Evaluation of Fish Models of Soluble Epoxide Hydrolase Inhibition

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Substituted ureas and carbamates are mechanistic inhibitors of the soluble epoxide hydrolase (sEH). We screened a set of chemicals containing these functionalities in larval fathead minnow (*Pimphales promelas*) and embryo/larval golden medaka (*Oryzias latipes*) models to evaluate the utility of these systems for investigating sEH inhibition *in vivo*. Both fathead minnow and medaka sEHs were functionally similar to the tested mammalian orthologs (murine and human) with respect to substrate hydrolysis and inhibitor susceptibility. Low lethality was observed in either larval or embryonic fish exposed to diuron [*N*-(3,4-dichlorophenyl), *N´*-dimethyl urea], desmethyl diuron [*N*-(3,4-dichlorophenyl), *N´*-methyl urea], or siduron [*N*-(1-methylcyclohexyl), *N´*-phenyl urea]. Dose-dependent inhibition of sEH was a sublethal effect of substituted urea exposure with the potency of siduron < desmethyl diuron = diuron, differing from the observed *in vitro* sEH inhibition potency of siduron > desmethyl diuron > diuron. Further, siduron exposure synergized the toxicity of *trans*-stilbene oxide in fathead minnows. Medaka embryos exposed to diuron, desmethyl diuron, or siduron displayed dose-dependent delays in hatch, and elevated concentrations of diuron and desmethyl diuron produced developmental toxicity. The dose-dependent toxicity and *in vivo* sEH inhibition correlated, suggesting a potential, albeit undefined, relationship between these factors. Additionally, the observed inversion of *in vitro* to *in vivo* potency suggests that these fish models may provide tools for investigating the *in vivo* stability of *in vitro* inhibitors while screening for untoward effects. *Key words:* epoxide hydrolase, fish, *in vivo* models, mammals, urea pesticides. *Environ Health Perspect* 109:61–66 (2001). [Online 13 December 2000]
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Chemicals containing urea, carbamate, and amide moieties flanked by hydrophobic residues can be potent *in vitro* and *in vivo* inhibitors of mammalian soluble epoxide hydrolases (sEH; EC 3.3.2.3) (*1*). Several pesticides contain these structures. Soluble epoxide hydrolase is a highly conserved, ubiquitous enzyme that hydrolyzes aliphatic epoxides to their corresponding vicinal diols (*2*). The identification of lipid epoxides as endogenous sEH substrates (*3-5*) suggests a central role for this enzyme in cellular ion balance, vascular permeability, and inflammation (*6-9*) in addition to its role in xenobiotic metabolism. Therefore, sEH inhibition by environmental contaminants or therapeutics may present a subtle yet pertinent health risk. A model system allowing *in vivo* effects of sEH inhibition to be rapidly evaluated could accelerate identification of such risks while assisting research into the endogenous role of this enzyme. Additionally, the investigation of sEH as a therapeutic target could benefit from a cost-efficient, rapid *in vivo* sEH inhibition model.

It is well recognized that metabolic transformations can alter the relative potency of bioactive molecules when assessed under *in vivo* versus *in vitro* scenarios. Unfortunately, the cost of rigorous *in vivo* toxicity testing in rodent models often prohibits comprehensive mammalian testing. Alternative *in vivo* models may provide invaluable tools for prioritizing further studies in mammalian systems. A precursor model would use easily reared and handled organisms that have a complement of drug-metabolizing enzymes and the biochemical target of toxicant action. Fish models of mammalian responses to toxicants exist for investigations of carcinogenicity (*10*), oxidative stress (*11*), and immunotoxicity (*12*). Among the benefits of these fish models are the ease of husbandry, the potential for large experimental sample sizes with increased statistical power, the ability to investigate early developmental life stages, and the similarity between mammalian and piscine xenobiotic metabolizing enzymes.

We assessed two larval and embryo/larval fish models for the investigation of xenobiotic interactions with soluble epoxide hydrolase. Considerable quantities of sEH have been shown in the liver, kidney, and gill of the rainbow trout (*Salmo gairdneri*) (*13*). In addition, gender-specific and temporal changes in epoxy and dihydroxy eicosatrienoic acids reported in the marine scup (*Stenotomus chrysops*) (*14*) suggest a dynamic physiological role for sEH in teleost species. The functional conservation of sEH between mammals and fish was first evaluated by investigating the *in vitro* sEH inhibition capacity of the chemicals listed in Table 1 with appropriate sEH orthologs. Further, two phenyl urea herbicides, diuron (II) and siduron (VI) were selected as model *in vivo* toxicants and evaluated in both larval fathead minnow (*Pimphales promelas*) and embryo/larval golden medaka (*Oryzias latipes*) assays. In the medaka, sublethal toxicity end points were investigated as markers of developmental toxicity linked to sEH inhibition *in vivo*. Further, we assess the utility of these fish models to investigate *in vivo* versus *in vitro* sEH inhibitor potency.

Materials and Methods

Chemicals. Compounds listed in Table 1 were either purchased or synthesized in our laboratory. Compounds were purchased at > 99% purity from ChemServices Inc. (Philadelphia, PA; II, VI, VII, and VIII) and Thompson Hayward (Kansas City, KS; V) or > 98% purity from Chevron Chemical Co. (Richmond, CA; IX and XI) and Stauffer Chemical Co. (Richmond, CA; X). The diuron (II) used had less than 0.05% of *bis*-3,4-dichloro-phenyl urea (IV) by weight as determined by HPLC analysis (*1*). Siduron (VI) was a technical mixture of positional methyl cyclohexyl isomers. The *trans*-stilbene oxide (TSO) was purchased from Aldrich Chemical Co. (Milwaukee, WI). The *cis*-stilbene oxide ([^{3}H]-CSO), [^{3}H]-*trans*-diphenyl propene oxide ([^{3}H]-tDPPO), and racemic 4-nitrophenyl-*trans*-2,3-epoxy-3-phenyl-propyl carbonate (NEPC) were previously synthesized in our laboratory (*15–17*). Chemical stock solutions for enzyme assays and *in vivo* exposures were prepared in either dimethyl formamide (Aldrich) or methanol (Optima grade; Fisher Scientific, Pittsburgh, PA), respectively.

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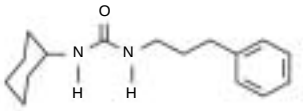
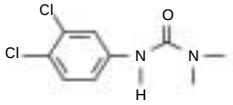
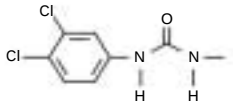
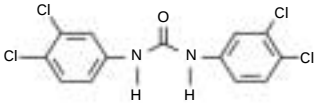
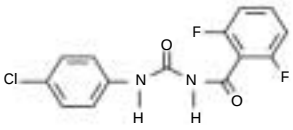
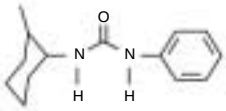
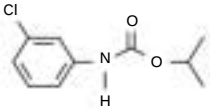
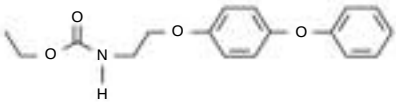
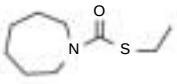
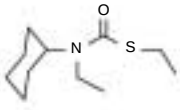
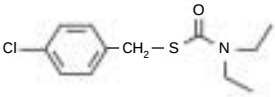
We synthesized ureas I, III, and IV by mixing isocyanates with corresponding amines and isolated them by recrystallization. Structural confirmation and purity assessments were made by melting-point (mp) determinations, proton nuclear mass resonance (NMR), and mass spectral analyses. The syntheses of compounds I (mp 103.5–104°C) and III (mp 155–156°C) have been previously reported (*1,18*). Compound IV was synthesized by adding a 10% molar excess of 3,4-dichloro amine (Aldrich) to 3,4-dichlorophenyl isocyanate dissolved in chloroform. Crystallization was promoted by hexane addition. Crystals were isolated by filtration and further purified by recrystallization from *n*-hexane. The isolated product displayed a 349 Da molecular ion under positive mode electrospray ionization on a Quattro-BQ (Fisons, Altrancham, England) and exhibited a decomposition point of 270–271°C. Proton NMR on a QE-300 (General Electric Co., Fairfield, CT) provided complete proton assignments: 2 $^{1}H_{Aromatic}$ - *d* (7.743 ppm; J = 8.9 Hz), 2 $^{1}H_{Aromatic}$ - *d* (7.928 ppm; J = 9.0 Hz), 2 $^{1}H_{Aromatic}$ - *s* (8.263 ppm), 2 $^{1}H_{Urea}$ - *s* (9.535 ppm).

Test organisms. Larval fathead minnows (7 days old) were purchased from Aquatox (Hot Springs, AR) and acclimated for 24 hr in aerated test water (see "Exposure water preparation and handling"). Golden medaka embryos were obtained from our colony maintained at the University of California Davis, Institute of Ecology. Embryonated egg masses were separated by rolling them between moistened fingertips; then they were rinsed in 25 mg/L NaCl and aerated vigorously to prevent fungal infections. We isolated embryos in early to late blastula stage (20–24 hr postfertilization) under a dissecting scope for exposures. The care and treatment of fish were in accordance with UC Davis animal use protocols 7357 and 8450 for fathead minnow and medaka, respectively.

Fish protein preparations. We pooled either 150 fathead minnows (7 days old) or 125 medaka (21–28 days old) for initial enzyme assays. Pooled larvae were rinsed with water, transferred to polyethylene vials, flash frozen in liquid nitrogen, and stored at –80°C. Frozen larvae were subsequently thawed and homogenized with a 1-mL glass homogenizer in amended phosphate buffer [0.1 M, pH 7.4 with 1 mM each of phenyl methyl sulfonyl fluoride (PMSF), disodium ethylenediamine tetracetate, and dithiothreitol]. Homogenates were centrifuged at 9,000 *g* at 4°C for 10 min. Supernatants were subaliquoted and stored at –80° C. Subsequently, aliquots were thawed for the assessment of total protein content and selected enzyme activities. We quantified total protein concentrations using the bicinchonic acid assay (Pierce, Rockford, IL) with fraction V bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as the calibrating compound.

Mammalian soluble epoxide hydrolase preparations. Recombinant mouse and human sEH were expressed and purified as previously reported using a baculovirus expression system and affinity chromatography (*19–21*). The preparations were at least 97% pure as judged by Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis and scanning densitometry.

Table 1. Usage of selected urea, carbamate, and thiocarbamate pesticides.

Compound	Chemical structure	AI (kg)	Common name (description)
I			*N*-Cyclohexyl, *N´*-(3-phenyl) propyl urea
II		472,298	Diuron (herbicide)
III			Desmethyl diuron (diuron metabolite)
IV		1–5%	Bis-dichlorophenyl urea (diuron impurity)
V		35,418	Diflubenzuron (insecticide)
VI		112	Siduron (herbicide)
VII		202[a]	Chlorpropham (herbicide)
VIII		765	Fenoxycarb (insecticide)
IX		2,226,055	Molinate (herbicide)
X		412,325	Cycloate (herbicide)
XI		652,109	Thiobencarb (herbicide)

AI, active ingredient. Annual use data from the U.S. Geological Survey (*35*) unless otherwise noted.
[a]Annual use data from the U.S. Department of Agriculture (*36*).

Enzyme assays. We assessed activity of sEH using either a radiometric assay with [^{3}H]-tDPPO (*16*) or a spectrophotometric assay employing NEPC (*17*) for purified and crude protein preparations, respectively. Unlike NEPC, [^{3}H]-tDPPO allows determination of sEH activity in the presence of active esterases and glutathione-*S*-transferases (*16*). Glutathione conjugates of [^{3}H]-tDPPO were not detected in crude preparations. Microsomal epoxide hydrolase (mEH) activity was determined using [^{3}H]-CSO as previously described (*13*). To determine compound concentrations that inhibited diol formation by 50% (IC_{50}), protein preparations were preincubated with potential inhibitors (Table 1) for 5 min at 30°C before reporting substrate addition. Fathead minnow, mouse, and human enzyme dilutions (1 nmol diol formed/min/mg of total protein) were assayed in triplicate with 10-min incubations. Medaka enzyme (0.4 nmol/min/mg) assays were run in duplicate with 15-min incubations due to limited enzyme availability. IC_{50} values were determined by linear regression ($r^2 > 0.97$) of the percent inhibition with respect to the log-transformed inhibitor concentration using values between 20 and 80% inhibition. The curve was generated from at least three separate runs to obtain the standard deviations in Table 2.

Exposure water preparation and handling. All tests were conducted in moderately hard water (i.e., test water) as defined by the U.S. Environmental Protection Agency. Test water met described pH, dissolved oxygen, and conductivity criteria (*22*). Sufficient volumes of test water containing diluted chemicals (i.e., exposure water) were prepared within 8 hr of initial organism exposure. For static renewal experiments, excess exposure water was stored at 4°C until use. Renewal aliquots were brought to assay temperature daily within 4 hr of solution renewal. We prepared exposure levels as independent dilutions of methanolic stock solutions, with final methanol concentrations not exceeding 0.05%. We achieved siduron concentrations of 5 mg/L or greater by elevating mixture temperature to 85°C with stirring to dissolve the compound and then reducing the temperature before use. For dual exposures, matched *trans*-stilbene oxide exposures were treated similarly.

Fathead minnow acute toxicity tests. Three replicates ($n = 3$) of 10 larval fathead minnows (7 days old) were exposed for 96 hr in 250 mL test water held at 20 ± 1°C. Larval mortality was counted, general condition of survivors was recorded, and 80% (200 mL) of exposure water was replaced daily. Fish were fed brine shrimp (*Artemia sp.*) daily 2 hr before exposure water renewal. Exposure water temperature, pH, dissolved oxygen content, and conductivity were recorded daily and maintained in acceptable ranges according to standard acute toxicity test methods (*22*). Fathead minnows were exposed to compound II (4.1, 8.2, 16, or 32 µM), compound VI (5.4, 11, 22, 43, or 65 µM), or TSO (7.6, 13, 18, 24, or 28 µM). We repeated *trans*-stilbene oxide exposures in the presence of 40 µM compound VI. Concentrations causing 50% lethality (LC_{50}) were calculated from linear regressions of log-transformed concentration-versus-mortality curves. In the case of urea herbicide exposures, larval minnows surviving at 96 hr within each exposure replicate were prepared for enzyme assays as described above. Linear regressions of log-transformed data allowed the calculation of 50% effect concentrations (EC_{50}).

Medaka sEH activity and developmental toxicity. To identify the developmental stage of sEH appearance, pooled individuals (n = 18–25) were assayed at 2-day intervals throughout development through 1 day posthatch. We then measured sEH activity in homogenized embryo/larval preparations as described above. Toxicity tests were conducted with five replicates ($n = 5$) of five medaka embryos in early to late blastula stage (20–24 hr postfertilization) in capped, acid-washed, 20-mL borosilicate vials (Fisher Scientific, Pittsburgh, PA) containing 2 mL of exposure water, described above. Vehicle and reconstituted water controls were included for each exposure scenario (*22*). The oxygen supply in a 2-mL static bath exposure is sufficient for embryos to complete normal development (*23,24*). We inspected embryos for mortality and developmental deformities at 24 ± 2 hr intervals using a dissecting scope. Observations of gross morphologic alterations, edematous changes, skeletal deformities, abnormal hatching, and delayed hatching were collected and used to calculate toxicity scores as previously described (*25*). In initial experiments exposing embryos to compound II (4.1, 8.6, or 17 µM) or VI (22, 43, or 65 µM), hatched fry were transferred to clean water at day 15 postexposure and observed for 4–5 days. We assessed larvae for viability and the presence of persistent morphologic changes, including swim bladder inflation state. In subsequent experiments, 15 days postexposure with 8.6, 35, or 65 µM of either compound II or III, surviving embryos/larvae were pooled (n = 17–20) and prepared for sEH activity assays as described above.

Statistics. We considered observations statistically significant if comparisons yielded p-values < 0.05. Means were compared using Student's t-test. For medaka embryo/larval results, differences from controls and between treatments were assessed using a Tukey-Kramer HSD test with the student version of Minitab (Minitab Inc., State College, PA).

Results

In vitro *inhibition of sEH.* The chemicals listed in Table 1 were used to assess sEH inhibition with the fathead minnow, medaka, mouse, and human enzymes (Table 2). Both sEH reporting substrates (i.e., NEPC and [^{3}H]-tDPPO) afforded the same rank potency for compounds I, II, and IV tested against the mouse enzyme, indicating that these data can be compared. With [^{3}H]-tDPPO, the IC_{50} was 0.01 ± 0.003 µM, 0.5 ± 0.1 µM, and 55 ± 5 µM for compounds I, IV, and II, respectively. The radiolabeled substrate [^{3}H]-tDPPO was used to discriminate sEH from mEH inhibition *in vitro* (*16*), while mEH activity was determined in the presence of sEH by measuring [^{3}H]-CSO hydrolysis (*13,26*). The sEH inhibitors I, II, III, and VI failed to inhibit mEH (IC_{50} > 500 µM), suggesting inhibitor specificity for sEH, which agrees with our results in mammalian systems (*1*). Enzyme concentration and enzyme affinity for the reporting substrate are important factors affecting IC_{50} results; therefore, the IC_{50} values in Table 2 should not be directly compared between species. However, the relative inhibition potency for a given enzyme can be compared. As seen in Table 2, potent urea inhibitors of mammalian enzymes were potent inhibitors

Table 2. Soluble epoxide hydrolase IC_{50} values for selected pesticides.

Compound	Minnow[a]	Medaka[b]	Mouse[c]	Human[c]
I	0.014 ± 0.001	0.09 ± 0.01	0.06 ± 0.01	0.29 ± 0.01
II	78 ± 7	170 ± 30	120 ± 4	130 ± 6
III	5.5 ± 0.16	63 ± 8	110 ± 4	47 ± 2
IV	0.36 ± 0.01	1.3 ± 0.3	0.9 ± 0.1	1.5 ± 0.07
V	> 500	> 500	270 ± 10	160 ± 10
VI	0.25 ± 0.01	1.0 ± 0.2	0.16 ± 0.02	0.36 ± 0.04
VII	130 ± 1	380 ± 62	210 ± 4	280 ± 10
VIII	97 ± 5	220 ± 10	110 ± 3	65 ± 4
IX	160 ± 20	460 ± 12	230 ± 20	130 ± 10
X	130 ± 2	260 ± 14	200 ± 15	82 ± 9
XI	350 ± 10	> 500	190 ± 10	40 ± 3

IC_{50} values are the means ± the SDs of three separate experiments at 30°C expressed in µM concentrations.
[a]Incubated 15 min with 50 µM tDPPO (3,000 dpm/assay). [b]Incubated 10 min with 50 µM tDPPO (3,000 dpm/assay).
[c]Incubated 1 min with 40 µM NEPC detected at Δ405nm (14 sec/scan).

of fish enzymes (compounds I, IV, and VI). Similarly, carbamates and thiocarbamates were weak inhibitors in all tested species. In general, the relative inhibitor strength of chemicals within a given class (ureas: I–VI; carbamates: VII and VIII; thiocarbamates: IX–XI) were equivalent in all species with the exception of thiobencarb (XI), which was weaker in fish than in mammals. Among the ureas, the inhibitor potency rank was comparable except for desmethyl diuron (III). The potency enhancement afforded by the *N*-dealkylation of diuron (II) to III produced a 14-, 2.7-, 2.8-, and 1.1-fold increased potency for the fathead minnow, medaka, human, and mouse enzymes, respectively.

Fathead minnow toxicity and sEH activity. To determine if *in vitro* sEH inhibition equated to *in vivo* inhibition, fathead minnows were exposed to increasing doses of two model compounds, II and VI. Mortality due to urea herbicide exposure was observed only at 32 µM of compound II, which reached 70% by 96 hr (16 µM < LC_{50} < 32 µM). Although compound VI did not produce larval mortality, the highest exposure produced behavioral effects. Control larvae feeding on brine shrimp had a pronounced pink coloration of the gastrointestinal tract by 72 hr. However, about 10% of the fry exposed to 64 µM compound VI were lethargic and had clear gastrointestinal tracts, suggesting poor food consumption. Larvae exposed to 40 µM compound VI, however, were indistinguishable from controls at 96 hr. Split aliquots of protein preparations from fathead minnows surviving exposures of either compounds II or VI were assayed for sEH activity and normalized to sample protein content. Soluble epoxide hydrolase activity exhibited dose-dependent suppression under both exposure scenarios (Figure 1). An EC_{50} of 12 µM and 28 µM was observed for compounds II and VI, respectively. Because fish tissues were diluted upon homogenization, and inhibition of sEH with these competitive inhibitors is rapidly reversible under such conditions (*1*), the determined inhibition values should be considered the lowest possible *in vivo* inhibition. It is also possible that the inhibition measured in sample homogenates was due either totally or partly to inhibitor accumulated *in vivo* that gained access to sEH *in situ* after sample homogenization.

To verify that *in vitro* sEH inhibition after exposure was an organismal event, not an artifact of homogenization, we investigated the dose-dependent toxicity of TSO in the presence or absence of 40 µM compound VI. *trans*-Stilbene oxide is a moderately reactive epoxide-containing molecule and good surrogate substrate for soluble epoxide hydrolase (*27*). Figure 2 displays larval mortality results for exposures to compound VI and TSO, either alone or in combination. *trans*-Stilbene oxide induced a dose-dependent acute toxicity in exposed fish, with an apparent LC_{50} of 27 ± 3 µM. The addition of 40 µM compound VI reduced the TSO LC_{50} 2.5-fold (Figure 2).

Medaka embryo/larval sEH activity and developmental toxicity. Significant sEH activity was observed from day 0 (stage 11, 1 day postfertilization; 2-fold over background), increased linearly with time (r^2 = 0.98), and attained an 8-fold enhancement at 1 day posthatch (11 days postfertilization). To assess the potential effect of sEH inhibitors on developing vertebrates, we exposed medaka embryos to increasing concentrations of either compound II or VI. Herbicide concentrations were selected to avoid the acute toxic responses observed in fathead minnows. Although morphologic alterations were intermittently observed, significant delays in hatching time were noted for compound II at 8.6 and 17 µM and at all tested concentrations of compound VI (Figure 3).

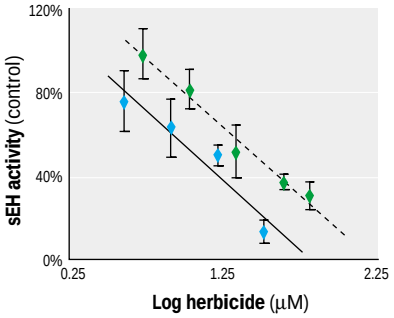


Figure 1. *In vivo* exposure to aryl ureas produced *in vitro* sEH inhibition. Larval fathead minnows were exposed to diuron (0–32 µM) and siduron (0–65 µM) for 96 hr at 20°C. Survivors were pooled, sEH activity was measured in triplicate, and the means ± SDs were calculated. Diuron: r^2 = 0.92; EC_{50} = 12 µm. Siduron: r^2 = 0.98; EC_{50} = 28 µm.

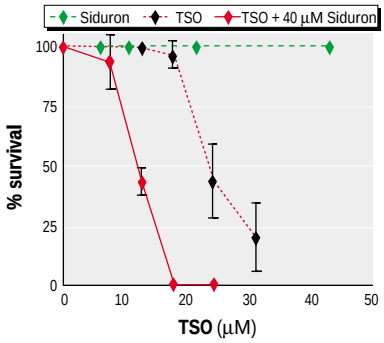


Figure 2. Siduron exposure synergized *trans*-stilbene oxide (TSO) toxicity *in vivo*. Larval fathead minnows were exposed to siduron (5–65 µM) and TSO (7.6–28 µM) with or without 40 µM siduron for 96 hr. The potency of TSO measured by larval mortality (LC_{50}) was increased from 27 ± 3 µM to 11 ± 0.7 µM (p < 0.01) by siduron.

Embryo/larval assays were then conducted with either compound II or its *N*-demethylation product (compound III), the primary metabolite of II in humans (*28*). At the 8.6 µM exposures, hatch delays for larvae exposed to compound II but not to compound III were significant (Figure 4); however, < 3% of the embryos failed to hatch within 14 days. At exposures ≥ 32 µM, delays in the time to embryonic hatch were saturated (3.5 ± 0.9 day) for both compounds II and III, and 20% of the embryos failed to hatch within 15 days, yet retained a visible heartbeat.

Developmental toxicity was dose dependent and similar in embryos exposed to either compound II or III (Figure 5). Similarly, sEH activity was depressed in a dose-dependent manner at hatch (II: r^2 = 0.91; III: r^2 = 0.80; 70 ± 14 % maximum inhibition), and the EC_{50} for compounds II and III were not

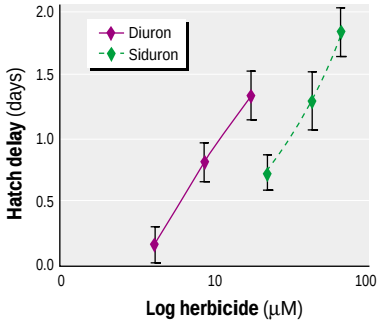


Figure 3. Medaka embryos exposed to diuron and siduron displayed dose-dependent delays in time to hatch. The difference in days to hatch between exposed and 0.05% methanol control groups is displayed as the means ± SEs. Day zero equals initiation of exposure. Test water and methanol control hatch times were 10 ± 0.3 and 9.7 ± 0.2 days, respectively.

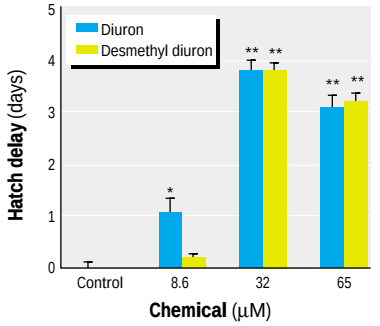


Figure 4. *N*-Demethylation of diuron reduced the potency, but not the efficacy, of hatch delay. Embryos were simultaneously collected and exposed to diuron and desmethyl diuron. Hatch delays were calculated as the difference in days to hatch between exposed and 0.05% methanol control groups. Day zero equals exposure initiation. Test water and methanol control hatch times were 10.1 ± 0.2 and 9.9 ± 0.6 days, respectively. Results are reported as the means ± SEs (n = 5). *p < 0.05; **p < 0.001).

statistically different. A single control embryo died in one of five replicates during this experiment, and no other control toxicity was observed. The result was deemed aberrant and excluded using a *Q*-test. At low doses, toxicity scores were composed of > 90% mild edematous injuries and minor delays in hatch. Embryos exposed to ≥ 32 µM showed numerous morphologic changes *in ovo* that persisted after hatching. At these high doses, the swim bladders in all exposed larvae failed to inflate, and 90% displayed edema of the yolk sac. Increases of the dorsal to ventral axis in exposed larvae constituted additional evidence of edematous injury (Figure 6). Inspection of morphologic alterations, restricted here to those seen under a dissecting scope, suggested alterations of the gall bladder (dilation) and liver (increased area). Previous correlated microscopic versus dissecting scope investigations of medaka larvae in our laboratory were the basis for anatomical assignments at the organ level (*25*). In the developing medaka, the gall bladder is a single vacuole, on the left side of the midline, bearing a transparent, yellow-green appearance. In controls this structure can be found at the caudal-most margin of the liver (Figure 6). In exposed larvae this structure was enlarged, forming a large, yellow-green vacuole in the abdominal cavity. This gall bladder enlargement appeared *in ovo* in all embryos exposed to ≥ 32 µM of either compound II or III. This finding may indicate excessive hepatic bile production or dysfunction in the gall bladder mucosa, impairing bile concentration normally achieved by water removal.

Exposure to 65 µM of compound II or III produced additional effects or lesions that were more severe: apparent hepatomegaly (i.e., enlargement of the liver; Figure 6), mild to moderate pericardial edema, and hatch failure and/or late stage embryonic death. Pericardial edema brought the normally twisted and coiled heart tube (i.e., initial chamber formation) into a single pulsatile tube and darkened the embryonic vasculature, suggesting decreased blood flow. At 65 µM, edema-related changes in cardiac morphology and severe delays in time to hatch accounted for about 10% and about 25% of the total toxicity scores, respectively. Skeletal abnormalities were rare (< 2%) and showed a random distribution across doses. The pronounced tilt of the head in exposed fry was interpreted as a reflection of the abdominal edema rather than as a skeletal defect (Figure 6).

Discussion

The primary goal of this study was to evaluate two fish models as tools to investigate the *in vivo* fate and effect of novel sEH inhibitors. Ureas, carbamates, and amides containing a protonated nitrogen alpha to a carbonyl [i.e., R-NH(C = O)-*R´*] represent a novel class of mechanistic sEH inhibitors (*1*). Therefore, we compared the *in vitro* and *in vivo* potency of a series of chemicals containing this pharmacophore, including a number of pesticides and related compounds. In this way, we made a preliminary risk assessment of pesticide interactions with this biochemical target. We found that, like rainbow trout (*13*), both fathead minnow and medaka sEH were functionally similar to the tested mammalian orthologs with respect to substrate hydrolysis and inhibitor susceptibility.

Three aryl ureas (compounds I, IV, and VI) proved to be potent *in vitro* inhibitors for all enzymes tested, whereas the screened carbamates (VII and VIII) and thiocarbamates (IX–XI) were poor inhibitors. One notable difference in observed potency was for the mono-*N*-demethylation product of compound II (i.e., compound III). With the fathead minnow sEH, compound III was a 14-fold more potent inhibitor than compound II, while the other tested orthologs showed only a 2.2 ± 0.9-fold enhancement. Because *N*-dealkylation is a dominant metabolic transformation and degradation route of many alkylureas (*28–30*), this finding suggests that medaka may provide a better model for mouse and human sEH inhibition than fathead minnow.

Exposure to compounds II and VI produced little to no acute mortality in either larval fathead minnows or embryonic medaka. Similarly, a low incidence of late-stage medaka embryo death was observed at the highest concentration of compound III. However, dose-dependent reductions in sEH activity were induced by exposure to compounds II, III, and VI in both fish models. Further, compound VI synergized *trans*-stilbene oxide toxicity in co-exposed fathead minnow larvae (Figure 2), confirming the *in vitro* suppression of sEH activity as an *in vivo* effect. Although we attempted to assess changes in sEH protein level as a source of the reduced activity, available antibodies against the mammalian sEH orthologs displayed weak cross reactivity with the fish proteins (data not shown). Nevertheless, these data clearly demonstrate that the tested fish models can identify chemicals that reduce *in vivo* sEH activity.

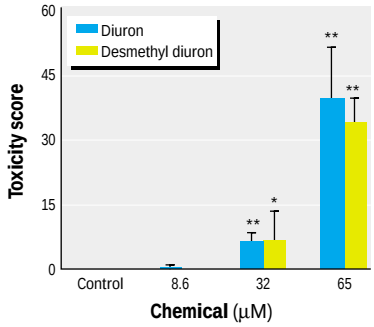


Figure 5. Medaka embryos exposed to either diuron or desmethyl diuron displayed indistinguishable dose-dependent developmental toxicity. Toxicity scores were calculated as previously described (*25*) and represent the means ± SDs of the sum of ranked effects observed in each exposure replicate ($n = 5$). Data that differed significantly from control values are noted (*$p < 0.05$; **$p < 0.001$).

In fathead minnow larvae exposed for 96 hr, compound II reduced sEH activity more than did compound VI, a reversal of the *in vitro* IC_{50} results. *In vitro* inhibition describes the relative inhibitor activity with a biochemical target, while *in vivo* inhibitor behavior integrates factors including chemical uptake, distribution, and clearance. Therefore, the shift of *in vivo* potency indicates that the processes governing the fate and/or transport of compounds II and VI *in vivo* are not equivalent. Theoretically, either difference in the physical chemical partitioning or metabolic fate of these compounds could lead to the observed discrepancy. The uptake of a chemical from water by an organism can be related to the log of the octanol:water partitioning coefficient (log K_{OW}) of that

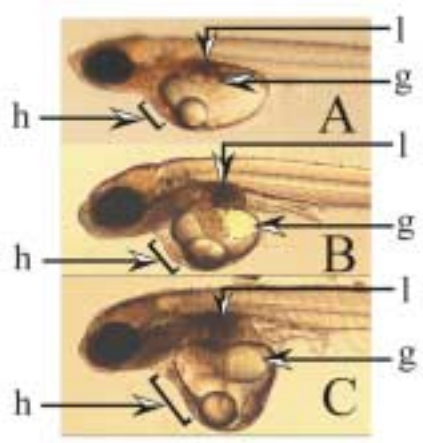


Figure 6. Morphologic observation of medaka larvae. (*A*) Intact control. (*B*) 65 µM diuron (compound II) exposed. (*C*) 65 µM desmethyl diuron (compound III) exposed. Morphologic alterations proved similar in both exposures. At 35 µM or greater, gall bladder (g) enlargement was a common response [compare (*A*) vs. (*B*) and (*C*)]. The gall bladder was identified by the yellow-green coloration and stage of first appearance during embryo development. The photographic filter used to provide contrast improved structural definition but obscured the distinct coloration. The dashed line in (*B*) delineates the margin of the observed coloration. The central dark mass was identified as the liver (l) based on time of appearance in development and position in larvae as described in the text. Edematous injury to the pericardium induced elongation of the heart (h) in a subset of larvae exposed to 65 µM herbicide [compare (*A*) and (*B*) vs. (*C*)]. Relative sizes can be estimated using the ocular orbital height as reference.

compound (*31*). The log K_{OW} of compounds II and VI are 2.58 and 3.09, respectively (*32*). If solubility were driving the observed *in vivo* differences, higher tissue concentrations of compound VI would be expected. However, based on the *in vitro* results, higher concentrations of compound VI relative to compound II should produce lower sEH activity, rather than the observed increase. Therefore, either sequestration of compound VI into a non-sEH–containing compartment occurs; or, alternatively, compounds II and VI may exhibit different modes and/or time dependence of metabolic clearance. Although the metabolic transformation of compound II has been described (*28*), similar information for compound VI is lacking. Considering the structure of compound VI, cytochrome P450-dependent oxidation of either the phenyl ring or the methyl cyclohexyl moiety would be expected. Hydroxylation of either functionality would significantly reduce the hydrophobic character of these side chains, a modification that dramatically reduces sEH inhibition potency in a related series of compounds (*33*). Therefore, metabolic activation of compound II by *N*-demethylation and inactivation of compound VI by side-chain hydroxylation could explain the inversion of *in vitro* versus *in vivo* potency, suggesting the presence of competent xenobiotic metabolizing systems in the exposed embryonic and larval fish.

Medaka embryos displayed toxicity; therefore, the semipermeable embryonic protein coat, the chorion, did not prevent embryonic exposure. Dose-dependent delays in hatch were observed in exposures to both compounds II and VI. As observed for the *in vivo* sEH inhibition potency in fathead minnows, compound II was a more potent inducer of hatch delays. The severe developmental effects at exposure concentrations of 65 µM (16 mg/L) of compound II compare well with concentrations reported to cause frog embryo developmental abnormalities and retardation of tadpole growth (*34*). The inability to discriminate either the toxicity or the *in vivo* sEH inhibition potency of compound II from III will require further investigations to elucidate fully; however, they suggest metabolic transformation of compound II occurs *in vivo*. Specifically, compound II may be activated by demethylation, or both compounds may be transformed to a downstream toxic metabolite, producing the similarity of the observed organismal responses.

We have shown that extrapolating some observations of sEH inhibition between mammalian and teleost species is valid. The presence of sEH activity in early life-stage fish and their ability to modulate the potency of sEH inhibitors supports their use as model systems to identify discrepancies between *in vitro* and *in vivo* sEH inhibition. Specifically, postexposure sEH activity determinations and/or shifts in *trans*-stilbene oxide toxicity curves provide indices of *in vivo* sEH activity suppression. Using these systems we have identified diuron and desmethyl diuron as weak *in vivo* sEH inhibitors. However, their low potency indicates that the risk of sEH-dependent untoward effects from environmental diuron exposure is low. Nonetheless, both depression of sEH activity and embryo toxicity showed similar dose dependence, suggesting a potential link between these factors. This finding further suggests that new pesticides and therapeutics containing the pharmacophores discussed here should be evaluated as sEH inhibitors before they are used commercially.

References and Notes

1. Morisseau C, Goodrow M, Dowdy D, Jiang J, Greene JF, Sanborn JR, Hammock BD. Ureas, carbamates and related compounds are potent inhibitors of soluble epoxide hydrolases. Proc Natl Acad Sci U.S.A. 96:8849–8854 (1999).
2. Grant DF, Moody DE, Beetham J, Storms DH, Moghaddam MF, Borhan B, Pinot F, Winder B, Hammock BD. The response of soluble epoxide hydrolase and other hydrolytic enzymes to peroxisome prolferators. In: Peroxisome Proliferators: Unique Inducers of Drug-Metabolizing Enzymes, (Moody DE, ed). Boca Raton, FL:CRC Press, 1994;113–121.
3. Chacos N, Capdevila J, Falck JR, Martin-Wixtrom C, Gill SS, Hammock BD, Estabrook RA. The reaction of arachidonic acid epoxides (epoxyeicosatrienoic acids) with cytosolic epoxide hydrolase. Arch Biochem Biophys 233:639–648 (1983).
4. Moghaddam MF, Motoba K, Borhan B, Pinot F, Hammock BD. Novel metabolic pathways for linoleic and arachidonic acid metabolism. Biochim Biophys Acta 1290:327–339 (1996).
5. Moghaddam MF, Grant DF, Cheek JM, Greene JF, Williamson KC, Hammock BD. Bioactivation of leukotoxin to their toxic diols by epoxide hydrolase. Nat Med 3:562–566 (1997).
6. McGiff JC, Steinberg M, Quilley J. Missing links: cytochrome P450 arachidonate products; a new class of lipid mediators. Trends Cardiovasc Med 6:4–10 (1996).
7. Oltman CL, Weintraub NL, Vanrollins M, Dellsperger KC. Epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids are potent vasodilators in the canine coronary microcirculation. Circ Res 83:932–939 (1998).
8. Weintraub NL, Fang X, Kaduce TL, Vanrollins M, Chatterjee P, Spector AA. Potential of endothelium-dependent relaxation by epoxyeicosatrienoic acids. Circ Res 81:258–267 (1997).
9. Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, Liao JK. Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. Science 285:1276–1279 (1999).
10. Bailey GS, Williams DE, Hendricks JD. Fish models for environmental carcinogenesis: the rainbow trout. Environ Health Perspect 104(suppl 1):5–21 (1996).
11. Kelly SA, Havrilla CM, Brady TC, Abramo KH, Levin ED. Oxidative stress in toxicology: established mammalian and emerging piscine model systems. Environ Health Perspect 106:375–384 (1998).
12. Gogal RM, Ahmed SA, Smith SA, Holladay SD. Mandates to develop non-mammalian models for chemical immunotoxicity evaluation: are fish a viable alternate to rodents? Toxicol Lett 106:89–92 (1999).
13. Lauren DJ, Halankar PP, Hammock BD, Hinton DE. Microsomal and cytosolic epoxide hydrolase and glutathione *S*-transferase activities in the gill, liver, and kidney of the rainbow trout, *Salmo gairdneri*: Baseline levels and optimization of assay conditions. Biochem Pharmacol 38:881–887 (1989).
14. Schlezinger JJ, Parker C, Zeldin DC, Stegeman JJ. Arachidonic acid metabolism in the marine fish *Stenotomus chrysops* (Scup) and the effects of cytochrome P450 1A inducers. Arch Biochem Biophys 353:265–275 (1998).
15. Gill SS, Ota K, Hammock BD. Radiometric assays for mammalian epoxide hydrolases and glutathione *S*-transferase. Anal Biochem 131:273–282 (1982).
16. Borhan B, Mebrahtu T, Nazarian S, Kurth MJ, Hammock BD. Improved radiolabeled substrates for soluble epoxide hydrolase. Anal Biochem 231:188–200 (1995).
17. Dietze EC, Kuwano E, Hammock BD. Spectrophotometric substrates for cytosolic epoxide hydrolase. Anal Biochem 216:176–187 (1994).
18. Kaur A, Goodrow MH, Schmidt DJ, Hammock BD, Bigelow MW. Synthesis of haptens and derivation of monoclonal antibodies for immunoassay of the phenylurea herbicide diuron. J Agric Food Chem 42:301–309 (1994).
19. Grant DF, Storms DH, Hammock BD. Molecular cloning and expression of murine liver soluble epoxide hydrolase. J Biol Chem 268:17628–17633 (1993).
20. Beetham JK, Tian T, Hammock BD. cDNA cloning and expression of a soluble epoxide hydrolase from human liver. Arch Biochem Biophys 305:197–201 (1993).
21. Wixtrom RN, Silva MH, Hammock BD. Affinity purification of cytosolic epoxide hydrolase using derivatized epoxy activated Sepharose gels. Anal Biochem 169:71–80 (1988).
22. U.S. EPA. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. 4th ed. EPS/600/4-90/027F. Cincinnati, OH:U.S. Environmental Protection Agency, 1993.
23. Marty GD, Cech JJ, Hinton DE. Effect of incubation temperature on oxygen consumption and ammonia production by Japanese medaka (*Oryzias latipes*) eggs and newly hatched larva. Environ Toxicol Chem 9:1399–1405 (1990).
24. Villalobos SA, Anderson MJ, Denison MS, Hinton DE, Tullis K, Kennedy IM, Jones AD, Chang DPY, Yang G, Kelly P. Dioxinlike properties of a trichloroethylene combustion-generated aerosol. Environ Health Perspect 104:734–743 (1996).
25. Villalobos SA, Hamm JT, Teh SJ, Hinton DE. Thiobencarb-induced embryotoxicity in medaka (*Oryzias latipes*): stage-specific toxicity and the protective role of chorion. Aquat Toxicol 48:309–326 (2000).
26. Hammock BD, Grant DF, Storms D. Epoxide hydrolases. In: Comprehensive Toxicology, Vol 3 (Guengerich FP, ed). Oxford:Pergamon, 1997;283–305.
27. Gill SS, Ota K, Hammock BD. A rapid radiometric assay for mammalian cytosolic epoxide hydrolase. Anal Biochem 131:273–282 (1983).
28. Van Boven M, Laruelle L, Daenens P. HPLC analysis of diuron metabolites in blood and urine. J Anal Toxicol 14:231–234 (1990).
29. Jirkovsky J, Faure V, Boule P. Photolysis of diuron. Pestic Sci 50:42–52 (1997).
30. Zablotowicz RM, Schrader KK, Locke MA. Algal transformation of fluometuron and atrazine by N-dealkylation. J Environ Sci Health Part B Pestic Food Contam Agric Wastes B33:511–528 (1998).
31. Meylan W, Howard PH, Boethling RS, Aronson D, Printup H, Gouchie S. Improved method for estimating bioconcentration/bioaccumulation factor from octanol/water partition coefficient. Environ Toxicol Chem 18:664–672 (1999).
32. Liu J, Chuanfan Q. Hydrophobic coefficiants of S-triazine and phenylurea herbicides. Chemosphere 31:3951–3959 (1995).
33. Morriseau C, Hammock BD. Unpublished data.
34. Schuytema GS, Necbeker AV. Comparative toxicity of diuron on survival and growth of pacific treefrog, bullfrog, red-legged frog, and African clawed frog embryos and tadpoles. Arch Environ Contam Toxicol 34:370–376 (1998).
35. USGS. NAWQA Pesticide National Synthesis Project: National Assessment of Pesticides in the Streams, Rivers, and Ground Water of the United States. Available: http://water.wr.usgs.gov/pnsp/ [cited 27 August 2000].
36. AMS-USDA. Pesticide Data Program: 1992-1998 Data and Summary Reports. Available: http://www.ams.usda.gov/science/pdp/index.htm [cited 27 August 2000].