Universiteit
Antwerpen

This item is the archived peer-reviewed author-version of:

# 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

uantwerpen.be
Institutional repository IRUA

1 **Effects of primary metabolites of organophosphate flame retardants on transcriptional**

2 **activity via human nuclear receptors**

3

4 Hiroyuki Kojima[a,*], Shinji Takeuchi[a], Nele Van den Eede[b], Adrian Covaci[b]

5

6 [a]Hokkaido Institute of Public Health, Kita-19, Nishi-12, Kita-ku, Sapporo 060-0819, Japan;

7 [b]Toxicological Center, Department of Pharmaceutical Sciences, University of Antwerp,

8 Universiteitsplein 1, 2610 Wilrijk, Antwerp, Belgium

9

10 [*]Corresponding author. Tel.: +81-11-747-2733; fax: +81-11-736-9476.

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

12

13

14 **Abstract**

15 Organophosphate flame retardants (OPFRs) have been used in a wide variety of applications
16 and detected in several environmental matrices, including indoor air and dust. Continuous
17 human exposure to these chemicals is of growing concern. In this study, the agonistic and/or
18 antagonistic activities of 12 primary OPFR-metabolites against ten human nuclear receptors
19 were examined using cell-based transcriptional assays, and compared to those of their parent
20 compounds. As a result, 3-hydroxylphenyl diphenyl phosphate and 4-hydroxylphenyl
21 diphenyl phosphate showed more potent estrogen receptor $\alpha$ (ER$\alpha$) and ER$\beta$ agonistic
22 activity than did their parent, triphenyl phosphate (TPHP). In addition, these hydroxylated
23 TPHP-metabolites also showed ER$\beta$ antagonistic activity at higher concentrations and
24 exhibited pregnane X receptor (PXR) agonistic activity as well as androgen receptor (AR) and
25 glucocorticoid receptor (GR) antagonistic activities at similar levels to those of TPHP.
26 Bis(2-butoxyethyl) 3'-hydroxy-2-butoxyethyl phosphate and 2-hydroxyethyl
27 bis(2-butoxyethyl) phosphate act as PXR agonists at similar levels to their parent,
28 tris(2-butoxyethyl) phosphate. On the other hand, seven diester OPFR-metabolites and
29 1-hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate did not show any receptor activity.
30 Taken together, these results suggest that hydroxylated TPHP-metabolites show increased
31 estrogenicity compared to the parent compound, whereas the diester OPFR-metabolites may
32 have limited nuclear receptor activity compared to their parent triester OPFRs.



33 **Key words**: flame retardants, human, nuclear receptor, organophosphate, reporter gene assay,
34 urinary metabolite.


35

36 **1. Introduction**

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

53 Two previous studies using rodents showed that TDCIPP is quickly metabolized to
54 bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), which is excreted primarily in the urine
55 (Lynn et al., 1981; Nomeir et al., 1981). Recent human biomonitoring studies have also
56 reported that OPFRs are readily metabolized, via hydrolysis or hydroxylation, and excreted in

57 the urine as their dialkyl and diaryl compounds, which function as biomarkers for OPFR
58 exposure (Butt et al., 2014; Cooper et al., 2011; Dodson et al., 2014; Van den Eede et al.,
59 2013b). Van den Eede et al. (2015b) reported that 1-hydroxy-2-propyl bis(1-chloro-2-propyl)
60 phosphate (BCIPHIPP), diphenyl phosphate (DPHP), and BDCIPP were detected in >95% of
61 human urine samples and bis(2-butoxyethyl) 2-hydroxyethyl phosphate (BBOEHEP) was
62 found in >80% of those. In particular, DPHP, a primary metabolite of TPHP, was frequently
63 detected at ppb levels in human urine (Cooper et al., 2011; Meeker et al., 2013; Reemtsma et
64 al., 2011; Schindler et al., 2009). Thus, although various OPFR-metabolites are frequently
65 detected in human urine and are probably quickly eliminated from the human body, it remains
66 unclear whether these metabolites have adverse effects on human health during their residence
67 time in the body.

68 The nuclear receptor superfamily is comprised of ligand-inducible transcription factors
69 that specifically regulate the expression of target genes involved in metabolism, development,
70 and reproduction (McKenna et al., 1999). Their primary function is to mediate the
71 transcriptional response in target cells to hormones, such as sex steroids, adrenal steroids,
72 vitamin D3, and thyroid and retinoid hormones, in addition to a variety of other metabolic
73 ligands. Recent *in vitro* studies have suggested that several OPFRs act as endocrine disruptors
74 via nuclear receptors, including hormone receptors (Kojima et al., 2013; Suzuki et al., 2013).
75 In particular, our previous study using cell-based transactivation assays revealed that several
76 OPFRs have agonistic and/or antagonistic activity against estrogen receptors (ERs), androgen
77 receptor (AR), glucocorticoid receptor (GR) and pregnane X receptor (PXR) (Kojima et al.,

78 2013). In addition, other studies have shown that TPHP could act as an activator of the
79 peroxisome proliferators-activated receptor (PPAR)$\gamma$ to induce adipogenesis (Belcher et al.,
80 2014; Pillai et al., 2014). In the present study, we characterized the agonistic and antagonistic
81 activity of twelve metabolites of TBOEP, TCIPP, TPHP, TCEP, TDCIPP, and TNBP, which
82 are predominately found in indoor dust (Tajima et al., 2014; Van den Eede et al., 2011),
83 against the following human nuclear receptors; ER$\alpha/\beta$, AR, GR, TR$\alpha_1$, retinoic acid receptor
84 (RAR)$\alpha$, retinoic X receptor (RXR)$\alpha$, PPAR$\alpha/\gamma$ and PXR. In this article, we provide the first
85 evidence that hydroxylated TPHP-metabolites might possess multiple effects on
86 transcriptional activity via human nuclear receptors, whereas dealkylated OPFR-metabolites
87 do not show these activities.
88

89 **2. Materials and methods**

90 ***2.1. Chemicals, biochemicals and cells***

91 Di-*n*-butyl phosphate (DNBP; >97% pure), diphenyl phosphate (DPHP; >99% pure) and
92 TPHP (>99% pure) were purchased from Sigma-Aldrich (Bornem, Belgium). TDCIPP, TCIPP,
93 TBOEP, TNBP, and TCEP (each >99% pure) were purchased from Wako Pure Chemical
94 Industries, Ltd. (Wako; Osaka, Japan). Bis(2-chloro-1-methylethyl) phosphate (BCIPP; >91%
95 pure), bis(2-chloroethyl) phosphate (BCEP; >97.5% pure), bis(2-butoxyethyl) phosphate
96 (BBOEP; >97.5% pure), and BDCIPP (>97.5% pure) were synthesized in the Max Planck
97 Institute (Göttingen, Germany). 4-Hydroxylphenyl diphenyl phosphate (HO-*p*-TPHP; >90%
98 pure), 4-hydroxylphenyl phenyl phosphate (HO-DPHP; >90% pure), bis(2-butoxyethyl)

99 3'-hydroxy-2-butoxyethyl phosphate (HO-TBOEP; >90% pure), BBOEHEP (>95% pure),
100 and BCIPHIPP (>90% pure) were also synthesized in the Max Planck Institute.
101 3-Hydroxylphenyl diphenyl phosphate (HO-*m*-TPHP; >90% pure) was kindly provided from
102 Dr. Heather Stapleton (Duke University, USA). The chemical structures of the 12
103 OPFR-metabolites and their parent compounds are shown in Fig. 1.

104 17β-Estradiol ($E_2$; >97% pure), 5α-dihydrotestosterone (DHT; 95% pure),
105 hydrocortisone (HC; >98% pure), 9-*cis* retinoic acid (9-*cis* RA; 98% pure), and
106 hydroxyflutamide (HF; >99% pure) were purchased from Wako. 3,3',5-triiodo-L-thyronine
107 ($T_3$; 99% pure), all *trans*-retinoic acid (at-RA; >98% pure), rifampicin (RIF; >97% pure),
108 ciprofibrate (>99% pure), rosiglitazone (>99% pure), and mifepristone (RU-486; 98% pure)
109 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was
110 used as a vehicle and purchased from Wako, and all compounds used were dissolved in
111 DMSO at a concentration of $1 \times 10^{-2}$ M.

112 Dulbecco's modified Eagle's medium (D-MEM), and D-MEM plus Ham's F-12 nutrient
113 mixture (D-MEM/F-12) were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine
114 serum (FBS) and charcoal/dextran-treated FBS (CD-FBS) were obtained from Hyclone
115 (Logan, UT, USA). The penicillin-streptomycin solution (antibiotics) was obtained from
116 Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Bovine serum albumin (BSA) and
117 4-methylumbelliferyl-β-D-galactoside (4-MUG) were obtained from Sigma-Aldrich.

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

120 FBS and antibiotics, and COS-7 cells were maintained in D-MEM supplemented with 10%

121 FBS and antibiotics.

122 ***2.2. Plasmids***

123 The expression plasmids, pcDNAERα, pcDNAERβ, pZeoSV2AR, pSG5-GR,

124 pZeo-TR$\alpha_1$, pcDNA-RXRα, pcDNA-RARα, pSG5-hPXR, pcDNA-PPARα, and

125 pTransExpress-PPARγ1, encoding full-length receptor proteins, as well as the reporter

126 plasmids, pGL3-tkERE, pIND-ARE, pGRE-tk-Luc, pIND-TREpal, pXREM-3A4-Luc, and

127 pGL4-10xAOXluc2, were prepared as previously described (Kojima et al., 2009; 2011; 2013).

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

129 USA).

130 ***2.3. Reporter gene assays***

131 For detection of human ERα, ERβ, AR, GR, TR$\alpha_1$, RARα, and RXRα activity, we

132 plated the CHO-K1 cells in 96-well microtiter plates (Nalge, Nunc, Denmark) at a density of

133 8,400 cells per well in phenol red-free D-MEM/F-12 containing 5% CD-FBS (complete

134 medium) one day before transfection. COS-7 cells (8,400 cells/well) in D-MEM containing

135 10% CD-FBS were used as host cells for the detection of human PXR, PPARα, and PPARγ1

136 activity. These cells were transiently transfected with the receptor expression plasmid, the

137 reporter plasmid, and the internal control plasmid (pCMVβ-Gal) using the FuGENE 6

138 Transfection Reagent (Promega, Madison, WI, USA) (Kojima et al., 2009; 2011; 2013). After

139 a 3-hr transfection period, cells were dosed with various concentrations of the test compounds

140 or with 0.3% DMSO (vehicle control) in complete medium. To avoid any cytotoxic effects

141 associated with the test compounds, assays were performed for test compounds at
142 concentrations of less than $3 \times 10^{-5}$ M. After an incubation period of 24 hr, cells were rinsed
143 with phosphate-buffered saline (pH 7.4) and lysed with passive lysis buffer (50 μl/well;
144 Promega).

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

151 ***2.4. β-Galactosidase activity assay***

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

154 ***2.5. Evaluation of agonistic and antagonistic activities***

155 To estimate the potency of the receptor agonistic activities of the tested compounds, the
156 luminescence intensity of the assay was represented in a dose-response curve. The
157 concentration of the compound equal to 20% of the maximal response of the positive control
158 was obtained from the dose-response curve of the luminescence intensity and expressed as an
159 $REC_{20}$ value (20% relative effective concentration). The results for the receptor antagonistic
160 activities of each compound were expressed as $RIC_{20}$ values (20% relative inhibitory
161 concentration), which is the concentration of the test compounds showing 20% inhibition of

162 the activities induced by the endogenous hormones. These values can be compared to those of
163 the positive controls and other compounds described in previous our reports (Kojima et al.
164 2009; 2011; 2013).

165 ***2.6. Statistical Analysis***

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

170

171 **3. Results**

172 ***3.1. Agonistic activities of the OPFR-metabolites against ERα/β, AR, GR, and TR$\alpha_1$***

173 We found that HO-*m*-TPHP and HO-*p*-TPHP among the 12 OPFR-metabolites tested
174 induced ERα- and ERβ-mediated estrogenic activity of greater than 20% that of the
175 $E_2$–induced maximal activity. However, none of the 12 OPFR-metabolites showed AR, GR or
176 TR$\alpha_1$ agonistic activity in these assays (data not shown).

177 Figure 2A shows the dose-response curves of ERα agonistic activities for TPHP and four
178 TPHP-metabolites. HO-*m*-TPHP and HO-*p*-TPHP showed more potent ERα agonistic activity
179 than did their precursor TPHP, whereas DPHP and HO-DPHP did not show any ERα
180 agonistic activity. The order of the relative ERα agonistic activity of these compounds was
181 HO-*p*-TPHP > HO-*m*-TPHP > TPHP > DPHP = HO-DPHP. From a comparison of $REC_{20}$
182 values, the estrogenic activity via ERα of HO-*p*-TPHP, the most potent ERα agonist in this

183 study, was about 100,000-fold lower than that of $E_2$, but over 10-fold higher than that of

184 TPHP (Table 1).

185 Figure 2B shows the dose-response curves of ERβ agonistic activities for TPHP and four

186 TPHP-metabolites. These results indicated that HO-*m*-TPHP, HO-*p*-TPHP, and TPHP could

187 act as ERβ agonists while DPHP and HO-DPHP did not show any ERβ agonistic activity, as

188 also seen in above results for the ERα agonistic assay. A comparison of $REC_{20}$ values

189 revealed that the estrogenic activity via ERβ of HO-*p*-TPHP was about 57,000-fold lower

190 than that of $E_2$, but HO-*m*-TPHP and HO-*p*-TPHP induced about 2- and 20-fold higher

191 responses, respectively, than did TPHP (Table 1).

192

193 ***3.2. Antagonistic activities of the OPFR-metabolites against ERα/β, AR, GR, and TR$\alpha_1$***

194 We found that HO-*m*-TPHP and HO-*p*-TPHP among the 12 OPFR-metabolites tested had

195 antagonistic activity against ERβ, AR and GR. However, none of the 12 OPFR-metabolites

196 showed antagonistic activity against ERα or TRα1 (data not shown).

197 Figure 3a shows the dose-response curves of ERβ antagonistic activities for HO-*m*-TPHP,

198 HO-*p*-TPHP, TPHP and TAM, a well-known ER antagonist. HO-*m*-TPHP and HO-*p*-TPHP

199 inhibited ERβ-mediated estrogenic activity of greater than 20% that of the activity induced by

200 $1 \times 10^{-10}$ M $E_2$, while TPHP showed slight ERβ antagonistic activity of less than 20% that of

201 the activity induced by $1 \times 10^{-10}$ M $E_2$. Although HO-*m*-TPHP and HO-*p*-TPHP were ERβ

202 agonists, they could also act as ERβ antagonists at higher concentrations. As shown in Table 1,

203 the $RIC_{20}$ value for HO-*p*-TPHP, the most potent ERβ antagonist in this study, was $5.4 \times 10^{-6}$

204 M and its antiestrogenic activity via ERβ was 900-fold lower than that of TAM ($RIC_{20}$: $6.0 \times$

205 $10^{-9}$ M).

206 Figure 3b shows the AR antagonistic effects of HO-*m*-TPHP and HO-*p*-TPHP at high

207 concentrations as well as that of TPHP. A comparison of $RIC_{20}$ values showed that the

208 anti-androgenic activities of the hydroxylated TPHP-metabolites and TPHP were about

209 650-fold lower than that of HF, a known AR antagonist (Table 1).

210 Figure 3c shows that HO-*m*-TPHP and HO-*p*-TPHP as well as TPHP had weak inhibitory

211 effects on the GR-mediated transcriptional activity induced by $3 \times 10^{-8}$ M of HC. A

212 comparison of $RIC_{20}$ values revealed that the GR antagonistic activity of HO-*p*-TPHP, the

213 most potent GR antagonist in this study, was an estimated 75-fold lower than that of RU-486,

214 a known GR antagonist (Table 1).

215 Based on their β-galactocidase activity, except for TPHP, HO-*m*-TPHP and HO-*p*-TPHP,

216 which were slightly cytotoxic at a concentration of $3 \times 10^{-5}$ M, none of the six OPFRs or 12

217 OPFR-metabolites tested in this study showed any cytotoxic effects against CHO-K1 cells at

218 the doses tested ($\leq 3 \times 10^{-5}$ M).

219

220 ***3.3. Agonistic activities of the OPFR-metabolites against RARα, RXRα, PXR, and***

221 ***PPARα/γ1***

222 We found that HO-TBOEP, BBOEHEP, HO-*m*-TPHP and HO-*p*-TPHP among the 12

223 OPFR-metabolites possess PXR agonistic activity. However, none of the 12

224 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-*m*-TPHP and HO-*p*-TPHP showing PXR agonistic activity greater than 20% that of the maximum activity of RIF. The $REC_{20}$ 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-*m*-TPHP > TPHP > HO-*p*-TPHP > TNBP > HO-TBOEP > BBOEHEP. Comparisons of their $REC_{20}$ 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 PPARγ1 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 PPARγ1 assay. Although TPHP showed week PPARγ1 agonistic activity of about 15% that of the maximum response of rosiglitazone, the activities of the two hydroxylated TPHP metabolites (HO-*m*-TPHP and HO-*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 PPARγ1 agonistic activity.

Based on their β-galactosidase activity, except for HO-*m*-TPHP and HO-*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).

## 4. Discussion

246 Recently, environmental pollution by OPFRs has been of increasing concern in terms of
247 public health and the need for risk assessment (van der Veen and de Boer, 2012). Our previous
248 study provided evidence that several OPFRs show potential endocrine disruption activity via
249 human nuclear receptors (Kojima et al., 2013). In this study, to further elucidate the
250 endocrine-disrupting properties of OPFRs in humans, we characterized activities of 12
251 primary metabolites (Fig. 1) derived from TBOEP, TCIPP, TPHP, TCEP, TDCIPP, and TNBP
252 against 10 nuclear receptors using cell-based reporter gene assays with CHO-K1 and COS-7
253 cells. The results summarized in Table 1 reveal that two hydroxylated TPHP-metabolites
254 among the 12 compounds tested exhibited ERα/β and PXR agonistic activity as well as ERβ,
255 AR, and GR antagonistic activity, while two TBOEP-metabolites showed only PXR agonistic
256 activity. However, it was also found that none of the 12 OPFR-metabolites, together with the
257 11 OPFRs reported in our previous paper (Kojima et al., 2013), possess $TR\alpha_1$, RARα, RXRα,
258 or PPARα/γ activity.

259 The metabolization of TPHP by human liver microsomes has been reported to produce
260 several primary metabolites, HO-*p*-TPHP, DPHP and HO-DPHP, through the hydroxylation
261 and *O*-dealkylation of TPHP (Fig. 1) (Sasaki et al., 1984; Van den Eede et al., 2013a). Recent
262 studies have shown that *in vitro* metabolism of TPHP using chicken embryonic hepatocytes
263 induced the formation of HO-*m*-TPHP, together with DPHP and HO-*p*-TPHP (Su et al., 2014;
264 2015), although HO-*m*-TPHP has not been confirmed as a metabolite formed by human liver
265 enzymes. However, as HO-*m*-TPHP is present as an impurity/hydrolysis product of the flame
266 retardant resorcinol bis(diphenylphosphate) (RDP), humans could be directly exposed to this

267 compound (Ballesteros-Gomes et al., 2015). Several analytical studies have shown that DPHP
268 was frequently detected as a major metabolite of TPHP at levels in order of a few ng/ml in
269 human urine samples (Cooper et al., 2011; Meeker et al., 2013; Reemtsma et al., 2011;
270 Schindler et al., 2009;). In the assays of the 12 OPFR-metabolites against nuclear receptors,
271 HO-*m*-TPHP and HO-*p*-TPHP showed multiple ERα, ERβ, and PXR agonistic activities, as
272 well as ERβ, AR and GR antagonistic activity. In particular, HO-*p*-TPHP activated ERα and
273 ERβ at lower concentrations than did the parent, TPHP, and HO-*m*-TPHP (Figs. 3a and b).
274 This suggests that a *para*-substituted OH group connected to a benzene ring may be important
275 for the induction of high levels of estrogen activity, a notion also supported by several studies
276 reporting that hydroxylated polychlorinated biphenyls (HO-PCBs) with *para*-substituted
277 HO-groups possess the most potent estrogenic effects via ERα, followed by *meta*- and
278 *ortho*-substituted HO-PCBs (Arulmozhiraja et al., 2005; Kitamura et al., 2005). Additionally,
279 this result implies that the metabolism of TPHP to HO-*p*-TPHP and HO-*m*-TPHP by oxidation
280 enzymes enhances their endocrine-disrupting effects, such as estrogenic activity, suggesting
281 metabolic activation against ERs. Interestingly, we found that HO-*p*-TPHP and HO-*m*-TPHP
282 induce ERα- and ERβ-mediated transcriptional activities, but simultaneously inhibit ERβ-,
283 AR- and GR-mediated transcriptional activities (Table 1). Such an activity pattern in same the
284 assay systems using CHO-K1 cells is similar to those of 2',3',4',5'-tetrachlorobiphenyl-2-ol
285 (2'-HO-CB61) and 2',3,3',4',5'-pentachlorobiphenyl-4-ol (4'-HO-CB106) (Takeuchi et al.,
286 2011) and those of butylbenzyl phthalate (plasticizer) and the methoxychlor (insecticide)
287 metabolite, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) (Takeuchi et al., 2005).

288 These findings, together with those of the present study, indicate that chemicals showing such
289 pleiotropic effects may unexpectedly exist in the environment. Furthermore, hydroxylated
290 TPHP-metabolites also showed PXR agonistic activity similar to that of TPHP (Fig. 6). Thus,
291 HO-*p*-TPHP and HO-*m*-TPHP are thought to act as modulators against various nuclear
292 receptors. On the other hand, DPHP and HO-DPHP did not exhibit any receptor activity.
293 These results suggest that TPHP and hydroxylated TPHP-metabolites (triaryl phosphates)
294 have multiple and pleiotropic effects against nuclear receptors, whereas DPHP and HO-DPHP
295 (diaryl phosphates) were inactive against nuclear receptors. This difference might reflect the
296 fact that diaryl phosphates show increased hydrophilicity, an important factor in their
297 interactions with nuclear receptor, compared to triaryl phosphates.

298 TBOEP is an OPFR produced in large volumes and used in a broad range of applications,
299 and is detectable at high concentrations in indoor dust (Tajima et al., 2014; Van den Eede et al.,
300 2011). TBOEP-metabolizing studies using human liver preparations revealed the presence of
301 several primary metabolites, such as BBOEP, HO-TBOEP and BBOEHEP (Fig. 1) (Van den
302 Eede et al, 2013a; 2015a). BBOEHEP, in particular, is frequently detected in human urine
303 (Van den Eede et al., 2015b). Although our previous study showed that the parent TBOEP has
304 only PXR agonistic activity among various nuclear receptor assays (Kojima et al., 2013), we
305 found that HO-TBOEP and BBOEHEP exhibited weaker PXR agonistic activity than did
306 TBOEP, while an *O*-dealkylated metabolite, BBOEP, did not show any activity (Fig. 5). This
307 implies that TBOEP-OH and BBOEHEP are structurally similar to TBOEP, allowing them to
308 act as PXR activators, whereas the structural properties of BBOEP might lead to reduction in

309 its binding to PXR (Fig. 1).

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

322 The *in vitro* biotransformation of TCIPP, TCEP and TNBP by human liver preparations
323 showed that these compounds are predominately metabolized to BCIPP, BCEP and DNBP,
324 respectively (Van den Eede et al., 2013a). In this study, we also examined the nuclear receptor
325 activity of the dechlorinated and hydroxylated TCIPP-metabolite, BCIPHIPP. The results
326 revealed that these four metabolites did not exhibit any nuclear receptor activity, although
327 TCIPP is a PXR agonist, and TNBP is a PXR agonist as well as an AR and GR antagonist
328 (Kojima et al., 2013). These results suggest that the diester phosphates BCIPP, BCEP and
329 DNBP lose their activity against nuclear receptors as also seen in DPHP, BBOEP and BDCIPP.

330 These decreases in receptor activity commonly observed in the diester phosphates appear to
331 resemble those with regard to PXR agonistic activity observed in several oxon-forms of
332 phosphorus insecticides (Kojima et al., 2011). Thus, the metabolization of a triester phosphate
333 ($R_3$-P=O) to a diester phosphate ($R_2$-P(=O)-OH) by liver esterase increases the hydrophilicity
334 of the OPFRs, which might consequently lead to the loss of nuclear receptor activity.

335 We also found that none of the OPFRs or their metabolites possesses $TR\alpha_1$, $RAR\alpha$,
336 $RXR\alpha$, or $PPAR\alpha/\gamma$ activity. On the other hand, more recent studies have shown that TPHP
337 and other environmental chemicals, including brominated flame retardants, can act as $PPAR\gamma$
338 agonists to induce adipogenesis (Pillai et al., 2014; Fang et al, 2015a; 2015b). Based on
339 COS-7 reporter gene and BMS2 cell protein expression assays together with lipid
340 accumulation, Pillai et al. (2014) reported that FM550 and TPHP have $PPAR\gamma$ agonistic
341 activity. Fang et al. (2015b) showed that several of compounds in indoor dust can potentially
342 activate $PPAR\gamma1$ on the basis of a highly sensitive reporter assay with a human embryo kidney
343 293H cell line containing $PPAR\gamma1$ ligand-binding domain/GAL4 DNA-binding domain. In
344 this study, we showed that TPHP-metabolites had little $PPAR\gamma1$ agonistic activity, although
345 the parent TPHP showed $PPAR\gamma1$ activity at a level 15% that of the maximum activity of
346 rosiglitazone (Fig. 6). Our reporter assay in which a full-length $PPAR\gamma1$ protein was
347 expressed in COS-7 cells may be less sensitive by one order of magnitude than the assay
348 reported by Fang et al. (2015b), but is as sensitive as the assays reported in other studies
349 (Belcher et al., 2014; Pillai et al., 2014; Suzuki et al., 2013). Nevertheless, our findings
350 suggest that TPHP is a weak $PPAR\gamma1$ 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.

368 **References**

369 Abdallah, M.A., Zhang, J., Pawar, G., Viant, M.R., Chipman, J.K., D'Silva, K., Bromirski, M.,
370 Harrad, S. 2015. High-resolution mass spectrometry provides novel insights into products
371 of human metabolism of organophosphate and brominated flame retardants. Anal. Bioanal.
372 Chem. 407, 1871-1883.

373 Ali, N., Van den Eede, N., Dirtu, A.C., Neels, H., Covaci, A., 2012. Assessment of human
374 exposure to indoor organic contaminants via dust ingestion in Pakistan. Indoor Air 22,
375 200-211.

376 Arulmozhiraja, S., Shiraishi, F., Okumura, T., Iida, M., Takigami, H., Edmonds, J.S., Morita,
377 M., 2005. Structural requirements for the interaction of 91 hydroxylated polychlorinated
378 biphenyls with estrogen and thyroid hormone receptors. Toxicol. Sci. 84, 49-62.

379 Ballesteros-Gómez, A., Van den Eede, N., Covaci, A., 2015. In vitro human metabolism of the
380 flame retardant resorcinol bis(diphenylphosphate) (RDP). Environ. Sci. Techol. 49,
381 3897-3904.

382 Belcher, S.M., Cookman, C.J., Patisaul, H.B., Stapleton, H.M., 2014. In vitro assessment of
383 human nuclear hormone receptor activity and cytotoxicity of the flame retardant mixture
384 FM 550 and its triarylphosphate and brominated components. Toxicol. Lett. 228, 93-102.

385 Butt, C.M., Congleton, J., Hoffman, K., Fang, M., Stapleton, H.M., 2014. Metabolites of
386 organophosphate flame retardants and 2-ethylhexyl tetrabromobenzoate in urine from
387 paired mothers and toddlers. Environ. Sci. Tecnol. 48, 10432-10438.

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

389 W., Stapleton, H.M., Webster, T.F., 2013. Predictors of tris(1,3-dichloro-2-propyl)
390 phosphate metabolite in the urine of office workers. Environ. Int. 55, 56-61.
391 Cooper, E.M., Covaci, A., van Nuijs, A.L., Webster, T.F., Stapleton, H.M., 2011. Analysis of
392 the flame retardant metabolites bis(1,3-dichloro-2-propyl) phosphate (BDCPP) and
393 diphenyl phosphate (DPP) in urine using liquid chromatography-tandem mass spectrometry.
394 Anal. Bioanal. Chem. 401, 2123-2132.
395 Dodson, R.E., Van den Eede, N., Covaci, A., Perovich, L.J., Brody, J.G., Rudel, R.A., 2014.
396 Urinary biomonitoring of phosphate flame retardants: Levels in California adults and
397 recommendations for future studies. Environ. Sci. Technol. 48, 13625-13633.
398 Fang, M., Webster, T.F., Ferguson, P.L., Stapleton, H.M., 2015a. Characterizing the
399 peroxisome proliferator-activated receptor (PPARγ) ligand binding potential of several
400 major flame retardants, their metabolites, and chemical mixtures in house dust. Environ.
401 Health Perspect. 123, 166-172.
402 Fang, M., Webster, T.F., Stapleton, H.M., 2015b. Activation of human peroxisome
403 proliferator-activated nuclear receptors (PPARγ1) by semi-volatile compounds (SVOCs)
404 and chemical mixtures in indoor dust. Environ. Sci. Technol. 49, 10057-10064.
405 Hartmann, P.C., Burgi, D., Giger, W., 2004. Organophosphate flame retardants and
406 plasticizers in indoor air. Chemosphere 57, 781-787.
407 Hoffman, K., Daniels, J.L., Stapleton, H.M., 2014. Urinary metabolites of organophosphate
408 flame retardants and their variability in pregnant women. Environ. Int. 63, 169-172.
409 Kitamura, S., Jinno, N., Suzuki, T., Sugihara, K., Ohta, S., Kuroki, H., Fujimoto, N., 2005.

410 Thyroid hormone-like and estrogenic activity of hydroxylated PCBs in cell culture.
411 Toxicology 208, 377-387.
412 Kojima, H., Sata, F., Takeuchi, S., Sueyoshi, T., Nagai, T., 2011. Comparative study of human
413 and mouse pregnane X receptor agonistic activity in 200 pesticides using *in vitro* reporter
414 gene assays. Toxicology 280, 77-87.
415 Kojima, H., Takeuchi, S., Itoh, T., Iida, M., Kobayashi, S., Yoshida, T., 2013. In vitro
416 endocrine disruption potential of organophosphate flame retardants via human nuclear
417 receptors. Toxicology 314, 76-83.
418 Kojima, H., Takeuchi, S., Uramaru, N., Sugihara, K., Yoshida, T., Kitamura, S., 2009. Nuclear
419 hormone receptor activity of polybrominated diphenyl ethers and their hydroxylated and
420 methoxylated metabolites in transactivation assays using Chinese hamster ovary cells.
421 Environ. Health Perspect. 117, 1210-1218.
422 Lynn, R.K., Wong, K., Garviegould, C., Kennish, J.M., 1981. Disposition of the
423 flame-retardant, tris(1,3-dichloro-2-propyl) phosphate, in the rat. Drug Metab. Dispos. 9,
424 434-441.
425 Marklund, A., Andersson, B., Haglund, P., 2003. Screening of organophosphorus compounds
426 and their distribution in various indoor environments. Chemosphere 53, 1137-1146.
427 McKenna, N.J., Lanz, R.B., O'Malley, B.W., 1999. Nuclear receptor coregulators: cellular
428 and molecular biology. Endocr. Rev. 20, 321-344.
429 Meeker, J.D., Cooper, E.M., Stapleton, H.M., Hauser, R., 2013. Urinary metabolites of
430 organophosphate flame retardants: temporal variability and correlations with house dust

431 concentrations. Environ. Health Perspect. 121, 580-585.

432 Meeker, J.D., Stapleton, H.M., 2010. House dust concentrations of organophosphate flame
433 retardants in relation to hormone levels and semen quality parameters. Environ. Health
434 Perspect. 118, 318-323.

435 Nomeir, A.A., Kato, S., Matthews, H.B., 1981. The metabolism and disposition of
436 tris(1,3-dichloro-2-propyl) phosphate (Fyrol FR-2) in the rat. Toxicol. Appl. Pharmacol. 57,
437 401-413.

438 Pillai, H.K., Fang, M., Beglov, D., Kozakov, D., Vajda, S., Stapleton, H.M., Webster, T.F.,
439 Schlezinger, J.J., 2014. Ligand binding and activation of PPARγ by Firemaster® 550:
440 effects on adipogenesis and osteogenesis in vitro. Environ. Health Perspect. 122,
441 1225-1232.

442 Reemtsma, T., Quintana, J.B., Rodil, R., Garcia-Lopez, M., Rodriguez, I., 2008.
443 Organophosphorus flame retardants and plasticizers in water and air I. Occurrence and fate.
444 Trends Anal. Chem. 27, 727-737.

445 Reemtsma, T., Lingott, J., Roegler, S., 2011. Determination of 14 monoalkyl phosphates,
446 dialkyl phosphates and dialkyl thiophosphates by LC-MS/MS in human urinary samples.
447 Sci. Total Environ. 409, 1990-1993.

448 Sasaki, K., Suzuki, T., Takeda, M., Uchiyama, M., 1984. Metabolism of phosphoric acid
449 triester by rat liver homogenate. Bull. Environ. Contam. Toxicol. 33, 281-288.

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

452 chromatography-tandem mass spectrometry. J. Chromatogr. B 877, 375-381.
453 Stapleton, H.M., Klosterhaus, S., Eagle, S., Fuh, J., Meeker, J.D., Blum, A., Webster, T.F.,
454 2009. Detection of organophosphate flame retardants in furniture foam and US house dust.
455 Environ. Sci. Technol. 43, 7490-7495.
456 Stapleton, H.M., Sharma, S., Getzinger, G., Ferguson, P.L., Gabriel, M., Webster, T.F., et al.
457 2012. Novel and high volume use flame retardants in US couches reflective of the 2005
458 PentaBDE phase out. Environ. Sci. Technol. 46, 13432-13439.
459 Su, G., Crump, D., Letcher, R.J., Kennedy, S.W., 2014. Rapid in vitro metabolism of the flame
460 retardant triphenyl phosphate and effects on cytotoxicity and mRNA expression in chicken
461 embryonic hepatocytes. Environ. Sci. Technol. 48, 13511–13519.
462 Su, G., Letcher, R.J., Crump, D., Gooden, D.M., Stapleton, H.M., 2015. In vitro metabolism
463 of the flame retardant triphenyl phosphate in chicken embryonic hepatocytes and the
464 importance of the hydroxylation pathway. Environ. Sci. Technol. Lett. 2, 100-104.
465 Suzuki, G., Tue, N.M., Malarvannan, G., Sudaryanto, A., Takahashi, S., Tanabe, S., Sakai, S.,
466 Brouwer, A., Uramaru, N., Kitamura, S., Takigami, H., 2013. Similarities in the
467 endocrine-disrupting potencies of indoor dust and flame retardants by using human
468 osteosarcoma (U2OS) cell-based reporter gene assays. Environ. Sci. Technol. 47,
469 2898-2908.
470 Tajima, S., Araki, A., Kawai, T., Tsuboi, T., Ait Bamai, Y., Yoshioka, E., Kanazawa, A., Cong,
471 S., Kishi, R., 2014. Detection and intake assessment of organophosphate flame retardants in
472 house dust in Japanese dwellings. Sci. Total Environ. 478, 190-199.

473 Takeuchi, S., Iida, M., Kobayashi, S., Jin, K., Matsuda, T., Kojima, H., 2005. Differential
474 effects of phthalate esters on transcriptional activities via human estrogen receptors α and β,
475 and androgen receptor. Toxicology 210, 223-233.
476 Takeuchi, S., Shiraishi, F., Kitamura, S., Kuroki, H., Jin, K., Kojima, H., 2011.
477 Characterization of steroid hormone receptor activities in 100 hydroxylated polychlorinated
478 biphenyls, including congeners identified in humans. Toxicology 289, 112-121.
479 Takigami, H., Suzuki, G., Hirai, Y., Ishikawa, Y., Sunami, M., Sakai, S., 2009. Flame
480 retardants in indoor dust and air of a hotel in Japan. Environ. Int. 35, 688-693.
481 Van den Eede, N., Dirtu, A.C., Neels, H., Covaci, A., 2011. Analytical developments and
482 preliminary assessment of human exposure to organophosphate flame retardants from
483 indoor dust. Environ Int. 37, 454-61.
484 Van den Eede, N., Erratico, C., Exarchou, V., Maho, W., Neels, H., Covaci, A., 2015a. In vitro
485 biotransformation of tris(2-butoxyethyl) phosphate (TBOEP) in human liver and serum.
486 Toxicol. Appl. Pharmacol. 284, 246-253.
487 Van den Eede, N., Heffernan, A.L., Aylward, L.L., Hobson, P., Neels, H., Mueller, J.F., Covaci,
488 A., 2015b. Age as a determinant of phosphate flame retardant exposure of the Australian
489 population and identification of novel urinary PFR metabolites. Environ. Int. 74, 1-8.
490 Van den Eede, N., Maho, W., Erratico, C., Neels, H., Covaci, A., 2013a. First insights in the
491 metabolism of phosphate flame retardants and plasticizers using human liver fractions.
492 Toxicol. Lett. 223, 9-15.
493 Van den Eede, N., Neels, H., Jorens, P.G., Covaci, A., 2013b. Analysis of organophosphate

494 flame retardant diester metabolites in human urine by liquid chromatography electrospray
495 ionisation tandem mass spectrometry. J. Chromatogr. A 1303, 48-53.
496 van der Veen, I., de Boer, J., 2012. Phosphorus flame retardants: Properties, production,
497 environmental occurrence, toxicity and analysis. Chemoshere 88, 1119-1153.
498 WHO, 1998. Flame retardants: tris(chloropropyl) phosphate and tris(2-chloroethyl) phosphate.
499 Environmental Health Criteria. World Health Organization, Geneva, pp. 209.
500 WHO, 2000. Flame retardants: tris(2-butoxyethyl) phosphate and tris(2-ethylhexyl) phosphate.
501 Environmental Health Criteria. World Health Organization, Geneva, pp. 218.
502

503 **Figure captions**

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

505 *HO-*m*-TPHP was confirmed as a metabolite of TPHP in chicken embryonic hepatocytes (Su

506 et al., 2015), but not in *in vitro* metabolizing study using human liver preparations.

507

508 **Fig. 2.** Estrogenic effects of TPHP and its metabolites in the hERα and hERβ transactivation

509 assays. CHO cells were transiently transfected with an expression plasmid for human ERα (a)

510 or ERβ (b) as well as a reporter-responsive *firefly* luciferase plasmid and a constitutively

511 active β-galactosidase expression plasmid. Cells were treated with $1 \times 10^{-7}$ to $3 \times 10^{-5}$ M of

512 TPHP or its metabolites. The *firefly* luciferase activity was normalized based on the

513 β-galactosidase activity. Values represent the means ± SD of three independent experiments

514 and are presented as the percentage of the response, taking the activity obtained using $1 \times 10^{-9}$

515 M $E_2$ as 100%.

516

517 **Fig. 3.** Antagonistic activities of TPHP-hydroxylated metabolites against hERβ, hAR and

518 hGR. CHO cells were transiently transfected with an expression plasmid for human ERβ (a),

519 AR (b) or GR (c) as well as a reporter-responsive *firefly* luciferase plasmid and a

520 constitutively active β-galactosidase expression plasmid. In ERβ antagonist assay (a), cells

521 were treated with $1 \times 10^{-7}$ M to $3 \times 10^{-5}$ M of TPHP or its hydroxylated metabolites, or with 1

522 $\times 10^{-9}$ M to $1 \times 10^{-7}$ M of tamoxifen (TAM) in the presence of $1 \times 10^{-10}$ M $E_2$ to detect ERβ

523 antagonistic activity. In AR antagonist assay (b), cells were treated with $1 \times 10^{-7}$ M to $3 \times 10^{-5}$

524 M of TPHP or its hydroxylated metabolites, or with 1 × $10^{-9}$ M to 1 × $10^{-7}$ M of

525 hydroxyflutamide (HF) in the presence of 1 × $10^{-10}$ M DHT to detect AR antagonistic activity.

526 In GR antagonist assay (c), cells were treated with 1 × $10^{-7}$ M to 3 × $10^{-5}$ M of TPHP or its

527 hydroxylated metabolites, or with 1 × $10^{-8}$ M to 1 × $10^{-6}$ M of RU-486 in the presence of 3 ×

528 $10^{-8}$ M HC to detect GR antagonistic activity. The *firefly* luciferase activity was normalized

529 based on the β-galactosidase activity. Values represent the means ± SD of three independent

530 experiments and are presented as the percentage of the response, taking the activity obtained

531 using 1 × $10^{-10}$ M $E_2$ (a), 1 × $10^{-10}$ M DHT (b) or 3 × $10^{-8}$ M HC (c) as 100%.

532 *Significant difference ($p<0.05$; ANOVA) from 1 × $10^{-10}$ M $E_2$ (a), 1 × $10^{-10}$ M DHT (b) or 3

533 × $10^{-8}$ M HC (c) alone.

534

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

536 COS-7 cells were transiently transfected with an expression plasmid for human PXR as well

537 as a reporter-responsive *firefly* luciferase plasmid and a constitutively active β-galactosidase

538 expression plasmid. Cells were treated with 1 × $10^{-6}$ M to 3 × $10^{-5}$ M of OPFRs, their

539 metabolites, or rifampicin (RF) to detect PXR agonistic activity. The *firefly* luciferase activity

540 was normalized based on the β-galactosidase activity. Values represent the means ± SD of

541 three independent experiments and are presented as the percentage of the response, taking the

542 activity obtained using 1 × $10^{-5}$ M RF as 100%.

543

544 **Fig. 5.** Effects of TPHP and its 4 metabolites on transcriptional activity via hPPARγ. COS-7

545 cells were transiently transfected with an expression plasmid for human PPARγ as well as a

546 reporter-responsive *firefly* luciferase plasmid and a constitutively active β-galactosidase

547 expression plasmid. Cells were treated with $1 \times 10^{-8}$ M to $3 \times 10^{-5}$ M of TPHP or its 4

548 metabolites, or with $1 \times 10^{-9}$ M to $1 \times 10^{-5}$ M of rosiglitazone to detect PPARγ agonistic

549 activity. The *firefly* luciferase activity was normalized based on the β-galactosidase activity.

550 Values represent the means ± SD of three independent experiments and are presented as the

551 percentage of the response, taking the activity obtained using $1 \times 10^{-6}$ M rosiglitazone as

552 100%.