Published in final edited form as:

Chem Biol Interact. 2013 March 25; 203(1): 257–264. doi:10.1016/j.cbi.2012.10.005.

Identifying Safer Anti-Wear Triaryl Phosphate Additives for Jet Engine Lubricants

Paul E. Baker^{a,b}, Toby B. Cole^{a,c}, Megan Cartwright^c, Stephanie M. Suzuki^{a,b}, Kenneth E. Thummel^d, Yvonne S. Lin^d, Aila L. Co^{a,b}, Allan E. Rettie^e, Jerry H. Kim^f, and Clement E. Furlong^{a,b}

Paul E. Baker: bakerp@uw.edu; Toby B. Cole: tobycole@uw.edu; Megan Cartwright: mcartwri@uw.edu; Stephanie M. Suzuki: stephis@uw.edu; Kenneth E. Thummel: thummel@uw.edu; Yvonne S. Lin: yvonlin@uw.edu; Aila L. Co: ailaco@uw.edu; Allan E. Rettie: rettie@uw.edu; Jerry H. Kim: jerkim@uw.edu

^aDepartment of Medicine-Division of Medical Genetics, University of Washington, Seattle, WA, 98195

^bDepartment of Genome Sciences, University of Washington, Seattle, WA, 98195

^cDepartment of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA, 98195

^dDepartment of Pharmaceutics, University of Washington, Seattle, WA, 98195

^eDepartment of Medicinal Chemistry, University of Washington, Seattle, WA, 98195

^fDepartment of Anesthesiology and Pain Medicine, University of Washington, Seattle, WA, 98195

Abstract

Individuals aboard jet aircraft may be exposed to potentially toxic triaryl organophosphate anti-wear lubricant additives (TAPs) that are converted by cytochromes P450 into toxic metabolites. Consequences of exposure could be reduced by using less toxic TAPs. Our goal was to determine whether an *in vitro* assay for inhibition of butyrylcholinesterase (BChE) by bioactivated TAPs would be predictive of inhibition of serine active-site enzymes *in vivo*. The *in vitro* assay involved TAP bioactivation with liver microsomes and NADPH, followed by incubation with human BChE and measurement of BChE activity. Of 19 TAPs tested, tert-butylated isomers produced the least BChE inhibition. To determine the relevance of these results *in vivo*, mice were exposed to Durad 125 (D125; a commercial mixture of TAP esters) or to TAPs demonstrating low or no BChE inhibition when assayed *in vitro*. Inhibition of BChE by bioactivated TAPs *in vitro* correlated well with inhibition of other serine active-site enzymes *in vivo*, with the exception of brain acetylcholinesterase and neuropathy target esterase (NTE), which were not inhibited by any TAP tested following single exposures. A recombinant catalytic domain of NTE (rNEST) exhibited classical kinetic properties of NTE. The metabolite of tri-(*o*-cresyl) phosphate (T_oCP), 2-(*o*-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one (CBDP), inhibited rNEST *in vitro*, but with an

© 2012 Elsevier Ireland Ltd. All rights reserved.

Corresponding author: Clement E. Furlong, Medical Genetics, Box 357720, University of Washington, 1959 N.E. Pacific St., Seattle, WA 98195-7720, tel: (206) 543-1193, fax: (206) 543-3050, clem@uw.edu.

Toby Cole and Paul Baker contributed equally to research in this paper.

Conflict of Interest Statement

Clement Furlong, Paul Baker, Toby Cole and Aila Co have been funded in part by Pilot Unions, Flight Attendant Unions, the Royal Australian Air Force, Norwegian Union of Energy Workers (SAFE) and NYCO S.A.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

IC₅₀ value almost 6-times higher than for inhibition of BChE. Physiologically-relevant concentrations of the flavonoid, naringenin, dramatically reduced D125 bioconversion *in vitro*. The *in vitro* assay should provide a valuable tool for prescreening candidate TAP anti-wear additives, identifying safer additives and reducing the number of animals required for *in vivo* toxicity testing.

Keywords

biomarker; butyrylcholinesterase; CBDP; exposure; hydrolases; serine active-site esterases; triaryl phosphate; ToCP

1. Introduction

On most jet turbine aircraft, unfiltered engine bleed air is fed into the cabin, providing oxygen for those aboard [1]. Exposure of passengers and crew to some level of triaryl phosphates (TAPs) occurs in approximately 23% of monitored flights [2, 3], whereas higher levels of exposure can occur when engine seals wear or fail. Symptoms of aerotoxic syndrome resulting from such exposures can include extreme mental impairment [4], an acute flight safety issue when crew exposure to contaminated air is significant. Material safety data sheets for synthetic jet lubricants list TAP contents of 1-10%.

Neurotoxicity of aromatic phosphate esters to humans was first reported in 1899, following treatment of tuberculosis patients with phosphocresote [5]. Studies in 1930 examining the cause of Ginger Jake paralysis, which affected 10,000 to 50,000 individuals consuming extracts of ginger adulterated with tri-cresyl phosphate (TCP), identified tri-(*o*-cresyl) phosphate (ToCP) as the paralytic agent [6, 7]. Epidemic poisonings have since been associated with consumption of food adulterated with TCPs [8-13].

In the early 1950s, Aldridge reported that liver metabolism of ToCP was required to generate toxic metabolite (s) [14]. In 1961, Casida et al. [15] reported the toxic metabolite of ToCP to be 2-(*o*-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one (CBDP, or cyclic saligenin cresyl phosphate), which has since been shown to be an inhibitor of several serine active-site esterases including butyrylcholinesterase (BChE) [16], acetylcholinesterase (AChE) [17], carboxylesterase (CES) [18-20] and acylpeptide hydrolase (APH) [21]. The *in vitro* role of microsomes in the metabolism of TAPs, including ToCP, was established by Sprague and Castles in 1985 [22]. An analogue of CBDP, phenyl saligenin phosphate [23], is a potent inhibitor of neuropathy target esterase (NTE) [24], the inhibition and aging of which results in organophosphate (OP)-induced delayed neuropathy (OPIDN) [25]. Toxic metabolites of other jet engine TAP additives, including those evaluated in this study, have not yet been described in the literature.

The purpose of the present study was to develop an *in vitro* assay for assessing the inhibitory potential of TAPs using the biomarker esterase, BChE [26], and to verify the *in vitro* results with *in vivo* exposures of mice. Mice were exposed by gavage to a commercial TCP mixed-isomer formulation, Durad 125 (D125), and to two TAPs found not to inhibit BChE with the *in vitro* bioactivation assay, tri-(*o*-tert-butylphenyl) phosphate (TpBP) and tri-*p*-cresyl phosphate (TpCP). The three compounds were compared for their ability to inhibit serine active-site enzymes from plasma, RBCs, liver and brain. Finally, because the flavonoid compound, naringenin, has been shown to inhibit cytochromes P450 [27] we examined its effect on bioactivation of D125.

2. Materials and methods

2.1 Chemicals and supplies

N-Acetyl-L-alanyl-*p*-nitroanilide (AcAlaPNA) was from Bachem (Torrance, CA); Paraoxon from Chem Service (West Chester, PA); NADPH from RPI (Mt. Prospect, IL); anti-poly-His antibody from Invitrogen (Grand Island, NY); acetylthiocholine, S-butyrylthiocholine iodide, 5,5'-dithio-bis-nitrobenzoic, *p*-nitrophenyl valerate (PNV), phenyl valerate (PV), alpha-naphthyl butyrate (α NB), methyl umbelliferyl acetate (MUA), potassium ferricyanide, and 4-aminoantipyrine from Sigma (St. Louis, MO); microplates (96-well, flat bottom) for visible light or UV range (Greiner Bio-One) were purchased from Fisher Scientific (Hanover Park, IL). Mice were from Taconic Farms (Germantown, NY). Mipafox and CBDP were gifts from Drs. Marion Ehrich and Oksana Lockridge, respectively. Table 1 shows the source and purity for TAPs that were used.

2.2 Rat liver microsomes (RLMs)

RLMs were leftover samples from male Sprague-Dawley rats (150 – 200 g) injected intraperitoneally for four days with 80 mg/kg/day phenobarbital [28] and stored at -80° C in 50 mM sodium phosphate, pH 7.4 (buffer A).

2.3. Microsomal bioactivation of TAPs

Solutions of TAPs were prepared at 2.5 mg/ml in ETOH, then diluted 1:62.5 (to 40 μ g/ml) prior to making serial dilutions and addition to RLMs and NADPH in buffer A. Final concentrations in the bioactivation step were 14 mg/ml RLMs, 1 mM NADPH and TAPs at concentrations, up to 20 μ g/ml. Bioactivation proceeded for 25 min at 25°C, when 10 μ l of purified human BChE [29] (1.33 μ g/ml in DD H₂O) were added, followed by incubation for an additional 25 min.

2.4. Measurement of BChE activity

BChE activity was determined by a kinetic modification of the Ellman procedure [30], adapted for continuous monitoring with a SpectraMax Plus 384 plate reader (Molecular Devices). Kinetic data were acquired at 405 nm for 4 min using SoftMax Pro software, with path length correction. Only linear initial reaction rates (< 4 min) were used for analyses.

2.5. Expression and properties of the rNEST domain of NTE

Cloned rNEST was expressed (with a C-terminal His₆ tag), purified, and incorporated into dioleoylphosphatidyl-choline liposomes as previously described [31], except without an N-terminal tag. Since RLMs contained high levels of PV-hydrolyzing enzyme (s), interfering with measurement of rNEST activity, CBDP (the metabolite of bioactivated ToCP) was used to determine the direct effect TCP metabolites on BChE and rNEST.

2.6. Suppression of D125-mediated inhibition of BChE by naringenin

RLMs and NADPH were pre-incubated (20-25 min) with naringenin (0, 0.2, 2.0, 20 and 200 nM) in buffer A, then D125 (0.36 μ g/ml; equivalent to its IC₅₀ for BChE inhibition) was added to the mixture for an additional 20-25 min. One set of controls contained no naringenin and another contained no D125. BChE activity was determined as described above.

2.7. Administration of TAPs to mice

All animal experiments were in compliance with the Guiding Principles in the Use of Animals in Toxicology [32], and approved by the University of Washington IACUC. Mice

(7-9 weeks old male C57Bl/6) were housed in a SPF facility with food and water *ad libitum*. TAPs were dissolved at 25 mg/ml directly in warm corn oil (55°C), diluted in corn oil to the desired concentration, cooled (25°C) and administered to mice by gavage (10 µl/g body weight). Control mice received vehicle (corn oil) alone.

2.8. Preparation of tissue samples

At 6 hr post-exposure, mice were assessed for signs of morbidity and blood was collected from the saphenous vein in heparinized capillary tubes. Plasma and RBCs were separated by centrifugation (1000 rcf for 10 min). RBCs were washed 3X with PBS, and both plasma and RBCs were stored at -80°C. At 24 hr post-exposure, mice were euthanized by CO₂ asphyxiation. Following decapitation, trunk blood was collected in heparinized tubes and processed as above. Brains and livers were also harvested and homogenized (4°C) in Potter-Elvehjem homogenizers. Brains were homogenized in 5 volumes (w/v) of sodium phosphate (50 mM, pH 8.0, 4°C), while livers were homogenized in 10 volumes (w/v) of Tris (100 mM, pH 8.0, 4°C). Both homogenates were centrifuged (700 rcf for 10 min) and supernatants stored at -80°C.

2.9. Enzyme assays in mouse blood and tissues

BChE activity of mouse plasma samples was measured as described in section 2.4 above, except 100 µl of plasma samples (1:50 dilution in buffer A) were used. APH activity in RBCs was measured by hydrolysis of αNB [33]. Ten µl of thawed blood cell lysate (1:20 dilution in 20 mM Tris, pH 7.0) were added to 200 µl αNB reaction mix (200 µM α-naphthyl butyrate in the same buffer). Substrate hydrolysis was measured continuously at 235 nm (4 min at 25°C). Liver APH activity was determined with a modified assay of Quistad et al. [21]. Liver homogenate supernatant (above) was diluted to 100 µg/ml (100 mM Tris, pH 7.5) and 185 µl was added to microplate wells along with 1.5 mg/ml AcAlaPNA (100 µl in the same buffer). Formation of *p*-nitroanilide was measured continuously at 405 nm (10 min at 37°C). Plasma CES or CES1C [34] activity was measured with two different substrates, PNV and MUA. Rates of PNV hydrolysis were quantified by a modification of the protocol of Munger et al. [35]. Twenty µl of plasma (1:50 in 50 mM Tris, pH 8.0) were added to microplate wells, followed by 200 µl of freshly-prepared PNV substrate mix (1 mM PNV in 50 mM Tris, pH 8.0). Formation of *p*-nitrophenol was monitored continuously (10 min at 25°C) at 405 nm. The MUA hydrolysis assay for plasma and liver CES was adapted from Brzezinski et al. [36], with kinetic modifications. Twenty µl of liver supernatant, adjusted to 1 mg/ml (50 mM Tris, pH 8.0) or diluted plasma (1:50, same buffer) were added to microplate wells, followed by 200 µl of MUA (5 µg/ml in the same buffer containing 1 mM EDTA). Rates of hydrolysis were monitored continuously at 354 nm for 4 min. The brain NTE assay was adapted from Winrow et al. [37] with kinetic modifications. Brain supernatants were incubated with mipafox (50 µM) and/or paraoxon (PO, 40 µM) for 20 min at 37°C, then 60 µl were added to microplate wells with 150 µl PV assay mix, also at 37°C. Formation of 4-*N*-(1,4-benzoquinoneimine)-antipyrine was monitored continuously at 510 nm (15 min 37°C). NTE activity was considered to be that portion of PV-hydrolyzing activity that was insensitive to PO inhibition but sensitive to mipafox inhibition.

Initial linear rates of product formation (mOD/min) for each enzyme were converted to Units per ml (U/ml) using pathlength correction and the respective extinction coefficients: α-naphthol, 24.2 mM⁻¹ cm⁻¹; *p*-nitroaniline, 8.8 mM⁻¹ cm⁻¹; *p*-nitrophenol, 18 mM⁻¹ cm⁻¹; 4-methylumbelliferone, 12.2 mM⁻¹ cm⁻¹; 4-*N*-(1,4-benzoquinoneimine)-antipyrine, 13.9 mM⁻¹ cm⁻¹.

2.10. Statistical analyses

In vitro data were graphed using Microsoft Excel. Results are presented as percent of control and are shown as the mean $\pm$ SEM or mean $\pm$ SD, as indicated. Differences in enzyme inhibition among TAP compounds *in vivo* were tested for statistical significance with Student's *t*-test followed by a Bonferroni correction for multiple testing. Values for half-maximal inhibitory concentrations (IC₅₀) *in vitro* and half-maximal effective doses (ED₅₀) *in vivo* were calculated with Prism software (GraphPad v. 5.03, San Diego, CA) using non-linear regression (curve fit) vs. normalized responses.

3. Results

3.1. Development and testing of the *in vitro* BChE inhibition assay

Initial experiments, performed to optimize concentrations of RLMs and NADPH for bioactivation of TAPs (25°C for 25–30 min), were evaluated by determining IC₅₀ values (data not shown). D125 bioactivation, measured by BChE inhibition under optimized conditions, had a mean IC₅₀ value ($\pm$ SD) of 0.36 ± 0.06 μ g/ml (9 experiments, each in triplicate) (Fig. 1A). Identical conditions were used for testing 18 additional TAPs (Table 1), where D125 was included as a positive control for inhibition with each set of TAPs assayed. An example of using D125 as a standard across individual experiments is shown in Figure 1B, where T α CP and T p BP were bioactivated along with D125. Bioactivated T α CP (IC₅₀ = 0.12 μ g/ml) was approximately three times more inhibitory to BChE than bioactivated D125 (IC₅₀ = 0.36 μ g/ml), whereas bioactivated T p BP did not inhibit BChE at levels up to 20 μ g/ml. BChE was not inhibited by any TAP in the absence of NADPH (Fig. 1C). Bioactivation of T p CP or any tert-butylphenyl phosphate (TBP) isomer (tri-*o*-, tri-*m*- or tri-*p*-) generated no BChE inhibitor (s) (Fig. 1D). The IC₅₀ values and additional information for the 19 different TAPs examined are summarized in Table 1.

3.2. Inhibition of the rNEST domain of NTE

Three OP compounds were tested for inhibition of rNEST. Exposure of rNEST to 40 μ M paraoxon (PO) and 50 μ M mipafox demonstrated that purified rNEST exhibited the classic catalytic properties of NTE, inhibition insensitive to PO but sensitive to mipafox inhibition (not shown). CBDP inhibited rNEST with an IC₅₀ value of 40 ng/ml (109 nM) (Fig. 2A). BChE was more sensitive to CBDP inhibition with an IC₅₀ value = 7 ng/ml (19 nM) (Fig. 2B).

3.3. Naringenin inhibition of D125 bioactivation

The protocol developed to examine TAP inhibition of BChE *in vitro* was modified to examine the effect of pre-incubation with varying concentrations of naringenin. Pre-incubation of RLMs/NADPH with naringenin resulted in a concentration-dependent reduction of D125 bioactivation from (Fig. 3). In the absence of D125, naringenin had no effect on BChE activity.

3.4. Effects on serine active-site hydrolases

Pilot studies in mice established the dose range and most efficacious route of TAP exposure for *in vivo* studies. None of the routes of TAP exposure (IP, dermal, or gavage) or dosage level examined (as great as 240 mg/kg body weight) resulted in overt toxicity following single exposures. Exposure by gavage produced the greatest and most consistent inhibition of enzyme activity (data not shown) and was used for further experiments.

Three TAPs (D125, T p BP and T p CP) were administered to mice by gavage at doses of 10, 60, 120, and 240 mg/kg in corn oil. Vehicle (corn oil) alone was administered to the control

group. Effects of TAP exposure on blood, brain and liver enzymes were examined 6 hr post-exposure (for plasma and RBCs enzyme levels) and 24 hr post-exposure (for plasma, RBCs, liver and brain levels). For RBC and plasma enzymes, inhibition differences between the 6 and 24 hr time points were statistically insignificant, so only the latter data are reported.

D125 exposure inhibited > 60% of plasma BChE at all but the lowest dose tested, whereas T_pCP inhibited only 20% plasma BChE at the highest exposure dose (240 mg/kg), and T_pBP did not inhibit BChE at any dosage level (Fig. 4A). D125 and T_pCP exposure resulted in modest but significant inhibition of RBC APH activity (measured by α NB hydrolysis) at 240 mg/kg, whereas T_pBP did not inhibit RBC APH at any dosage level (Fig. 4B). All three TAPs significantly reduced plasma CES activity measured by PNV (Fig. 4C) or MUA (Fig. 4D) hydrolysis; however, plasma CES inhibition by T_pBP was again less than that observed with D125 or T_pCP. D125 and T_pCP significantly reduced liver APH activity (measured by AcAlaPNA hydrolysis, a more specific APH substrate than α NB) (Fig. 4E), and CES activity (measured by MUA hydrolysis) (Fig. 4F) at all exposure levels. T_pBP exposure did not significantly inhibit liver APH (measured by AcAlaPNA hydrolysis) (Fig. 4E) or CES activity (measured by MUA hydrolysis) (Fig. 4F) at any exposure level.

ED₅₀ values from the *in vivo* exposures are shown in Table 2. In plasma, the ED₅₀ value for D125 was 63 mg/kg for BChE, whereas for T_pCP and T_pBP the ED₅₀ values were >240 mg/kg (Fig. 4A, Table 2). The ED₅₀ values for all three TAPs were also >240 mg/kg for RBC APH (measured by α NB hydrolysis) (Fig. 4B). In RBCs, the ED₅₀ values for all TAPs were > 240 mg/kg for APH (Table 2). The ED₅₀ value for D125 inhibition of plasma CES was 19 mg/kg (measured by MUA or PNV hydrolysis) (Figs. 4C, D; Table 2). In plasma, ED₅₀ values for CES inhibition by T_pCP (measured by PNV or MUA) were 27 and 17 mg/kg, respectively. The ED₅₀ values for T_pBP inhibition of plasma CES, measured by PNV (93 mg/kg) or MUA (86 mg/kg), were significantly greater than those of T_pCP or D125 (Fig. 4D, Table 2). In liver, the ED₅₀ values for T_pBP were >240 mg/kg for both APH and CES (Figs. 4E, F, Table 2). D125 and T_pCP both significantly inhibited liver CES (ED₅₀ = 85 and 52 mg/kg, respectively) and APH (ED₅₀ = 33 and 14 mg/kg, respectively).

Brain homogenates from control and TAP-treated mice were compared for NTE activity by measuring the PV-hydrolyzing activity that was insensitive to 40 μ M PO but sensitive to 50 μ M mipafox. NTE was not significantly inhibited by these single exposures at any dosage level (not shown). Similarly, brain AChE activity was not inhibited by any of the TAPs following the single *in vivo* exposure (not shown).

4. Discussion

Exposures of individuals aboard jet aircraft to aerosolized TAPs represent a serious health concern [1, 29, 38, 39]. Because pressurization is necessary for proper engine seal function, exposure to aircraft engine lubricants containing TAPs occurs during “fume events”, engine start-up/shut-down on the ground, and throughout “uneventful” flights during normal acceleration or deceleration [1-3]. Exposures to aerosolized jet engine lubricants are poorly documented and are often overlooked by airline industries [38]. Boeing’s new 787 “Dreamliner” does not use bleed air to condition the cabin [40]. For the foreseeable future, however, there will be of thousands of aircraft still using bleed air to provide air for passengers and crew, with accompanying possibilities of TAP exposure. Therefore, it makes sense to develop lubricant formulations that are less toxic than current formulations.

We describe here a rapid *in vitro* assay for identifying potentially safer TAP lubricant additives, and have validated the potential safety of some of these TAPs by *in vivo* exposure of mice. To date, almost all studies on the safety of TAP-containing compounds involved chronic exposure of chickens followed by evaluation of neuropathology and/or symptoms of

OPIDN [22, 41-44], although rodents have also been used to examine the effect (s) of TAP exposure [42, 45] TAP bioactivation provided a rapid evaluation of 19 different parent compounds *in vitro*, using inhibition of BChE. None of the TAPs or commercial OP additive formulations inhibited BChE without both RLMs and NADPH (Figs. 1B, C), implicating the cytochrome P450 superfamily in the bioactivation of the TAPs. Nine TAP formulations tested were not bioactivated at their limits of solubility (~20 µg/ml) (Table 1). Bioactivated T α CP produced the most potent BChE inhibition (IC₅₀ values = 0.12 µg/ml - 0.15 µg/ml, or ~330 nM). The D125 IC₅₀ value was 0.36 µg/ml ($\pm$ 0.06). Human liver microsomes (University of Washington, School of Pharmacy Human Tissue Bank) also bioactivated TAPs in the presence of NADPH (not shown), but limited availability precluded their use for extensive analyses.

For *in vivo* validation, T p CP, T p BP, and D125 were administered to mice by gavage. Gavage exposure was determined in pilot studies to be a more appropriate route than dermal or IP exposure, and administration by aerosol was not feasible because of regulatory restrictions on inhalation exposures in our shared animal housing. D125 was chosen as a positive control for the *in vivo* studies, since it is used commercially and was also used as the *in vitro* control. T p CP and T p BP were selected because they generated no BChE inhibition *in vitro* (Table 1). Further, others reported tri-*p*-substituted phenyl phosphates like T p CP to be less toxic than *m*- and *o*-substituted TAPs, and chronic exposure studies using the hen model of OPIDN reported relatively low toxicity of butylated TAP-containing formulations [46]. T p BP was especially interesting because none of the tert-butylated TAPs inhibited BChE *in vitro*, and T p BP exposures produced no BChE inhibition *in vivo*.

Efficacy of target enzyme inhibition is likely related to TAP bulkiness or substituted aryl group (s) geometry, which may interfere with access to active-site serines [47]. TAPs with bulkier substitutions (e.g., T p BP and the tri-isopropyl-phenyl phosphates) were generally less inhibitory for esterases while bioactivated methyl-substituted TAPs were more efficient esterase inhibitors (Table 1). However, the product (s) of bioactivated T p CP did not readily reach/bind to the BChE serine active-site, but effectively inhibited the active-site serine of other enzymes (e.g., plasma and liver CESs and liver APH) (Table 2).

Different enzymes exhibit dramatically different sensitivities to inhibitory TAP metabolites. Consistent with the CES inhibition by TAPs in this study (Figs. 4C,D,F, Table 2), Quistad and Casida reported the high sensitivity of CES to CBDP inhibition [24]. APH, reported to be involved in cognition [48, 49], was inhibited *in vivo* by TAP metabolite (s) (Fig. 4E, Table 2). Inhibition and subsequent aging of NTE, a protein important for neuronal transport, may lead to paralysis if significantly inhibited following repeated exposure of animals to neurotoxic OPs [25, 46, 50-52]. The rNEST catalytic domain of NTE [31, 53] was sensitive to CBDP (IC₅₀ = 109 nM) (Fig. 2A), although BChE was even more sensitive to CBDP (IC₅₀ = 19 nM) (Fig. 2B), in close agreement with the 30 nM IC₅₀ of the related compound, phenyl saligenin cyclic phosphonate [24]. While CBDP was ~6 times more effective at inhibiting BChE than rNEST, it was nonetheless a potent inhibitor of both enzymes.

Since it is clear from this study and interpretation of earlier studies of Aldridge with rat liver slices [14] that the P450 system is required for conversion of TAPs into toxic metabolites, we investigated whether naturally occurring inhibitors would block the conversion of TAPs to toxic metabolites. As show in Figure 3, naringenin, a compound found in grapefruit, inhibited conversion of D125 into BChE inhibitor (s) in a concentration-dependent manner. The inhibition was observed at concentrations that fall well below the reported ~6 µM concentration of naringenin observed in plasma following ingestion of grapefruit juice [54]. Lu et al. [27] recently described the naringenin enantiomer-dependent inhibition of

cytochromes CYP19, CYP2C9, CYP2C19, and CYP3A by the (*R*)- and (*S*)-enantiomers of naringenin. Additional research will be required to determine which of the P450 isomers are involved in the bioactivation of each of the TAPs and which naringenin enantiomer is the most potent inhibitor of the bioactivation of a specific TAP by a relevant P450 isozyme. While consumption of grapefruit juice following an exposure may possibly block the conversion of TAP to toxic inhibitor, an important caveat comes from the observation that the microsomal/NADPH system is also involved in the inactivation of active metabolite (s) (data not shown). Aldridge [14] had observed in his earlier studies that incubation of T α CP with liver slices for extended periods of time decreased toxicity following bioactivation.

As normal neuronal function depends on many serine active-site enzymes, *in vitro* analysis of a representative panel of these enzymes would be informative. The *in vivo* data reported here suggest that such a panel should include, at least, APH, CES (s) and BChE. Because rNEST is not stable over time and must be incorporated into lipid bilayers for functionality [31], it would not yet be included in such a panel. The metabolites of TAPs that maximally inhibit the panel of enzymes would merit further toxicological testing. Availability of human cDNAs encoding additional enzymes should facilitate generation of future screening panels.

5. Conclusions

In summary, *in vitro* testing of 19 TAPs for bioactivation into esterase inhibitors narrowed the focus of this study to three compounds for further *in vivo* testing. T p BP was significantly less inhibitory than T p CP or D125, suggesting that T p BP, or a similar compound based on its molecular characteristics, should be a safer anti-wear additive for jet engine lubricants, provided its performance characteristics meet those of similar TAP-containing formulations currently in use (e.g., D125). An inhibitory effect of the flavonoid naringenin on the bioactivation of D125 into BChE inhibitor (s) was also demonstrated, however, additional studies are required to determine if it might help modulate the effects of TAP exposures.

Acknowledgments

We thank Rebecca Richter for technical assistance and Judit Marsillach-Lopez for constructive comments and advice. We also thank Drs. Oksana Lockridge for providing the CDBP and Marion Ehrich for providing mipafox. Stephanie Suzuki was supported by an NIEHS Environmental Pathology/Toxicology Training Grant, P30ES007032. This work was also supported by NIH Grants R01ES09883, P42ES04696, NIEHS P30ES07033, GM32165 and funding from Pilot and Flight Attendant Unions, the Royal Australian Air Force, Norwegian Union of Energy Workers (SAFE), the CDC National Center for Environmental Health Division of Laboratory Sciences, and NYCO S.A.

References

1. Michaelis S. Contaminated aircraft cabin air. *J Biol Phys Chem*. 2011; 11:132–145.
2. Crump, D.; Harrison, P.; Walton, C. Aircraft Cabin Air Sampling Study; Part 1 of the Final Report Publisher. Cranfield University; Cranford (Bedfordshire), UK: 2011.
3. Crump, D.; Harrison, P.; Walton, C. Aircraft Cabin Air Sampling Study; Part 2 of the Final Report Publisher. Institute of Environment and Health; Cranfield (Bedfordshire), UK: 2011.
4. Winder C, Balouet JC. Aerotoxic syndrome. *Toxicology*. 2001; 164:47–47.
5. Roger H, Poursines Y, Recordier M. Polyneuritis after curative antitetanic serotherapy, with participation of the central nervous system and of the meninges (Anatomo-clinical observation). *Rev Neurol-France*. 1934; 1:1078–1088.
6. Abeyratne R. Forensic aspects of the aerotoxic syndrome. *Med Law*. 2002; 21:179–199. [PubMed: 12017442]

7. Hale MA, Al-Seffar JA. Preliminary report on aerotoxic syndrome (AS) and the need for diagnostic neurophysiological tests. *Am J Electroneurodiagnostic Technol.* 2009; 49:260–279. [PubMed: 19891417]
8. Smith HV, Spalding JM. Outbreak of paralysis in Morocco due to ortho-cresyl phosphate poisoning. *Lancet.* 1959; 2:1019–1021. [PubMed: 13831987]
9. Vasilescu C, Florescu A. Clinical and electrophysiological study of neuropathy after organophosphorus compounds poisoning. *Arch Toxicol.* 1980; 43:305–315. [PubMed: 7387390]
10. Susser M, Stein Z. An outbreak of tri-ortho-cresyl phosphate (T.O.C.P.) poisoning in Durban. *British journal of industrial medicine.* 1957; 14:111–120. [PubMed: 13426434]
11. Senanayake N. Tri-cresyl phosphate neuropathy in Sri Lanka: a clinical and neurophysiological study with a three year follow up. *J Neurol Neurosurg Psychiatry.* 1981; 44:775–780. [PubMed: 7310416]
12. Srivastava AK, Das M, Khanna SK. An outbreak of tricresyl phosphate poisoning in Calcutta, India. *Food Chem Toxicol.* 1990; 28:303–304. [PubMed: 2358255]
13. Wang D, Tao Y, Li Z. Toxic polyneuropathy due to flour contaminated with tricresyl phosphate in China. *J Toxicol Clin Toxicol.* 1995; 33:373–374. [PubMed: 7629908]
14. Aldridge WN. Tricresyl Phosphates and Cholinesterase. *Biochemical Journal.* 1954; 56:185–189. [PubMed: 13140171]
15. Casida JE, Eto M, Baron RL. Biological activity of a trio-cresyl phosphate metabolite. *Nature.* 1961; 191:1396–1397. [PubMed: 13877086]
16. Clement JG. Importance of alioesterase as a detoxification mechanism for soman (Pinacolyl methylphosphonofluoridate) in mice. *Biochem Pharmacol.* 1984; 33:3807–3811. [PubMed: 6508835]
17. Boskovic B. The influence of 2-/o-cresyl/-4 H-1 : 3 : 2-benzodioxaphosphorin-2-oxide (CBDP) on organophosphate poisoning and its therapy. *Arch Toxicol.* 1979; 42:207–216. [PubMed: 475595]
18. Garrett TL, Rapp CM, Grubbs RD, Schlager JJ, Lucot JB. A murine model for sarin exposure using the carboxylesterase inhibitor CBDP. *Neurotoxicology.* 2010; 31:502–508. [PubMed: 20510297]
19. Maxwell DM. The specificity of carboxylesterase protection against the toxicity of organophosphorus compounds. *Toxicol Appl Pharm.* 1992; 114:306–312.
20. Jimmerson VR, Shih TM, Maxwell DM, Kaminskis A, Mailman RB. The effect of 2-(o-cresyl)-4H-1:3: 2-benzodioxaphosphorin-2-oxide on tissue cholinesterase and carboxylesterase activities of the rat. *Fundamental and applied toxicology : official journal of the Society of Toxicology.* 1989; 13:568–575. [PubMed: 2612789]
21. Quistad GB, Klintenberg R, Casida JE. Blood acylpeptide hydrolase activity is a sensitive marker for exposure to some organophosphate toxicants. *Toxicological Sciences.* 2005; 86:291–299. [PubMed: 15888665]
22. Sprague GL, Castles TR. Estimation of the delayed neurotoxic potential and potency for a series of triaryl phosphates using an in vitro test with metabolic activation. *Neurotoxicology.* 1985; 6:79–86. [PubMed: 3873039]
23. Kim JH, Stevens RC, Maccoss MJ, Goodlett DR, Scherl A, Richter RJ, Suzuki SM, Furlong CE. Identification and characterization of biomarkers of organophosphorus exposures in humans. *Adv Exp Med Biol.* 2010; 660:61–71. [PubMed: 20221871]
24. Quistad GB, Casida JE. Sensitivity of blood-clotting factors and digestive enzymes to inhibition by organophosphorus pesticides. *J Biochem Mol Toxicol.* 2000; 14:51–56. [PubMed: 10561082]
25. Johnson MK. Organophosphorus esters causing delayed neurotoxic effects: mechanism of action and structure activity studies. *Arch Toxicol.* 1975; 34:259–288. [PubMed: 766733]
26. Stefanidou M, Athanaselis S, Spiliopoulou H. Butyrylcholinesterase: biomarker for exposure to organophosphorus insecticides. *Intern Med J.* 2009; 39:57–60. [PubMed: 19290984]
27. Lu WJ, Ferlito V, Xu C, Flockhart DA, Caccamese S. Enantiomers of Naringenin as Pleiotropic, Stereoselective Inhibitors of Cytochrome P450 Isoforms. *Chirality.* 2011; 23:891–896.
28. Schilter B, Andersen MR, Acharya C, Omiecinski CJ. Activation of cytochrome P450 gene expression in the rat brain by phenobarbital-like inducers. *Journal of Pharmacology and Experimental Therapeutics.* 2000; 294:916–922. [PubMed: 10945841]

29. Marsillach J, Richter RJ, Kim JH, Stevens RC, MacCoss MJ, Tomazela D, Suzuki SM, Schopfer LM, Lockridge O, Furlong CE. Biomarkers of organophosphorus (OP) exposures in humans. *Neurotoxicology*. 2011; 32:656–660. [PubMed: 21767566]
30. Ellman GL, Courtney KD, Andres V, Featherstone RM. A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. *Biochem Pharmacol*. 1961; 7:88. [PubMed: 13726518]
31. Atkins J, Glynn P. Membrane association of and critical residues in the catalytic domain of human neuropathy target esterase. *J Biol Chem*. 2000; 275:24477–24483. [PubMed: 10816586]
32. Mattsson JL, Albee RR, Eisenbrandt DL. Neurological Approach to Neurotoxicological Evaluation in Laboratory-Animals. *J Am Coll Toxicol*. 1989; 8:271–286.
33. Mastropaolo W, Yourno J. An ultraviolet spectrophotometric assay for alpha-naphthyl acetate and alpha-naphthyl butyrate esterases. *Anal Biochem*. 1981; 115:188–193. [PubMed: 7304943]
34. Duysen EG, Koentgen F, Williams GR, Timperley CM, Schopfer LM, Cerasoli DM, Lockridge O. Production of ES1 plasma carboxylesterase knockout mice for toxicity studies. *Chem Res Toxicol*. 2011; 24:1891–1898. [PubMed: 21875074]
35. Munger JS, Shi GP, Mark EA, Chin DT, Gerard C, Chapman HA. A serine esterase released by human alveolar macrophages is closely related to liver microsomal carboxylesterases. *J Biol Chem*. 1991; 266:18832–18838. [PubMed: 1918003]
36. Brzezinski MR, Spink BJ, Dean RA, Berkman CE, Cashman JR, Bosron WF. Human liver carboxylesterase hCE-1: binding specificity for cocaine, heroin, and their metabolites and analogs. *Drug Metab Dispos*. 1997; 25:1089–1096. [PubMed: 9311626]
37. Winrow CJ, Hemming ML, Allen DM, Quistad GB, Casida JE, Barlow C. Loss of neuropathy target esterase in mice links organophosphate exposure to hyperactivity. *Nat Genet*. 2003; 33:477–485. [PubMed: 12640454]
38. Murawski, JTL. *Internat Conf Environ Syst*. Vol. 2011. Portland: Case study: Analysis of reported contaminated air events at one major US airline in 2009-2010.
39. Liyasova M, Li B, Schopfer LM, Nachon F, Masson P, Furlong CE, Lockridge O. Exposure to tri-o-cresyl phosphate detected in jet airplane passengers. *Toxicol Appl Pharm*. 2011; 256:337–347.
40. Sinnott M. 787 No-Bleed Systems. *Aeromagazine*. 2007; 4:1–4.
41. Weiner ML, Jortner BS. Organophosphate-induced delayed neurotoxicity of triarylphosphates. *Neurotoxicology*. 1999; 20:653–673. [PubMed: 10499364]
42. Mackerer CR, Barth ML, Krueger AJ, Chawla B, Roy TA. Comparison of neurotoxic effects and potential risks from oral administration or ingestion of tricresyl phosphate and jet engine oil containing tricresyl phosphate. *J Toxicol Environ Health A*. 1999; 57:293–328. [PubMed: 10405186]
43. Durham HD, Ecobichon DJ. The function of motor nerves innervating slow tonic skeletal muscle in hens with delayed neuropathy induced by tri-o-tolyl phosphate. *Can J Physiol Pharmacol*. 1984; 62:1268–1273. [PubMed: 6509374]
44. Carrington CD, Brown HR, Abou-Donia MB. Histopathological assessment of triphenyl phosphite neurotoxicity in the hen. *Neurotoxicology*. 1988; 9:223–233. [PubMed: 3205432]
45. Veronesi B. A rodent model of organophosphorus-induced delayed neuropathy: distribution of central (spinal cord) and peripheral nerve damage. *Neuropathol Appl Neurobiol*. 1984; 10:357–368. [PubMed: 6521845]
46. Daughtrey W, Biles R, Jortner B, Ehrich M. Subchronic delayed neurotoxicity evaluation of jet engine lubricants containing phosphorus additives. *Fund Appl Toxicol*. 1996; 32:244–249.
47. Saboori AM, Lang DM, Newcombe DS. Structural Requirements for the Inhibition of Human Monocyte Carboxylesterase by Organophosphorus Compounds. *Chem Biol Interact*. 1991; 80:327–338. [PubMed: 1954660]
48. Richards PG, Johnson MK, Ray DE. Identification of acylpeptide hydrolase as a sensitive site for reaction with organophosphorus compounds and a potential target for cognitive enhancing drugs. *Mol Pharmacol*. 2000; 58:577–583. [PubMed: 10953051]
49. Pancetti F, Olmos C, Dagnino-Subiabre A, Rozas C, Morales B. Noncholinesterase effects induced by organophosphate pesticides and their relationship to cognitive processes: implication for the

action of acylpeptide hydrolase. J Toxicol Environ Health B Crit Rev. 2007; 10:623–630. [PubMed: 18049927]

50. Fioroni F, Moretto A, Lotti M. Triphenylphosphite neuropathy in hens. Arch Toxicol. 1995; 69:705–711. [PubMed: 8572929]
51. Glynn P. Axonal degeneration and neuropathy target esterase. Arh Hig Rada Toksikol. 2007; 58:355–358. [PubMed: 18050888]
52. Read DJ, Li Y, Chao MV, Cavanagh JB, Glynn P. Neuropathy target esterase is required for adult vertebrate axon maintenance. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009; 29:11594–11600. [PubMed: 19759306]
53. Glynn P. Neuropathy target esterase. Biochem J. 1999; 344(Pt 3):625–631. [PubMed: 10585848]
54. Erlund I, Meririnne E, Alfthan G, Aro A. Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestion of orange juice and grapefruit juice. The Journal of nutrition. 2001; 131:235–241. [PubMed: 11160539]

Abbreviations

AcAlaPNA	<i>N</i> -Acetyl-L-alanyl- <i>p</i> -nitroanilide
AChE	acetylcholinesterase
APH	acylpeptide hydrolase
αNB	alpha-naphthyl butyrate
BChE	butyrylcholinesterase
CBDP	2-(<i>o</i> -cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one
CES	carboxylesterase
D125	Durad 125
DD H₂O	double-distilled water
ED₅₀	<i>in vivo</i> half-maximal effective dose
ETOH	ethanol
IC₅₀	<i>in vitro</i> half-maximal inhibitory concentration
MUA	methyl umbelliferyl acetate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NTE	neuropathy target esterase
OP	organophosphate
OPIDN	organophosphate-induced delayed neuropathy
PO	paraoxon
PNV	<i>p</i> -nitrophenyl valerate
PV	phenyl valerate
rNEST	recombinant catalytic NTE esterase domain
RBCs	red blood cells
RLMs	rat liver microsomes
TAP	triaryl phosphate
TBP	tert-butylphenyl phosphate
TCP	tri-cresyl phosphate

<i>TmBP</i>	tri-(<i>m</i> -tert-butylphenyl) phosphate
<i>ToBP</i>	tri-(<i>o</i> -tert-butylphenyl) phosphate
<i>TpBP</i>	tri-(<i>p</i> -tert-butylphenyl) phosphate
<i>ToCP</i>	tri-(<i>o</i> -cresyl) phosphate
<i>TpCP</i>	tri-(<i>p</i> -cresyl) phosphate

Highlights

An *In vitro* screening protocol for potential toxicity of triaryl phosphates was developed.

Isomers of tert-butyl phenyl phosphate produced the least BChE inhibition *in vitro*.

BChE inhibition by *in vitro* bioactivated TAPS correlated with esterase inhibition *in vivo*.

The flavonoid naringenin reduced bioactivation of TCPs into BChE inhibitor (s).

Fig. 1.

(A) BChE inhibition (mean $\pm$ SD) by RLM-bioactivated D125 (9 separate experiments, each in triplicate). The mean IC_{50} value was $0.36 \mu\text{g/ml} \pm 0.06$ (SD). **Inset** SDS-PAGE analysis of purified human plasma BChE stained with Coomassie blue: Lane 1, molecular weight markers; Lane 2, 875 ng purified human BChE, showing the 85 kDa monomer and 170 kDa dimer. (B) Concentration dependence of BChE inhibition by RLM-bioactivated ToCP, D125 and TpBP in the presence of NADPH, and (C) in the absence of NADPH [performed concurrently with conditions for (B)]. BChE activity measurements are expressed as percent of control (mean $\pm$ SD; error bars not visible due to low SD). (D) Inhibition of BChE by RLM/NADPH bioactivation of the three isomers of TBP (tri-*o*-, tri-*m*- and tri-*p*-), TpCP and D125. TpBP, ToBP, TmBP and TpCP did not generate statistically significant inhibition of BChE at levels $\leq 20 \mu\text{g/ml}$.

Fig. 2.

(**A**) Concentration dependence of rNEST inhibition by CDBP (as percent of control $\pm$ SD) (IC_{50} value = 40 ng/ml $\pm$ 1.21, SD, or 145 nM) of triplicate assays. **Inset:** Left box: Lane 1, MW markers; Lane 2, SDS-PAGE, of *E. coli* lysate containing rNEST, stained with Coomassie blue; Lane 3, column-purified rNEST-His₆ domain of NTE ($\approx$ 55 kDa), stained with Coomassie blue. Right Box, Western blots (using anti-His₆ as primary antibody) staining nickel column flow-through (Lane 4) or nickel column-purified eluate (Lane 5). (**B**) Concentration dependence of BChE inhibition by CDBP (as percent of control $\pm$ SD) (IC_{50} value = 7 ng/ml $\pm$ 0.03, SD) in triplicate assays.

Fig. 3. Concentration dependence of D125 bioactivation by naringenin inhibition *in vitro*. Triplicate samples of RLMs containing NADPH were treated in the following order: 1) naringenin incubation (concentration on x axis); 2) incubation with D125 at its IC₅₀ level for BChE inhibition, 0.36 μ g/ml; 3) incubation with purified human BChE; 4) assay for BChE activity. One triplicate set of assays contained no D125 to serve as a control value. An additional triplicate set of assays contained D125 but no naringenin (not shown). Data points represent percent of the no-D125 control (mean $\pm$ SD).

Fig. 4.

Inhibition of enzyme activities from mice exposed to TAPs. Activity measurements were made 24 hr post-gavage exposure of mice. Data points represent percent of control (mean $\pm$ SEM) from tissues of 6 mice at each TAP dosage level, assayed in triplicate. Dose response of TAP exposures: (A) plasma BChE activity; (B) RBC APH activity measured by α NB hydrolysis; (C) plasma CES activity measured by PNV hydrolysis; (D) plasma CES activity measured by MUA hydrolysis; (E) liver APH activity measured by AcAlaPNA hydrolysis, and; (F) liver CES activity measured by MUA hydrolysis. Values that were significantly different from controls by Student's *t*-test are indicated with * ($p < 0.02$), ** ($p < 0.01$) or *** ($p < 0.001$).

Table 1

IC₅₀ Values of TAPs Bioactivated with Rat Liver Microsomes, *in vitro*.

TRIARYL PHOSPHATE	Referred To as:	ID No.	Source	% Purity ^a	IC ₅₀ ^b (μg/ml)
tri-(<i>o</i> -cresyl) PO ₄	ToCP	433-89B	Chem Service ^c	98.8	0.12
tri-(<i>o</i> -cresyl) PO ₄	ToCP	60M108	City Chem ^d	Unknown	0.15
Syn-O-Add 8484	N/A	E10-234	Supresta ^e	Mixed Isomers	0.32
Durad 125	D125	E09-652	Chemtura	Mixed Isomers	0.36
mono-(<i>p</i> -isopropyl phenyl) diphenyl PO ₄	N/A	106-83A	Chem Service	Unknown	0.41
mono-(<i>p</i> -isopropyl phenyl) diphenyl PO ₄	N/A	E08-338	NYCO ^g	99.8	0.51
<i>p</i> -isopropyl phenyl PO ₄ [mixture of tri-, di-, & mono-(<i>p</i> -isopropyl phenyl) PO ₄]	N/A	E08-337	NYCO	99.9	0.56
mono-(<i>t</i> -butyl phenyl) diphenyl PO ₄	N/A	E10-225	NYCO	>99.9	0.63
mono-(dodecyl phenyl) diphenyl PO ₄	N/A	E10-228	NYCO	99.3	1.22
tri-(<i>m</i> -cresyl) PO ₄	N/A	AO1090 99	ACROS ^h	97	2.52
mono-(1-methyl-nonyl phenyl) diphenyl PO ₄ (Mixture of isomers)	N/A	E10-227	NYCO	Mixed Isomers	4.23
tri-(<i>p</i> -cresyl) PO ₄	TpCP	60M107	City Chem	Unknown	>20.0
tri-(<i>p</i> -cresyl) PO ₄	TpCP	E08-292	NYCO	99.8	>20.0
di-(isopropyl phenyl) phenyl PO ₄	N/A	E10-229	NYCO	99.7	>20.0
tri-(<i>o</i> -isopropyl-phenyl) PO ₄	N/A	E08-293	NYCO	99.8	>20.0
tri-(<i>p</i> -isopropyl-phenyl) PO ₄	N/A	E08-292	NYCO	99.8	>20.0
di-(<i>tert</i> -butyl phenyl) phenyl PO ₄	N/A	E10-226	NYCO	99.5	>20.0
tri-butyl PO ₄	N/A	90818	Fluka ⁱ	>98	>20.0
tri-(<i>p</i> - <i>tert</i> -butyl phenyl) PO ₄	TpBP	E09-649	NYCO	>99.9	>20.0
tri-(<i>o</i> - <i>tert</i> -butyl phenyl)PO ₄	ToBP	E09-650	NYCO	>99.9	>20.0
tri-(<i>m</i> - <i>tert</i> -butyl phenyl) PO ₄	TmBP	E10-036	NYCO	>99.9	>20.0

^a Percent purity was determined by gas chromatography.

^b IC₅₀ based on *in vitro* bioactivation using rat liver microsomes. When TAPs were assessed repeatedly, the lowest value is reported.

^c Chemical Service, West Chester, PA

^dCity Chemical, West Haven, CT

^eSupresta, c/o Clearon Corporation, Charleston, WV

^fChemtura Corporation, Middlebury, CT

^gNYCO S.A., Paris

^hACROS Organics, Geel, Belgium

ⁱFluka/Sigma-Aldridge, Buchs, Switzerland

Table 2

ED₅₀ Values from Blood and Liver of Mice Exposed to D125, TpCP or TpBP^a.

Assay Substrate	Enzyme Assayed					
	Blood Enzymes			Liver Enzymes		
	BChE	APH	CES	APH	CES	
Triaryl Phosphate	BTC	αNB	PNV	MUA	AcAla	MUA
D125	63	>240	19	19	33	85
TpCP	>240	>240	27	17	14	52
TpBP	>240	>240	93	86	>240	>240

^aED₅₀ values (mg/kg body weight) were based on enzyme activity from blood plasma (for BChE and CES) or RBC homogenates (for APH) and liver homogenates (for liver APH and CES) 24 hr after exposure to TAPs (TpCP and TpBP) or TAP-containing D125.