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EFFECT OF THE FUNGICIDE CLOTRIMAZOLE ON THE BIOCONCENTRATION OF BENZO[*a*]PYRENE IN GIZZARD SHAD (*DOROSOMA CEPEDIANUM*): IN VIVO AND IN VITRO INHIBITION OF CYTOCHROME P4501A ACTIVITY

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Abstract—Clotrimazole was found to inhibit in vivo and in vitro hepatic microsomal ethoxyresorufin *O*-deethylase (EROD) activity in gizzard shad (*Dorosoma cepedianum*). Gizzard shad pretreated with 50 mg clotrimazole/kg and then exposed for 1 or 3 d to benzo[*a*]pyrene (B*a*P) (0.86 μg/L) had significantly lower EROD activity compared to fish that were exposed to B*a*P alone. Following 1 and 3 d of B*a*P exposure, groups pretreated with clotrimazole had a 14- and 4-fold decrease in EROD activity and had bioconcentrated 8 and 11 times more parent B*a*P, respectively, compared to groups exposed to B*a*P alone. Addition of clotrimazole to B*a*P-induced microsomes produced a type II binding spectrum and was an effective in vitro inhibitor of EROD activity. The median inhibitory concentration for EROD activity was 0.51 μM clotrimazole. Kinetic and spectral experiments suggest that the mechanism of inhibition by clotrimazole is noncompetitive. Reduction in the rate of oxidative B*a*P metabolism is hypothesized to result from noncompetitive inhibition of cytochrome P4501A and other P450 enzymes that metabolize B*a*P.

Keywords—P4501A Inhibition Clotrimazole Benzo[*a*]pyrene Gizzard shad

INTRODUCTION

Over the past two decades a number of antifungal imidazole derivatives have been approved for use in agricultural and clinical applications. These fungicides were designed with the intent to inhibit cytochrome P450-mediated ergosterol biosynthesis [1,2]. However, a number of these derivatives have been shown to inhibit cytochrome P450-mediated steroid metabolism in rats [3–6] and fish [7] as well as xenobiotic metabolism in rats [8–10] and fish [11,12]. The clinically used fungicide clotrimazole has been shown to prevent the formation of benzo[*a*]pyrene (B*a*P) metabolite–DNA adducts in vitro [10,13] and has been associated with a reduction in B*a*P-induced skin tumors in neonatal rats through a cytochrome P450 inhibitory mechanism [10]. Inhibition of cytochrome P450 activity has been reported to occur through an interaction between the heme moiety on cytochrome P450 with the nonbonded electrons of the nitrogen in the imidazole ring [14,15], and competitive inhibition at the substrate binding site [16,17].

The main role of cytochromes in the P450 subfamily P4501A is to enhance the elimination of water-insoluble compounds [18]. A single atom of oxygen is added to the foreign molecule, resulting in the addition of a hydroxyl group, which is the first phase for increasing water solubility and hence the excretability of the compound. Cytochrome P4501A activity accounts for the majority of B*a*P hydroxylase activity in mammals [19–21] and fish [18]. A well-established characteristic of cytochrome P4501A systems is their inducibility. Induction of the cytochrome P4501A isoenzyme in fish liver has been successfully used to identify exposure to polycyclic aromatic hydrocarbons [22] and other planar environmental contaminants [23].

Although numerous studies have demonstrated the inhibition of xenobiotic metabolism by imidazole compounds, no study to date has investigated the influence of one of these compounds on the bioaccumulation rates for a xenobiotic that is readily metabolized by cytochrome P450s. Based on mechanistic grounds we chose clotrimazole as a model imidazole fungicide to test the hypothesis that inhibition of cytochrome P450 activity by an imidazole fungicide could increase bioconcentration of B*a*P by decreasing its rate of oxidative metabolism. The present study examined the effect of clotrimazole pretreatment on in vivo gizzard shad (*Dorosoma cepedianum*) hepatic cytochrome P4501A enzyme activity and B*a*P metabolism following waterborne-B*a*P exposures, and the effect of clotrimazole on in vitro gizzard shad hepatic cytochrome P4501A activity following waterborne B*a*P exposures. In addition, the mechanism of interaction between clotrimazole and cytochrome P4501A activity was characterized with in vitro kinetic and spectral binding experiments.

MATERIALS AND METHODS

Chemicals

Ethoxyresorufin, resorufin, reduced nicotinamide adenine dinucleotide phosphate (NADPH) (cofactor), B*a*P, sodium dithionite, tetrabutyl ammonium bromide, ammonium formate, Trizma hydrochloride and base, sucrose, potassium chloride, glycerol, and clotrimazole (>99% purity) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All solvents were high-pressure liquid chromatography (HPLC) grade and were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Care and handling of fish

Age II gizzard shad (weight range 20–25 g) were collected from Acton Lake, Preble Co., Ohio, USA, by electroshocking and were held in dechlorinated water in 500-L, flow-through tanks on a 12L:12D photoperiod for approximately 1 year prior to use. Fish were acclimated to 16 ± 1°C for 8 to 10 weeks prior to experimentation and all experiments were conducted at 16 ± 1°C. Previous work in our laboratory has shown that

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gizzard shad from Acton Lake held for this period of time have low basal rates of cytochrome P4501A-catalyzed enzyme activity [24].

Effect of clotrimazole alone on basal in vivo P4501A activity

In order to examine the effect of clotrimazole alone on in vivo P4501A activity, fish were given intraperitoneal injections with clotrimazole alone (2 μl/g fish) or corn oil alone (2 μl/g fish). Groups were then held in separate 50-L, flow-through glass tanks until sacrificed 3 and 5 d postinjection.

Effect of co-exposure to clotrimazole and BaP on in vivo P4501A activity

Fish that received coexposure to B*a*P and clotrimazole were pretreated with 50 mg clotrimazole/kg 2 d prior to the start of waterborne-B*a*P exposure. Fish that were exposed to B*a*P alone received corn oil injections (2 μl/g) 2 d prior to the start of waterborne-B*a*P exposure. The B*a*P exposure concentration (0.86 ± 0.02 μg/L) was maintained with a dilutor system [25] under cool-white fluorescent lighting. Benzo[*a*]pyrene water concentrations were measured using reverse-phase HPLC with a fluorescence detector at an excitation wavelength of 285 nm and an emission wavelength of 405 nm [22] with a limit of detection of 0.1 μg B*a*P/L. Benzo[*a*]pyrene was delivered to the water in methanol, and the methanol concentration in the exposure tanks was <0.03%. This methanol concentration had no effect on hepatic ethoxyresorufin *O*-deethylase (EROD) activity [22]. The water solubility for B*a*P at the exposure temperature was approximately 1 μg/L [26] and this was the target exposure concentration. Following 1 and 3 d of waterborne exposure, fish were sacrificed and livers were removed for microsomal isolation. Weighed carcasses were stored in zip-closure plastic bags at −74°C until analyzed for parent B*a*P concentration. Parent B*a*P concentration was measured using reverse-phase HPLC with an ion-pairing buffer and a fluorescence detector at an excitation wavelength of 285 nm and an emission wavelength of 405 nm [22].

Microsomal isolation and measurement of gizzard shad hepatic EROD activity

Three sample groups (two fish per group) were used for all treatment and control groups. It was necessary to pool livers from two fish in order to have enough tissue for a microsomal isolation. Microsomes were isolated as described previously [22], resuspended in 700 μl of 0.1 M Tris and 20% (v/v) glycerol (pH 7.8), and stored at −74°C for no longer than 4 weeks. A modification of the catalytic method described by Burke and Mayer [27] was performed to measure EROD activity. Incubations contained 1.8 ml of 0.1 M Tris (pH 7.8), 100 μl microsomal protein (0.5 to 1 mg/mL), and 100 μl of 50 μM ethoxyresorufin. Assays were carried out in duplicate for each group and were incubated at the ambient exposure temperature. The assays were initiated after a 1-min preincubation with 10 μl of 50 mM NADPH and were ended after 10 min with the addition of 2 ml of ice-cold methanol. Incubation vials were centrifuged to remove precipitated microsomal protein and supernatants were transferred to HPLC vials for measurement of resorufin concentrations [24]. The limit of detection was 0.05 nM resorufin. Formation of resorufin was linear over the 10-min incubation period and <15% of the substrate was transformed. Incubations without NADPH and incubations without protein served as negative controls.

Binding difference spectra of microsomal cytochrome P450

Hepatic microsomes isolated from gizzard shad that were given a 3-d waterborne-B*a*P exposure (1.01 ± 0.03 μg/L) were diluted to a concentration of 1.0 mg protein/ml in 50 mM Tris (pH 7.8) and divided equally between the sample and the reference cuvettes. Clotrimazole dissolved in methanol was added to the sample cuvette in 2-μl aliquots and equal amounts of methanol were added to the reference cuvette. Clotrimazole was added to produce final concentrations of 0.1 and then 1 μM. Binding spectra were recorded following a 10-min equilibration period. To determine if cytochrome P450-bound clotrimazole could be displaced by CO, the sample and reference cuvettes were bubbled with CO for 30 sec, the sample cuvette was reduced with 1 mg of sodium dithionite, and the binding spectra were recorded following a 10-min equilibration period. Spectral binding assays were conducted at 16°C using a Varian (Harbor City, CA, USA) DMS-100 dual beam spectrophotometer.

In vitro EROD inhibition and in vitro EROD kinetic assays

The median inhibitory concentration (IC50) value for in vitro hepatic EROD activity and the apparent maximal reaction velocity (V_{max}) and apparent Michaelis constant (K_m) values for hepatic EROD activity were measured with microsomes isolated from gizzard shad that received 3 d of waterborne-B*a*P exposure (1.01 ± 0.03 μg/L). The intrinsically low basal activity of nontreated microsomes precluded detection of decreases in EROD activity over a large range of inhibitor concentrations. Six clotrimazole incubation concentrations (10^{-3} to 10^{2} μM) were used to determine the IC50 value for EROD activity and the ethoxyresorufin concentration was held constant at 2.5 μM. Ethoxyresorufin *O*-deethylase kinetic assays were performed with three different clotrimazole concentrations (10^{-3} to 10^{-1} μM) and seven ethoxyresorufin substrate concentrations (31–2,500 nM). Control incubation mixtures contained the same volume of vehicle (10 μl of methanol) that was used for additions of clotrimazole. Ethoxyresorufin *O*-deethylase assays for inhibition and kinetic assays were performed as described above with the following differences: clotrimazole was added to the incubation mixture prior to the addition of protein, ethoxyresorufin, and NADPH; incubations contained 1 mg protein/ml; and kinetic assays were stopped after a 3-min incubation period. Formation of resorufin in the kinetic assays was linear with each substrate concentration over the 3-min incubation period and <15% of the substrate was transformed.

Statistics

Benzo[*a*]pyrene exposure concentrations and EROD activity values are reported as means ± standard error of the mean (SEM). Analysis of variance (ANOVA) and Duncan's multiple range test were performed to test for statistical differences in EROD activity and whole-fish parent B*a*P concentrations among treatment groups using the Statistical Analysis System (SAS) [28] on an IBM mainframe computer. The sigmoidal EROD inhibition curve and its IC50 value ± asymptotic SEM were estimated using a four-parameter logistic model [29]. Apparent V_{max} and apparent K_m values ± asymptotic SEM were calculated using a nonlinear regression model to fit the Michaelis–Menten equation using the SAS NLIN procedure [28]. Statistical differences between apparent V_{max} and apparent K_m values were determined by comparing asymptotic 95% confidence intervals.

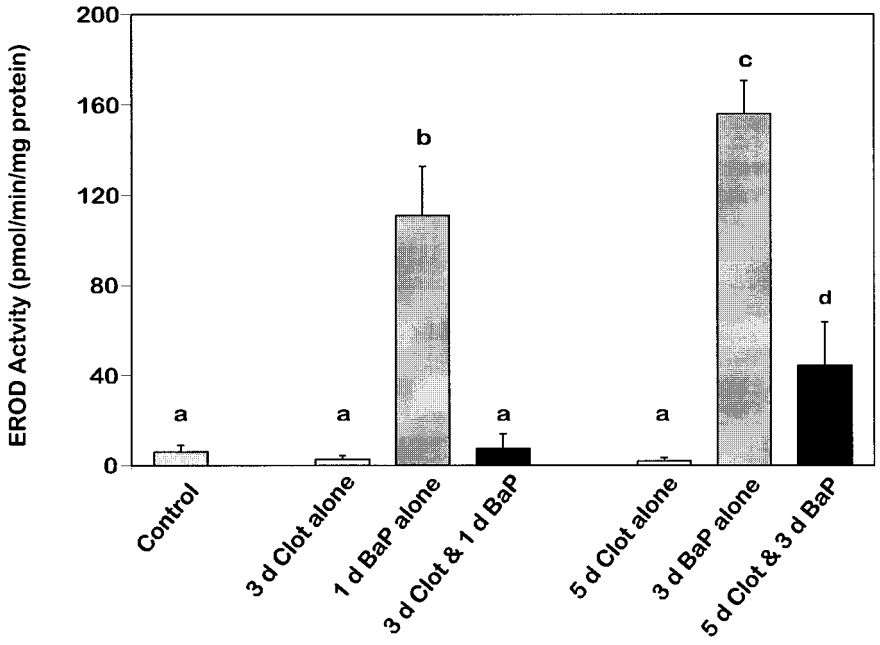

Fig. 1. Effect of clotrimazole on in vivo gizzard shad hepatic ethoxyresorufin *O*-deethylase (EROD) activity. Ethoxyresorufin *O*-deethylase activities are presented as means ± SEM for three observations (each observation represents two pooled livers). Gizzard shad were pretreated with clotrimazole (Clot) 2 d prior to either 1 or 3 d of benzo[*a*]pyrene (BaP) exposure. Values from treatment groups with the same letter were not significantly different as determined with analysis of variance (ANOVA) and Duncan's multiple range test ($\alpha = 0.05$).

RESULTS

Effect of clotrimazole alone on basal in vivo P4501A activity

Gizzard shad EROD activity 3 and 5 d postinjection with 50 mg clotrimazole/kg was on average half of the EROD activity of gizzard shad that received corn oil injections alone (Fig. 1). The intrinsically low basal EROD activity of nontreated microsomes precluded detection of significant inhibition of basal in vivo activity.

Effect of coexposure to clotrimazole and BaP on in vivo P4501A activity

To investigate the effect of clotrimazole on in vivo EROD activity, gizzard shad were pretreated with 50 mg clotrimazole/kg and then exposed for 1 or 3 d to B*a*P (0.86 ± 0.02 μg/L). Fish that were pretreated with clotrimazole and then exposed to B*a*P had significantly lower EROD activity compared to gizzard shad that were exposed to B*a*P alone (Fig. 1). Following 1 and 3 d of B*a*P exposure, groups pretreated with clotrimazole had a 14- and 4-fold decrease, respectively, in EROD activity compared to groups exposed to B*a*P alone.

BaP tissue levels of waterborne-exposed gizzard shad

Lower levels of parent B*a*P were found in fish exposed to B*a*P alone compared to fish that were pretreated with clotrimazole and then exposed to B*a*P (Table 1). Gizzard shad pretreated with clotrimazole had bioconcentrated 8 and 11 times more parent B*a*P after 1 and 3 d of B*a*P exposure, respectively, compared to fish that were exposed to B*a*P alone. Bioconcentration values were calculated to index the concentration of parent B*a*P in fish relative to waterborne B*a*P exposure concentration. Bioconcentration of parent B*a*P in fish exposed for 1 or 3 d to B*a*P alone ranged from zero to four times the waterborne-B*a*P concentration. Bioconcentration of parent B*a*P in gizzard shad pretreated with clotrimazole and then exposed for 1 or 3 d to B*a*P ranged from 30 to 35 times the waterborne B*a*P concentration (Table 1).

Binding difference spectra of microsomal cytochrome P450

Addition of clotrimazole to gizzard shad hepatic microsomes resulted in the formation of a type II binding spectrum (Fig. 2, curves 2 and 3). Addition of clotrimazole to a final concentration of 0.1 μM then followed by addition of clotri-

Table 1. Benzo[*a*]pyrene (B*a*P) tissue levels for waterborne-exposed gizzard shad. Whole-fish parent-B*a*P tissue concentrations following 1 and 3 d of waterborne exposures are shown as means ± SEM. Treatment groups (B*a*P or B*a*P + clotrimazole [CLOT]) with the same letters were not significantly different as determined with analysis of variance (ANOVA) and Duncan's multiple range test ($\alpha = 0.05$)[a]

Length of B*a*P exposure (d)	Treatment: B*a*P (Whole-fish parent B*a*P)		Treatment: B*a*P bioconcentration	Treatment: B*a*P + CLOT (Whole-fish parent B*a*P)		Treatment: B*a*P + CLOT bioconcentration
	N	μg/kg		*N*	μg/kg	
1	3	3.1 ± 0.2[a]	3.7 ± 0.2	3	26.1 ± 6.7[b]	30.4 ± 7.7
3	4	2.8 ± 0.3[a]	3.2 ± 0.2	4	29.9 ± 10.1[b]	34.8 ± 11.7

[a] Bioconcentration values are expressed as means ± SEM; numbers indicate whole-fish parent-B*a*P concentration relative to water.

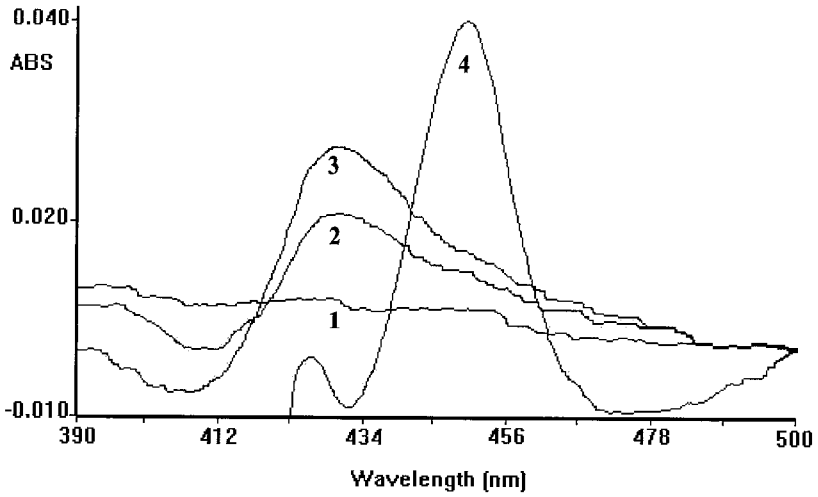

Fig. 2. Binding spectra of gizzard shad hepatic microsomes after the addition of clotrimazole. A baseline was recorded between the reference cuvette and sample cuvette (curve 1). Clotrimazole was added to a final concentration of 0.1 μM in the sample cuvette and an equal volume of carrier solvent was added to the reference cuvette (curve 2). Additional clotrimazole was then added to a final concentration of 1 μM in the sample cuvette and an equal volume of carrier solvent was added to the reference cuvette (curve 3). The sample cuvette containing 1 μM clotrimazole and the reference cuvette were bubbled with CO for 30 sec, and the sample cuvette was then reduced with sodium dithionite (curve 4).

mazole to a final concentration of 1 μM resulted in a progressive spectral change. The spectral change was characterized by a gain in absorbance at 427 nm and a loss of absorbance from 400 to 412 nm. The isobetic point for the two binding curves occurred at 417 nm. Bubbling the reference cuvette and the sample cuvette, which contained 1 μM clotrimazole, with CO for 30 sec, followed by reducing the sample cuvette with sodium dithionite, decreased the absorbance at 427 nm and produced an additional peak at 448 nm (Fig. 2, curve 4). The presence of two distinct peaks, with the first at 448 nm and the second at 426 to 427 nm, may indicate that a portion of the clotrimazole may have remained bound to the cytochrome P450s because the absorbance maximum of the type II binding spectra was at 427 nm. For comparison, a second batch of the same microsomal preparation without the addition of clotrimazole was bubbled with CO for 30 sec and the sample cuvette was reduced with sodium dithionite. The change in absorbance between 490 nm and 448 nm from preparations without clotrimazole was 10 to 20% greater than the change in absorbance between 490 and 448 nm from preparations that had a final clotrimazole concentration of 1 μM (not shown in Fig. 2). Furthermore, there was no second absorbance peak at 426 to 427 nm for preparations without clotrimazole.

Inhibition of in vitro EROD activity

Clotrimazole was a potent inhibitor of gizzard shad hepatic in vitro EROD activity (Fig. 3). The IC50 value for EROD activity was estimated to be 0.51 ± 0.01 μM clotrimazole. When percent EROD inhibition was plotted against concentrations of clotrimazole there was a concentration-dependent decrease in reaction velocity with a plateau at >99% inhibition by concentrations equal to or greater than 10 μM (Fig. 3). The ability of clotrimazole to cause nearly 100% inhibition of EROD activity demonstrates that EROD activity is not partially inhibited by clotrimazole. Although this inhibition curve demonstrates that clotrimazole can cause total inhibition of EROD activity, it does not provide conclusive information concerning the type of inhibition.

Ethoxyresorufin *O*-deethylase kinetic assays were performed to characterize the mechanism of P4501A inhibition by clotrimazole. Ethoxyresorufin *O*-deethylase activity in the presence and absence of clotrimazole exhibited Michaelis–Menten-type kinetics (Fig. 4). Increases in clotrimazole con-

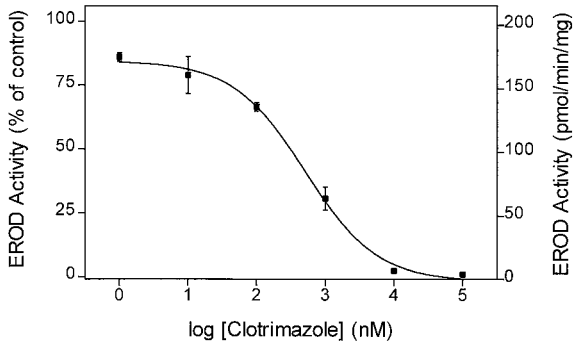

Fig. 3. Concentration–response curve for in vitro inhibition of ethoxyresorufin *O*-deethylase (EROD) activity by clotrimazole. The median inhibitory concentration (IC50) value was estimated to be 0.51 ± 0.01 μM clotrimazole. Each point represents the mean of two separate determinations ± SEM.

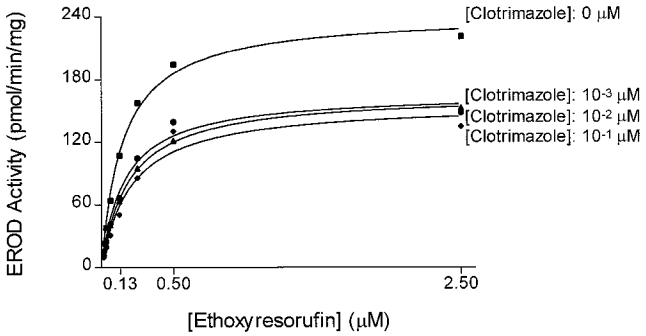

Fig. 4. Michaelis–Menten plot for clotrimazole inhibition of in vitro gizzard shad hepatic ethoxyresorufin *O*-deethylase (EROD) activity. Each point represents the mean of two separate determinations. Clotrimazole was added in the following concentrations to the incubations: (■) 0 μM, (●) 10^{-3} μM, (▲) 10^{-2} μM, (♦) 10^{-1} μM.

Table 2. In vitro apparent maximal reaction velocity (V_{max}) (±asymptotic SEM) and apparent Michaelis constant (K_m) (±asymptotic SEM) values for gizzard shad ethoxyresorufin *O*-deethylase (EROD) activity. Gizzard shad were exposed for 3 d to waterborne benzo[*a*]pyrene prior to microsomal isolation. Apparent K_m values with the same letter are not significantly different ($\alpha = 0.05$) and apparent V_{max} values with the same numeral are not significantly different ($\alpha = 0.05$)

		[Clotrimazole] (μM)		
EROD kinetic parameter	No inhibitor	10^{-3}	10^{-2}	10^{-1}
Apparent K_m (nM)	151.1 ± 14.1[a]	161.8 ± 26.2[a]	190.8 ± 5.8[a]	205.5 ± 49.9[a]
Apparent V_{max} (pmol/min/mg protein)	241.9 ± 7.2[1]	166.4 ± 8.7[2]	165.6 ± 1.7[2]	156.0 ± 12.8[2]

centration caused apparent V_{max} values to significantly decrease and caused a trend toward increasing apparent K_m values with increasing inhibitor concentration (Table 2). The absence of a statistically significant difference in apparent K_m values and of a concomitant decrease in apparent V_{max} values suggests that clotrimazole was a noncompetitive inhibitor of gizzard shad EROD activity [30]. For noncompetitive inhibition, the inhibition rate constant (K_I) equals the IC50 value [31].

DISCUSSION

This study characterized the effect of the imidazole antifungal compound clotrimazole on cytochrome P4501A activity in gizzard shad. Fish pretreated with clotrimazole and then exposed to B*a*P for either 1 or 3 d showed significant inhibition of the *O*-deethylation of ethoxyresorufin. Concomitant with EROD inhibition was a significantly higher bioconcentration of parent B*a*P in tissues. Increased parent B*a*P concentration in tissues is consistent with inhibition of cytochrome P4501A, the form responsible for the majority of B*a*P metabolism. The rate of B*a*P metabolism for gizzard shad exposed to B*a*P alone was similar to findings with mosquito fish (*Gambusia affinis*) that rapidly metabolized B*a*P during a waterborne-B*a*P exposure [32]. In that study, mosquito fish bioconcentrated parent B*a*P only in the presence of the cytochrome P450 monooxygenase inhibitor piperonyl butoxide (PBO). Following 3 d of coexposure to waterborne PBO and B*a*P, mosquito fish had bioconcentrated parent B*a*P 22 times above the water concentration. Similarly, in the present study gizzard shad pretreated with clotrimazole followed by a 3-d waterborne-B*a*P exposure had bioconcentrated parent B*a*P 35 times above the waterborne-B*a*P concentration.

Reduction of microsomal hepatic EROD activity can in part be attributed to clotrimazole directly binding to the heme of cytochrome P450, as illustrated by the formation of a type II binding spectrum. As a result of this direct binding, cytochrome P450 was converted to its low-spin form, thereby slowing reduction to ferrous cytochrome P450. Furthermore, because clotrimazole was occupying the site of oxygen binding, the rate of cytochrome P450-catalyzed oxidations was decreased. Displacement of the type II binding spectra by bubbling with CO provided further evidence that clotrimazole directly and reversibly bound to the heme moiety of cytochrome P450s. Similar spectral studies have shown that clotrimazole [13] and ketoconazole [33] compete with CO for reduced cytochrome P450s from rats.

The mechanism of EROD inhibition by clotrimazole appears to be noncompetitive based on cytochrome P450-binding spectra and apparent EROD kinetic values. Previous work investigating the inhibitory mechanism of clotrimazole on cytochrome P4501A activity concluded that the majority of inhibition was caused by interaction with the heme, but part of the inhibitory effect was mediated through interaction at the substrate binding site [16]. Therefore, the trend toward increasing K_m values with higher inhibitor concentration in the present study may reflect additional competitive inhibition at the substrate binding site (Table 2). Noncompetitive inhibition of P4501A-catalyzed reactions by imidazole fungicides has been demonstrated with clotrimazole using mammalian microsomes [3,13] and with the agricultural imidazole fungicide prochloraz using rainbow trout microsomes [12].

Although the dose of the imidazole inhibitor used in this study was greater than doses utilized agriculturally and clinically, there is the potential for significant effects on monooxygenase activities occurring at much lower concentrations. This could especially be the case where lower levels of P450 induction are coupled with lower levels of direct inhibition by the fungicides. Therefore, the potential exists for organisms that are coexposed to imidazole fungicides and to organic environmental contaminants to reach higher steady-state contaminant levels. The implication of increased steady-state levels is to increase the likelihood of trophic transfer of contaminants to top predator species and to humans. Furthermore, these findings are of relevance for the use of cytochrome P450 as a biomarker in environmental monitoring. For example, the inhibitory effects of imidazole derivatives could mask the induction response, causing underestimation of the actual exposure to wild fish populations.

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