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Selective Inhibitors of Fatty Acid Amide Hydrolase Relative to Neuropathy Target Esterase and Acetylcholinesterase: Toxicological Implications

Gary B. Quistad, Susan E. Sparks, Yoffi Segall, Daniel K. Nomura, and John E. Casida[1]

Environmental Chemistry and Toxicology Laboratory, Department of Environmental Science, Policy, and Management, University of California, Berkeley, California 94720-3112

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Fatty acid amide hydrolase (FAAH) plays an important role in nerve function by regulating the action of endocannabinoids (e.g., anandamide) and hydrolyzing a sleep-inducing factor (oleamide). Several organophosphorus pesticides and related compounds are shown in this study to be more potent in vivo inhibitors of mouse brain FAAH than neuropathy target esterase (NTE), raising the question of the potential toxicological relevance of FAAH inhibition. These FAAH-selective compounds include tribufos and (R)-octylbenzodioxaphosphorin oxide with delayed neurotoxic effects in mice and hens plus several organophosphorus pesticides (e.g., fenthion) implicated as delayed neurotoxicants in humans. The search for a highly potent and selective inhibitor for FAAH relative to NTE for use as a toxicological probe culminated in the discovery that octylsulfonyl fluoride inhibits FAAH by 50% at 2 nM in vitro and 0.2 mg/kg in vivo and NTE is at least 100-fold less sensitive in each case. More generally, the studies revealed 12 selective in vitro inhibitors for FAAH (mostly octylsulfonyl and octylphosphonyl derivatives) and 9 for NTE (mostly benzodioxaphosphorin oxides and organophosphorus fluoridates). The overall in vivo findings with 16 compounds indicate the expected association of AChE inhibition with acute or cholinergic syndrome and >70% brain NTE inhibition with delayed neurotoxic action. Surprisingly, 75–99% brain FAAH inhibition does not lead to any overt neurotoxicity or change in behavior (other than potentiation of exogenous anandamide action). Thus, FAAH inhibition in mouse brain does not appear to be a primary target for organophosphorus pesticide-induced neurotoxic action (cholinergic or intermediate syndrome or delayed neurotoxicity).

Neurotoxic effects of organophosphorus (OP)[2] pesticides and related compounds fall into three categories: (1) acute or cholinergic syndrome from inhibition of acetylcholinesterase (AChE), (2) intermediate syndrome of unknown molecular target and with no experimental model, and (3) OP-induced delayed neurotoxicity (OPIDN) or polyneuropathy from inhibition of neuropathy target esterase (NTE) (Fig. 1). In humans OP-induced cholinergic syndrome and mortality primarily result during the first day after exposure while the intermediate syndrome characterized by temporary limb weakness occurs after 1–4 days for survivors of high acute OP insecticidal poisoning (Brown and Brix, 1998). OPIDN involves permanent lower-limb paralysis with onset 2–3 weeks after OP exposure to agents such as tri-(2-tolyl) phosphate via an active metabolite (thousands of human cases in the 1930s) (Lotti, 2000) and methamidophos (currently a main causal agent of intermediate syndrome and OPIDN) (Senanayake and Karalliedde, 1987).

A newly recognized and very sensitive target for OPs in brain is fatty acid amide hydrolase (FAAH) (also called anandamide amidohydrolase), which regulates important natural endocannabinoids (anandamide, 2-arachidonyl glycerol) and hydrolyzes a sleep-inducing factor (oleamide) (Ueda *et al.*, 2000). Methyl arachidonylfluorophosphonate (MAFP) (Deutsch *et al.*, 1997b) and ethyl oleylfluorophosphonate (Patricelli *et al.*, 1999) were critical probes in elucidating the structure of FAAH and methyl fluorophosphonate analogs are potent inhibitors (Martin *et al.*, 2000). Many OP inhibitors have similar high potency for both FAAH and NTE (Quistad *et al.*, 2001), although these enzymes belong to different protein families (Atkins and Glynn, 2000). Methamidophos strongly inhibits

[1] To whom correspondence should be addressed. Fax: (510) 642-6497; E-mail: ectl@nature.berkeley.edu.

[2] Abbreviations used: AChE, acetylcholinesterase; BDPO, benzodioxaphosphorin oxide or 2-substituted-4*H*-1,3,2-benzodioxaphosphorin 2-oxide; DFP, diisopropyl phosphorofluoridate; DMSO, dimethyl sulfoxide; ED50, effective dose for 50% inhibition; EOPF, ethyl octylphosphonofluoridate; FAAH, fatty acid amide hydrolase; IC50, concentration inhibiting 50% of activity; MAFP, methyl arachidonylphosphonofluoridate; NTE, neuropathy target esterase; OP, organophosphate or organophosphonate; OPIDN, OP-induced delayed neurotoxicity; OS, organosulfonate; OSF, octylsulfonyl fluoride; PMSF, phenylmethylsulfonyl fluoride; $t_{1/2}$, recovery half-time.

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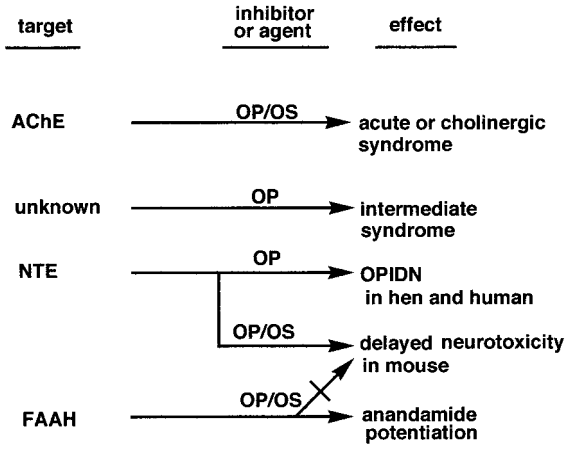

FIG. 1. Toxicological relevance of various targets for OS and OP compounds. This study shows an association between OP- and OS-induced inhibition of NTE but not FAAH and delayed neurotoxic action in the mouse.

FAAH at near lethal levels in mice, whereas other insecticides causing OPIDN and intermediate syndrome have not been assayed on FAAH. Selective inhibitors are needed for FAAH and NTE to evaluate the relative importance of these enzymes for neurotoxic effects. Although selective long-chain alkylsulfonyl fluorides and OPs have been reported for FAAH inhibition relative to binding at the cannabinoid receptor (Deutsch *et al.*, 1997a,b; Martin *et al.*, 2000), these compounds have not been evaluated for selectivity with NTE or AChE. This study uses selective inhibitors of FAAH (compared to NTE and AChE) to determine the possible toxicological relevance of FAAH inhibition in the neurotoxic action of organosulfonate (OS) and OP esters with emphasis on octylsulfonyl fluoride (OSF) and OP insecticides known to cause delayed neurotoxicity (intermediate syndrome or OPIDN) (Fig. 1).

MATERIALS AND METHODS

Chemicals. Caution: some of the test compounds have high acute toxicity and others are delayed neurotoxicants in mice (Wu and Casida, 1996). Candidate inhibitors (each >95% pure) were obtained as follows: MAFP from Cayman Chemical (Ann Arbor, MI); dichlorvos, dimethoate, fenitrothion, fenthion, leptofos, methyl parathion, monocrotophos, paraoxon, and tribufos from Chem Service (West Chester, PA); methamidophos from Chevron Chemical (Richmond, CA); (*R,S*)-profenofos from Syngenta (Greensboro, NC); chlorpyrifos from Dow AgroSciences (Indianapolis, IN); octylphosphonic dichloride and difluoride, octylphosphonothioic dichloride, and octylsulfonyl imidazole synthesized by conventional methods; anandamide, phenylmethylsulfonyl fluoride (PMSF), and diisopropyl fluorophosphate (DFP) from Sigma (St. Louis, MO); octylsulfonyl chloride from Aldrich (Milwaukee, WI). The following chemicals were available from previous studies in this laboratory: OSF, ethyl octylphosphonofluoridate (EOPF), (*R,S*)-, (*R*)-, and (*S*)-2-octyl-4*H*-1,3,2-benzodioxaphosphorin 2-oxide (octyl-BDPO), dodecyl-BDPO, phenyl-BDPO, 2-tolyl-BDPO, dipentyl 2,2-dichlorovinyl phosphate, mipafox, and EPN oxon (Wu and Casida, 1995, 1996) and (*R*)- and (*S*)-profenofos (Leader and Casida, 1982).

Animal studies. Male Swiss-Webster mice (22–28 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. The test compounds were administered ip using dimethyl sulfoxide (DMSO) as the carrier solvent (10–100 μl) or DMSO alone was injected (control). For toxic effects, mice were observed for poisoning signs and maintained up to 6 days.

Inhibition of FAAH, NTE, and AChE in vitro. Brain was homogenized (20% w/v) in 50 mM Tris buffer (pH 8) with 0.2 mM EDTA. Homogenates were centrifuged at 700*g* for 10 min (pellet discarded). Half of the supernatant was used for assays of NTE and AChE. The other half was centrifuged at 10,000*g* for 20 min and the pellet used for FAAH assays at 25°C with [^{14}C]oleamide as substrate (Quistad *et al.*, 2001). NTE is considered to be that portion of the phenyl valerate-hydrolyzing activity that is insensitive to paraoxon (40 μM) but sensitive to mipafox (50 μM). Inhibition of mouse brain NTE activity was determined at 37°C by the modified procedure for hen brain NTE (Johnson, 1977; Wu and Casida, 1996). Inhibition of AChE activity was assayed with acetylthiocholine as substrate in 100 mM phosphate (pH 7.4, 25°C) (Ellman *et al.*, 1961; Quistad *et al.*, 2000). Candidate inhibitors were introduced in DMSO or tetrahydrofuran (for compounds with high chemical reactivity) (1% final level). The concentration of OS or OP inhibiting 50% of the enzyme activity (IC50) was derived usually from threefold concentration differentials giving 15–85% inhibition. Mean IC50 and SE values reported represent at least three experiments.

Inhibition of FAAH, NTE, and AChE in vivo. Four hours after treatment mice were euthanized by cervical dislocation followed by removal of brain for preparation of homogenates and FAAH, NTE, and AChE activity assays as above. The effective dose for 50% inhibition (ED50) was estimated from dose–response data. Recovery rates for FAAH inhibited in brain were determined by assays at 4, 24, 48, and 120 h posttreatment.

Relationship of FAAH inhibition to potentiation of cannabinoid effects. Mice were treated ip with OS and OP compounds as above, and then, after 15 min or 24 h, anandamide was administered ip at 30 mg/kg in DMSO (50 μl) for observation of depression of activity (hypomotility) and recumbent posture at 15 min (Quistad *et al.*, 2001). These signs are consistent with those produced by anandamide administered to FAAH-knockout mice, which adopt a flattened, rigid posture and remain completely motionless (Cravatt *et al.*, 2001).

RESULTS

Potency and Selectivity of in Vitro Inhibitors of Mouse Brain FAAH, NTE, and AChE (Table 1)

Four OS compounds are selective for FAAH versus NTE and AChE. OSF has high potency (IC50, 1.9 nM) combined with high selectivity for FAAH versus NTE (390-fold) and AChE (>250,000); it is also less potent for butyrylcholinesterase and carboxylesterase than for NTE. Octylsulfonyl chloride is 4-fold less active than OSF for FAAH and 35-fold less potent for NTE (3200-fold preference for FAAH). Octylsulfonyl imidazole is much less potent for FAAH (IC50, 5900 nM). PMSF is both less active with FAAH (IC50, 12,000 nM) and less selective (7-fold) compared to NTE.

There are also eight OPs selective for FAAH versus NTE. MAFP is the most potent (IC50, 0.1 nM) and 6-fold selective. Octylphosphonic dichloride is less potent (IC50, 3.9 nM) but more selective for FAAH relative to NTE (4600-fold). The corresponding difluoro and thiono analogs retain activity for FAAH (IC50, 9 and 13 nM, respectively) but are less selective compared to NTE (5- and 550-fold, respectively). Paraoxon is the most selective (>65-fold), while a 3- to 17-fold preference

TABLE 1
Potency and Selectivity of OS and OP Compounds as *in Vitro* Inhibitors of Mouse Brain FAAH, NTE, and AChE

Compound	IC50 ± SE (nM) (n = 3–5)			IC50 ratio
	FAAH	NTE	AChE	
FAAH-selective OS compounds				*NTE/FAAH*
Octyl-SO_2F (OSF)[a]	1.9 ± 0.2	740 ± 40	>500,000	390
Octyl-SO_2Cl[b]	8.1 ± 0.9	26,000 ± 9,000	>100,000	3200
Octyl-SO_2-imidazole	5,900 ± 700	>100,000	>100,000	>17
Phenylmethyl-SO_2F (PMSF)	12,000 ± 1,000	84,000 ± 23,000	320,000	7.0
FAAH-selective OP compounds				*NTE/FAAH*
MAFP	0.10 ± 0.02[c]	0.60 ± 0.10	124 ± 17[c]	6.0
Octyl-P(O)Cl_2[b]	3.9 ± 0.4	18,000 ± 7,000	>100,000	4600
Octyl-P(O)F_2[b]	9.0 ± 1.0	43 ± 19	>100,000	4.8
Octyl-P(S)Cl_2[b]	13 ± 2	7,100 ± 600	>100,000	550
(*R*)-Profenofos	77 ± 13			
(*R*,*S*)-Profenofos	270 ± 13[c]	2,300 ± 400	5,000 ± 1,000[c]	8.5
(*S*)-Profenofos	680 ± 140			
Paraoxon	770 ± 200[c]	>50,000	13 ± 1[c]	>65
EPN oxon	1,200 ± 300	3,000 ± 1,000	800 ± 170	2.5
Dichlorvos	1,800 ± 300	30,000 ± 300	5,100 ± 920	17
NTE-selective OP compounds				*FAAH/NTE*
EOPF	0.60 ± 0.05[c]	0.02 ± 0.00[d]	120 ± 7[c]	30
(*R*,*S*)-Octyl-BDPO	8.1 ± 0.9[c]	0.12 ± 0.03[d]	300 ± 51[c]	68
(*R*)-Octyl-BDPO	8.8 ± 3.0	2.3 ± 0.2[d]	11,600 ± 280	3.8
(*S*)-Octyl-BDPO	21 ± 5	0.05 ± 0.01[d]	137 ± 39	420
Dipentyl dichlorovinyl phosphate	86 ± 12	2.4 ± 0.1	250 ± 10	36
2-Tolyl-BDPO	690 ± 310	150[e]	3,600 ± 300	4.6
Phenyl-BDPO	11,000 ± 2,000[c]	85 ± 30	9,500 ± 2,000[c]	130
DFP	48,000 ± 10,000[c]	1,200 ± 100	9,000 ± 1,000[c]	40
Mipafox	>10,000	5,700 ± 1,900		>1.8

[a] IC50 values (nM) for OSF and assay methods with other enzymes: carboxylesterase, 1400 ± 140 for porcine liver (Quistad and Casida, 2000), 1900 ± 1500 and 8800 ± 3700 for mouse brain and liver homogenates (87 and 6 μg protein), respectively; butyrylcholinesterase, 7800 ± 300 for mouse plasma (Sparks *et al.*, 1999).
[b] Chemically reactive and introduced in tetrahydrofuran.
[c] Quistad *et al.* (2001).
[d] Wu and Casida (1996).
[e] Veronesi *et al.* (1991).

occurs for profenofos, EPN oxon, and dichlorvos. The *R* isomer of profenofos is 9-fold more potent than the corresponding *S* isomer as an FAAH inhibitor.

Nine OP compounds are selective for NTE versus FAAH. EOPF and (*R*,*S*)-octyl-BDPO have high potency (IC50, 0.02–0.12 nM) and selectivity (30- to 68-fold). The resolved isomers of octyl-BDPO differ in their selectivity, with the *R* isomer more potent than the *S* on FAAH and the *S* isomer more potent than the *R* on NTE. (*S*)-Octyl-BDPO favors NTE by 420-fold.

Potency and Selectivity of in Vivo Inhibitors of Mouse Brain FAAH, NTE, and AChE (Tables 2 and 3)

Two OS and 14 OP compounds were examined at various doses for *in vivo* inhibition of FAAH, NTE, and AChE and the potency and selectivity were compared for the most potent compounds as ED50 values and NTE/FAAH ratios. OSF is the most potent and selective for FAAH of the compounds examined (Fig. 2). PMSF, a series of BDPOs, and EOPF are also potent *in vivo* FAAH inhibitors (ED50, 0.5–6 mg/kg) but with only moderate selectivity for FAAH versus NTE (ED50 ratio, 0.6–10) except for (*R*)-octyl-BDPO (ED50 ratio, 15). The selectivity of 10 OP insecticides was compared at discriminating doses with the finding that 7 of them gave higher inhibition for FAAH than NTE or AChE and 3 others for AChE versus FAAH and NTE.

Toxicological Relevance of AChE, NTE, and FAAH Inhibition in Vivo (Table 2)

Two criteria were used in evaluating the poisoning signs, the symptoms shortly after treatment (from AChE inhibition), and delayed death (from NTE inhibition). On this basis, the target for OSF is NTE and for PMSF is AChE. NTE is also the target for the following compounds with brain NTE inhibition of ≥67% and delayed toxicity: octyl- and phenyl-BDPOs, EOPF,

TABLE 2
Potency and Selectivity of OS and OP Compounds as *in Vivo* Inhibitors of Mouse Brain FAAH, NTE, and AChE in Relation to Poisoning Signs

Compound	Dose (mg/kg)	Percent inhibition[a] (4 h)			Proposed target based on poisoning signs[b]
		FAAH	NTE	AChE	
OS compounds					
OSF	100		87 ± 4	4 ± 5	NTE
	30	100, 97[c]	55 ± 4	0 ± 0	NTE
	10	100, 100[c]	46, 39[c]	5 ± 6	None
PMSF	200		100, 96[c]	100, 89[c]	AChE (severe)
	80	99, 95[c]	98 ± 2	66 ± 2	AChE (mild)
	30	99, 98[c]	64 ± 6	7 ± 5	None
OP delayed neurotoxicants					
Octyl-BDPO	10	76[c]	80 ± 4[d]	14 ± 2[d]	NTE
	3	55 ± 10	51 ± 12[d]		None
	1	32 ± 8	14 ± 5[d]		None
Phenyl-BDPO	100	92 ± 2[e]	100, 100[c]	68, 66[c]	NTE
	30	75 ± 5[e]			NTE
EOPF	10	82 ± 6[e]	83 ± 13	26, 15[c]	NTE
Tribufos	100	95 ± 3	67 ± 19	0 ± 0	NTE
	30	86 ± 5[e]	8 ± 6	0 ± 0	None
	10	75 ± 16[e]			None
OP insecticides					
FAAH selective					
Fenitrothion	100	77 ± 12	0 ± 0	20 ± 11	None
	30	44 ± 20		5 ± 6	None
	10	29 ± 13		0 ± 0	None
Profenofos	100	97 ± 1	11 ± 4	68 ± 9	AChE (mild)
	30	80 ± 6[e]	13, 3[c]	28, 26	None
Fenthion	300	79 ± 7	7 ± 9	60 ± 4	AChE (mild)
	100	65 ± 8	0, 0[c]	38 ± 5	AChE (mild)
Methyl parathion	10	78 ± 8	25 ± 3	18 ± 7	AChE (severe)
Methamidophos	3	63 ± 15[e]	39, 29[c]	49, 40[c]	AChE (mild)
Diazinon	30	61 ± 16[e]	34, 25[c]	38, 4[c]	AChE (mild)
Chlorpyrifos	30	61 ± 5[e]	32, 28[c]	29, 0[c]	AChE (mild)
AChE selective					
Dimethoate	300	45 ± 13	35 ± 1	81 ± 2	AChE (mild)
Leptofos	10	17 ± 11	43, 32[c]	88, 72[c]	AChE (mild)
Monocrotophos	10	4 ± 5	51, 18[c]	57, 49[c]	AChE (mild)

[a] Mean ± SE, $n = 3–7$.
[b] Criteria for proposed target: NTE, 55–100% inhibition at 4 h with 30–100% mortality at 0.3–6 days ($n = 5–15$). AChE (severe), 67–100% mortality at 0–0.3 days ($n = 3–5$) with cholinergic signs. AChE (mild), no mortality but typically depression, some tremors, and ataxia.
[c] Individual values.
[d] Wu and Casida (1996).
[e] Quistad *et al.* (2001).

and tribufos. Although phenyl-BDPO initially causes cholinergic signs, the mortality is delayed and associated with NTE inhibition. Nine insecticides poison as AChE inhibitors, six of which are selective for FAAH versus AChE (profenofos, fenthion, methyl parathion, methamidophos, diazinon, and chlorpyrifos) and the other three are selective for AChE (dimethoate, leptofos, and monocrotophos). High inhibition (≥75%) of FAAH only does not cause delayed neurotoxicity in mice as evidenced by OSF (10 mg/kg), PMSF (30 mg/kg), tribufos (10–30 mg/kg), fenitrothion (100 mg/kg), profenofos (30 mg/kg), and fenthion (300 mg/kg). The action of OSF is particularly noteworthy since ≥98% inhibition of FAAH fails to cause delayed death.

Recovery from Brain FAAH Inhibition Relative to Potentiation of Exogenous Anandamide Activity (Table 4)

OSF and four OP compounds potentiate the cannabinoid activity of anandamide (mice rendered recumbent within 15 min, $n = 3–6$ each). The half-time ($t½$) for recovery of FAAH activity in brain of mice is 2–5 days after inhibition by OSF, tribufos, profenofos, and two BDPOs (phenyl, dodecyl). Po-

TABLE 3
Selective Inhibition of FAAH Relative to NTE and AChE in Mouse Brain *in Vivo*

Compound	ED50 (mg/kg)[a]			ED50 ratio
	FAAH	NTE	AChE	NTE/FAAH
OS compounds				
OSF	0.2	20	>100 (4 ± 5)[b]	100
PMSF	1	10	60	10
OP compounds				
(*R*,*S*)-Octyl-BDPO	2	3[c]	>10[c] (14 ± 2)[b]	1.5
(*R*)-Octyl-BDPO	2	30	>10 (17 ± 7)[b]	15
(*S*)-Octyl-BDPO	1	0.6	>3 (30 ± 4)[b]	0.6
Phenyl-BDPO	6[d]	<100 (100, 100)[b]	<100 (66, 68)[b]	
EOPF	0.5[d]	3[c]	>10 (26, 15)[b]	6

[a] Estimated from log dose versus percent inhibition plots of data in Table 2 and additional data not shown.
[b] Percent inhibition at indicated dose; mean ± SE or individual values.
[c] Wu and Casida (1996).
[d] Quistad *et al.* (2001).

tentiation of the cannabinoid activity of anandamide only occurs when FAAH inhibition is 75% or higher, i.e., at 4 and 24 h with OSF and at 4 (but not 24) h with the OP compounds at the doses examined.

DISCUSSION

Potent and Selective Inhibitors of FAAH

The principal goal of this study is to establish the toxicological relevance of FAAH inhibition in the neurotoxic action of OP pesticides and related compounds (Fig. 1). The approach involves the use of potent and selective inhibitors for FAAH relative to NTE and AChE in mouse brain *in vitro* and particularly *in vivo*.

Potency and Selectivity of OSF and Analogs

Long-chain ($C_{12,}$ C_{14}, C_{16}, and C_{18}) alkylsulfonyl fluorides are potent *in vitro* inhibitors of rat brain FAAH (IC50, 3–7 nM) (Deutsch *et al.*, 1997a), whereas butylsulfonyl fluoride is of relatively low activity with hen brain NTE (IC50, 60 μM) (Osman *et al.*, 1996). OSF in the present study was both highly potent (IC50, 1.9 nM) and selective for mouse brain FAAH relative to other enzymes in brain (390-fold over NTE, >250,000-fold over AChE), liver (4600-fold over carboxylesterase), and plasma (4100-fold over butyrylcholinesterase). Four octyl analogs (sulfonyl chloride, phosphonic dichloride, phosphonic difluoride, and phosphonothioic dichloride) were only slightly less active *in vitro* inhibitors of FAAH (4–13 nM). *In vivo* studies including OP compounds (see below) established OSF as the most potent and selective inhibitor of FAAH in mouse brain.

Potency and Selectivity of OP Compounds

Many OP pesticides and related compounds are potent inhibitors of FAAH and AChE (Quistad *et al.*, 2001). Only a few of the OP compounds examined are selective *in vitro* inhibitors of FAAH versus NTE (MAFP, profenofos, paraoxon, EPN oxon, and dichlorvos) and, except for paraoxon, the selectivity ratios are fairly low. (*R*,*S*)-Profenofos with 9-fold selectivity for FAAH over NTE and 19-fold over AChE was projected as a good probe for *in vivo* selectivity, whereas dichlorvos and two activated metabolites of pesticides (paraoxon and EPN oxon) were less active with FAAH and expected to be too toxic via AChE inhibition. The selectivity for NTE relative to FAAH *in vitro* is highest for (*S*)-octyl-BDPO and phenyl-BDPO, but moderate selectivity is observed for the other BDPOs, dipentyl dichlorovinyl phosphate, and several organophosphorus fluoridates (EOPF, DFP, and mipafox). Interestingly, each of these NTE-selective compounds is known to cause OPIDN in hens or delayed neurotoxicity in mice (Wu and Casida, 1996).

Chiral specificity is also a factor in potency and selectivity. The IC50s for FAAH and NTE *in vitro* with the *S* isomer of octyl-BDPO differ by 420-fold, favoring mouse brain NTE, while (*R*)-octyl-BDPO is highly selective for FAAH *in vivo*. The potency for the enantiomers of both octyl-BDPO and profenofos with FAAH *in vitro* is reversed ($R > S$) compared to NTE (mouse, hen), AChE (bovine erythrocyte), and butyrylcholinesterase (Wu and Casida, 1996; Leader and Casida, 1982), implying structural differences in the active site of FAAH compared to these other serine hydrolases.

Acute or Cholinergic and Intermediate Syndromes

Typical acute cholinergic symptoms occurred with high doses for 9 of 10 OP insecticides examined, PMSF, and phenyl-BDPO, but poisoning was not always associated with extensive inhibition of brain AChE (e.g., methyl parathion). This lack of correlation for brain AChE inhibition with acute toxicity is known for certain OP toxicants (Kewitz and Nachmansohn, 1957) and inhibition of peripheral AChE in muscle

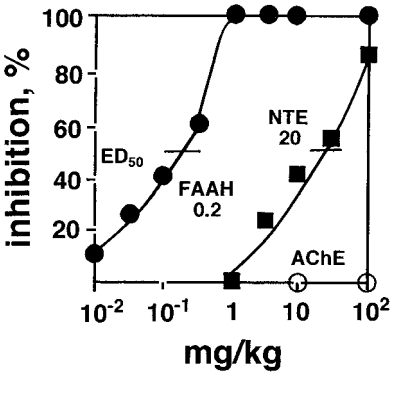

FIG. 2. Potency and selectivity of OSF as an *in vivo* inhibitor of mouse brain FAAH, NTE, and AChE.

TABLE 4
Recovery Rates from Brain FAAH Inhibition Relative to Potentiation of Exogenous Anandamide Activity in Mice at Various Times after Treatment with OSF and Four OP Compounds

Compound	Dose (mg/kg)	Inhibition (%)[a] 4 h	24 h	48 h	120 h	Recovery $t_{\frac{1}{2}}$, days[b]	Potentiation rating[c] 4 h	24 h
OSF	3	99 ± 1	75 ± 5	57 ± 1	19 ± 12	2–3	3 (3)	1 (1)
Tribufos	30	86 ± 5[d]	61 ± 11	56 ± 4	19 ± 4	2–3	3 (3)[d]	0 (0)
Profenofos	30	80 ± 6[d]	71 ± 5	73 ± 2	26 ± 15	3–4	3 (2–3)[d]	0 (0)
Phenyl-BDPO	100	92 ± 2[d]	87 ± 3	60 ± 9	42 ± 7	4–5	[e]	[e]
	30	75 ± 5[d]	60 ± 5	71 ± 3	39 ± 5		3 (3)[d]	0 (0)
Dodecyl-BDPO	10	81 ± 10[d]	31 ± 21	36 ± 2		2	1.5 (1–2)[d]	0 (0)

[a] Mean ± SE ($n = 3$).
[b] Estimated from first-order plots of time versus percent inhibition on a log scale. The inhibition value at 4 h was normalized to 100% and later times increased proportionately.
[c] Median followed in parentheses by the range (a single number indicates same rating for all mice), $n = 3–6$. Mice administered anandamide ip at 30 mg/kg 4 or 24 h after test compound and then scored individually at 15 min on a scale of 0–3: 0, no effect; 1, minimal activity depression; 2, moderate activity depression; 3, recumbent posture.
[d] Quistad *et al.* (2001).
[e] Anandamide potentiation not observed because of OP poisoning effects.

may contribute more to mortality. The intermediate syndrome for humans recovering from poisoning by OP pesticides occurs in about 8–20% of the cases, a frequency more common than initially appreciated (He *et al.*, 1998; Lotti, 2000). The limb weakness may be elicited by excess acetylcholine from AChE inhibition (Eyer, 1995). A high incidence of intermediate syndrome is associated with overexposure to fenthion, methyl parathion, parathion, omethoate, and dimethoate (De Bleeker *et al.*, 1993; He *et al.*, 1998), while methamidophos, fenitrothion, and diazinon also have been implicated as causal agents (Senanayake and Karalliedde, 1987; He, 2000; Groszek *et al.*, 1995). Each of these OP pesticides inhibits 45–79% FAAH in brain of mice and, except for fenitrothion at 100 mg/kg, there are cholinergic poisoning signs from AChE inhibition. Although the OP pesticides that cause intermediate syndrome in humans also effectively inhibit FAAH in the mouse model, this relationship appears to be coincidental with AChE inhibition as the biochemical lesion. More generally, the cause of the intermediate syndrome remains unknown, with no validated experimental model and no known molecular target.

OPIDN in Hen and Human and Delayed Neurotoxicity in Mouse

NTE inhibition and aging are the cause of OPIDN in hen and human (Johnson, 1975) and interestingly also appear to be associated with delayed neurotoxicity in mouse (Wu and Casida, 1996), a proposal supported by the present study. The sensitivity of NTE to seven representative inhibitors [EOPF, three BDPOs (octyl, phenyl, and 2-tolyl), DFP, and mipafox)] is similar in mouse compared to hen (this study; Wu and Casida, 1992, 1996; Veronesi *et al.*, 1991; Johnson, 1975; Novak and Padilla, 1986); the correlation coefficient determined by linear regression analysis for IC50 values of mouse and hen brain NTE is $r = 0.99$, indicating conserved specificity of their active sites. NTE inhibition of ≥75% in mice without high AChE inhibition causes no overt signs of toxicity within 7 h, but mortality occurs at 1–3 days associated with NTE inhibition (Wu and Casida, 1996; this study). Two of the classical causal agents for OPIDN in hen (2-tolyl-BDPO and DFP) are 5- to 40-fold better *in vitro* inhibitors of NTE than FAAH in mouse brain. This investigation establishes for the first time that tribufos at high doses greatly depresses NTE activity and is a delayed neurotoxicant in mice, consistent with the reported delayed neurotoxicity from tribufos in rats (Murphy and DuBois, 1959) and hens (Gaines, 1969).

Toxicological Relevance of FAAH Inhibition

High inhibition of brain FAAH alone in mice causes neither death nor behavioral changes. The lack of overt toxicity after selective FAAH inhibition is consistent with observations of knockout mice without FAAH expression (Cravatt *et al.*, 2001). The recovery *t*½ of FAAH activity in brain after inhibition by five compounds with diverse structures was 2–5 days, similar to that of OP-inhibited rat brain AChE (1 week) and hen brain NTE (4–6 days) (Lotti, 1992, 2000). FAAH inhibition by OSF (this study) and many OPs of ≥75% in brain depresses movement of mice administered anandamide ip at 30 mg/kg (Quistad *et al.*, 2001). In summary, this study with selective inhibitors in mice shows that, on an overall basis, the inhibition of each target enzyme in brain by 70–80% or more leads to the relevant toxicological effect, i.e., AChE for acute or cholinergic syndrome, NTE for delayed neurotoxicity, and FAAH for exogenous anandamide potentiation (Fig. 1).

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