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In vitro endocrine disruption potential of organophosphate flame retardants via human nuclear receptors

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ABSTRACT

Various organophosphate flame retardants (OPFRs) are widely used in building materials, textiles and electric appliances, and have been reported to cause indoor environmental pollution in houses and office buildings. In this study, using cell-based transactivation assays, we characterized the agonistic and/or antagonistic activities of 11 OPFRs against human nuclear receptors; estrogen receptor α (ERα), ERβ, androgen receptor (AR), glucocorticoid receptor (GR), thyroid hormone receptor α_1 (TRα_1), TRβ_1, retinoic acid receptor α (RARα), retinoid X receptor α (RXRα), pregnane X receptor (PXR), peroxisome proliferator-activated receptor α (PPARα), and PPARγ. Of the 11 OPFRs tested, triphenyl phosphate (TPhP) and tricrecyl phosphate (TCP) showed ERα and/or ERβ agonistic activity. In addition, tributyl phosphate (TBP), tris(1,3-dichloro-2-propyl) phosphate (TDCPP), TPhP and TCP showed AR antagonistic activity, and TBP, tris(2-ethylhexyl) phosphate (TEHP), TDCPP, TPhP and TCP showed GR antagonistic activity. Furthermore, we found that seven compounds, TBP, tris(2-chloro-1-methylethyl) phosphate (TCPP), TEHP, tris(2-butoxyethyl) phosphate (TBEP), TDCPP, TPhP, and TCP, display PXR agonistic activity. However, none of test compounds showed agonistic or antagonistic activity against TRα/β, or agonistic activity against RARα, RXRα or PPARα/γ. Taken together, these results suggest that several OPFRs may have potential endocrine disrupting effects via ERα, ERβ, AR, GR and PXR.

1. Introduction

Flame retardants are used in large quantities worldwide in plastic materials, paints, textile fabrics, and so on (Alaee et al., 2003). Although a major series of flame retardants, polybrominated diphenyl ethers (PBDEs), remain in widespread use, common PBDE mixtures (i.e., pentaBDE and octaBDE) have been banned or voluntarily phased out in many countries due to their leachability from various materials, persistence, bioaccumulation, and evidence for adverse health effects (Darnerud et al., 2001). On the other hand, organophosphate compounds continue to be used as flame retardants and plasticizers in a wide variety of applications, resulting in widespread environmental dispersion (Reemtsma et al., 2008). According to several reports, the production and use of organophosphate flame retardants (OPFRs) has surpassed that of PBDEs in Europe, and the total consumption of OPFRs in Europe in 2006 was 20% of the total 465,000-ton total consumption of flame retardants (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 (Marklund et al., 2003; Hartmann et al., 2004; Stapleton et al., 2009). In Japan, OPFRs have been used in larger amounts than PBDEs, and, indeed, recent studies have reported that various OPFRs are detectable in indoor environmental samples from houses and office buildings (Saito et al., 2007; Takigami et al., 2009). Thus, indoor environmental pollution resulting from OPFRs has been of increasing concern recently.

Nuclear receptors are 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 the sex steroids, adrenal steroids, vitamin D3, and thyroid and retinoid hormones, in addition to a variety of other metabolic ligands. Forty-eight nuclear receptors are known to exist in humans, and these proteins comprise the single largest family of metazoan transcription factors, the nuclear receptor superfamily. Recent studies have revealed that PBDEs affect transactivation via nuclear receptors

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Table 1
Abbreviation, CAS number, source, and purity of organophosphorus compounds tested in the present study.

Compound name	Abbreviation	CAS number	Source	Purity (%)
Trimethyl phosphate	TMP	512-56-1	Wako[a]	>99
Triethyl phosphate	TEP	78-40-0	Wako	>97
Tripropyl phosphate	TPrP	513-08-6	Aldrich[b]	99
Tributyl phosphate	TBP	126-73-8	Wako	>97
Tris(2-chloro-1-methylethyl) phosphate	TCPP	13674-84-5	Wako	>98
Tris(2-chloroethyl) phosphate	TCEP	115-96-8	Wako	>98
Tris(2-butoxyethyl) phosphate	TBEP	78-51-3	Wako	>98
Tris(1,3-dichloro-2-propyl) phosphate	TDCPP	13674-87-8	Wako	>98
Tris(2-ethylhexyl) phosphate	TEHP	78-42-2	Wako	>98
Triphenyl phosphate	TPhP	115-86-6	Wako	>97
Tricresyl phosphate	TCP	78-30-8	Wako	>98

[a] Wako Pure Chemical Industries Ltd.
[b] Aldrich Chemical company.

such as estrogen receptors (ERs), androgen receptor (AR), and pregnane X receptor (PXR) (Hamers et al., 2006; Kojima et al., 2009; Pacyniak et al., 2007). However, it remains unclear as to whether OPFRs have any potential nuclear receptor activity.

Transactivation assays, such as reporter gene assays, have an advantage in detecting the agonistic and antagonistic activity of various chemicals against nuclear receptors. We have previously provided evidence that a variety of pesticides (Kojima et al., 2003, 2004), phthalates (Takeuchi et al., 2005), phytochemicals (Takeuchi et al., 2009), hydroxylated and methoxylated PBDEs (Kojima et al., 2009), and hydroxylated polychlorinated biphenyls (OH-PCBs) (Takeuchi et al., 2011) have agonistic and/or antagonistic activities against ERα, ERβ, AR, glucocorticoid receptor (GR), or thyroid hormone receptors (TR) α_1 and β_1 using Chinese hamster ovary (CHO-K1) cell-based transactivation assays. In addition, we provided data on peroxisome proliferator-activated receptor (PPAR)α and γ, and PXR activity for 200 pesticides using simian kidney CV-1 and COS-7 cell-based transactivation assays, respectively (Takeuchi et al., 2006; Kojima et al., 2011). In the present study, we have characterized the agonistic and antagonistic activity of eleven OPFRs against human nuclear receptors ERα/β, AR, GR, TRα$_1$/β$_1$, retinoic acid receptor (RAR)α, retinoid X receptor (RXR)α, PPARα/γ and PXR. Consequently, we found that several of the 11 tested OPFRs have ERα, ERβ, AR, or PXR activity, suggesting that these compounds might act as endocrine disruptors. Here, we also provide the first evidence that several OPFRs have multiple effects on transcriptional activity via nuclear receptors.

2. Materials and methods

2.1. Chemicals, biochemicals and cells

The abbreviation, CAS No., source, and purity of the OPFRs tested in this study are shown in Table 1, and their chemical structures are shown in Fig. 1. 17β-Estradiol (E_2; >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 Pure Chemical Industries Ltd. (Wako; Osaka, Japan). 3,3′,5-Triiodo-L-thyronine (T_3; 99% pure), all *trans*-retinoic acid (at-RA; >98% pure), rifampicin (RIF; >97% pure), ciprofibrate (>99% pure), pioglitazone (>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 3×10^{-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). 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-TRα$_1$ and pZeo-TRβ$_1$, as well as the reporter plasmids, pGL3-tkERE, pIND-ARE, pGRE-tk-Luc and pIND-TREpal, were prepared as previously described (Kojima et al., 2009). We used the expression plasmids, pcDNA-PPARα, pTransExpress-PPARγ1, pcDNA-RXRα and pcDNA-RARα encoding full-length receptor proteins, and the reporter plasmid, pGL4-10xAOXluc2 (Iida, M., unpublished). For determining PXR activity, we used the expression plasmid, pSG5-hPXR, and the reporter plasmid pXREM-3A4-Luc (Kojima et al., 2011). The internal control plasmid, pCMVβ-Gal, was purchased from Clontech (Palo Alto, CA, USA).

2.3. Reporter gene assays for ERα/β, AR, GR, TRα$_1$/β$_1$, RARα and RXRα

We previously described the procedures for CHO-K1 cell-based reporter gene assays to detect the agonistic and antagonistic activities of chemicals against ERα, ERβ, AR, GR, TRα$_1$, or TRβ$_1$ (Kojima et al., 2009; Takeuchi et al., 2009). In brief, we transiently transfected with three plasmids (the receptor expression plasmid, the reporter plasmid and the internal control plasmid) into CHO-K1 cells using the FuGENE 6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN, USA). For detection of RARα or RXRα activity, we plated the CHO-K1 cells in 96-well microtiter plates (Nalge, Nunc, Denmark) at a density of 8400 cells/well in phenol red-free D-MEM/F-12 containing 5% CD-FBS (complete medium) one day before transfection. The cells were transfected with either 0.63 ng pcDNA-RARα or 0.63 ng pcDNA-RXRα, 50 ng pIND-TREpal and 5 ng pCMVβ-Gal per well using the FuGENE 6 Transfection Reagent. After a 3-h transfection period, cells were dosed with various concentrations of the test compounds or with 0.1% 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 3×10^{-5} M. The RARα and RXRα agonists, at-RA and 9-cis RA, were utilized as positive controls in these RARα and RXRα assays, respectively. After an incubation period of 24 h, cells were rinsed with phosphate-buffered saline (pH 7.4) and lysed with passive lysis buffer (50 μl/well; Promega, Madison, WI, USA).

We measured the *firefly* luciferase activity with 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. Reporter gene assays for PXR and PPARα/γ

We previously described the procedures for COS-7 cell-based reporter gene assay to detect the agonistic activity of chemicals against PXR (Kojima et al., 2011). In brief, we transiently transfected with three plasmids (pSG5-hPXR, pXREM-3A4-Luc and pCMVβ-Gal) into COS-7 cells using the FuGENE 6 Transfection Reagent, and measured luciferase activity and β-galactosidase activity after reaction with compound as described above. The PPARα and PPARγ assays were performed using the same procedure as that for the PXR assay. Namely, COS-7 cells (8400 cells/well) were transfected with 50 ng of either pcDNA-PPARα or pTransExpress-PPARγ1, 50 ng pGL4-10 × AOXluc2 and 10 ng pCMV-βGal per well using the FuGene6 transfection reagent. The PPARα and PPARγ agonists, ciprofibrate and pioglitazone, were utilized as positive controls in these PPARα/γ assays, respectively.

2.5. β-Galactosidase activity assay

The measurement of β-galactosidase activity was performed by a fluorescence method as described in our previous papers (Takeuchi et al., 2005, 2006).