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Identifying safer anti-wear triaryl phosphate additives for jet engine lubricants

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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 (ToCP), 2-(*o*-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one (CBDP), inhibited rNEST *in vitro*, but with an IC_{50} 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.

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 phosphocreosote [5]. Studies in 1930 examining the cause of Ginger Jake paralysis, which affected 10,000–50,000 individuals consuming extracts of ginger adulterated with tri-cresyl phosphate (TCP), identified tri-(*o*-cresyl) phosphate (ToCP) as the paralytic

Abbreviations: AcAlaPNA, *N*-Acetyl-L-alanyl-*p*-nitroanilide; AChE, acetylcholinesterase; APH, acylpeptide hydrolase; αNB, alpha-naphthyl butyrate; BChE, butyrylcholinesterase; BTC, S-butyrylthiocholine iodide; CBDP, [2-(*o*-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one]; CES, carboxylesterase; D125, Durad 125; DD H_2O, double-distilled water; ED_{50}, *in vivo* half-maximal effective dose; ETOH, ethanol; IC_{50}, *in vitro* 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, *p*-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; *TmBP*, tri-(*m*-*tert*-butylphenyl) phosphate; *ToBP*, tri-(*o*-*tert*-butylphenyl) phosphate; TpBP, tri-(*p*-*tert*-butylphenyl) phosphate; ToCP, tri-(*o*-cresyl) phosphate; TpCP, tri-(*p*-cresyl) phosphate.

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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 T*o*CP was required to generate toxic metabolite (s) [14]. In 1961, Casida et al. [15] reported the toxic metabolite of T*o*CP 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 T*o*CP, 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 (T*p*BP) and tri-*p*-cresyl phosphate (T*p*CP). 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 (PO) 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 (αNB), 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 μg/ml) prior to making serial dilutions and addition to RLMs and NADPH in buffer A. Final concentrations in the bioactivation step were 14 μg/ml RLMs, 1 mM NADPH and TAPs at concentrations up to 20 μg/ml. Bioactivation proceeded for 25 min at 25 °C, when 10 μl of purified human BChE [29] (1.33 μg/ml in DD H_2O) 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

A recombinant catalytic domain of NTE (rNEST) was cloned (with a *C*-terminal His_6 tag), expressed, 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 T*o*CP) was used to determine the direct effect of 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 μg/ml; equivalent to its IC_{50} 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 an 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 h post-exposure, mice were assessed for signs of morbidity and blood was collected from a 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 h post-exposure, mice were euthanized by CO_2 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 HCl (100 mM, pH 8.0, 4 °C). Both homogenates were centrifuged (700 rcf for 10 min at 4 °C) 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 microliters of thawed blood cell lysate (1:20 dilution in 20 mM Tris HCl, pH 7.0) were