of the OPFRs, which might consequently lead to the loss of nuclear receptor activity.

We also found that none of the OPFRs or their metabolites possesses TRα₁, RARα, RXRα, or PPARα/γ activity. On the other hand, more recent studies have shown that TPHP and other environmental chemicals, including brominated flame retardants, can act as PPARγ agonists to induce adipogenesis (Pillai et al., 2014; Fang et al., 2015a; 2015b). Based on COS-7 reporter gene and BMS2 cell protein expression assays together with lipid accumulation, Pillai et al. (2014) reported that FM550 and TPHP have PPARγ agonistic activity. Fang et al. (2015b) showed that several of compounds in indoor dust can potentially activate PPARγ1 on the basis of a highly sensitive reporter assay with a human embryo kidney 293H cell line containing PPARγ1 ligand-binding domain/GAL4 DNA-binding domain. In this study, we showed that TPHP-metabolites had little PPARγ1 agonistic activity, although the parent TPHP showed PPARγ1 activity at a level 15% that of the maximum activity of rosiglitazone (Fig. 6). Our reporter assay in which a full-length PPARγ1 protein was expressed in COS-7 cells may be less sensitive by one order of magnitude than the assay reported by Fang et al. (2015b), but is as sensitive as the assays reported in other studies (Belcher et al., 2014; Pillai et al., 2014; Suzuki et al., 2013). Nevertheless, our findings suggest that TPHP is a weak PPARγ1 activator, and its metabolization to hydroxylated and
dealkylated TPHP lead to a further decrease in PPARγ1 agonistic activity.

5. Conclusions

We demonstrated for first time that monohydroxylated TPHPs (HO-m-TPHP and HO-p-TPHP) possessed ERα/β and PXR agonistic activities as well as ERβ, AR and GR antagonistic activities, whereas none of the diester OPFR-metabolites tested showed any receptor activity. The diverse effects of these OPFR-metabolites against nuclear receptors might be due to their structural properties.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors thank Dr. Heather Stapleton (Duke University, USA) for the generous donation of the HO-m-TPHP standard. This study was supported by JSPS KAKENHI Grant Numbers 21590675, 15K08791.
References


Carignan, C.C., McClean, M.D., Cooper, E.M., Watkins, D.J., Fraser, A.J., Heiger-Bernays,


Thyroid hormone-like and estrogenic activity of hydroxylated PCBs in cell culture. Toxicology 208, 377-387.


Schindler, B.K., Förster, K., Angerer, J., 2009. Determination of human urinary organophosphate flame retardant metabolites by solid-phase extraction and gas


**Figure captions**

**Fig. 1.** Chemical structures of the 12 OPFR-metabolites used in the present study.

*HO-\textit{m}-TPHP was confirmed as a metabolite of TPHP in chicken embryonic hepatocytes (Su et al., 2015), but not in \textit{in vitro} metabolizing study using human liver preparations.

**Fig. 2.** Estrogenic effects of TPHP and its metabolites in the hER\textalpha{} and hER\textbeta{} transactivation assays. CHO cells were transiently transfected with an expression plasmid for human ER\textalpha{} (a) or ER\textbeta{} (b) as well as a reporter-responsive \textit{firefly} luciferase plasmid and a constitutively active \textbeta{}-galactosidase expression plasmid. Cells were treated with 1 \times 10^{-7} to 3 \times 10^{-5} M of TPHP or its metabolites. The \textit{firefly} luciferase activity was normalized based on the \textbeta{}-galactosidase activity. Values represent the means \pm SD of three independent experiments and are presented as the percentage of the response, taking the activity obtained using 1 \times 10^{-9} M \textit{E}_2 as 100%.

**Fig. 3.** Antagonistic activities of TPHP-hydroxylated metabolites against hER\textbeta{}, hAR and hGR. CHO cells were transiently transfected with an expression plasmid for human ER\textbeta{} (a), AR (b) or GR (c) as well as a reporter-responsive \textit{firefly} luciferase plasmid and a constitutively active \textbeta{}-galactosidase expression plasmid. In ER\textbeta{} antagonist assay (a), cells were treated with 1 \times 10^{-7} M to 3 \times 10^{-5} M of TPHP or its hydroxylated metabolites, or with 1 \times 10^{-9} M to 1 \times 10^{-7} M of tamoxifen (TAM) in the presence of 1 \times 10^{-10} M \textit{E}_2 to detect ER\textbeta{} antagonistic activity. In AR antagonist assay (b), cells were treated with 1 \times 10^{-7} M to 3 \times 10^{-5}
M of TPHP or its hydroxylated metabolites, or with $1 \times 10^{-9}$ M to $1 \times 10^{-7}$ M of hydroxyflutamide (HF) in the presence of $1 \times 10^{-10}$ M DHT to detect AR antagonistic activity. In GR antagonist assay (c), cells were treated with $1 \times 10^{-7}$ M to $3 \times 10^{-5}$ M of TPHP or its hydroxylated metabolites, or with $1 \times 10^{-8}$ M to $1 \times 10^{-6}$ M of RU-486 in the presence of $3 \times 10^{-8}$ M HC to detect GR antagonistic activity. The *firefly* luciferase activity was normalized based on the β-galactosidase activity. Values represent the means ± SD of three independent experiments and are presented as the percentage of the response, taking the activity obtained using $1 \times 10^{-10}$ M E$_2$ (a), $1 \times 10^{-10}$ M DHT (b) or $3 \times 10^{-8}$ M HC (c) alone.

*Significant difference (p<0.05; ANOVA) from $1 \times 10^{-10}$ M E$_2$ (a), $1 \times 10^{-10}$ M DHT (b) or $3 \times 10^{-8}$ M HC (c) alone.

**Fig. 4.** Comparison of hPXR agonistic activities between 6 OPFRs and their metabolites.

COS-7 cells were transiently transfected with an expression plasmid for human PXR as well as a reporter-responsive *firefly* luciferase plasmid and a constitutively active β-galactosidase expression plasmid. Cells were treated with $1 \times 10^{-6}$ M to $3 \times 10^{-5}$ M of OPFRs, their metabolites, or rifampicin (RF) to detect PXR agonistic activity. The *firefly* luciferase activity was normalized based on the β-galactosidase activity. Values represent the means ± SD of three independent experiments and are presented as the percentage of the response, taking the activity obtained using $1 \times 10^{-5}$ M RF as 100%.

**Fig. 5.** Effects of TPHP and its 4 metabolites on transcriptional activity via hPPARγ. COS-7
cells were transiently transfected with an expression plasmid for human PPARγ as well as a reporter-responsive firefly luciferase plasmid and a constitutively active β-galactosidase expression plasmid. Cells were treated with $1 \times 10^{-8}$ M to $3 \times 10^{-5}$ M of TPHP or its 4 metabolites, or with $1 \times 10^{-9}$ M to $1 \times 10^{-5}$ M of rosiglitazone to detect PPARγ agonistic activity. The firefly luciferase activity was normalized based on the β-galactosidase activity. Values represent the means ± SD of three independent experiments and are presented as the percentage of the response, taking the activity obtained using $1 \times 10^{-6}$ M rosiglitazone as 100%. 
