

# Inhibition of Human Liver Carboxylesterase (hCE1) by Organophosphate Ester Flame Retardants and Plasticizers: Implications for Pharmacotherapy

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## ABSTRACT

Organophosphate ester (OPE) flame retardants and plasticizers, consumer product additives with widespread human exposure, were evaluated for their effect on the activity of purified human liver carboxylesterase (hCE1). Four of the 15 OPEs tested had  $IC_{50}$  values lower than 100 nM, including triphenyl phosphate (TPHP), 2-ethylhexyl diphenyl phosphate (EHDPHP), 4-isopropylphenyl diphenyl phosphate (4IPDP), and 4-*tert*-butylphenyl diphenyl phosphate (4tBPDPP), as did 4 of the commercial flame retardant mixtures tested. Because hCE1 is critical for the activation of imidapril, an angiotensin-converting enzyme-inhibitor prodrug prescribed to treat hypertension, the most potent inhibitors, TPHP and 4tBPDPP, and an environmentally relevant mixture (house dust) were further evaluated for their effect on imidapril bioactivation *in vitro*. TPHP and 4tBPDPP were potent inhibitors of hCE1-mediated imidapril activation ( $K_i = 49.0$  and  $17.9$  nM, respectively). House dust extracts (100  $\mu$ g/ml) also caused significant reductions (up to 33%) in imidapril activation. Combined, these data suggest that exposure to OPEs may affect pharmacotherapy.

**Key words:** organophosphate ester; flame retardant; carboxylesterase; house dust; enzyme inhibition.

Current evidence suggests that human exposure to isopropylated and *tert*-butylated triarylphosphate esters (ITPs and TBPPs) is common; however, there are very limited data available on their fate and effects in the human body (Behl *et al.*, 2015; Hammel *et al.*, 2016). ITP and TBPP isomers are used as plasticizers in polymers and as flame retardants in polyurethane foam commonly applied to residential furniture (McGee *et al.*, 2013; Phillips *et al.*, 2017). Toxicology and epidemiological studies have demonstrated potential hazards associated with ITP exposure, ranging from developmental neurotoxicity in zebrafish assays to the reduced probability of successful fertilization, implantation, clinical pregnancy, and live birth in humans (Carignan *et al.*, 2017; Jarema *et al.*, 2015). Indoor house dust is a known conduit of exposure for flame retardants, and organophosphate esters (OPEs), like ITPs and TBPPs, are commonly detected at high levels ( $\mu$ g/g) in this matrix (Hoffman *et al.*, 2015; Phillips *et al.*, 2018). Due to their widespread exposure and potentially toxic properties, the U.S. EPA has prioritized their risk assessment under the Frank R. Lautenberg

Chemical Safety for the 21st Century Act, with a statutory deadline for proposed action by June 22, 2019 (U.S. EPA, 2016).

Although ITPs and TBPPs are structurally similar to organophosphate pesticides, OPEs have not been found to act as potent acetylcholinesterase inhibitors (Eldefrawi *et al.*, 1977). However, multiple studies have shown that triphenyl phosphate (TPHP), an organophosphate chemical with an analogous structure to ITPs and TBPPs, inhibits mammalian liver carboxylesterase enzymes (Ces) (Brandt *et al.*, 1980; Morris *et al.*, 2014). For instance, a recent study found that mice treated with an intraperitoneal injection of 100 mg/kg TPHP had diminished hepatic Ces activity (43% decrease) compared to untreated controls 4 h following administration (Morris *et al.*, 2014). Another study conducted in our laboratory found that rats dosed orally with 1000  $\mu$ g/day Firemaster 550, an ITP and TPHP-containing flame retardant mixture, had significantly reduced hepatic carboxylesterase activity (62% decrease) compared to controls (Patisaul *et al.*, 2013). Previous studies have indicated that increasing hydrophobicity is positively correlated with Ces inhibitor potency,



below 1%. The nominal concentration of each dosing stock was quantified via gas chromatography mass spectrometry (GC/MS) using previously published methods (Phillips et al., 2017; Stapleton et al., 2009).

**Screening for hCE1 inhibition.** Prior to screening experiments, the specific activity of hCE1 (16 µg/ml) for PNPA (1 mM) was evaluated. The observed specific activity agreed well with the value reported by the manufacturer and appeared to be linear for a 25-min incubation period (Supplementary Figure 1). Each chemical and commercial mixture was then individually evaluated for their ability to inhibit hCE1 (16 µg/ml) at a range of concentrations (0.1 nM–100 µM). Total hCE1 activity was determined by measuring the formation of *p*-nitrophenol (PNP) at 405 nm from the hydrolysis of *p*-nitrophenyl acetate (1 mM) (Ross and Borazjani, 2007). Following 5-min incubations in phosphate buffer (100 mM, pH 7.4) at 37°C, an equal volume of ice-cold acetonitrile was used to stop the reaction and total carboxylesterase activity was measured. Bis-*p*-nitrophenyl phosphate (BNPP), a known Ces inhibitor, was included as a positive control. Experiments included 3 replicates per plate and were run in triplicate. Results were corrected for spontaneous hydrolysis using controls containing PNPA but no hCE1, and inhibition curves were generated.

**Effect on hCE1-mediated imidapril activation.** Due to their widespread exposure and potency in screening assays, TPHP and 4tBPDP were also tested for their effect on the bioactivation of imidapril. An LC-MS/MS method to quantify imidapril and imidaprilat was created based on the one described by Mabuchi et al. (1999). More information on this method can be found in the Supplementary Table 1. Initial range-finding experiments were performed to determine incubation duration and which concentrations of imidapril and hCE1 should be used to produce detectable amounts of imidaprilat (Supplementary Figure 2). Preliminary experiments were also conducted to establish a mass balance between imidapril and imidaprilat after the incubation of imidapril with hCE1 (Supplementary Figure 3). Following 150-min hCE1 (160 µg/ml) incubations with 10 µM imidapril and either TPHP or 4tBPDP (0.1 nM–100 µM), an equal volume of ice-cold acetonitrile containing d3-imidapril and d5-imidaprilat was used to terminate the reaction. The incubation mixture was then cleaned using Costar Spin-X centrifuge filters (nylon membrane, pore size 0.22 µm, Corning). Extracts were transferred to autosampler vials, imidaprilat formation was monitored via LC-MS/MS, and inhibition curves were generated. Spike/recovery experiments were performed to determine the recovery of imidapril and imidaprilat following clean up (see Supplementary Material). In addition to TPHP and 4tBPDP, house dust sample extracts ( $n = 20$ ; collected in 2014–2016) were tested for their effect on imidapril activation at 10 and 100 µg/ml doses. TPHP and 4tBPDP concentrations in dust extracts were previously quantified and their associations with the inhibition of imidapril activation were assessed (Phillips et al., 2018). All imidapril activation experiments were run in duplicate.

**Kinetic and mode of inhibition experiments.** Michaelis-Menten kinetic parameters were also determined using a range of imidapril concentrations (1 µM–2.5 mM) in 60-min incubations with 160 µg/ml hCE1. Mode of inhibition experiments were carried out for TPHP (30, 60, and 200 nM) and 4tBPDP (10, 20, and 75 nM) at a range of imidapril concentrations (10–500 µM). After the addition of d3-imidapril and d5-imidaprilat in an equal volume of acetonitrile, samples were cleaned using the centrifuge filter method described above. Imidaprilat formation was quantified via LC-MS/MS.

**Statistical analyses.** All IC<sub>50</sub> values were calculated using a 3-parameter nonlinear model in JMP Pro (version 11; SAS Institute Inc, Cary, North Carolina) (Roberts et al., 2015). IC<sub>50</sub> values presented represent mean ± standard error. All analyses involving house dust extracts were conducted using SAS statistical software (version 9.4; SAS Institute Inc). One-factor ANOVA indicated a significant effect of dose ( $p < .01$ ). Significant effects were further tested using Tukey's post hoc test. Imidapril activation was log-transformed before statistical analysis. Preliminary analyses indicated that OPE concentrations in dust, as well as the percent control imidapril activation were normally-distributed. Accordingly, Pearson correlations were used to assess relationships between these 2 parameters. The  $K_m$  and  $V_{max}$  values and inhibition types were determined by fitting data to a competitive, noncompetitive, uncompetitive, or mixed inhibition model by nonlinear regression analysis using GraphPad Prism (version 7; GraphPad Software, Inc, San Diego, California) (Fukami et al., 2010). The  $K_m$  and  $V_{max}$  values depict mean ± standard error. Statistical significance was set to  $\alpha = .05$ . More information regarding model equations used for data analysis can be found in the Supplementary Material.

## RESULTS

### Dosing Stock Confirmation

Test chemicals were dissolved in methanol, and the final organic concentration was kept below 1% in all assays. All nominal dosing stock concentrations were within 10% of measured concentrations, as measured by GC/MS. Nominal concentrations were adjusted according to measured concentrations for data analysis (Supplementary Table 2).

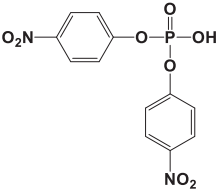
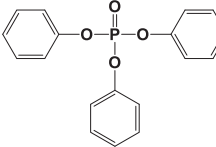
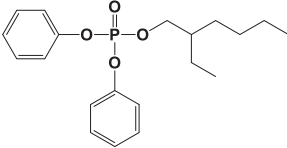
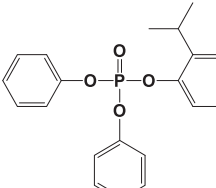
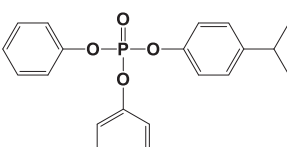
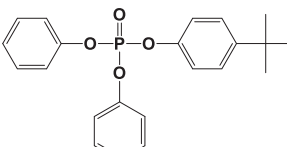
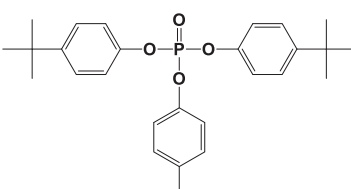
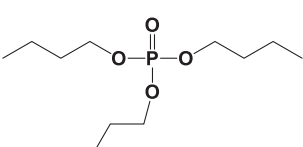
### Screening for hCE1 Inhibition

The inhibitory effects of various OPE flame retardants and related commercial mixtures on total hCE1 activity were screened in an absorbance-based assay that measured *p*-nitrophenol formation from *p*-nitrophenyl acetate hydrolysis (Ross and Borazjani, 2007). BNPP, a known carboxylesterase inhibitor, was included as a positive control for hCE1 inhibition. The measured IC<sub>50</sub> value for BNPP in our assay, 69.3 ± 9.4 nM, agreed well with the IC<sub>50</sub> value reported by Corning (24 nM), the enzyme's manufacturer (Wang et al., 2011). IC<sub>50</sub> values for the test chemicals and commercial mixtures ranged from 22.3 nM to > 90 400 nM. The TBPP commercial flame retardant mixture had the lowest IC<sub>50</sub> value (17.6 ± 6.9 nM), and FM 550, K 50, the ITP Mix, TPHP, EHDPP, 4IPDP, 4tBPDP all had IC<sub>50</sub> values lower than 100 nM (Table 1).

### Imidaprilat Formation Kinetics

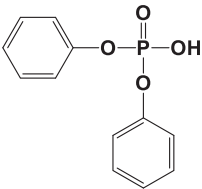
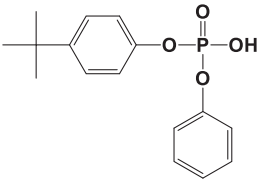
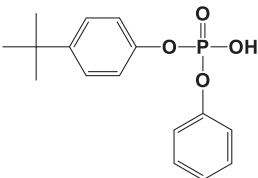
The kinetics of imidaprilat formation was investigated using purified hCE1. Imidaprilat formation showed typical Michaelis-Menten enzyme kinetics, and the model fit was excellent ( $r^2 = .993$ ). The apparent  $K_m$  for the formation of imidaprilat from imidapril was determined to be 113.0 ± 8.2 µM, and the  $V_{max}$  was estimated to be 2.01 nmol/min/mg hCE1 (Figure 2). Although these values were not identical to previously-reported values obtained using recombinant enzyme expressed in Sf21 cells ( $K_m = 3.2 \pm 8.2$  mM,  $V_{max} = 24.5$  nmol/mg/min), their semblance was deemed acceptable due to the difference in enzyme source (Fukami et al., 2010). The interday variation was assessed by performing the assay on 2 separate days ( $n = 2$  per day) using optimized conditions (100 µM imidapril and 160 µg/ml hCE1 for a 60-min incubation). The results showed no statistical difference among the 2 days (ANOVA;  $p > .05$ ).

**Table 1.** hCE1b IC<sub>50</sub> Values of ITP and TBPP Isomers and Related FR Mixtures

Test Chemical	Structure or Description	Presence in FR Mixture <sup>a</sup>	IC <sub>50</sub> Value (nM) <sup>b</sup>	R <sup>2</sup> of Model
BNPP		NA	69.3 ± 9.4 <sup>c</sup>	0.998
TPHP		19.8% in FM 550 44.6% in ITP Mix 1.7% in FM 600 24.0% in TBPP Mix	65.4 ± 8.3	0.993
EHDPPH		NA	99.4 ± 65.6	0.975
FM 550	EH-TBB, BEH-TEBP and ITP mixture	NA	31.2 ± 2.9	0.999
BZ-54	EH-TBB and BEH-TEBP only	NA	> 35 000	NA
ITP Mix	ITP mixture	NA	28.0 ± 15.4	0.979
K 50	ITP mixture	NA	27.6 ± 16.9	0.978
2IPDPDP		11.8% in FM 550 26.9% in ITP Mix	574 ± 127	0.996
4IPDPDP		2.3% in FM 550 4.9% in ITP Mix	49.9 ± 21.9	0.984
TBPP Mix	TBPP mixture	NA	17.9 ± 6.9	0.988
4tBPDPDP		2.6% in FM 600 35.5% in TBPP Mix	22.3 ± 6.9	0.977
T4tBPP		22.0% in FM 600 2.5% in TBPP Mix	1740 ± 1010	0.979
TBP		NA	> 19 000	NA

(continued)

Table 1. (continued)

Test Chemical	Structure or Description	Presence in FR Mixture <sup>a</sup>	IC <sub>50</sub> Value (nM) <sup>b</sup>	R <sup>2</sup> of Model
DPHP		NA	> 90 400	NA
ip-PPP		NA	3420 ± 1620	0.985
tb-PPP		NA	4820 ± 2690	0.979

Abbreviations: hCE1b, human hepatic carboxylesterase; ITP and TBPP, isopropylated and *tert*-butylated triarylphosphate esters; FR, flame retardant; BNPP, bis(4-nitrophenyl) phosphate; TPHP, triphenyl phosphate; EHDPHP, 2-ethylhexyl diphenyl phosphate; FM 550, Firemaster 550; BZ-54, Firemaster BZ-54; ITP, isopropylated triaryl phosphate commercial mixture; 2IPDPDP, 2-isopropylphenyl diphenyl phosphate; 4IPDPDP, 4-isopropylphenyl diphenyl phosphate; TBPP, *tert*-butylated triarylphosphate commercial mixture; 4tBPDPP, 4-*tert*-butylphenyl diphenyl phosphate; T4tBPP, tris(4-*tert*-butylphenyl) phosphate; TBP, tributyl phosphate; DPHP, diphenyl phosphate; ip-PPP, isopropylated phenyl phenylphosphate; tb-PPP, *tert*-butylated phenyl phenylphosphate; NA, not applicable.

<sup>a</sup>Percent composition is expressed on a wt/wt basis (Phillips et al., 2017).

<sup>b</sup>IC<sub>50</sub> values were calculated using a 3 parameter nonlinear model in JMP Pro 11 (Roberts et al., 2015). More information regarding the model equation can be found in the [Supplementary Material](#).

<sup>c</sup>This value is comparable to the IC<sub>50</sub> value determined by the manufacturer (24 nM) (Wang et al., 2011).

#### Effect of TPHP and 4tBPDPP on hCE1-mediated Imidapril Activation

The inhibitory effects on imidapril hydrolase activity by hCE1 were investigated using TPHP and 4tBPDPP. TPHP was chosen due to its ubiquity in the indoor environment and 4tBPDPP was chosen due to its potency in the screening assay. TPHP and 4tBPDPP were dissolved in methanol (< 1% vol/vol), and inhibition was calculated as percent inhibition of the control activity (ie, imidaprilat formed without presence of inhibitors).

TPHP and 4tBPDPP were found to be potent inhibitors of imidapril activation (IC<sub>50</sub> = 57.7 and 15.8 nM, respectively) (Figure 3).

#### Mode of Inhibition of TPHP and 4tBPDPP on Imidapril Activation

The type of inhibition (competitive, noncompetitive, uncompetitive, or mixed inhibition) was investigated by calculating the Michaelis-Menten parameters ( $V_{max}$  and  $K_m$  values) using various concentrations of the OPE inhibitor (either TPHP or 4tBPDPP) (Figure 4). The  $V_{max}$  rate was statistically lower with both increasing TPHP and 4tBPDPP concentrations but the apparent  $K_m$  did not significantly vary with inhibitor concentration. For example, the maximal rate of imidaprilat formation decreased from 2.14 to 0.32 nmol/mg/min upon addition of 200 nM TPHP, although the  $K_m$  did not change significantly (98.9 vs 115.1  $\mu$ M,  $p > .05$ ). Similarly, the  $K_m$  did not vary significantly upon the addition of 30 nM 4tBPDPP (85.8 vs 109.3  $\mu$ M,  $p > .05$ ) but the maximal rate of imidaprilat formation decreased from 2.28 to 0.57. These observations in enzymatic constants are indicative of noncompetitive inhibition.

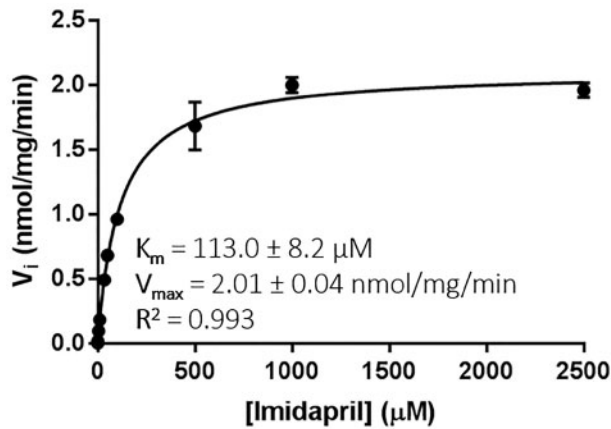
#### Effect of House Dust Extracts on hCE1-mediated Imidapril Activation

To evaluate potential inhibition with environmentally relevant mixtures, imidapril was incubated with hCE1 in the presence of 2 doses (10 and 100  $\mu$ g/ml) of individual house dust extracts ( $n = 20$  per dose). Although limited hCE1b inhibition was observed by dust extracts at 10  $\mu$ g/ml, significant inhibition (reductions of up to 33%) was observed for 10 of the 20 extracts tested at 100  $\mu$ g/ml. Both doses tested were environmentally relevant concentrations (Figure 5). The interday variation was assessed by performing the assay on 2 separate days ( $n = 2$  per day) using optimized conditions (10  $\mu$ M imidapril and 160  $\mu$ g/ml hCE1 for a 150-min incubation). The results showed no statistical difference among the 2 days (ANOVA;  $p > .05$ ). Percent inhibition was negatively correlated with both  $\log_{10}$ TPHP and  $\log_{10}$ 4tBPDPP concentrations measured in the dust using mass spectrometry ( $r_p = -.65$  and  $r_p = -.49$ ,  $p < .05$ ), suggesting that a large proportion of the observed hCE1 inhibition from house dust was driven by these chemicals (Figure 6).

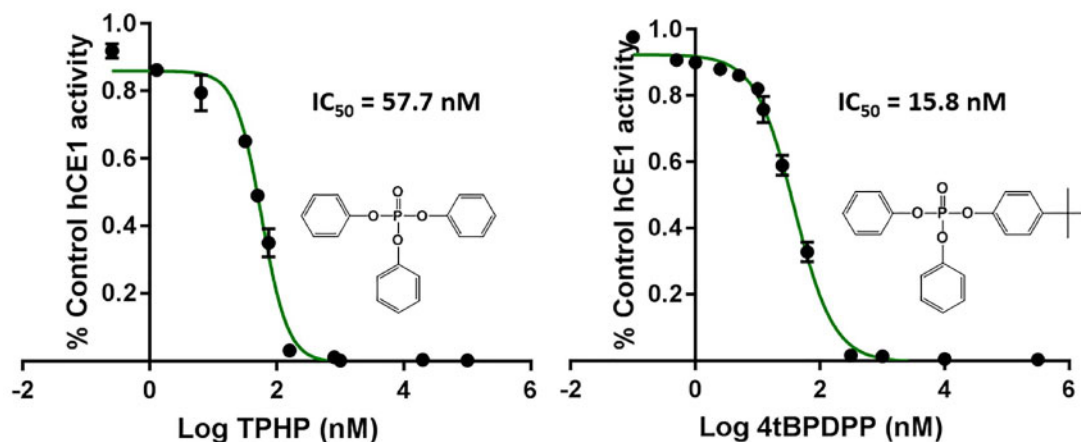
## DISCUSSION

#### Screening for hCE1 Inhibition

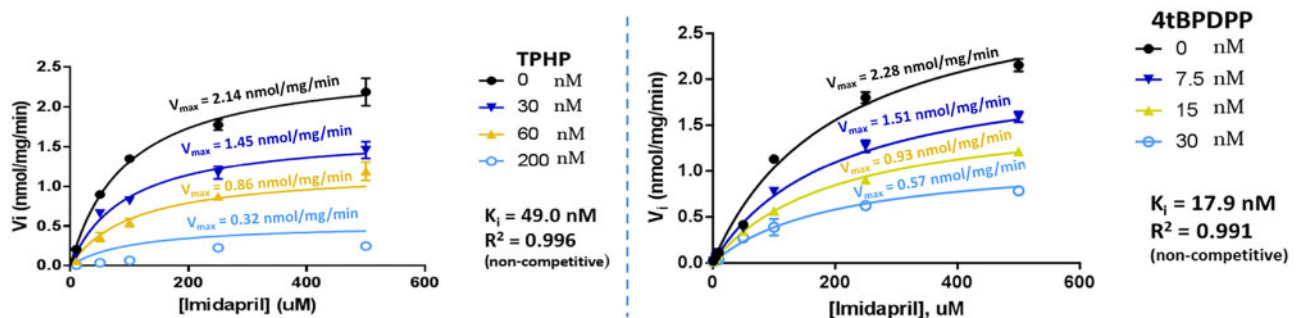
Of 2IPDPDP and 4IPDPDP, the 2 ITP isomers tested, 4IPDPDP had a much lower IC<sub>50</sub> value compared to 2IPDPDP, suggesting that steric effects (ie, alkyl substitution position on the ring) may contribute to inhibitor potency. Of 4tBPDPP and T4tBPP, the mono- and tri-substituted TBPP isomers, the measured IC<sub>50</sub>



**Figure 2.** The rate of formation of imidaprilat (nmol/mg/min) resulting from the incubation of imidapril with 160  $\mu\text{g/ml}$  human hepatic carboxylesterase (hCE1b) for 60 min. The Michaelis constant ( $K_m$ ) and the maximal reaction velocity ( $V_{\max}$ ) were obtained from nonlinear regression analysis in GraphPad Prism 7 (see [Supplementary Material](#) for model equation). The experiment was run in duplicate and each point depicts mean  $\pm$  SEM.



**Figure 3.** Inhibition curves for triphenyl phosphate (TPHP) and 4-*tert*-butylphenyl diphenyl phosphate (4tBDPDP) using 10  $\mu\text{M}$  imidapril and 160  $\mu\text{g/ml}$  human hepatic carboxylesterase (hCE1b). Activity was normalized to control and was determined by measuring imidaprilat formation following 150-min incubations. Experiments were run in duplicate and each point depicts mean  $\pm$  SEM.  $\text{IC}_{50}$  values were calculated using a 3 parameter linear model in JMP Pro 11 (Roberts et al., 2015). More information regarding the model equation can be found in the [Supplementary Material](#).



**Figure 4.** Imidaprilat formation rate resulting from the incubation of imidapril (10–500  $\mu\text{M}$ ) with varying concentrations of triphenyl phosphate (TPHP) (0, 30, 60, and 200 nM) or 4-*tert*-butylphenyl diphenyl phosphate (4tBDPDP) (0, 7.5, 15, or 30 nM) with 160  $\mu\text{g/ml}$  human hepatic carboxylesterase (hCE1b) for 60 min. Kinetic data were fit to competitive, noncompetitive, uncompetitive, and mixed inhibition models by nonlinear regression in GraphPad Prism and were best modeled by noncompetitive inhibition ( $K_i = 49.0$  nM,  $R^2 = .996$  for TPHP;  $K_i = 17.9$  nM,  $R^2 = .991$  for 4tBDPDP; see [Supplementary Material](#) for model equations).  $K_m$  values ranged from 98.9 to 115.1  $\mu\text{M}$  for TPHP and from 85.8 to 109.3  $\mu\text{M}$  for 4tBDPDP. The experiment was run in duplicate and each point depicts mean  $\pm$  SEM.

value for 4tBDPDP was multiple orders of magnitude lower than  $\text{IC}_{50}$  for T4tBPP, suggesting that 4tBDPDP and possibly B4tBPPP (not tested in this study) were the main contributors to the observed potency of the TBPP mixture (Table 1). Consistent with the previously observed positive trend between inhibitor potency and hydrophobicity, 4tBDPDP (*para*-substituted, *tert*-butylated isomer,  $\log K_{\text{OW}} = 6.3$ ) had an  $\text{IC}_{50}$  value that was approximately half of the  $\text{IC}_{50}$  value found for 4IPDPDP (*para*-substituted, isopropylated isomer,  $\log K_{\text{OW}} = 5.7$ ) (Wadkins et al., 2006). Because the active site of hCE1 is lined with mostly hydrophobic amino acids, it is unsurprising that an inhibitor's potency is related to its  $\log K_{\text{OW}}$  value, the octanol-water partitioning coefficient that is correlated with hydrophobicity (Wang et al., 2018). Interestingly, hCE1 activity was unaffected even at the highest test concentration for TBP ( $\log K_{\text{OW}} = 2.9$ ). The diester metabolites that were tested (DPHP, ip-PPP, and tBPPP;  $\log K_{\text{OW}} = 1.4$ –3.1) were also less potent hCE1 inhibitors compared to their corresponding triester parent compounds (TPHP, 4IPDPDP, and 4tBDPDP;  $\log K_{\text{OW}} = 4.6$ –6.3), potentially reflecting the previously noted positive correlation between hCE1 inhibitor potency and hydrophobicity. Although most of

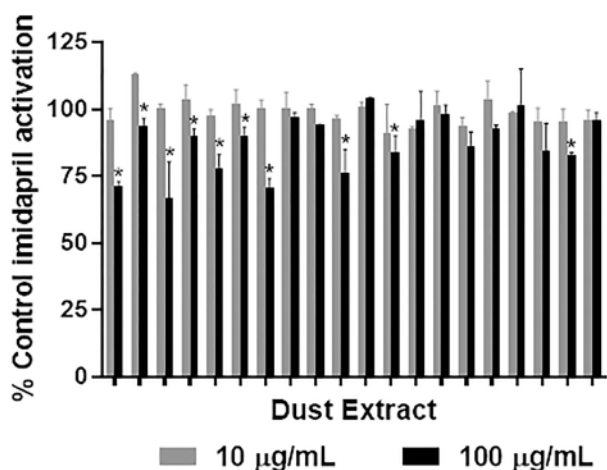


Figure 5. Following the incubation of dust extracts (10 or 100 µg/ml,  $n=20$  per dose) with 160 µg/ml human hepatic carboxylesterase (hCE1b) and 10 µM imidapril for 150 min, imidaprilat formation was monitored via LC-MS/MS. Samples were run in duplicate on separate days and bars depict mean  $\pm$  SEM. \* $p < .05$  indicates a significant difference from control.

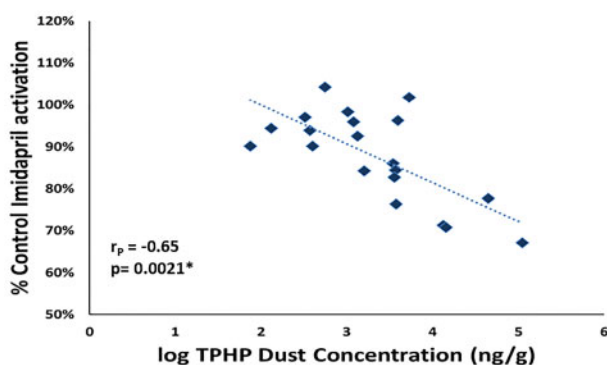


Figure 6. Following the incubation of dust extracts (100 µg/ml,  $n=20$ ) with 160 µg/ml hCE1b and 10 µM imidapril for 150 min, imidaprilat formation was monitored via LC-MS/MS. Triphenyl phosphate (TPHP) concentration in dust extracts were previously quantified (Phillips et al., 2018). Pearson analysis showed that percent control imidapril activation was negatively correlated ( $r_p = -.65$ ,  $p = .0021$ ,  $n = 20$ ) with  $\log_{10}$ TPHP dust concentration.

the chemicals tested in this study are additives in construction materials and consumer products, EHDPHP has long been approved by the Food and Drug Administration for use in food packaging and has been detected in food on numerous occasions (Castle et al., 1988; Poma et al., 2017). This is particularly concerning due to the high potential for human exposure and its potency as an hCE1 inhibitor in our assay ( $IC_{50} = 99.4$  nM).

Notably, the FM 550 and BZ-54 mixtures, which both contain the brominated compounds EH-TBB and BEH-TEBP, had different effects on hCE1 activity. BZ-54, which only contains EH-TBB and BEH-TEBP had no effect on hCE1 activity even at the highest test concentration, whereas FM 550, which contains TPHP and ITP isomers in addition to EH-TBB and BEH-TEBP was a potent inhibitor of hCE1 activity. This indicates that organophosphate components of the FM 550 drove the reduction of liver microsomal carboxylesterase activity observed in previous *in vivo* experiments (Patisaul et al., 2013). Both of the ITP mixtures that were tested, ITP Mix and K 50, had  $IC_{50}$  values just under 30 nM, reflecting their nearly identical ITP isomer composition profiles. Because the main components of the TBPP mixture are 4tBPDP

and TPHP (24% and 36% wt/wt, respectively) and T4tBPP is relatively minor component (3% wt/wt), it is unsurprising that the  $IC_{50}$  for the TBPP mixture more closely resembles the  $IC_{50}$  value for 4tBPDP and TPHP than the  $IC_{50}$  value for T4tBPP (Phillips et al., 2017).

#### Imidaprilat Formation Kinetics

The measured  $V_{max}$  value for imidaprilat formation using purified hCE1 (2.01 nmol/min/mg) was an order of magnitude lower than the  $V_{max}$  value reported using human liver microsomes (39.1 nmol/min/mg) by Fukami et al. (2010), which is in line with values reported by the enzyme's manufacturer for fluorescein diacetate (purified hCE1 = 0.31 µmol/min/mg; HLM = 18.5 µmol/min/mg) (Wang et al., 2011). However, Takahashi et al. (2009) reported a  $V_{max}$  of 2.4 nmol/min/mg for imidaprilat formation using human liver microsomes, which closely matches the observed value in this study. Different  $V_{max}$  values could reflect the substantial interindividual variability (31-fold) in imidaprilat hydrolysis by human liver microsomes that has been reported previously (Takahashi et al., 2009). It is also possible that the recombinant enzyme purchased from Corning had lower activity than the carboxylesterases found in human liver microsomes, or that other enzymes present in human liver microsomes also participate in imidapril activation.

#### Effect of TPHP and 4tPDPP on hCE1-mediated Imidapril Activation

TPHP and 4tBPDP were found to inhibit hCE1-mediated imidapril activation at environmentally-relevant levels, highlighting the potential for these chemicals to interfere with certain pharmacotherapies. TPHP is used as both a flame retardant and plasticizer and is one of the primary components of multiple currently-used commercial flame retardant mixtures including FM 550, ITP mixtures, and TBPP mixtures (Phillips et al., 2017). TPHP is also found in some nail polishes, hydraulic fluids, lubricating oils, electrical equipment, and lacquers (Carlsson et al., 2000; Mendelsohn et al., 2016). TPHP has been detected in indoor and outdoor air, house dust, surface water, fish, and human hair, nails, and breastmilk (Liu et al., 2016; Phillips et al., 2018; Sundkvist et al., 2010; Wilford et al., 2004; Xu et al., 2016). Similarly, 4tBPDP has been detected in the indoor environment, and has been detected frequently in indoor house dust (Phillips et al., 2018). Geometric mean and maximum levels of ip-PPP, a urinary metabolite of ITPs, have been measured to range from 6 to 200 nM, concentrations which are well within  $IC_{50}$  values found for TPHP and 4tBPDP in our imidapril assay (Butt et al., 2016; Phillips et al., 2018).

#### Mode of Inhibition of TPHP and 4tBPDP on Imidapril Activation

Our kinetic experiments suggest that TPHP and 4tBPDP are noncompetitive inhibitors of hCE1 ( $K_i = 49.0$  and 17.9 nM, respectively), further raising concern about their ability to compromise certain drug treatments. Although competitive inhibition in the context of xenobiotic-drug interactions likely has little relevance due to the stark differences in substrate concentration (ie, intentionally-administered drugs are found at much higher levels in the human body than xenobiotics), non-competitive enzyme inhibition by a pervasive xenobiotic, like TPHP, could potentially affect a pharmaceutical's efficacy. In the case of noncompetitive inhibition of hCE1 by TPHP or 4tBPDP, the formation of imidaprilat from imidapril in the liver would be reduced, resulting in a lower concentration of imidaprilat in circulation than the intended dose and potentially, the attenuation of the therapeutic effect (reduction in blood pressure). As TPHP and 4tBPDP are likely noncompetitive inhibitors

of hCE1, increasing the dose of imidapril will not necessarily lead to increased imidaprilat formation. This is because the liver of each individual has a finite amount of hCE1 content, and a fixed percentage of hCE1 will always be inactivated by a noncompetitive inhibitor, such as a TPHP or 4tBPDP (Bisswanger, 2011).

#### Effect of House Dust Extracts on hCE1-mediated Imidapril Activation

Because inadvertent ingestion of contaminated house dust is a primary route of exposure for these OPEs, testing house dust extracts for their effects on imidapril bioactivation is especially relevant. The U.S. EPA recommended values for the central tendency of the general population for dust ingestion range from 30 to 60 mg/day, depending on age (U.S. EPA, 2011). Recent exposure assessments estimate a total daily intake for TPHP attributed to house dust to be 37–54 ng/kg bw/day for the 95th percentile of toddlers and measured urinary levels of DPHP, a metabolite of TPHP, range from 27 to 153 nM (geometric mean-maximum concentrations) (Phillips et al., 2018; Tajima et al., 2014). Importantly, imidapril activation takes place in the liver where the levels of parent compound are likely to be the highest prior to metabolism. Although 4tBPDP was a more potent inhibitor than TPHP, a stronger correlation was observed for TPHP perhaps because TPHP is present in house dust at much higher levels compared to 4tBPDP. To the authors' knowledge, other known carboxylesterase inhibitors such as benzil, BNPP, and trifluoromethyl ketones have not been detected at substantial levels in house dust.

#### Limitations and Future Directions

Because OPEs are known to be rapidly metabolized and the diester metabolites were less potent hCE1 inhibitors than the triester parents,  $IC_{50}$  values measured in our experiments with purified hCE1 may be lower than they would have been had experiments been performed with human liver microsomes or in cell culture (Cooper et al., 2011). Although multiple *in vivo* experiments have noted hepatic carboxylesterase activity reductions resulting from OPE administration, the doses used often exceed environmental relevance and significant interspecies variation in microsomal carboxylesterase activity may limit the translation of these findings to humans (Hosokawa et al., 1995). Although human and rodent carboxylesterases share a high degree of sequence homology (approximately 70%), different species express distinct carboxylesterase isoforms which have been shown to exhibit different inhibitor sensitivities, making comparison across species difficult (Xie et al., 2002). Future studies should focus on using human-derived cell culture lines or other metabolically active model systems to evaluate hCE1 inhibition by OPE flame retardants and plasticizers. Additional studies should also be conducted to evaluate the potential reversibility of carboxylesterase inhibition by OPEs. For example, uncharged oximes reactivate carboxylesterases inactivated by some organophosphate compounds, but certain organophosphate compounds cause inhibition that is refractory to oxime reactivation (Maxwell et al., 1994). The data presented herein highlight the potential for drug-xenobiotic interactions via hCE1 inhibition, but future studies should be conducted to confirm that the inhibition observed *in vitro* is biologically meaningful *in vivo*.

#### Implications

Taken together, these results highlight the possibility for Ces inhibition by ITP and TBPP isomers at environmentally relevant concentrations. Although TPHP and other organophosphate have been shown to inhibit Ces previously, these data add to our current knowledge of hCE1 inhibitors. Unfortunately, ITPs

and TBPPs are rapidly metabolized and direct measurement of ITP and TBPP concentrations at the target (ie, human liver tissue) is not feasible. To approximate what a relevant concentration in the liver might be, urinary metabolite levels can be used as a surrogate. Geometric mean concentrations of isopropyl phenyl diphenyl phosphate (ip-PPP), a urinary metabolite of ITPs, range from 2 to 7 ng/ml in recent human exposure assessments, with reported maximum levels as high as 61 ng/ml (Butt et al., 2016; Phillips et al., 2018).  $IC_{50}$  values measured in this study for TPHP, EHDPHP, FM 550, ITP Mix, K 50, 4IPDP, and 4tBPDP fall within this urinary concentration range (6–200 nM). It is unknown whether urinary metabolite concentrations under- or over-estimate the levels of ITPs and TBPPs in the liver following exposure, and the present study should be interpreted in the context of this uncertainty. For example, urinary metabolite levels may exceed corresponding liver concentrations (Bankir and Yang, 2012). However, because OPEs also undergo biliary excretion in addition to urinary elimination, urinary metabolite levels might also underestimate total exposure. Although biochemical experiments sometimes overstate actual cellular potency, our study illustrates biological plausibility for Ces inhibition by OPEs at doses that might occur in real life exposure scenarios.

Our data also demonstrate the potential of OPE flame retardants and plasticizers to interfere with pharmaceuticals that rely on bioactivation by hCE1b. Because plasticizers are often used as excipients in drug formulations, plasticizer excipients should be evaluated for hCE1 inhibition, especially when used in drugs that require hCE1-mediated activation (Bhyan et al., 2011). Other drugs that are activated by hCE1b include Tamiflu, antihyperlipidemic agents including simvastatin and lovastatin, mycophenolate, an immunosuppressant drug used to prevent rejection during organ transplantation, and capecitabine, a chemotherapy agent (Laizure et al., 2013). Similarly, xenobiotics and drugs inactivated by hCE1b include Ritalin, cocaine, meperidine (an opioid pain medication), and pyrethroid pesticides like bioresmethrin (Ross et al., 2006). As such, inhibition of hCE1b by organophosphate aryl esters at environmentally relevant levels has far-reaching public health ramifications and brings attention to xenobiotic-drug interactions, an idea that receives little attention in the current literature but could have profound implications for the effective use of pharmaceuticals.

## SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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## DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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