Mechanisms for Selective Toxicity of Fipronil Insecticide and Its Sulfone Metabolite and Desulfinyl Photoproduct

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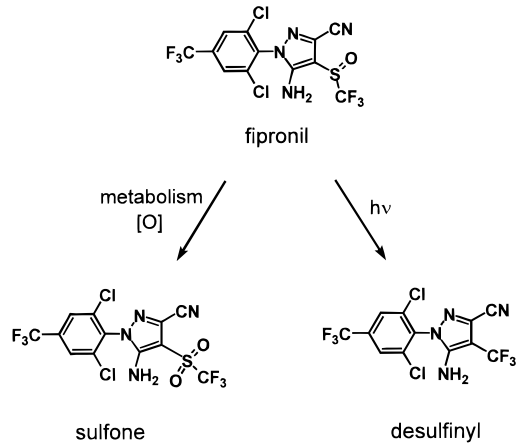
Fipronil, an *N*-phenylpyrazole with a trifluoromethylsulfinyl substituent, initiated the second generation of insecticides acting at the γ-aminobutyric acid (GABA) receptor to block the chloride channel. The first generation includes the polychlorocycloalkanes α-endosulfan and lindane. In this study, we examine the mechanisms for selective toxicity of the sulfoxide fipronil and its sulfone metabolite and desulfinyl photoproduct relative to their target site interactions in vitro and ex vivo and the importance in fipronil action of biooxidation to the sulfone. Differences in GABA receptor sensitivity, assayed by displacement of 4′-ethynyl-4-*n*-[2,3-$^3H_2$]-propylbicycloorthobenzoate ([^{3}H]EBOB) from the noncompetitive blocker site, appear to be a major factor in fipronil being much more toxic to the insects (housefly and fruit fly) than to the vertebrates (humans, dogs, mice, chickens, quail, and salmon) examined; in insects, the IC_{50}s range from 3 to 12 nM for fipronil and its sulfone and desulfinyl derivatives, while in vertebrates, the IC_{50} average values are 1103, 175, and 129 nM for fipronil, fipronil sulfone, and desulfinyl fipronil, respectively. The insect relative to the vertebrate specificity decreases in the following order: fipronil > lindane > desulfinyl fipronil > fipronil sulfone > α-endosulfan. Ex vivo inhibition of [^{3}H]EBOB binding in mouse brain is similar for fipronil and its sulfone and desulfinyl derivatives at the LD_{50} dose, but surprisingly, at higher doses fipronil can be lethal without detectably blocking the [^{3}H]EBOB site. The P450 inhibitor piperonyl butoxide, acting in houseflies, increases the metabolic stability and effectiveness of fipronil and the sulfone but not those of the desulfinyl compound, and in mice it completely blocks the sulfoxide to sulfone conversion without altering the poisoning. Thus, the selective toxicity of fipronil and fipronil-derived residues is due in part to the higher potency of the parent compound at the insect versus the mammalian GABA receptor but is also dependent on the relative rates of conversion to the more persistent and less selective sulfone metabolite and desulfinyl photoproduct.

Introduction

Fipronil, an *N*-phenylpyrazole with a trifluoromethylsulfinyl substituent (Scheme 1), represents a new chemical class of insecticides (*1*, *2*) acting at the γ-aminobutyric acid (GABA)[1] receptor as a noncompetitive blocker of the GABA-gated chloride channel (*3*, *4*). It is very effective on a wide range of economically important pests (*2*, *3*, *5*), including the boll weevil (*6*) and plant bugs (*7*) on cotton and cockroaches (*8*, *9*) and grasshoppers (*10*). Other sensitive species are the housefly (*Musca domestica* L.) (*3*, *11*) and the fruit fly (*Drosophila melanogaster* Meigen) (*12*). Fipronil has a more favorable selective toxicity for insects relative to mammals than most of the first generation of insecticidal chloride channel blockers, i.e., the chlorinated cyclodienes and other polychlorocycloalkanes (PCCAs) (*2*, *13*). One form of selective toxicity is target site specificity between the GABA receptors of insects and mammals. This is conveniently assayed with the radioligand 4′-ethynyl-4-*n*-[2,3-$^3H_2$]-propylbicycloorthobenzoate ([^{3}H]EBOB) (*14*). Binding

Scheme 1. Structures of Fipronil and Its Major Metabolite (sulfone) and Photoproduct (desulfinyl)[a]



[a] The chemical name for fipronil is 5-amino-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[1(*R*,*S*)-(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile.

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[1] Abbreviations: [^{3}H]EBOB, 4′-ethynyl-4-*n*-[2,3-$^3H_2$]propylbicycloorthobenzoate; ECD, electron capture detector; GABA, γ-aminobutyric acid; IC_{50}, concentration of the test compound required for 50% inhibition; methimazole, 2-mercapto-1-methylimidazole; NBI, *N*-benzylimidazole; PB, piperonyl butoxide; PCCAs, polychlorocycloalkanes; PPP, *O*-propyl *O*-(2-propynyl) phenylphosphonate.

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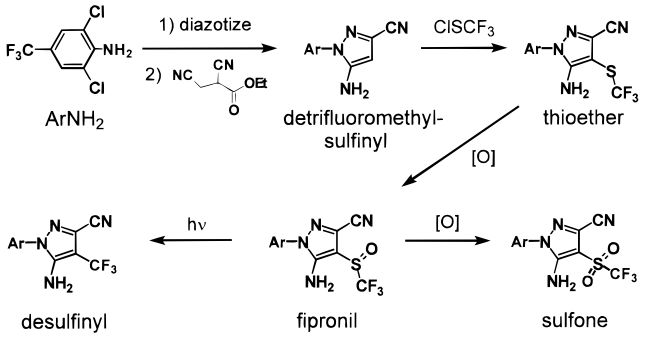
Scheme 2. Synthesis of Selected Fipronil Derivatives



studies with [^{3}H]EBOB indicate fipronil and lindane are much more selective than α-endosulfan in brain membrane preparations of mice and houseflies (*4*, *15*), and target site insensitivity to fipronil is conferred by the Ala → Ser or Gly mutants of the *Rdl* subunit in *D. melanogaster* (*12*, *16*, *17*).

Under normal use conditions for fipronil, there are three toxicants to consider, the parent compound, its major sulfone metabolite, and the desulfinyl photoproduct (*11*, *18*). Fipronil sulfone is the major metabolite of fipronil in Southern armyworm larvae (*18*) and mice (*11*) and presumably other insects and vertebrates. The sulfone is more toxic than the sulfoxide to all birds, freshwater fish, and freshwater invertebrates examined (*19*), and it is 9-fold more potent than fipronil in blocking [^{3}H]EBOB binding in mouse brain membranes (*20*). It is not clear if metabolic oxidation to the sulfone is a required bioactivation step in vertebrates or just yields an additional toxicant. Desulfinyl fipronil, although not a metabolite, is the principal photoproduct on plants and soils and is as potent as or more potent than fipronil in toxicity to mice and houseflies and at the [^{3}H]EBOB binding site (*11*).

In this study, we consider the mechanisms for selective toxicity of fipronil and its sulfone and desulfinyl derivatives in vertebrates and insects. The [^{3}H]EBOB assay is used to compare the target site selectivity of two commercial PCCAs (α-endosulfan and lindane) with that of fipronil and its sulfone and desulfinyl derivatives in membrane preparations of vertebrate brain and insect head. Mice and houseflies are then used for ex vivo [^{3}H]-EBOB binding assays to evaluate the contribution of the GABA-gated chloride channel in the selective toxicity. The importance of fipronil sulfone in fipronil action is also evaluated by determining the effects of totally blocking in vivo oxidation of the parent compound on the toxicity in mice and houseflies and the ex vivo [^{3}H]EBOB binding in houseflies.

Materials and Methods

Fipronil and Derivatives. The required compounds were synthesized as shown in Scheme 2.

(A) Ethyl 2,3-dicyanopropionate (*21*) was made by adding ethyl cyanoacetate (19.8 g, 175 mmol) in portions to sodium ethoxide (11.9 g, 175 mmol) in ethanol (60 mL) under reflux conditions. The mixture was refluxed for another 30 min, allowed to cool to room temperature, and added to a solution of glycolonitrile (10 g, 175 mmol; freshly extracted from an aqueous solution with ether and dried with $MgSO_4$) in ethanol (25 mL). This suspension was refluxed for 1 h, stirred overnight at 40 °C, poured onto crushed ice (acidified with concentrated HCl), diluted with water, and extracted with ether. The combined organic layers were dried (Na_2SO_4), evaporated to dryness, and distilled to give ethyl 2,3-dicyanopropionate (18.9 g, 47%, purity of >90%): bp 130−142 °C (1 mmHg); ^{1}H NMR ($CDCl_3$) δ 4.30 (q, OCH_2), 3.88 [t, CH(CN)], 3.00 [d, CH_2(CN)], 1.33 (t, CH_3).

(B) 5-Amino-3-cyano-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]pyrazole (the detrifluoromethylsulfinyl intermediate) was prepared by the reaction of ethyl 2,3-dicyanopropionate with the diazo salt of 2,6-dichloro-4-(trifluoromethyl)aniline (from treating the aniline with a mixture of $NaNO_2$ and concentrated H_2SO_4) as described previously (*1*). It was purified by chromatography on silica gel (7:1 hexane/ethyl acetate) and recrystallized from hexane/toluene (5:1) (purity of >99%): mp 117−118 °C (reported value of 140-142 °C). ^{1}H and ^{19}F NMR data are given in ref *11*; the structure was confirmed by X-ray crystallography (data not shown).

(C) 5-Amino-3-cyano-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-(trifluoromethyl)thiopyrazole (the thioether intermediate) was prepared by treating the detrifluoromethylsulfinyl compound (6.4 g, 20 mmol) in CH_2Cl_2 (30 mL) with trifluoromethylsulfenyl chloride (2.7 g, 20 mmol) in CH_2Cl_2 (15 mL) (*1*). After being stirred for 2 h under reflux conditions, the mixture was washed with water and dried over Na_2SO_4, the solvent evaporated, and the solid recrystallized from hexane/toluene (3:1) (purity of >99%): mp 166−167 °C (reported value of 169-171 °C); ^{1}H NMR ($CDCl_3$) δ 7.80 (s, 2× aryl-H), 4.39 (s, NH_2); ^{19}F NMR ($CDCl_3$, C_6F_6 as a reference at 0 ppm) δ −99.52 (aryl-CF_3), −118.37 (pyrazole-CF_3).

(D) Fipronil and the Sulfone. The thioether was oxidized either with *m*-chloroperbenzoic acid in CH_2Cl_2 to fipronil (*1*) or with $KMnO_4$ in aqueous acetone to the sulfone. In the latter case, the thioether (210 mg, 0.5 mmol) in acetone (10 mL) was combined with $MgSO_4$ (170 mg) in water/acetone (50 mL, 1:1). A solution of $KMnO_4$ (210 mg, 1.3 mmol) in water/acetone (20 mL, 1:1) was added while the solution was mixed thoroughly. After the mixture was stirred overnight at room temperature, concentrated HCl was added to destroy excess oxidant. Then the clear solution was extracted with ether, and the combined ether extracts were dried over Na_2SO_4; the solvent was evaporated and the white solid recrystallized from hexane/$CHCl_3$ (2:1) to give the sulfone in almost quantitative yield (215 mg, purity of >99%): mp 221−222 °C [reported value of 219−221.5 °C (*1*)]; ^{1}H NMR ($CDCl_3$) δ 7.84 (s, 2x aryl-H), 5.27 (s, NH_2); ^{19}F NMR ($CDCl_3$) δ −99.56 (aryl-CF_3), −82.70 (pyrazole-CF_3).

(E) 5-Amino-3-cyano-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-(trifluoromethyl)pyrazole (desulfinyl compound) was produced by irradiating a solution of fipronil (220 mg, 0.5 mmol) in ethanol/water/30% aqueous H_2O_2 (2:1:0.1) (40 mL) at 300 nm for 16 h in a Rayonet reactor equipped with four RPR-3000 lamps, followed by TLC separation (silica gel, 5:1 hexane/ethyl acetate, R_f = 0.45) and HPLC purification (reversed-phase C_{18} Ultrasphere column, 5 μm, 25 cm × 4.6 mm i.d., MeOH/water gradient) (yield of ∼40%, purity of >99%): mp 189−190 °C. ^{1}H and ^{19}F NMR data are given in ref *11*.

Other Chemicals. Lindane and α-endosulfan were from Chem Service (West Chester, PA), and [^{3}H]EBOB was synthesized and purified (>99% radiochemical purity) as reported previously (*14*). The P450 inhibitors piperonyl butoxide (PB), *N*-benzylimidazole (NBI), and 2-mercapto-1-methylimidazole (methimazole) were from Aldrich Chemical Co. (Milwaukee, WI), and *O*-propyl *O*-(2-propynyl) phenylphosphonate (PPP) was from FMC Corp. (Middleport, NY).

Organisms and Brains. Male albino Swiss-Webster mice were obtained from Charles River Laboratories (Wilmington, MA). Houseflies (adult females, SCR susceptible strain) and fruit flies were from cultures maintained in the Environmental Chemistry and Toxicology Laboratory and the Department of Molecular and Cell Biology, respectively, of the University of California at Berkeley. Vertebrate brains for preparation of membranes were obtained as follows: human brains from a medical examiner's office (*15*), dog and chicken brains from Pel-Freez Biologicals (Rogers, AZ), mouse brains as described above,

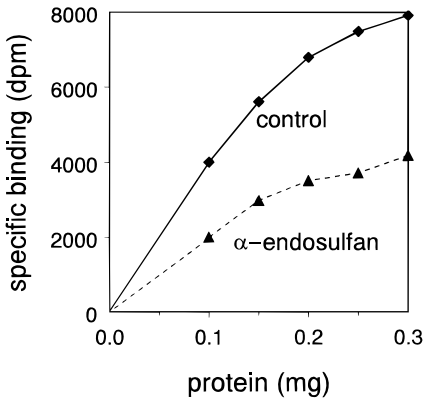


Figure 1. Effect of α-endosulfan on ex vivo [^{3}H]EBOB binding in mouse brain. Assays were carried out 20 min after ip administration of α-endosulfan at 8 mg/kg (the LD_{50}). In standard assays in other experiments, 0.15 mg of membrane protein was used.

Japanese quail brains (10-week-old males) provided by B. Wilson of the University of California at Davis, and Coho salmon brains [*Onchorhynchus kisutch* (Walbaum)] from local fresh fish markets in Berkeley, CA.

[^{3}H]EBOB Binding Assays. The vertebrate brain membranes were prepared according to Cole and Casida (*15*), including 1 mM EDTA/water dialysis to remove endogenous GABA. They were resuspended in 10 mM phosphate buffer (pH 7.5) containing 200 mM NaCl (referred to as assay buffer). Incubation mixtures consisted of 0.75 nM [^{3}H]EBOB (final concentration) in assay buffer (0.5 mL) to which was added the membrane preparation (0.2 mg of protein; *22*) in assay buffer (0.5 mL). After incubation for 90 min at 37 °C (samples from mammals and birds) or 45 min at 12 °C (samples from fish), the mixtures were filtered on Whatman GF/C glass-fiber filters followed by three 5.0 mL rinses with ice-cold assay buffer. The insect head membranes were prepared without EDTA/water dialysis as described by Deng et al. (*23*) and assayed as described above except with 300 mM NaCl in the assay buffer, incubation for 70 min at 22 °C, and filtration with Whatman GF/B filters. Specific binding was defined as the difference between the total amount of ^{3}H bound with 0.75 nM [^{3}H]EBOB and the nonspecific amount of ^{3}H bound on addition of 5 μM unlabeled EBOB.

In Vitro Inhibition of [^{3}H]EBOB Binding. The candidate inhibitor was added in Me_2SO (5 μL) to the assay buffer with [^{3}H]EBOB before introducing the membrane preparation; Me_2SO as a solvent control did not affect the binding. The concentration of the test compound for 50% inhibition (IC_{50}) was determined from three to five experiments carried out in duplicate.

Ex Vivo Inhibition of [^{3}H]EBOB Binding. Mice were treated ip with fipronil, fipronil sulfone, desulfinyl fipronil, or α-endosulfan at 8 mg/kg (the LD_{50} of α-endosulfan) and at 1, 2, and 4 times higher than their individual LD_{50} values using Me_2SO (40 μL) as the carrier vehicle and control. All mice treated with compounds at concentrations 2 and 4 times higher than the LD_{50} died in 6 ± 1 min, whereas mice treated with compounds at the LD_{50} usually survived for 20 min or longer and were sacrificed after 20 min. The brains removed following death or sacrifice were stored in pairs at −80 °C. For analysis, the pair of brains was homogenized in 0.32 M sucrose (24 mL) with a glass−Teflon homogenizer and centrifuged at 1000*g* for 10 min, and the supernatant was recentrifuged at 12000*g* for 20 min. To minimize the amount of endogenous GABA, the resulting pellet was washed three times with 1 mM EDTA (24 mL) with centrifugation at 12000*g* the first two times and 26000*g* the third time to obtain the final pellet stored at −80 °C until it was assayed for [^{3}H]EBOB binding with 0.15 mg of membrane protein. This method was validated and the 0.15 mg protein level (*22*) selected for standard assays in an experiment with α-endosulfan at the LD_{50} dose, showing ∼50% inhibition 20 min after treatment (Figure 1); the corresponding value 30 min after treatment is 51% ex vivo inhibition of [^{35}S]-*tert*-butylbicyclophosphorothionate binding (*24*).

Houseflies (in groups of ∼100) were treated topically (*23*) with fipronil or its sulfone or desulfinyl derivative (0.20 μg/g) using acetone (0.5 μL/fly) as the carrier vehicle. In some studies, PB was applied at 250 μg/g 1 h before the test compound was applied. After 24 h, the flies were placed in Erlenmeyer flasks surrounded by dry ice for quick freezing, and the frozen flies were shaken to break them into their body parts, which were almost completely separated by passing through a cold (dry ice) 2 mm diameter sieve, recovering the heads on a 1 mm sieve; the heads and bodies were stored separately at −80 °C (*25*). The bodies were used for analysis of fipronil, the sulfone, and the desulfinyl compound as described below. The heads were homogenized in 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose (*23*) and centrifuged at 500*g* for 5 min, and the supernatant was filtered through four layers of cheesecloth. The filtrate was centrifuged at 130000*g* for 1 h and the amount of protein determined (*22*), and the fresh membrane preparations were used directly for experiments of binding with [^{3}H]EBOB.

Analysis of Fipronil and Fipronil Sulfone in Mouse Brain and Liver and Housefly Bodies. Each mouse brain was homogenized in water (6 mL) and ethyl acetate (3 mL) and liver in water (9 mL) and ethyl acetate (6 mL). The organic layer was recovered (following centrifugation), washed once with water (1 mL), and dried over anhydrous Na_2SO_4. Housefly bodies (∼100) were ground with anhydrous Na_2SO_4 (2 g), and the resulting paste was extracted twice with 2:1 acetone/methanol (4 mL) in an ultrasonic bath (20 min). The extract was evaporated to dryness and the residue redissolved in 3:1 water/methanol (15 mL). Lipophilic components were removed by solid-phase extraction (3 mL low-displacement reversed-phase C_{18} bonded silica gel; "BAKER"-10 SPE system; J. T. Baker Research Products, Phillipsburg, NJ), eluting with methanol (600 μL) for GC analysis with an electron capture detector (ECD). The recovery values for each compound were >97% for brain and liver and >90% for houseflies.

For GC-ECD of mouse brain and liver and housefly extracts, following cleanup as above, we utilized the Hewlett-Packard 5480A instrument, equipped with an HP-1701 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness), helium as the carrier gas, and a temperature program from 150 to 250 °C at 10 °C/min and holding for 16 min. For quantitative analysis relative to standard curves, the individual response factors were determined to be 1.51 for fipronil sulfone and 0.83 for desulfinyl fipronil, both relative to fipronil (=1.0). Retention times were 11.23, 13.51, and 19.87 min for desulfinyl fipronil, fipronil, and fipronil sulfone, respectively. The identity of these compounds in mouse brain and liver extracts was confirmed by GC/mass spectrometry performed using the Hewlett-Packard 5985 system with the same column and temperature program that was used for GC-ECD.

Effect of Oxidase Inhibitors on Metabolism and Poisoning Signs of Fipronil in Mice and Houseflies. Mice were treated ip with PB or NBI (P450 inhibitors) or methimazole (a flavin-containing monooxygenase inhibitor) (each at 80 mg/kg) or with carrier solvent only (50 μL of Me_2SO) as the control, and after 40 min, various doses of fipronil were injected. The brain and liver were dissected at the time of death (5−7 min) or after sacrificing the mouse (20 min) and processed for GC analysis as above. Houseflies were treated topically with fipronil at a toxic but usually nonlethal dose (in the absence of synergist) of 0.10 μg/g 1 h after topical application of 200 μg/g PB, 500 μg/g NBI, 500 μg/g methimazole, or 250 μg/g PPP. Ten flies were used for each of two experiments to observe possible synergism of mortality at 24 h.

Results

Specificity of Fipronil Derivatives and Polychlorocycloalkanes as in Vitro Inhibitors of [^{3}H]-EBOB Binding in Vertebrate and Insect GABA

Table 1. Specificity of Fipronil Derivatives and Polychlorocycloalkanes as in Vitro Inhibitors of [^{3}H]EBOB Binding in Vertebrate and Insect GABA Receptors

	$IC_{50} \pm SE$ (nM)					
	fipronil derivatives			PCCAs		specific
receptor source	sulfoxide	sulfone	desulfinyl	α-endosulfan	lindane	binding (%)
vertebrate brain (number)						
human (2)	942 ± 51	155 ± 11	64 ± 6	11 ± 2	505 ± 36	88 ± 1
dog (3)	1177 ± 23	147 ± 10	75 ± 2	14 ± 1	575 ± 37	94 ± 1
mouse (50)	1014 ± 22[a]	181 ± 10	148 ± 18[a]	28 ± 5	833 ± 177	92 ± 1
chicken (4)	1391 ± 151	134 ± 11	94 ± 8	17 ± 2	665 ± 27	94 ± 1
quail (18)	1259 ± 112	258 ± 2	139 ± 9	16 ± 2	1234 ± 138	91 ± 1
salmon (48)	832 ± 64	174 ± 21	254 ± 38	33 ± 1	1636 ± 85	47 ± 1
composite[b]	1103 ± 86	175 ± 18	129 ± 29	20 ± 4	908 ± 180	84 ± 8
insect head						
housefly	6.3 ± 1.3[c]	11.9 ± 1.0	5.4 ± 1.0[c]	7.0 ± 1.0[d]	12 ± 2[d]	62 ± 2
fruit fly	7.7 ± 1.4	6.0 ± 1.5	2.8 ± 0.4	3.0 ± 0.1[d]	1.0 ± 0.1[d]	55 ± 3
composite[b]	7.0	9.0	4.1	5.0	6.5	59

[a] Reported earlier as 1010 ± 20 and 97 ± 4 for sulfoxide and desulfinyl, respectively (*11*). [b] Composite for six species of vertebrates (mean $IC_{50} \pm SE$) and two dipterous insects. [c] From ref *11*. [d] From ref *15*.

Table 2. Toxicity in Mice of Fipronil and Derivatives and α-Endosulfan in Relation to Their ex Vivo Inhibition of [^{3}H]EBOB Binding in Brain

	inhibition of [^{3}H]EBOB binding (%)[a]			
dose	fipronil	sulfone	desulfinyl	α-endosulfan
8 mg/kg	0	0	0	54 ± 3
LD_{50}[b]	22 ± 3	15 ± 3	26 ± 2	54 ± 3
$2LD_{50}$	0	35 ± 6	41, 43	61 ± 4
$4LD_{50}$	0	46, 22	49, 42	64 ± 2

[a] All values were determined 6 ± 1 min after treatment. Severe poisoning signs or death at the LD_{50} and death at $2LD_{50}$ and $4LD_{50}$. Means ± SE (n = 3−5 in duplicates) or values from two experiments. [b] LD_{50} values (milligrams per kilogram) 24 h after treatment are 41 for fipronil (*11*), 50 for the sulfone (this study), 23 for the desulfinyl (*11*), and 8 for α-endosulfan (this study; reported as 7 in ref *24*). These values are reproducible within 1.2-fold in repeated experiments.

Receptors (Table 1). The [^{3}H]EBOB assay allows direct comparisons of vertebrate (humans, dogs, mice, chickens, quail, and salmon) and insect (housefly and fruit fly) GABA receptors. The level of specific binding is 88−94% for the mammalian and avian brain membranes but still adequate at 47−62% for the salmon brain and insect head membranes. There are five principal observations. (a) Four of the five insecticides are much more potent with the two insect than with the five vertebrate GABA receptors; α-endosulfan, the exception, is less selective than the other compounds. (b) There is relatively little variation for any individual inhibitor in its potency among the vertebrates examined, with IC_{50} values generally ranging only about 2-fold among the six brain sources. (c) If the composite vertebrate data are considered, the sulfone is 6-fold and the desulfinyl 9-fold more potent than fipronil. (d) Similar potencies are observed for the five insecticides on the housefly and fruit fly receptors, with IC_{50}s varying from 4 to 9 nM for the composite results. (e) The vertebrate relative to insect selectivity of the fipronil series falls between that of lindane and α-endosulfan with IC_{50} ratios of 158 for fipronil, 140 for lindane, 31 for desulfinyl fipronil, 19 for fipronil sulfone, and 4 for α-endosulfan.

Toxicity in Mice of Fipronil and Derivatives and α-Endosulfan in Relation to Their ex Vivo Inhibition of [^{3}H]EBOB Binding in Brain (Table 2). The mouse ip LD_{50} 24 h after treatment is similar for fipronil and the sulfone (41 and 50 mg/kg), about $^1/_2$ the value for desulfinyl fipronil (23 mg/kg), and about $^1/_5$ of the value for α-endosulfan (8 mg/kg). The [^{3}H]EBOB site, assayed ex vivo 6 min after an 8 mg/kg dose, is inhibited 54% by α-endosulfan (its 24 h LD_{50}) but not detectably by the fipronil derivatives (16−35% of their 24 h LD_{50}s). Fipronil and its sulfone and desulfinyl derivatives at their LD_{50}s give 15−26% inhibition of the [^{3}H]EBOB site, a significant block although well below that observed for α-endosulfan at its LD_{50}. Higher doses generally increase the extent of blocking for fipronil sulfone, desulfinyl fipronil, and α-endosulfan but surprisingly not for fipronil where no inhibition is observed in repeated experiments at $2LD_{50}$ and $4LD_{50}$, doses which are lethal within 6 min. Thus, there are two apparent anomalies in the action of fipronil at the [^{3}H]EBOB site. First, fipronil is similar in effectiveness to the sulfone and desulfinyl derivatives on the basis of ex vivo assays at the LD_{50}s despite a 6−7-fold lower potency in vitro. Second, fipronil is the only compound examined that does not give ex vivo inhibition of [^{3}H]EBOB binding at a lethal dose.

Effect in Houseflies of Piperonyl Butoxide on the Recovery of Fipronil Derivatives and on Their Toxicity and ex Vivo Inhibition of [^{3}H]EBOB Binding in Brain (Table 3). Recovery values for fipronil and the sulfone are only $^1/_2$ of those of the desulfinyl compound 24 h after topical application, suggesting more rapid metabolism of the first two compounds than of the third compound. This is supported by the effect of PB, which essentially doubles the recovery of fipronil and its sulfone with much less effect on recovery of the desulfinyl compound. Fipronil is ∼90% converted to the sulfone under these test conditions, and this conversion is completely blocked by PB. The specificity of PB for enhancing the metabolic stability of fipronil and the sulfone but not the desulfinyl compound is also evident in the biological parameters indicated below.

The toxicity of fipronil is strongly synergized by PB (Table 3) and PPP but not by NBI or methimazole (data not shown). PB increases the mortality with fipronil and the sulfone to a greater extent than with the desulfinyl compound, which is evident as the percent kill at a standard dose or in the 4−7-fold synergism of the LD_{50} for the first two compounds but no synergism for the desulfinyl derivative. PB also increases the level of inhibition of ex vivo [^{3}H]EBOB binding by fipronil and the sulfone but not that by the desulfinyl compound. Clearly, the trifluoromethylsulfinyl or trifluoromethyl-

Table 3. Effect in Houseflies of Piperonyl Butoxide on the Recovery of Fipronil Derivatives and on Their Toxicity and ex Vivo Inhibition of [^{3}H]EBOB Binding in Brain

PB[a] pretreatment	treatment[b] fipronil	sulfone	desulfinyl
	Compound Recovery (%)		
−	28 ± 5[c]	21 ± 3[d]	51 ± 3[d]
+	68 ± 7[d]	44 ± 4[d]	64 ± 4[d]
	Mortality (%)[e]		
−	78 ± 4	44 ± 10	80 ± 6
+	99 ± 1	97 ± 1	88 ± 4
	Inhibition of [^{3}H]EBOB Binding (%)		
−	46 ± 6	48 ± 7	50 ± 11
+	70 ± 7	66 ± 4	55 ± 4

[a] PB at 250 μg/g 90 min before topical application of fipronil or derivative. [b] Values were determined 24 h after a dose of 0.20 μg/g was applied. Data are means ± SE (n = 3). [c] Sum of the values of fipronil (~10%) and sulfone (~90%). [d] Administered toxicant is the only product detected. [e] Flies that could not walk were considered dead. LD_{50} values (microgram per gram, 24 h) from a separate study (same treatment protocol) with the data given first alone and then with PB pretreatment were as follows: 0.16 ± 0.01 and 0.023 ± 0.004 for fipronil (similar to the values from ref *11*), 0.15 ± 0.01 and 0.041 ± 0.003 for the sulfone, and 0.053 ± 0.005 and 0.045 ± 0.004 for the desulfinyl (similar to the values from ref *11*).

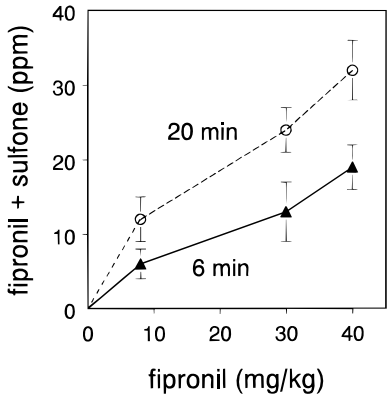


Figure 2. Relation in mice between fipronil dose and brain level of fipronil and its sulfone metabolite. Assays were carried out 6 or 20 min after ip treatment. The 6 min data for 40 mg/kg are at the time of death (5−7 min). The sulfone is 0−3% of the amount of total residue at 6 min and 20% at 20 min independent of the dose. The values are means ± SE (n = 5−8).

sulfonyl but not the trifluoromethyl substituent on the pyrazole is important for the synergistic effect.

Relation in Mice between the Fipronil Dose and the Brain Level of Fipronil and Its Sulfone Metabolite (Figure 2). The level of ip-administered fipronil increases in the brain as a function of the dose (10−40 mg/kg), and the level is almost doubled between 6 and 20 min. The contribution of the sulfone to the amount of total brain residue also increases from 0 to 3% at 6 min to ~20% at 20 min. Some of the mice at the LD_{50} (40 mg/kg) die in 6 ± 1 min with 19 ± 3 ppm fipronil in the brain, whereas the survivors at 20 min have a brain level of 32 ± 4 ppm. Clearly, the level of fipronil in the brain is not the only factor responsible for toxicity.

Effect in Mice of Piperonyl Butoxide, *N*-Benzylimidazole, and Methimazole on the Brain Level of Fipronil and Its Sulfone Metabolite (Figure 3). Following ip administration of fipronil at 30 mg/kg, the brain contains mostly fipronil at 0.12 and 0.33 h, equal amounts of fipronil and sulfone at 1 h, and 2 times as much sulfone as fipronil at 2 h. PB or NBI has no effect on the total level of brain residues but completely blocks sulfone formation, so only fipronil per se is evident in the brain. Methimazole only partially blocks sulfone formation. The effect of P450 inhibitors is even stronger in mouse liver, since in controls the sulfone is already detected within 2−4 min and its level increases approximately 2 times faster than in the brain, whereas PB- or NBI-pretreated mice form no sulfone for at least 2 h after fipronil treatment (data not shown). Although PB and NBI completely block conversion of fipronil to its sulfone, so that none is detected in the brain, they do

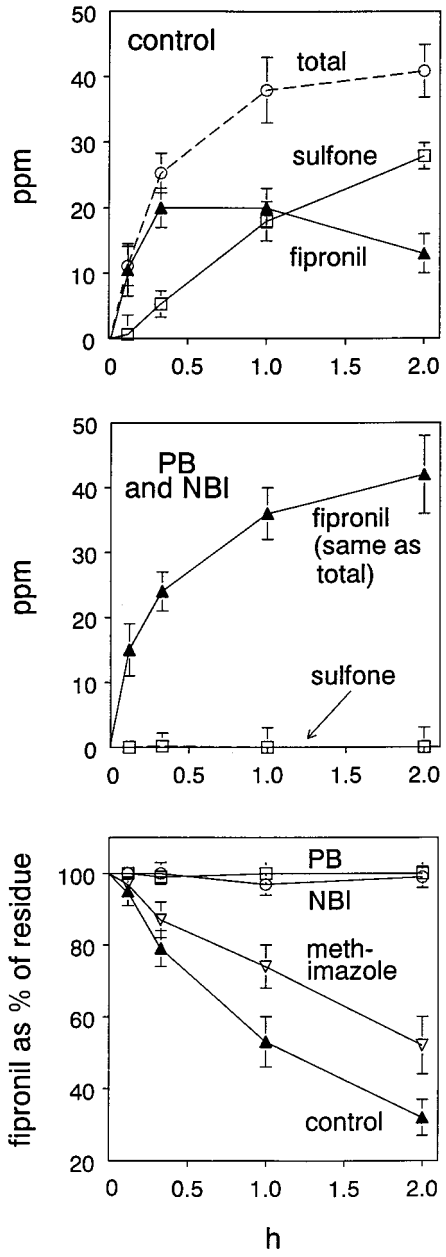


Figure 3. Effect in mice of piperonyl butoxide, *N*-benzylimidazole, and methimazole on the brain level of fipronil and its sulfone metabolite. Pretreatment (40 min) with PB, NBI, or methimazole (80 mg/kg) was carried out before ip administration of fipronil (30 mg/kg). The data are plotted as parts per million in brain for the control (top) and with oxidase inhibitors (middle) and as the percentage of fipronil of the combined fipronil and sulfone (bottom). Methimazole did not alter the total brain level of fipronil and its sulfone (data not shown). Values are means ± SE (n = 3−5) with the exception of those for the PB and NBI experiments where data are combined for plotting (n = 6−10).

not alter the poisoning signs or mortality from fipronil treatment at the LD_{50} dose.

Discussion

Fipronil is similar to α-endosulfan and lindane in its high potency at the *Drosophila* and *Musca* GABA receptors and in the cross resistance conferred by a low-affinity target site. Much of the knowledge on receptor specificity is derived from binding studies with [^{3}H]EBOB. The *Musca* and *Drosophila* GABA receptors are 70-fold more sensitive than the vertebrate receptors to the three fipronil derivatives and two PCCAs, on an overall basis, suggesting that a fundamental target site difference is a major factor in selective toxicity. More specifically, the selectivity ratio relative to the human GABA receptor (IC_{50} human/IC_{50} insect) is 135 for fipronil, 78 for lindane, 17 for the sulfone, 16 for the desulfinyl derivative, and 2.2 for α-endosulfan.

Fipronil sulfone is formed quickly from fipronil in biological systems and plays a major role in its toxicology. The sulfone, relative to fipronil, is more persistent, more potent in vitro at the vertebrate GABA receptor (6-fold overall in this study), and usually but not always more toxic, i.e., with freshwater invertebrates, freshwater fish, waterfowl, and upland game birds (*19*) but not mice (this study). The PB antagonism of fipronil toxicity in German cockroaches indicates that conversion to the sulfone is an activation process (*8*).

Fipronil itself is a toxicant for mammals and an insecticide even without oxidation to the sulfone. In mice, the conversion of fipronil to the sulfone can be completely blocked with PB (or NBI) for at least 2 h without changing the poisoning signs or mortality from fipronil treatment. However, the concentration of fipronil in the brain does not correlate with the mortality or poisoning signs since at an LD_{50} the mice that die at 6 min have 19 ppm fipronil in the brain and those that live for 20 min have 32 ppm. The *Musca* GABA receptor is more sensitive to fipronil than the sulfone, and PB strongly synergizes the toxicity of fipronil (*11*), preventing its oxidation to the sulfone and minimizing detoxification of both fipronil and the sulfone.

Fipronil, fipronil sulfone, desulfinyl fipronil, and α-endosulfan at their LD_{50} and higher doses inhibit [^{3}H]EBOB binding in brain on the basis of ex vivo assays in mice (this study) in agreement with an earlier investigation with many PCCAs using [^{35}S]-*tert*-butylbicyclophosphorothionate as the radioligand (*24*). However, there is one exception to this relationship since fipronil at a concentration 2 and 4 times the LD_{50} can be lethal without detectable inhibition of [^{3}H]EBOB binding in brain, possibly due to its lower affinity leading to dissociation during the ex vivo assay. No explanation is currently available for the greater inhibition level observed for fipronil at the LD_{50} than at 2 or 4 times this dose.

Desulfinyl fipronil is a photoproduct but not a metabolite, so its toxicological properties are particularly interesting. The desulfinyl compound is generally more toxic and more potent at the chloride channel than either fipronil or the sulfone. In houseflies, the desulfinyl compound is synergized little if at all by PB in contrast to the large synergism factors for fipronil and its sulfone, suggesting that P450-dependent detoxification is facilitated by the sulfinyl or sulfonyl moiety, but the mechanism and identity of the further metabolites are not defined. In mice, the metabolism is slow for the desulfinyl and sulfone derivatives relative to that for fipronil (*11*).

The toxicological properties of fipronil depend on the parent compound, the sulfone metabolite, and the desulfinyl photoproduct; i.e., each compound is a bioactive component of fipronil-derived residues. The selective toxicity of fipronil is due in part to the higher potency of the parent compound at the insect than the mammalian GABA receptor but is also dependent on the relative rates of conversion to the more persistent and less selective sulfone metabolite and desulfinyl photoproduct.

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