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Platelet-activating factor acetylhydrolase: selective inhibition by potent *n*-alkyl methylphosphonofluoridates

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Abstract

Platelet-activating factor (PAF) is a potent endogenous phospholipid modulator of diverse biological activities, including inflammation and shock. PAF levels are primarily regulated by PAF acetylhydrolases (PAF-AHs). These enzymes are candidate secondary targets of organophosphorus (OP) pesticides and related toxicants. Previously known OP inhibitors of other serine hydrolases were tested with PAF-AH from mouse brain and testes of established functional importance compared with the structurally different human plasma enzyme. Several key OP pesticides and their oxon metabolites were very poor inhibitors of mouse brain and human plasma PAF-AH in vitro but moderately active for mouse brain and blood PAF-AH in vivo (e.g., tribufos defoliant and profenofos insecticide, presumably following oxidative bioactivation). OP compounds were then designed for maximum in vitro potency and selectivity for mouse brain PAF-AH vs. acetylcholinesterase (AChE). Lead compounds were found in a series of benzodioxaphosphorin 2-oxides. Ultrahigh potency and selectivity were achieved with *n*-alkyl methylphosphonofluoridates (long-chain sarin analogs): mouse brain and testes IC50 ≤ 5 nM for C_8–C_{18} analogs and 0.1–0.6 nM for C_{13} and C_{14} compounds; human plasma IC50 ≤ 2 nM for C_{13}–C_{18} analogs. AChE inhibitory potency decreased as chain length increased with maximum brain PAF-AH/AChE selectivity (>3000-fold) for C_{13}–C_{18} compounds. The toxicity of i.p.-administered PAF (LD50 ca. 0.5 mg/kg) was increased less than 2-fold by pretreatment with tribufos or the C_{13} *n*-alkyl methylphosphonofluoridate. These studies with a mouse model indicate that PAF-AH is not a major secondary target of OP pesticide poisoning. The optimized PAF-AH inhibitors may facilitate investigations on other aspects of PAF metabolism and action.
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Keywords: Acetylhydrolase; Methylphosphonofluoridate; Organophosphorus inhibitor; Pesticide; Platelet-activating factor; Tribufos

Introduction

Platelet-activating factors (PAFs) are 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine phospholipids with potent, diverse physiological actions. They are strong mediators of inflammation (Karasawa et al., 2003; Prescott et al., 2000). Increased platelet activation triggers acute vascular syndromes, including ischemic stroke and myocardial infarction, and antiplatelet drugs are now a routine part of treatment to prevent additional occurrences (Zhao et al., 2001). PAF is highly toxic to mice, inducing severe shock and death at 40 μg/kg i.v. (Fukuda et al., 2000; Tanniere-Zeller et al., 1989).

PAF-acetylhydrolase (AH) plays an important role in human health by hydrolyzing the *sn*-2 ester bond and

Abbreviations: AChE, acetylcholinesterase; AH, acetylhydrolase; BDPO, benzodioxaphosphorin 2-oxide; CPO, chlorpyrifos oxon; DFP, diisopropyl fluorophosphate; ED50, dose inhibiting 50% of enzyme activity; IC50, concentration inhibiting 50% of enzyme activity; DMSO, dimethyl sulfoxide; 2-*N*-methylcarbamyl PAF, 1-*O*-palmityl-2-(*N*-methylcarbamyl)-*sn*-glycero-3-phosphocholine; OP, organophosphorus; PAF, platelet-activating factor; phenyl-BDPO, phenyl-benzodioxaphosphorin 2-oxide; PMSF, phenylmethanesulfonyl fluoride; SAR, structure–activity relationship; TMPF, *n*-tridecyl methylphosphonofluoridate.

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thereby attenuating the bioactivity of PAF (Tjoelker and Stafforini, 2000). This enzyme is expressed in many tissues with high abundance in brain, testes, and blood (Kidd et al., 2001; Koizumi et al., 2003; Stafforini et al., 1987). PAF-AHs are best characterized in brain and plasma, which show poor sequence homology; hence, they are more related in function rather than structure (Karasawa et al., 2003). The mammalian brain enzyme consists of two catalytic α subunits and one regulatory β subunit, whereas the blood enzyme is a single polypeptide (45 kDa for human) (Karasawa et al., 2003). Specific mutation of the β subunit is associated with Miller–Dieker syndrome involving brain malformation and seizures (Tjoelker and Stafforini, 2000). Mice lacking the catalytic α subunits display testicular shrinking and impairment in spermatogenesis (Koizumi et al., 2003).

PAF-AHs are serine hydrolases that are inhibited by organophosphorus (OP) compounds at the catalytic (e.g., α) subunit. They are almost completely inhibited by diisopropyl fluorophosphate (DFP) at 100 μM for bovine brain and methyl arachidonylphosphonofluoridate at 5 μM for human coronary artery endothelial cells (Hattori et al., 1993; Kell et al., 2003). Other OP compounds have not been examined and more potent inhibitors have not been reported. OP insecticides, chemical warfare agents, their metabolites and analogs inhibit not only acetylcholinesterase (AChE) as the primary target but also several secondary sites with toxicological ramifications (Casida and Quistad, 2004).

This investigation considers PAF-AH as a potential secondary target for OP pesticides and related toxicants. Mouse brain and testes PAF-AH, because of their established functional importance, are compared with the structurally different human plasma enzyme. OP pesticides are considered first, followed by other known serine hydrolase inhibitors (Casida and Quistad, 2004), emphasizing substituted benzodioxaphosphorin 2-oxides (BDPOs) (Wu and Casida, 1992) and *n*-alkyl alkylphosphonofluoridates (Wu and Casida, 1995). Lead compounds from this survey are then structurally optimized for potency and selectivity for PAF-AH vs. AChE. Finally, it examines whether the already high toxicity of PAF might be further increased by PAF-AH inhibitors. Overall, this study evaluates the toxicological relevance of OP-induced PAF-AH inhibition in mice.

Materials and methods

Chemicals. Caution: Some of the test compounds have high acute toxicity and others are delayed neurotoxicants in mice (Casida and Quistad, 2004; Wu and Casida, 1996). [^{3}H]PAF (1-*O*-hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine) from Perkin Elmer Life Sciences (Boston, MA) at 22 Ci/mmol was diluted with unlabeled PAF (Sigma, St. Louis, MO) to 11 mCi/mmol. 1-*O*-Palmityl-2-(*N*-methylcarbamyl)-*sn*-glycero-3-phosphocholine (2-*N*-methylcarbamyl PAF) was from Sigma. Chlorpyrifos, diazinon, tribufos, and chlorpyrifos-methyl oxon were from Chem Service (West Chester, PA). The other pesticides and candidate inhibitors were available from previous investigations in this laboratory (Quistad and Casida, 2004; Quistad et al., 2002; Segall et al., 2003; Wu and Casida, 1995, 1996). This included a series of BDPOs inhibitory to many esterases (Casida and Quistad, 2004; Casida et al., 1961, 1963; Wu and Casida, 1992, 1994). Other OPs were synthesized as required for structure optimization. Synthesis of *n*-tridecyl (*n*-$C_{13}H_{27}O$) methylphosphonofluoridate (TMPF) by the general method of Wu and Casida (1995) is described here as an example. Other phosphonofluoridates were prepared by an analogous procedure using the appropriate phosphonic dichloride and alcohol. They were identified by their ^{1}H and ^{31}P NMR spectra recorded for deuterochloroform solutions with a Bruker AM-300 spectrometer. Chemical shifts (δ) were determined for ^{1}H at 300 MHz relative to internal deuterochloroform referenced at 7.27 ppm and for ^{31}P at 121.5 MHz relative to external phosphoric acid.

Synthesis of TMPF was achieved by cooling a solution of methylphosphonic dichloride (1.56 g, 11.7 mmol) in methylene chloride (10 ml) in a dry ice–acetone bath, then adding *n*-tridecanol (2.18 g, 10.9 mmol) and triethylamine (2.0 ml, 14.3 mmol) in methylene chloride (30 ml) over 15 min. The cooling bath was removed and the brown solution was stirred at 25 °C for 2 h. A solution of benzyltriethylammonium chloride (24.2 mg, 0.106 mmol) in 20 ml of 20% potassium fluoride in water was added and the two phase system was stirred at 25 °C for 2 h. The methylene chloride phase was separated, dried over potassium carbonate, filtered, and concentrated to give 1.35 g of a light brown oil that solidified on storage at −15 °C. Purification by silica gel chromatography using a step gradient (20–50% ethyl acetate/hexanes) gave the desired compound (1.12 g, 4.01 mmol, 37% yield) as a light yellow oil. ^{1}H NMR: 2H, δ = 4.18 (dt, J = 15.9, 7.7), 3H, δ = 1.65 (dd, J = 18.5, 5.7), 22H, δ = 1.27 (m), 3H, δ = 0.89 (t, J = 6.7). ^{13}C NMR: $POCH_2$, δ = 67.5 (s), terminal CH_3, δ = 14.4 (s), CH_3-P, δ = 10.2 (dd, J = 149.2, 27.1). ^{31}P NMR: δ = 32.3 (d, J = 1048).

Inhibition of PAF-AH in vitro. Mouse brain, testes, and liver were from PEL Freez Biologicals (Rogers, AR). Plasma (human, mouse) was from Sigma. The general assay method for PAF-AH has been described for plasma and was modified minimally, e.g., 25 vs. 37 °C (Stafforini et al., 1990). It involves liberation of [^{3}H]acetate from [^{3}H]PAF. Brain, testes, and liver were homogenized (20% w/v) in 50 mM Tris buffer (pH 8, 5 °C) containing 0.2 mM EDTA. Homogenates were centrifuged at 700 × g for 10 min (pellet discarded) and 100-μl aliquots frozen (0 °C) with 10-fold dilution just prior to assay. Diluted homogenates (20 μl) were added to 0.1 M HEPES buffer (pH 7.2, 475 μl) containing 0.01% Triton X-100 in 4-ml

glass vials. Inhibitors were added in dimethyl sulfoxide (DMSO) (5 μl) followed by preincubation for 15 min at 25 °C (total volume 500 μl). Controls contained DMSO (5 μl), which inhibited less than 5%. [^{3}H]PAF (80 000 dpm, 8 μM final concentration) in DMSO (5 μl) was added and the mixture was incubated for 15 min at 25 °C. Hydrolysis was stopped by addition of 10 M acetic acid (100 μl) and 0.1 M sodium acetate (1.5 ml). [^{3}H]Acetic acid was separated from the reaction mixture using C_8 Bond Elut columns (1 × 2 cm) (Varian, Palo Alto, CA) rinsed with 0.1 M sodium acetate (1.5 ml). The eluant containing [^{3}H]acetic acid from [^{3}H]PAF hydrolysis was quantified by liquid scintillation counting. Columns were rinsed with methanol for reuse. Likewise, PAF-AH in human plasma was assayed after 8-fold dilution. Activity was measured with 36, 15, 150, and 7 μg protein for brain, testes, plasma, and liver, respectively. Under these conditions, control preparations hydrolyze about 8% of the [^{3}H]PAF and the reaction is linear with regard to protein level and time. PAF-AH activity was calculated relative to [^{3}H]acetate formation, correcting for controls. The concentration of compound inhibiting 50% of enzyme activity (IC50) was derived with two to three concentrations (above and below the IC50, each in triplicate) in the range of 20–80% enzyme inhibition. Results are reported as the mean ± SD.

Inhibition of AChE in vitro. The inhibition of mouse brain AChE activity by OPs with 15-min preincubation of OP and enzyme was assayed with acetylthiocholine as substrate in 100 mM phosphate (pH 7.4, 25 °C) (Ellman et al., 1961; Quistad et al., 2000).

Inhibition of PAF-AH in vivo. Male Swiss–Webster mice (27–30 g) from Harlan Laboratories (Indianapolis, IN) were maintained under standard conditions with access to water and food ad libitum. The studies were carried out in accordance with the Guiding Principles in the Use of Animals in Toxicology as adopted by the Society of Toxicology in 1989. Mice were treated i.p. (3–100 mg/kg) with the test compound in DMSO (30 μl) or carrier solvent alone as a control. After sufficient time for OP bioactivation and PAF-AH inhibition (2 h), mice were sacrificed by cervical dislocation. Serum was prepared from blood recovered by cardiac puncture. Brain and blood serum PAF-AH assays were made with 36 and 14 μg protein, respectively, to determine the approximate in vivo dose to inhibit 50% of PAF-AH activity (ED50).

Synergism of PAF toxicity. PAF, TMPF, and tribufos were administered i.p. in DMSO (30 μl). Possible synergism (toxicity enhancement) was assessed by pretreatment with TMPF and tribufos (100 mg/kg, 4 h) for mortality determination 2 h after PAF administration. At lethal i.v. doses of PAF, mice show labored respiration and general depression with death within 15–45 min (Young et al., 1985).

Results

OP and related compounds as in vitro PAF-AH inhibitors

Pesticides, their analogs, methylcarbamates, and sulfonyl fluorides

The first study examined four OP pesticides (tribufos, profenofos, dichlorvos, and monocrotophos), four activated oxon metabolites [chlorpyrifos oxon (CPO), CPO-methyl, diazoxon, and paraoxon], and two dipentyl analogs (of CPO and dichlorvos) for in vitro inhibition of PAF-AH in mouse brain and human plasma. Parent phosphorothionate pesticides (e.g., chlorpyrifos) were not studied in vitro since they require bioactivation to oxons. Tribufos inhibits at 100 μM, but the other three pesticides are inactive. CPO, CPO-methyl, and diazoxon have low activity for brain PAF-AH (IC50 21–80 μM), while CPO-pentyl is the most potent (IC50 1 μM) in this series. Dipentyl substitution increases inhibition also with dichlorvos (IC50 130 μM vs. inactive; data not shown). In general, PAF-AH in plasma is less sensitive and the structure–activity relationship (SAR) is reversed for the CPO series with CPO-methyl most active (IC50 25 μM). Two methylcarbamates are weak inhibitors (2-*N*-methylcarbamyl PAF, IC50 53–75 μM) or inactive (carbaryl, IC50 > 100 μM). Three sulfonyl fluorides are also weak inhibitors or inactive. Although the pesticides and activated oxons are fairly weak inhibitors of PAF-AH from mammalian brain and plasma, the greater potency of the

Table 1
Potency of OP pesticides, their analogs, methylcarbamates, and sulfonyl fluorides as inhibitors of PAF-AH in mouse brain and human plasma

Compound	IC50 (nM)[a]	
	Brain	Plasma
OP pesticides and their analogs		
Chlorpyrifos oxon		
pentyl $(n\text{-}C_5H_{11}O)_2$	1000 ± 400	>100 000 (27 ± 4)
ethyl $(C_2H_5O)_2$ (CPO)	21 000 ± 4000	89 000 ± 26 000
methyl $(CH_3O)_2$	80 000 ± 9000	25 000 ± 5000
Diazoxon	80 000 ± 25 000	>100 000 (8 ± 11)
Tribufos	ca. 100 000	ca. 100 000
Profenofos	>100 000 (13 ± 6)	>100 000 (4 ± 3)
Others[b]	>100 000	>100 000
Methylcarbamates		
2-*N*-methylcarbamyl PAF	53 000 ± 3000	75 000 ± 16 000
Carbaryl	>100 000 (0 ± 0)	>100 000 (19 ± 18)
Sulfonyl fluorides[c]	>100 000	>100 000

[a] Preincubation of enzyme and inhibitor for 15 min at 25 °C before substrate addition. Mean ± SD (n = 3–5). Values in parentheses are inhibition (%) at 100 000 nM.
[b] Paraoxon, dichlorvos, monocrotophos, and dipentyl analog of dichlorvos.
[c] $n\text{-}C_{12}H_{25}SO_2F$, $n\text{-}C_8H_{17}SO_2F$, and $C_6H_5CH_2SO_2F$.

dipentyl than diethyl or dimethyl derivatives for brain PAF-AH indicates that higher alkyl analogs may be of particular interest (Table 1).

Substituted benzodioxaphosphorin 2-oxides (BDPOs)

With the goal of optimizing PAF-AH-inhibitory activity, the effect of higher alkyl chain length was examined with 17 examples in the substituted BDPO series, again using mouse brain and human plasma. As before, PAF-AH from brain is generally more sensitive to inhibitors than that from plasma. Compounds with intermediate chain length are most active (C_7–C_{12} for brain and C_7–C_9 for plasma) with lower potency conferred by shorter or longer alkyl groups. The exception is methyl-BDPO with moderate activity despite the short chain length. Most interestingly, decyl-BDPO is about 200-fold more active for PAF-AH from brain (IC50 550 nM) than from plasma (Table 2).

n-Alkyl methylphosphonofluoridates and related compounds

Discovery of the potency of C_7–C_{12} *n*-alkyl-BDPOs prompted an extensive optimization study in the *n*-alkyl methylphosphonofluoridates and related compounds with emphasis on alkyl chain length. Twenty-four phosphofluoridates were obtained or synthesized and tested for potency with the three PAF-AH sources. With brain and testes PAF-AH, the C_8–C_{18} *n*-alkyl methylphosphonofluoridates are highly potent in vitro inhibitors (IC50 ≤ 5 nM) with maximal potency for C_{13} and C_{14} (IC50 0.1–0.6 nM). PAF-AH from testes is generally 2-fold less sensitive (n = 21) than that from brain, but the SAR is very similar (Fig. 1). With human plasma PAF-AH, the C_{13}–C_{18} *n*-alkyl methylphosphonofluoridates are potent inhibitors (IC50 < 2 nM), but shorter alkyl chains (C_8–C_{12}) are much less effective than with brain PAF-AH. Replacement of C by O in alkyl groups [R_2 = n-$C_9H_{19}O$ vs. n-$C_6H_{13}O(CH_2)_2O$] reduces PAF-AH inhibitory potency ≥10-fold. The C_{11} and C_{13} *n*-alkyl ethylphosphonofluoridates are highly active on brain and testes PAF-AH (IC50 0.6–2 nM) although C_5–C_9 compounds are much less active. Other alkyl phosphofluoridates, including DFP, C_8 and C_{12} phosphonates, two phosphates, and a biotin probe, are weak inhibitors (Table 3).

Table 2
Potency of substituted benzodioxaphosphorin 2-oxides as inhibitors of PAF-AH in mouse brain and human plasma

2-Substituent	IC50 (nM)[a]	
	Brain	Plasma
n-$C_{10}H_{21}$	550 ± 120	>100 000 (30 ± 3)
n-$C_8H_{17}O$	1700 ± 100	>100 000 (19 ± 6)
CH_3	3900 ± 2200	65 000 ± 8000
n-$C_7H_{15}S$	3400 ± 1300	>100 000 (29 ± 16)
n-$C_{11}H_{23}NH$	7400 ± 1700	>100 000 (32 ± 6)
n-C_8H_{17}	12 000 ± 6000	34 000 ± 15 000
n-C_7H_{15}	22 000 ± 2000	32 000 ± 5000
n-$C_{11}H_{23}$	18 000 ± 4000	>100 000 (17 ± 11)
n-$C_7H_{15}NH$	25 000 ± 6000	>100 000 (18 ± 5)
n-$C_{12}H_{25}$	26 000 ± 2000	>20 000 (23 ± 21)
n-C_9H_{19}	27 000 ± 5000	30 000 ± 4000
n-C_4H_9	66 000 ± 5000	>100 000 (10 ± 13)
n-C_6H_{13}	74 000 ± 13 000	55 000 ± 43 000
n-$C_{18}H_{37}$	100 000 ± 14 000	>20 000 (15 ± 15)
C_2H_5	102 000 ± 3000	>100 000 (21 ± 10)
CH_3O (salithion oxon)	>100 000 ± (27 ± 8)	
C_6H_5 (phenyl-BDPO)	>100 000 (22 ± 13)	>100 000 (32 ± 9)

[a] Preincubation of enzyme and inhibitor for 15 min at 25 °C before substrate addition. Mean ± SD (n = 3–5). Values in parentheses are inhibition (%) at 100 000 nM.

Selectivity as PAF-AH vs. AChE inhibitors (Table 3)

Having achieved potency as PAF-AH inhibitors, the next goal was to optimize for PAF-AH vs. AChE selectivity. There is considerable difference in chain length specificity of *n*-alkyl methylphosphonofluoridates for inhibition of mouse brain PAF-AH compared with mouse brain AChE. AChE potency decreases as chain length increases with maximum brain PAF-AH/AChE selectivity (>3000-fold) for C_{13}–C_{18} compounds.

OP compounds as in vivo PAF-AH inhibitors

The potency of OP compounds as in vivo PAF-AH inhibitors governs their potential ability to interfere with PAF action. PAF-AH inhibition in brain and blood was evaluated 2 h after OP treatment comparing four pesticides and TMPF (Table 4). Tribufos, profenofos, chlorpyrifos, and diazinon at 3–30 mg/kg inhibit the blood enzyme by 43–87% but only tribufos and profenofos at 100 mg/kg significantly inhibit brain PAF-AH.

The optimized in vitro PAF-AH inhibitor TMPF gives dose-dependent in vivo inhibition, but it is not remarkably active and with brain gives lower inhibition at 100 mg/kg than tribufos or profenofos (Table 4). The possibility that the lower than anticipated in vivo potency might be due to TMPF detoxification in liver was examined with the potent general esterase inhibitor phenyl-BDPO as a possible synergist blocking detoxification (Casida et al., 1961, 1963). Phenyl-BDPO (50 mg/kg, i.p., 2 h pretreatment) does not greatly affect the potency of TMPF for inhibiting PAF-AH in mouse brain or blood in vivo (data not shown) or PAF-AH in mouse brain or plasma or human plasma in vitro. However, phenyl-BDPO increases by 8-fold the potency for liver PAF-AH (Table 5), implying a key role for liver in TMPF detoxification.

Synergistic effect of OPs on PAF toxicity

The possible synergistic effect of OPs on PAF toxicity was examined by pretreating mice with the OP for 4 h,

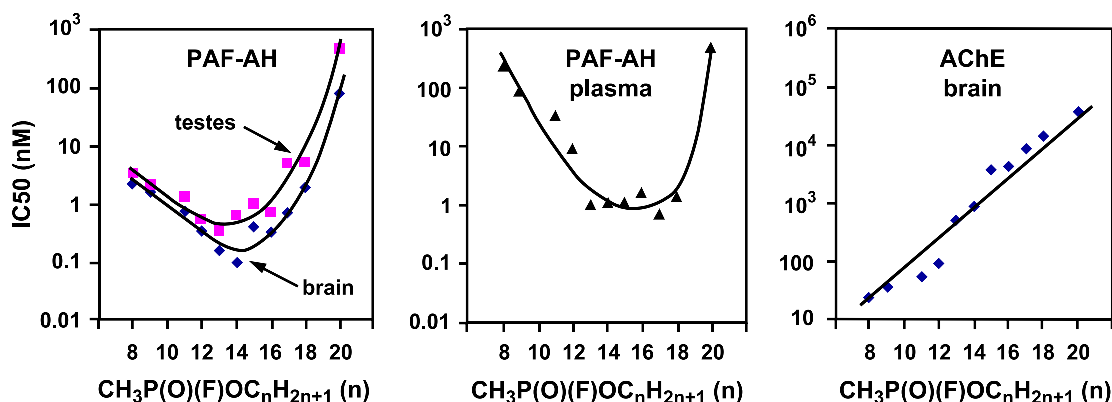

Fig. 1. Relationship between chain length for *n*-alkyl methylphosphonofluoridates and potency (IC50) in vitro for inhibition of PAF-AH (mouse brain and testes, human plasma) and AChE (mouse brain).

then administering PAF at 0.5 mg/kg using tribufos and TMPF, the two most potent OPs as in vivo blood PAF-AH inhibitors. With 10 mice per group, 5 survived with PAF alone and 5 with PAF plus TMPF whereas all died with PAF plus tribufos and none with tribufos or TMPF alone. Thus, TMPF has little effect on PAF toxicity and further

Table 3
Potency and selectivity of *n*-alkyl methylphosphonofluoridates and related compounds as inhibitors of PAF-AH (brain, testes and plasma) compared to AChE

$R_1R_2P(O)F$		IC50 (nM)[a]			
R_1	R_2	PAF-AH			AChE mouse brain
		Mouse		Human plasma	
		Brain	Testes		
Alkyl methylphosphonofluoridates					
CH_3	n-$C_8H_{17}O$	2.3 ± 0.2	3.3 ± 0.5	230 ± 45	24 ± 3
CH_3	n-$C_9H_{19}O$	1.6 ± 0.2	2.1 ± 0.6	89 ± 8	37 ± 1
CH_3	n-$C_{11}H_{23}O$	0.75 ± 0.16	1.3 ± 0.3	32 ± 22	56 ± 13
CH_3	n-$C_{12}H_{25}O$	0.35 ± 0.07	0.53 ± 0.01	8.7 ± 6.2	90 ± 10
CH_3	n-$C_{13}H_{27}O$ (TMPF)	0.16 ± 0.05	0.34 ± 0.14	1.0 ± 0.3	500 ± 130
CH_3	n-$C_{14}H_{29}O$	0.10 ± 0.02	0.64 ± 0.12	1.1 ± 0.5	900 ± 81
CH_3	n-$C_{15}H_{31}O$	0.41 ± 0.06	0.99 ± 0.27	1.1 ± 0.4	3800 ± 680
CH_3	n-$C_{16}H_{33}O$	0.33 ± 0.07	0.72 ± 0.14	1.6 ± 0.4	4200 ± 800
CH_3	n-$C_{17}H_{35}O$	0.72 ± 0.19	5.0 ± 1.1	0.69 ± 0.12	8400 ± 1400
CH_3	n-$C_{18}H_{37}O$	1.9 ± 0.6	5.2 ± 1.0	1.4 ± 0.6	14 000 ± 2400
CH_3	n-$C_{20}H_{41}O$	78 ± 20	460 ± 180	480 ± 53	37 000 ± 400
CH_3	n-$C_6H_{13}O(CH_2)_2O$	110 ± 33	170 ± 46	870 ± 25	140 ± 20
CH_3	$C_6H_5(CH_2)_2O$	1900 ± 600	5000 ± 2100	1600 ± 400	120 ± 3
Alkyl ethylphosphonofluoridates					
C_2H_5	n-$C_5H_{11}O$	720 ± 10	930 ± 210	12 000 ± 1800	120 ± 9
C_2H_5	n-$C_7H_{15}O$	32 ± 4	36 ± 8	730 ± 50	92 ± 5
C_2H_5	n-$C_9H_{19}O$	22 ± 1	43 ± 3	1800 ± 200	260 ± 40
C_2H_5	n-$C_{11}H_{23}O$	0.62 ± 0.01	1.3 ± 0.1	22 ± 4	200 ± 10
C_2H_5	n-$C_{13}H_{27}O$	0.80 ± 0.27	2.1 ± 0.5	17 ± 3	490 ± 41
Other O-alkyl phosphofluoridates					
i-C_3H_7O	i-C_3H_7O (DFP)	>100 000 (2 ± 4)	>100 000 (0 ± 0)	100000	9000[b]
n-C_8H_{17}	C_2H_5O	810 ± 150	1100 ± 100	24000 ± 1000	120[b]
n-$C_{12}H_{25}$	i-C_3H_7O	2100 ± 800	2200 ± 400	49000 ± 8000	700[c]
C_2H_5O	n-$C_9H_{19}O$	>100 000 (45 ± 8)	>100 000 (34 ± 3)	>100 000 (17 ± 16)	>100 000 (3 ± 2)
n-$C_5H_{11}O$	n-$C_5H_{11}O$	210 ± 29	220 ± 40	12000 ± 3000	210 ± 24
Biotin-$NH(CH_2)_6O$	i-C_3H_7O	39 000 ± 8000			

[a] Preincubation of enzyme and inhibitor for 15 min at 25 °C before substrate addition. Mean ± SD (n = 3–4). Values in parentheses are inhibition (%) at 100000 nM.
[b] Quistad et al., 2002.
[c] Segall et al., 2003.

Table 4
Potency of OP pesticides and TMPF as in vivo inhibitors of PAF-AH in mouse brain and blood

Compound and dose (mg/kg)	Inhibition (%)[a]	
	Brain	Blood
Tribufos		
100	50 ± 9	96 ± 2
30	12 ± 14	87 ± 1
10		67 ± 10
3		53 ± 12
Profenofos		
100	42 ± 5	66 ± 8
30	3 ± 5	52 ± 8
10		53 ± 6
Chlorpyrifos		
30	13 ± 12	65 ± 6
10		45 ± 2
Diazinon		
30	6 ± 11	43 ± 4
TMPF		
100	26 ± 2	88 ± 0
30	12 ± 13	71 ± 15
10	0 ± 0	47 ± 23

[a] Mean ± SD, n = 3–5.

study showed that tribufos synergizes the PAF toxicity by less than 2-fold.

Discussion

Substrate specificity of PAF-AH

An understanding of preferred PAF-AH substrates allows prediction of the effects of alkyl chain length on inhibitory potency by OPs. The PAF substrate used in this study was 1-*O*-hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine. For biological activity, acetyl is preferred in the *sn*-2 position with 50- and 10 000-fold reduction in potency for propionyl and butyryl, respectively (Hanahan, 1986). The substrate specificity of PAF-AH is best understood for plasma (Prescott et al., 2000). The *sn*-1 position can be either acyl- or ether-linked with appropriate C_{14}–C_{18} alkyl chains. Oxidized lecithins with medium length (up to C_9) *sn*-2 acyl groups terminated by an oxidized carbon are good substrates for PAF-AH in both plasma and brain (Hattori et al., 1993; Prescott et al., 2000; Stremler et al., 1989). The findings with the substrate and tissue sources used in the present study lay the background for understanding PAF-AH and its inhibitors in other species and tissues.

SAR and potency of PAF-AH inhibitors

The OP pesticides, in general, have little or no in vitro inhibitory effect for mouse brain or human plasma PAF-AH. The BDPO series has moderate activity with preference for methyl or intermediate-length *n*-alkyl substituents. The *n*-alkyl methylphosphonofluoridates are of highest potency with C_{12}–C_{16} while shorter and longer alkyl chains are increasingly less active. These findings are somewhat similar to, although not the same as, the substrate specificity of the PAF *sn*-2-acyl group.

The optimized PAF-AH inhibitors from this investigation are >1000-fold more potent than those known previously (i.e., subnanomolar vs. micromolar). Classical serine hydrolase inhibitors such as DFP and phenylmethanesulfonyl fluoride (PMSF) (Prescott et al., 2000) are poorly active with PAF-AH (IC50 ≥ 100 μM) (this study), but methyl arachidonylphosphonofluoridate is considerably more active (IC75 1 μM) (Kell et al., 2003). PAF-AH α subunits in rat testes have been affinity labeled successfully with a biotinylated phosphofluoridate (Kidd et al., 2001). Further optimized inhibitors of this type may lead to improved probes for localization and measurement of PAF-AH levels in tissues.

In vivo effects of OP PAF-AH inhibitors

Several OPs inhibit PAF-AH in brain and blood of mice, suggesting covalent derivatization in vivo. Since tribufos and profenofos are weak in vitro PAF-AH inhibitors, the moderate in vivo potency is likely from activation to *S*-oxides by microsomal oxidases (Hur et al., 1992; Wing et al., 1983). Although PAF-AH in blood is inhibited at a moderate level (ED50 ca. 3–10 mg/kg), the corresponding brain enzyme is only partially inhibited at 100 mg/kg. Perhaps metabolism or reaction with other serine hydrolase(s) might compromise the availability of inhibitors. Pretreatment with a general esterase inhibitor (phenyl-BDPO) (Casida et al., 1963) failed to improve PAF-AH inhibition by TMPF in brain or blood. However, since in vitro TMPF inhibition of liver PAF-AH was increased 8-fold by phenyl-BDPO pretreatment, other liver esterases probably degrade or react with TMPF and possibly other methylphosphonofluoridates, thereby reducing bioavailability.

Table 5
Effect of phenyl-BDPO on the potency of TMPF as an in vitro inhibitor of PAF-AH in mouse liver, brain, and plasma, and human plasma

PAF-AH source	TMPF IC50 (nM)[a]	
	None	Phenyl-BDPO
Mouse		
Liver	3.0 ± 0.5	0.38 ± 0.06
Brain	0.16 ± 0.05	0.37 ± 0.11
Plasma	8.9 ± 1.7	6.9 ± 1.6
Human		
Plasma	1.0 ± 0.3	1.2 ± 0.1

[a] Preincubation of enzyme with phenyl-BDPO (10 μM) or no inhibitor (none) for 15 min at 25 °C before TMPF addition. Mean ± S.D. (n = 3–4).

Mammalian toxicity relative to PAF-AH inhibition

n-Alkyl methylphosphonofluoridate inhibition of PAF-AH has several possible mammalian toxicity endpoints. These compounds are longer chain analogs of sarin with high acute lethality from AChE inhibition, but toxicity is lower for compounds with extended alkyl chains (e.g., LD50 at 24 h >100 mg/kg i.p. for TMPF). Related medium-chain alkylphosphonates are delayed toxicants in mice, acting through inhibition of neuropathy target esterase-lysophospholipase (Wu and Casida, 1996; Quistad and Casida, 2004). PAF levels presumably will increase on PAF-AH inhibition, resulting in exacerbation of its adverse properties. Less removal of oxidized lipid, e.g., hexadecyl azelaoyl phosphatidylcholine (Davies et al., 2001; Marathe et al., 2003), could potentiate inflammatory responses. Finally, extensive OP inhibition of PAF-AH (i.e., chemical knockout) may reduce spermatogenesis as known for genetic knockout (Koizumi et al., 2003).

Concluding comments

This study uses a mouse model to determine possible interactions between OP pesticides and related toxicants and the action of PAF via inhibition of PAF-AH in brain and blood. Several key OP pesticides and their oxon metabolites examined are very poor inhibitors in vitro but are moderately active in vivo (presumably following bioactivation of the thiophosphorus compounds). This survey indicates that potential OP pesticide-PAF interactions are probably not of toxicological concern. OP compounds were then designed for maximum potency and selectivity for PAF-AH vs. AChE. Ultrahigh potency was achieved for PAF-AH inhibition in vitro without improving the potency in vivo, probably due to detoxification. There was generally little interaction of the OP with PAF toxicity except in special cases. It therefore appears that residual PAF-AH activity following OP treatment is adequate to protect against PAF effects. Thus, the mouse model indicates that PAF-AH is not a major secondary target of OP poisoning.

The optimized PAF-AH inhibitors such as C_{12}–C_{16} *n*-alkyl methylphosphonofluoridates may be useful beyond the scope of the present study. The PAF receptors are assayed with [^{3}H]PAF as a radioligand under conditions of time and temperature where PAF-AH action is minimized (Valone, 1992). The new OP PAF-AH inhibitors may facilitate such assays by prolonging the stability of the intact radioligand. Rather than using *N*-methylcarbamyl PAF as a stabilized nonhydrolyzable probe (O'Flaherty et al., 1987), the endogenous ligand could be used more efficiently with the PAF-AH inhibitor providing that the OP itself is not a PAF receptor inhibitor. Finally, the potent OP inhibitors described here may be selective for PAF-AH, but they are not specific inhibitors so other systems may also be disrupted, once again as secondary targets.

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