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Title page:

Demethylation of the Pesticide Methoxychlor in Liver and Intestine from Untreated, Methoxychlor-Treated and 3-Methylcholanthrene-Treated Channel Catfish (*Ictalurus punctatus*): Evidence for roles of CYP1 and 3A family isozymes.

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Running title page

a) Methoxychlor demethylation in catfish liver and intestine

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Abstract:

Exposure to the organochlorine pesticide methoxychlor is associated with endocrine disruption in several species, through biotransformation to mono-desmethyl-MXC (OH-MXC) and bis-desmethyl-MXC (HPTE), which interact with estrogen receptors. The biotransformation of [^{14}C]-methoxychlor was examined in channel catfish (*Ictalurus punctatus*), a freshwater species found in the southern United States. Hepatic microsomes formed OH-MXC and HPTE, assessed by co-migration with authentic standards. The K_m for OH-MXC formation by control liver microsomes was 3.8 ± 1.3 μM (mean ± S.D., n = 4) and V_{max} was 131 ± 53 pmol/min/mg protein. These values were similar to those of catfish pretreated with 2 mg methoxychlor /kg ip, for 6 days (K_m 3.3 ± 0.8 μM and V_{max} 99 ± 17 pmol/min/mg), but lower ($p<0.05$) than the kinetic parameters for catfish treated with 3-methylcholanthrene (3-MC), which had K_m of 6.0 ± 1.1 μM and V_{max} 246 ± 6 pmol/min/mg protein. Liver microsomes from 3-MC-treated fish produced significantly more of the secondary metabolite and more potent estrogen, HPTE. Intestinal microsomes formed OH-MXC at lower rates than liver. Methoxychlor pretreatment significantly reduced intestinal metabolite formation from 32 ± 4 to 15 ± 6 pmol/min/mg (mean ± S.D., n=4) while 3-MC treatment significantly raised OH-MXC production to 72 ± 22 pmol/min/mg. Ketoconazole, clotrimazole and α-naphthoflavone all decreased the production of OH-MXC in liver microsomes, while α-naphthoflavone stimulated HPTE formation, suggesting CYP1 and CYP3 family isozymes demethylated methoxychlor. The results suggest that the formation of estrogenic metabolites from methoxychlor would be more rapid in catfish co-exposed to CYP1 inducers.

Introduction:

Methoxychlor (1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane) is an organochlorine pesticide that was developed as a substitute for DDT (1,1,1-trichloro-2,2-bis(chlorophenyl)ethane). In contrast to DDT, methoxychlor exhibits generally low overt toxicity and bioaccumulation in mammals. methoxychlor has been used as a pesticide on pets, livestock, crops, gardens, and in animal feed (Magliulo et al., 2002; Palanza et al., 2001). The use of methoxychlor in the United States legally ended in 2004 when the chemical was denied re-registration by the United States Environmental Protection Agency, however, widespread use over the past half century has produced considerable environmental contamination and exposure (Li and Kupfer, 1998). A recent study of house dust samples from 120 homes in Cape Cod, MA found a median methoxychlor concentration of 0.24 µg/g (Rudel et al. 2003). Tissues of several Arctic wildlife species, including snow crab and narwhal, had detectable residues of methoxychlor, indicating global distribution of the pesticide (Vorkamp et al., 2004).

As is the case with DDT, methoxychlor has estrogenic metabolites, and produces endocrine disruption in fish (Larkin et al., 2003, Borgert et al., 2004). Evidence has been presented which suggests that the demethylated metabolites of methoxychlor, 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane (OH-MXC) and 1,1,1- trichloro-2,2-bis(4-hydroxyphenyl)ethane (HPTE) are more potent estrogens in channel catfish than either o,p'-DDT or o,p'-DDE (Nimrod and Benson, 1997). Likewise, OH-MXC has been shown to be 16-43 times more potent than the parent methoxychlor for binding to hepatic estrogen receptors in channel catfish (Schlenk et al., 1998). Experiments with expressed human and rat estrogen receptors α and β indicated that both OH-MXC and HPTE acted as agonists at estrogen receptor α but antagonists at estrogen receptor β as well as at androgen receptors (Gaido et al., 1999;

Gaido et al., 2000). Thus, the biotransformation of methoxychlor to demethylated metabolites is an important toxication step.

Cytochrome P450 (P450)-catalyzed O-demethylation of methoxychlor results in the sequential formation of OH-MXC and HPTE, as shown in Figure 1. In human liver microsomes, several P450 isoforms, but predominantly CYP2C9 and CYP1A2 catalyze demethylation of methoxychlor to OH-MXC (Hazai and Kupfer, 2005; Hu and Kupfer, 2002, Hu et al., 2004). Demethylation of OH-MXC to form HPTE was catalyzed by human CYP1A2, CYP2C8, CYP2C19, CYP2D6 and to a lesser extent CYP3A4 (Hu and Kupfer, 2002). Although there have been several studies of the adverse effects of methoxychlor in various fish species, little is known of the biotransformation of methoxychlor in fish. Studies by Schlenk et al., (1997), with the environmentally relevant channel catfish model have examined the influence of methoxychlor pretreatment and P450 induction by β-naphthoflavone upon methoxychlor biotransformation in catfish hepatic microsomes. These studies showed that high dose exposures to methoxychlor (2 x 254 mg/kg ip) or this dose of methoxychlor in combination with β-naphthoflavone (50 mg/kg ip) both reduced the rates of methoxychlor demethylation to OH-MXC relative to untreated controls or fish receiving only β-naphthoflavone. β-Naphthoflavone treatment alone did not increase the rate of methoxychlor demethylation. The effects of lower, more environmentally relevant doses of methoxychlor, or the effects of other aryl hydrocarbon receptor inducers, were not examined.

Since Hu and Kupfer (2002) showed that human CYP1A2 readily metabolized methoxychlor, it was somewhat surprising that the CYP1 family inducer, β-naphthoflavone, did not increase methoxychlor metabolism in catfish (Schlenk et al., 1997). It has been shown, however, that residues of β-naphthoflavone can inhibit CYP1A (James et al., 1997), therefore it

was of interest to examine the effects of another polycyclic aromatic inducing agent, 3-methylcholanthrene (3-MC). Gaining a better understanding of the P450-dependent metabolism of methoxychlor to estrogenic metabolites would help establish which species were more susceptible to the pesticide and which exposures to other chemicals could induce or inhibit the production of estrogenic metabolites. As well as CYP1 family inducers, such as polyaromatic hydrocarbons and polychlorinated biphenyls, fish may be exposed to a variety of agricultural chemicals, some of which interact with P450 and may affect methoxychlor biotransformation. Imidazole and triazole-containing agrochemicals such as prochloraz, propiconazole and thiobendazole interact with P450 acutely to inhibit mono-oxygenation (Snegaroff and Bach, 1989), similarly to the antifungal drugs ketoconazole and clotrimazole, though there is evidence that chronic exposure to azoles induces P450 in fish (Egaas et al., 1999, Hegelund et al., 2004). This research aims to create a better understanding of the demethylation of methoxychlor in channel catfish and, subsequently, the factors creating the endocrine disrupting toxicity of methoxychlor.

MATERIALS AND METHODS

Chemicals: Methoxychlor used to treat catfish and in the preparation of metabolite standards was obtained from ICN (Aurora, OH) and was >99% pure. NADPH, clotrimazole, 3-MC and [^{14}C]-methoxychlor (9.6 mCi/mmole) were obtained from Sigma Chemical Co. (St. Louis, MO). Prior to use in assays, the [^{14}C-methoxychlor] was purified to >99.9% by normal phase silica gel thin layer chromatography on LK5DF plates (Whatman, Florham Park, NJ) developed in diethyl ether:*n*-heptane, 1:1 by volume. 7-Ethoxyresorufin was synthesized by ethylation of resorufin, and both 7-ethoxyresorufin and resorufin were purified by thin layer chromatography on

preparative silica gel plates developed in chloroform:toluene:ethyl acetate: acetone, 3:3:2:1 by volume (Prough et al., 1978). Ketoconazole was obtained from Janssen Pharmaceutica (Piscataway, NJ) and α-naphthoflavone was obtained from Aldrich (Milwaukee, WI). All chemicals were at least 98% pure.

Preparation of metabolite standards: OH-MXC and HPTE were prepared by boron tribromide-catalyzed demethylation of methoxychlor using the method of Hu and Kupfer (2002). The OH-MXC and HPTE products were separated by silica gel column chromatography and further purified by re-crystallization from diethyl ether:*n*-heptane. Their identities were established by melting point and proton NMR (Kapoor et al., 1970). OH-MXC had a melting point of 109°C, and ^{1}H NMR in $CDCl_3$ showed peaks at δ3.79 [s, 3H, O-CH_3], 4.84 [s, 1H, OH], 4.95 [s, 1H, $CHCl_3$], 6.78-6.89 [m, 4H, aromatic], 7.46-7.53 [m, 4H, aromatic]). HPTE had a melting point of 204°C and ^{1}H NMR in D_6-DMSO showed peaks at δ5.12 [s, 1H, $CHCl_3$], 6.71-6.73 [m, 4H, aromatic], 7.45-7.47 [m, 4H, aromatic], 9.46 [s, 2H, OH]). Reverse phase HPLC with UV detection at 245 nm was used to verify the purity of each methoxychlor metabolite. The column was a 25 cm x 4.6 mm, 5 µm C_{18} Discovery column fitted with a 2 cm x 4.6 mm, 5 µm C_{18} Discovery guard column (Supelco, Bellefonte, PA). The mobile phase was isocratic acetonitrile:water (70:30) at 1 ml/min. Both metabolite standards were shown by HPLC analysis to be >99% pure.

Animals: Male and female adult channel catfish weighing between 1100g and 2700g (average 1675g) were used in these studies. The catfish were fed a purified diet as described previously (James et al., 1997). Four catfish were used in each treatment group. These were untreated (control), treated daily for 6 days with 2 mg/kg methoxychlor in corn oil by ip injection before sacrifice on the seventh day (methoxychlor), or treated with a single ip injection of 10 mg/kg 3-

MC in corn oil 3-5 days before sacrifice (3-MC). The catfish were sacrificed by stunning with a blow to the head, following by severing the spinal cord behind the head. This procedure was approved by the University of Florida IACUC. The liver was removed, and following excision of the gall bladder, placed in ice-cold buffer 1 (0.15 M KCl, 0.05 M potassium phosphate pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride). After rinsing three times in this buffer, the liver was minced and homogenized in 4 volumes of buffer 1. The whole intestine was removed, rinsed with ice-cold buffer 2 (0.25 M sucrose, 5 mM EDTA, 0.05 M Tris-Cl, pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride) to remove particles and sliced open longitudinally. The intestinal mucosa was scraped with a scalpel into 10 ml buffer 2 and mucosal cells were sedimented by centrifugation at 2,000 x *g*. The mucosal cells were homogenized in 4 volumes of buffer 2. Washed hepatic and intestinal microsomes were prepared from all catfish as described previously and resuspended in a volume of buffer containing 0.25 M sucrose, 0.01 M Hepes buffer pH 7.4, 5% glycerol, 0.1 mM dithiothreitol, 0.1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride that was equal to the original wet weight of the liver or intestinal mucosa (James et al. 1997). The washed microsomes were stored under nitrogen at -80°C in 0.5 ml aliquots until use. Studies established that monooxygenase activity was stable for at least 6 months under these storage conditions.

Methoxychlor monoxygenation assay: A radiochemical method was developed, with extraction and TLC analysis of metabolites. The optimal conditions for solvent extraction of methoxychlor and metabolites, and for the incubation time and protein concentrations for linear product formation in hepatic and intestinal microsomes were determined experimentally. Assay tubes contained, in a final volume of 0.5 ml, [^{14}C]-methoxychlor (varying concentrations), 0.1M Hepes pH 7.6, 50 mM $MgCl_2$, liver microsomes (0.2 mg protein) or intestinal microsomes (0.3 mg

protein) and 2 mM NADPH. In all experiments the methoxychlor was added to empty tubes in ethanol solution (0.005 ml), and in most studies the ethanol was removed under nitrogen before addition of other components, except NADPH. The tubes were placed in a 35°C shaking water bath for two min then NADPH was added. After 15 min the reaction was stopped by the addition of 0.5 ml ice cold water and 2 ml water-saturated ethyl acetate. Each tube was vortex-mixed and the phases separated by centrifugation. The ethyl acetate layer was transferred to a clean tube. Two more extractions were performed with 2 ml ethyl acetate. The pooled ethyl acetate fractions were dried by addition of anhydrous sodium sulfate. The dry ethyl acetate was transferred to a clean tube, the solvent evaporated under nitrogen and the residue of methoxychlor and metabolites dissolved in 0.2 ml ethanol. Aliquots of each sample, 20-50 μL, were applied to LK6DF normal phase silica gel thin layer chromatography plates with fluorescence indicator (Whatman, Florham Park, NJ) along with standards for methoxychlor, OH-MXC and HPTE. The plates were developed in *n*-heptane: diethyl ether 1:1 by volume. The standards were visualized with UV light. The [^{14}C]-methoxychlor and metabolites were detected and quantitated by overnight electronic autoradiography with a Packard Instant Imager (Meriden, CT). Rates of formation of OH-MXC and HPTE were calculated from the percentage of each metabolite produced and the known concentration of methoxychlor present in assay tubes.

K_m and V_{max} determination: K_m, V_{max}, and clearance values for formation of OH-MXC by liver microsomes were calculated from the rates found at several concentrations of methoxychlor and the Michaelis-Menten equation using Prism software v 4.0 (GraphPad Software, Inc., San Diego, CA). In studies where 1% v/v ethanol remained in the assay tubes, final methoxychlor concentrations of 5, 10, 15, 50, and 100 μM were employed to determine K_m values. In

experiments where the ethanol was removed prior to addition of other assay components, final methoxychlor concentrations of 2, 5, 10, 15, and 25 μM were used.

Solvent Inhibition Studies: Ethanol and acetone were used to prepare solutions of methoxychlor and several chemical CYP inhibitors. The effects of these solvents on the demethylation of methoxychlor were examined. Solvents were added (1% ethanol v/v, 2% ethanol v/v, and 1% ethanol v/v with 1% acetone v/v) to assay tubes after addition of all other components except NADPH, and rates of formation of OH-MXC compared with rates in tubes without solvent.

Chemical inhibition Studies: The effects of ketoconazole, α-naphthoflavone and clotrimazole on methoxychlor demethylation were examined. The inhibitors were dissolved in ethanol and added to the assay at the same time as methoxychlor, 25 μM. The solvent was evaporated under nitrogen so that no ethanol remained in the tube before addition of other assay components. Five different concentrations of each inhibitor were used. From the inhibition of the mono-demethylation of methoxychlor an IC_{50} value was calculated for each inhibitor by plotting the log concentration of inhibitor versus the fraction of activity remaining. Where possible, the effects on HPTE formation were also examined.

Monoclonal antibody inhibition studies*:* Monoclonal antibody to scup CYP1A (MAb-123) was prepared as described previously (Stegeman et al., 1985, Park et al., 1986). Monoclonal antibody to egg lyzozyme (MAb Hy-Hel-9) in ascites fluid was kindly provided by K. Krausz, NIH and used as a control (Krausz et al., 2001). Ascites fluid containing monoclonal antibody to scup CYP1A was incubated on ice with microsomes from 3-MC-induced channel catfish, in the ratio 20 or 40 μg antibody per pmol total measured P450, for 15 min. The total P450 content of microsomes was measured spectrally, as described previously (James et al., 1997). Ascites fluid containing the MAb Hy-Hel-9 was similarly incubated with microsomes from 3-MC-

induced channel catfish in the ratio 1 µl per pmol total P450. The mixture of microsomes and monoclonal antibody was added to assay tubes prepared as described above, containing 25 µM methoxychlor. The tubes were placed in a shaking water bath at 35°C for 3 min before addition of NADPH.

Ethoxyresorufin O-deethylation (EROD) Activity: EROD activity was measured to establish that treatment of catfish with 3-MC induced CYP1A, and as a positive control for the effects of the monoclonal antibody to scup CYP1A on methoxychlor demethylation. A fluorescence assay was used as described previously (James et al., 1997). Assay tubes contained 2.5 µM ethoxyresorufin, 0.1 M Hepes buffer pH 7.6, 0.25 mg microsomal protein per ml (controls) or 0.005 mg microsomal protein per ml (3-MC-treated) and 2 mM NADPH in a total volume of 0.5 ml, and were incubated at 35 ºC for 5 min (controls) or 2 min (3-MC-treated). To study the effect on EROD activity of the monoclonal antibody to scup CYP1A, microsomes from 3-MC-induced fish were pre-incubated for 15 min on ice with 0.1M Hepes buffer pH 7.6 and MAb-123 in the ratio of 20 or 40 µg MAb per pmol total measured total P450, prior to use in assays. Three sets of controls were used. One set contained microsomes from 3-MC-induced fish that were pre-incubated on ice with 0.1 M Hepes buffer pH 7.6 and MAb Hy-Hel-9 in the ratio of 1 µl per pmol P450, a second contained microsomes from 3-MC-induced fish that were pre-incubated on ice for 15 min with 0.1 M Hepes buffer pH 7.6 and a volume of water equal to that of the ascites fluid, and a third set was not pre-incubated.

Western Blot Analysis of Liver CYP1A Protein: The induction status of 3-MC-treated fish and the amount of CYP1A in liver microsomes from control catfish was examined by immunoblotting. Monoclonal antibody to scup CYP1A (MAb 123) was used (Park et al., 1986). Hepatic microsomes from control (40 µg protein) and 3-MC-treated (5 µg protein) fish, as well

as unstained and pre-stained molecular weight standards in the range 14,400 to 97,000 were separated on a 4% stacking and 8.5% resolving SDS-polyacrylamide mini-gel as described by Laemmli (1970). The electrophoretically resolved proteins were transferred to a nitrocellulose membrane, blocked with non-fat milk and incubated with mouse anti-scup CYP1A (1:10,000) at 4°C overnight. The membrane was washed, incubated with 1:1000 sheep anti-mouse IgG conjugated with horseradish peroxidase re-washed and developed with Super Signal (Pierce) for 5 min. Membranes with control liver protein were exposed to film for 15 min and membranes with 3-MC treated protein were exposed to film for 15 sec. The intensities of the bands were compared with varying known amounts of rat CYP1A as reference standards (BD Gentest, Woburn, MA).

Statistical analysis: Differences between treatment groups were analysed using Student's t test and Excel software.

RESULTS

Assay development in liver microsomes

Incubations to investigate the demethylation of methoxychlor were initially performed with a procedure similar to those used by other investigators (Hu and Kupfer, 2002; Schlenk et al., 1997; Stresser and Kupfer, 1998). In the published procedures, and our initial studies, [^{14}C-methoxychlor] was dissolved in ethanol and added to the reaction mixture for a final concentration of 1% ethanol in the assay tubes. The metabolites were separated by thin layer chromatography. Methoxychlor displayed an R_F of 0.63, OH-MXC had an R_F of 0.33, and HPTE had an R_F of 0.17. No other products were identified, although a small amount of radioactivity remained on the pre-adsorbent band of the silica gel plates. The time course of

formation of metabolites was investigated by incubating tubes for 5, 10, 15, 20, 30 or 45 min. Production of OH-MXC by channel catfish liver microsomes was linear up to 30 min. After a lag phase of 10 min, HPTE production was linear for at least 45 min. The effect of varying protein concentration was investigated by conducting incubations for 15 min. The formation of OH-MXC was linear with up to 0.35 mg protein per tube, and HPTE formation was linear up to 0.6 mg protein per tube.

Solvent inhibition

In preparing to conduct studies with monooxygenase inhibitors, the possibility of solvent inhibition of methoxychlor metabolism was investigated. It was found that 1% ethanol in incubation tubes inhibited formation of OH-MXC in catfish liver microsomes by 44%. Addition of ethanol to 2% of the assay volume, or addition of 1% ethanol and 1% acetone inhibited monoxygenation by 71% and 64% respectively. To avoid solvent inhibition, all solvents were removed from assay tubes by evaporation under nitrogen before adding the other components of the reaction mixture.

Kinetics of methoxychlor mono-demethylation in liver, and effect of treatment

The kinetic constants K_m and V_{max} for OH-MXC formation in liver microsomes from control and methoxychlor-pretreated catfish were obtained in the presence of 1% ethanol, before the effect of ethanol on activity was recognized. Rates of OH-MXC formation fit the Michaelis-Menten equation in the range 2.5 to 100 µM methoxychlor in most fish, although in two fish, rates started to decline at methoxychlor concentrations above 50 µM (data not shown). For those fish that showed lower rates at 100 µM methoxychlor, this point was omitted from the calculations of K_m and V_{max} values. After discovery of the ethanol inhibition, K_m and V_{max} values for methoxychlor demethylation were obtained in the same liver samples without using ethanol

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in the procedure, and using a range of methoxychlor concentrations (2.5 to 25 μM) that bracketed the K_m and fit the Michaelis-Menten equation. The results for all kinetic studies are listed in Table 1. Pairwise comparisons of K_m and V_{max} values in the same control fish or fish pre-treated with methoxychlor revealed significantly lower K_m values in the absence of ethanol ($p<0.005$) than those obtained with ethanol, while V_{max} values were significantly higher ($p<0.05$) when ethanol was removed from the procedure.

Table 1 also shows the influence of treatment with methoxychlor or 3-MC on methoxychlor demethylation kinetics without the influence of ethanol. Figure 2 shows Michaelis-Menten graphs for representative control, methoxychlor-treated and 3-MC-treated fish. There were no significant differences in the K_m and V_{max} values for control and methoxychlor treated fish (Table 1), however there was a large spread in V_{max} values in both groups. Treatment of catfish with 3-MC significantly ($p<0.05$) altered the kinetics of OH-MXC production compared with control catfish, with increases in both K_m and V_{max}. The 3-MC treated fish showed less variability for V_{max} than control or methoxychlor-treated catfish.

The 3-MC-treated fish were shown to have induced CYP1A in hepatic microsomes. The EROD activity (mean ± SD, n = 4) for control fish was 19 ± 5 pmol/min/mg protein while 3-MC-treated fish had mean activity of 1078 ± 268 pmol/min/mg protein, a 58-fold increase ($p<0.001$). An increase of CYP1A protein was demonstrated by Western blot, shown in Figures 3A and B for control and 3-MC-treated fish, respectively. Control fish had an average of 1.7 ± 0.2 units CYP1A/mg protein while 3-MC-treated fish had an average of 1040 ± 225 units CYP1A/mg protein, where one unit was the signal density produced by 1 pmol rat CYP1A. The increase in CYP1A protein was significant ($p<0.001$).

Formation of HPTE in liver

The secondary metabolite, HPTE, was formed in incubations with channel catfish liver microsomes. Activity data from the three treatment groups of fish was compared for incubations with 25 μM [^{14}C]-methoxychlor (Table 2). HPTE formation in most fish treated with methoxychlor was below the detection limits (<1 pmol/min/mg protein). Fish treated with 3-MC formed HPTE at significantly higher rates ($p<0.05$) than the control. Under the assay conditions used, which were optimized for OH-MXC formation, the amounts of HPTE formed were significantly lower than OH-MXC in all groups of fish ($p<0.01$).

Intestinal demethylation of methoxychlor

The monooxygenation of methoxychlor in intestinal microsomes was examined in samples from control, methoxychlor, and 3-MC-treated fish using 15 μM [^{14}C]-methoxychlor. This concentration was selected as being low, yet saturating in liver microsomes from control fish (Figure 2). Fish treated with 3-MC had significantly higher intestinal activity ($p<0.05$) than the control fish (Table 2). The intestinal activity of the methoxychlor treated group was significantly lower than the control ($p<0.01$) (Table 2). Although HPTE was formed in some intestinal samples (data not shown), the amounts were generally too small for accurate quantitation under the incubation conditions used. Kinetic studies were not performed with intestinal microsomes because of the relatively small amount of intestinal mucosa obtained and the lower overall activity in intestines.

Inhibition of methoxychlor monooxygenation

Ketoconazole, clotrimazole and α-naphthoflavone all inhibited the formation of OH-MXC in hepatic microsomes from each of the catfish treatment groups, but with different

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inhibitory potencies. The mean IC_{50} values are summarized in Table 3. There was no significant treatment difference ($p>0.05$) in the IC_{50} values for ketoconazole or clotrimazole. Clotrimazole was slightly more potent than ketoconazole. α-Naphthoflavone was less potent than the other two inhibitors. Solubility for α-naphthoflavone was a limiting factor for accurate measurements in incubations containing more than 100 μM, thus it was not possible to demonstrate more than about 70% inhibition with this compound (data not shown). There was no significant difference ($p>0.05$) in the α-naphthoflavone IC_{50} between control and methoxychlor-treated catfish, but α-naphthoflavone was a more potent inhibitor ($p<0.05$) in 3-MC-treated catfish (Table 3). While increasing concentrations of α-naphthoflavone decreased the amount of OH-MXC produced by hepatic microsomes from 3-MC-treated catfish, the amount of HPTE produced increased. In a representative 3-MC-treated fish, HPTE formation was 14 pmol/min/mg protein in assays with no added modifier, increased to 22 pmol/min/mg protein in the presence of 25 μM α-naphthoflavone, and remained elevated at higher concentrations up to 100 μM, after which HPTE formation declined (Figure 4A). Similar results were found with hepatic microsomes from the other 3-MC-treated fish. Because α-naphthoflavone inhibited formation of OH-MXC, the rate of HPTE formation expressed as a percentage of OH-MXC formation increased with increasing amounts of α-naphthoflavone (Figure 4B).

Although 3-MC treatment increased methoxychlor monooxygenation, a monoclonal antibody to scup CYP1A did not inhibit the rate of formation of OH-MXC in liver microsomes from 3-MC-induced catfish, compared to control incubations conducted in the presence of anti-lyzozyme ascites fluid or incubations with no added antibody. Indeed, there was a slight increase in activity in the presence of MAb-123 (Figure 5A). Studies conducted to determine if this monoclonal antibody would inhibit catfish CYP1A demonstrated that MAb-123 did inhibit

EROD activity in hepatic microsomes from 3-MC-induced catfish. Tubes containing 20 µg antibody per pmol total CYP showed a 20% inhibition of EROD activity, and tubes containing 40 µg antibody per pmol total CYP were 42% inhibited (Figure 5B).

DISCUSSION

This study showed that ethanol inhibited methoxychlor demethylation in catfish liver microsomes (Table 1). The presence of 1% ethanol in assay tubes approximately doubled the K_m for methoxychlor in control and methoxychlor-pretreated fish, and on a fish by fish basis, decreased V_{max}. In the earlier study of methoxychlor metabolism in catfish liver microsomes, Schlenk et al. (1997) used 1% ethanol to add methoxychlor, making it difficult to compare our results with the earlier study. The ethanol effect on V_{max} as well as K_m that we observed suggests a mixed-type inhibition by ethanol, however detailed studies of the kinetics of ethanol inhibition were not carried out. The competitive portion of the inhibitory interaction may indicate the involvement of a CYP2E1-like isoform, which metabolizes ethanol, (Gonzalez, 2005) in methoxychlor demethylation in the catfish.

The finding that K_m values in untreated catfish were in the low µM range suggested that environmentally exposed catfish would rapidly metabolize methoxychlor to active estrogenic metabolites in the liver. Demethylation rates catalyzed by intestinal microsomes were lower than by liver microsomes, and since the intestine is a rather small organ in catfish, this suggests limited intestinal metabolism from dietary exposure to methoxychlor. We did not examine kinetics in the intestinal microsomes because of their lower methoxychlor demethylase activities and the relatively small amount of intestinal mucosa in catfish.

Considerably more OH-MXC than HPTE was formed by catfish liver microsomes in the 15 min incubation time used in most of these studies (Table 2). Human liver microsomes incubated under similar conditions (25 µM methoxychlor for 10 min) likewise formed less HPTE than OH-MXC, with amounts of HPTE ranging from non-detectable to about half the amount of OH-MXC formation (Stresser and Kupfer, 1998). As with human and rat liver microsomes, the

relative production of HPTE by catfish liver microsomes increased with longer incubation times, concurrent with increased formation of OH-MXC, the precursor of HPTE.

Treatment of catfish with 2 mg methoxychlor/kg for six days did not change the hepatic K_m or V_{max} values for OH-MXC formation, suggesting that sub-chronic exposure to low concentrations of methoxychlor will not change its initial biotransformation in the liver. In intestine, however, this treatment reduced demethylation activity to half that of untreated controls. The reduction in methoxychlor demethylation suggests that methoxychlor, or a metabolite, down-regulates or inhibits one or more of the CYP isoforms responsible for OH-MXC formation in the catfish intestine. This finding differs from the reported effects of methoxychlor in rats. Treatment of rats with high doses of methoxychlor reduced the formation of OH-MXC but increased the formation of HPTE and ring-hydroxylated metabolites by liver microsomes, suggesting no effect on the initial formation of OH-MXC, but rather more rapid further metabolism in the methoxychlor-treated rats (Li et al., 1995). In the rat, methoxychlor, OH-MXC, HPTE and other metabolites activated the constitutive androstane receptor (CAR) system and induced CYP2B and CYP3A, consistent with the observed increased metabolism of methoxychlor and OH-MXC (Blizard et al. 2001). There was also evidence in rats that methoxychlor induced synthesis of CYP3A through the pregnane X receptor (PXR) receptor (Mikamo et al., 2003). Although the effects of methoxychlor or its metabolites on the isoform composition of catfish liver or intestine are not known, induction of CYP2B isoforms through interaction with the CAR receptor has not been well established in any fish species, and there is an extensive literature showing that fish P450s are not inducible by mammalian phenobarbital-type inducers (Kleinow et al., 1987). There are reports of modulation of CYP3A levels in fish liver by diet and treatment with imidazole-containing compounds, however fish do not respond

to several known rat or human CYP3A inducers (Hasselburg et al., 2005; James et al., 2005). Species differences in binding and activation of the PXR, which is the first step in up-regulation of CYP3A, are well documented (Moore et al., 2002). The compounds that bind the catfish PXR are not known. Further research is needed to understand the effects of methoxychlor and its metabolites on P450-dependent biotransformation in catfish. Since methoxychlor has been found to bioaccumulate in fish (Krisfalusi et al., 1998), a longer exposure time may affect the hepatic metabolism. Similarly, if methoxychlor metabolites affect P450 enzymes in catfish, prolonged exposure may be needed to have an effect in liver.

Treatment of catfish with 3-MC increased the K_m and V_{max} of OH-MXC production in liver, and doubled the rate of intestinal formation of OH-MXC. EROD assays and Western blots confirmed that CYP1A protein was induced in all the 3-MC-treated catfish used. Very small amounts of CYP1A and low EROD activity were found in microsomes from control fish. The increase in V_{max} (liver) and measured rate (intestine) for OH-MXC formation in fish with elevated CYP1A initially suggested that CYP1A played a role in the primary metabolism of methoxychlor in channel catfish. Arguing against this conclusion is the finding that the monoclonal antibody to scup CYP1A, which inhibited EROD activity in hepatic microsomes from 3-MC-treated fish, had no effect on OH-MXC formation. Possibly, another CYP isoform, also induced by 3MC and susceptible to inhibition by α-naphthoflavone, was responsible for the increased production of OH-MXC. Multiple CYP1 family isoforms in the 1B and 1C sub-families have been cloned from other fish species (Godard et al., 2005). If present in catfish, one of these other CYP1 family isoforms may metabolize methoxychlor. HPTE formation was significantly elevated in liver microsomes from 3-MC-treated fish. Evidence from the effect of α-naphthoflavone on HPTE formation, discussed below, suggests that the increased formation of

HPTE in 3-MC-treated fish does not mean that a CYP1 family isoform catalyzes the demethylation of OH-MXC, but rather that the increase in formation of OH-MXC in liver microsomes augments HPTE production. The more rapid demethylation of methoxychlor in 3-MC-treated catfish suggests that environmental exposure of catfish to planar polycyclic compounds such as polycyclic aromatic hydrocarbons, dioxin and planar PCBs will increase their susceptibility to the endocrine-disrupting effects of methoxychlor, which are mediated by their estrogenic metabolites, OH-MXC and HPTE (Gaido et al., 1999, 2000).

Chemical inhibition studies performed with α-naphthoflavone, ketoconazole and clotrimazole showed that all three compounds inhibited methoxychlor demethylation (Table 3). Studies with liver microsomes from fish and humans showed that α-naphthoflavone selectively inhibited CYP1A-dependent benzo(a)pyrene monooxygenase activity, but stimulated CYP3A-dependent activity (James et al., 2005; Koley et al. 1997; Little et al., 1984). The inhibition of OH-MXC formation by α-naphthoflavone further suggests a role for a CYP1 family isoform in methoxychlor mono-demethylation. Interestingly, more HPTE was formed in the presence of α-naphthoflavone, and this was most readily observed in the 3-MC-treated fish, which produced more of the OH-MXC precursor. Even the lowest concentration of α-naphthoflavone added to assay tubes doubled the amount of HPTE formed (Fig 4A). This was similar to the effect of α-naphthoflavone on benzo(a)pyrene metabolism in catfish intestinal microsomes, attributed to CYP3A (James et al., 2005). The relative formation of HPTE, expressed as a percentage of the OH-MXC produced, increased with increasing concentrations of α-naphthoflavone in the assay tube, suggesting that CYP3A, which is stimulated by α-naphthoflavone, is at least partially responsible for production of HPTE in channel catfish liver. The results with the imidazole-containing compounds ketoconazole and clotrimazole were of interest because of their structural

similarity to fungicides used in agriculture, and their known effects on fish P450-dependent activity. In the catfish, ketoconazole was a very potent inhibitor of CYP3A-dependent testosterone 6-β-hydroxylase activity, with an IC_{50} value of 20 nM (James et al., 2005). Similarly, ketoconazole was a potent inhibitor of CYP3A-dependent 7-benzyloxy-4[trifluoromethyl]-coumarin dealkylation in the killifish and rainbow trout, with IC_{50} values of 10 and 100 nM respectively (Hegelund et al., 2004). Although ketoconazole inhibited OH-MXC formation in catfish liver microsomes, the potency of inhibition (around 5 μM) was much lower than was found for testosterone. Micromolar concentrations of ketoconazole can inhibit several forms of CYP (Zhang et al., 2002), so the relatively weak inhibitory potency suggests that CYP3A is not involved in the first demethylation of methoxychlor. Treatment with methoxychlor or 3-MC did not change the IC_{50} values for ketoconazole, further supporting a general inhibitory effect. Clotrimazole, which inhibited OH-MXC formation with IC_{50} values of 1-2 μM was a somewhat more potent inhibitor than ketoconazole in the catfish. There was no difference in clotrimazole IC_{50} values between the control and 3-MC-treated catfish. Since ketoconazole and clotrimazole structurally resemble other azole-containing fungicides used in agriculture, such as prochloraz, propiconazole and thiobendazole, these fungicides may inhibit methoxychlor metabolism, under conditions of co-exposure, and potentially decrease the endocrine-disrupting effects of methoxychlor exposure, at least acutely.

In summary, this work has shown that methoxychlor was readily demethylated to estrogenic metabolites in channel catfish liver, and to a lesser extent intestine. A P450 isoform induced by 3-MC was shown to play a role in the formation of OH-MXC, and there was evidence that CYP3A may catalyze the further metabolism of OH-MXC to HPTE. Induction of

methoxychlor metabolism by 3-MC suggested that co-exposure of fish to planar polycyclic compounds could increase the endocrine-disrupting effects of this pesticide in fish.

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FOOTNOTES

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Figure Legends

Figure 1 – The demethylation of methoxychlor.

Figure 2 – Rates of formation of OH-MXC at differing methoxychlor concentrations with hepatic microsomes from representative control (■), methoxychlor-treated (▲) and 3-MC-treated (●) channel catfish. Results shown are means of replicate samples from individual fish. The curves show the fit of the data to the Michaelis-Menten equation.

Figure 3 – Western blots showing cross-reactivity of catfish hepatic microsomal proteins and expressed rat CYP1A to a mouse monoclonal antibody to scup CYP1A. In Part A, the chemiluminescent signal was exposed to film for 15 min before developing. Lanes 1-4 contain 0.05, 0.1, 0.15 and 0.2 pmol expressed rat CYP1A, respectively. Lanes 5-8 contain 40 μg microsomal protein from control catfish liver. In part B, the chemiluminescent signal was exposed to film for 15 sec before developing. Lanes 1-4 contain 2, 4, 8 and 12 pmol rat CYP1A, respectively. Lanes 5-8 contain 5 μg microsomal protein from 3-MC-treated catfish liver.

Figure 4 – Effect of increasing concentrations of α-naphthoflavone on the demethylation of methoxychlor (25 μM) in hepatic microsomes from 3-MC-treated catfish. Panel A shows rates of formation of OH-MXC ● and HPTE ▲. Panel B shows HPTE formation expressed as a percentage of the OH-MXC produced. Results shown are the means of replicate samples from an individual fish. Similar results were observed with other fish.

Figure 5 – Effect of a monoclonal antibody to fish (scup) CYP1A (MAb-123) on OH-MXC formation (panel A) and EROD activity (panel B) in hepatic microsomes from a 3-MC-treated catfish. Similar results were observed with other fish.

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Table 1: Kinetic data for the formation of OH-MXC in hepatic microsomes from control, methoxychlor treated, and 3- methylcholanthrene treated channel catfish.

Treatment Group	1% ethanol present in assay tubes	K_m μM	V_{max} pmol/min/mg protein
Control	No	3.8 ± 1.3	131 ± 53
Control	Yes	8.1 ± 2.8*	84 ± 30*
Methoxychlor-treated	No	3.3 ± 0.8	99 ± 17
Methoxychlor-treated	Yes	6.7 ± 2.6*	56 ± 31*
3-MC-treated	No	6.0 ± 1.1*	246 ± 6**

Values shown are mean ± SD, n = 4 fish per treatment group. Methoxychlor-treated fish were injected ip with 2 mg/kg methoxychlor for 6 days prior to sacrifice on day 7. 3-MC-treated fish were injected ip with 10 mg/kg 3-MC three to five days prior to sacrifice.

* Significantly different from control, $p<0.05$. ** Significantly different from control, $p<0.01$.

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Table 2: Rates of formation of OH-MXC and HPTE in hepatic microsomes and OH-MXC in intestinal microsomes from control, methoxychlor-treated, and 3-MC-treated channel catfish.

Treatment group	Liver[a]		Intestine[b]
	OH-MXC	HPTE	OH-MXC
Control	120 ± 45	6 ± 5	32 ± 3.9
Methoxychlor-treated	87 ± 18	ND	15 ± 6**
3-MC-treated	200 ± 6*	15 ± 4**	72 ± 22**

Values shown are pmol/min/mg protein and are mean ± SD, n = 4 fish per treatment group. Fish were treated as described in Table 1.

[a] Incubations were carried out with 25 µM methoxychlor.

[b] Incubations were carried out with 15 µM methoxychlor.

* Significantly different from control, $p<0.05$. ** Significantly different from control, $p<0.01$.

ND – Formation of HPTE was below detection levels (<0.5 pmol/min/mg protein) in most samples.

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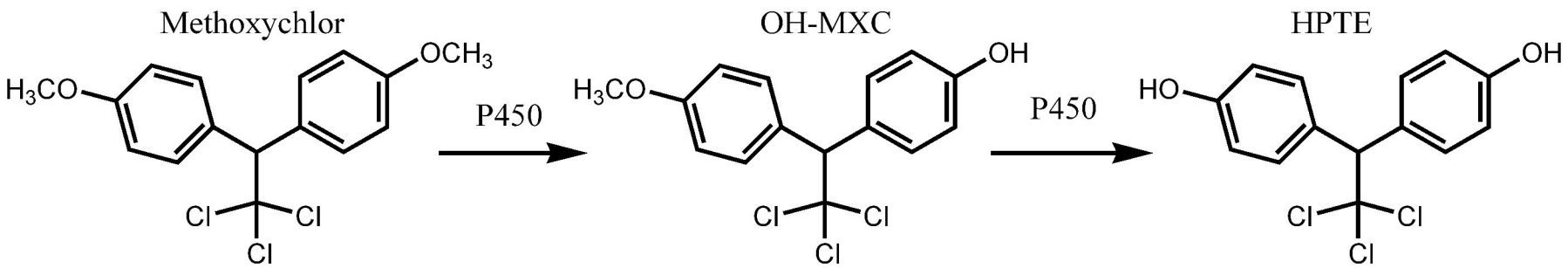
Table 3 – Ketoconazole, clotrimazole, and α-naphthoflavone IC_{50} values (μM) for inhibition of OH-MXC formation from 25 μM methoxychlor in hepatic microsomes from control, methoxychlor-treated, and 3-MC-treated channel catfish.

Treatment group	Ketoconazole	Clotrimazole	α-Naphthoflavone
Control	5.5 ± 3.9	1.7 ± 1.3	102 ± 9
Methoxychlor-treated	5.9 ± 3.0	ND	98 ± 12
3-MC-treated	3.4 ± 0.4	1.6 ± 1.8	80 ± 8*

Values are mean ± SD, n=4 fish per treatment group.

* Significantly different from control, $p<0.05$. ND – not determined

Figure 1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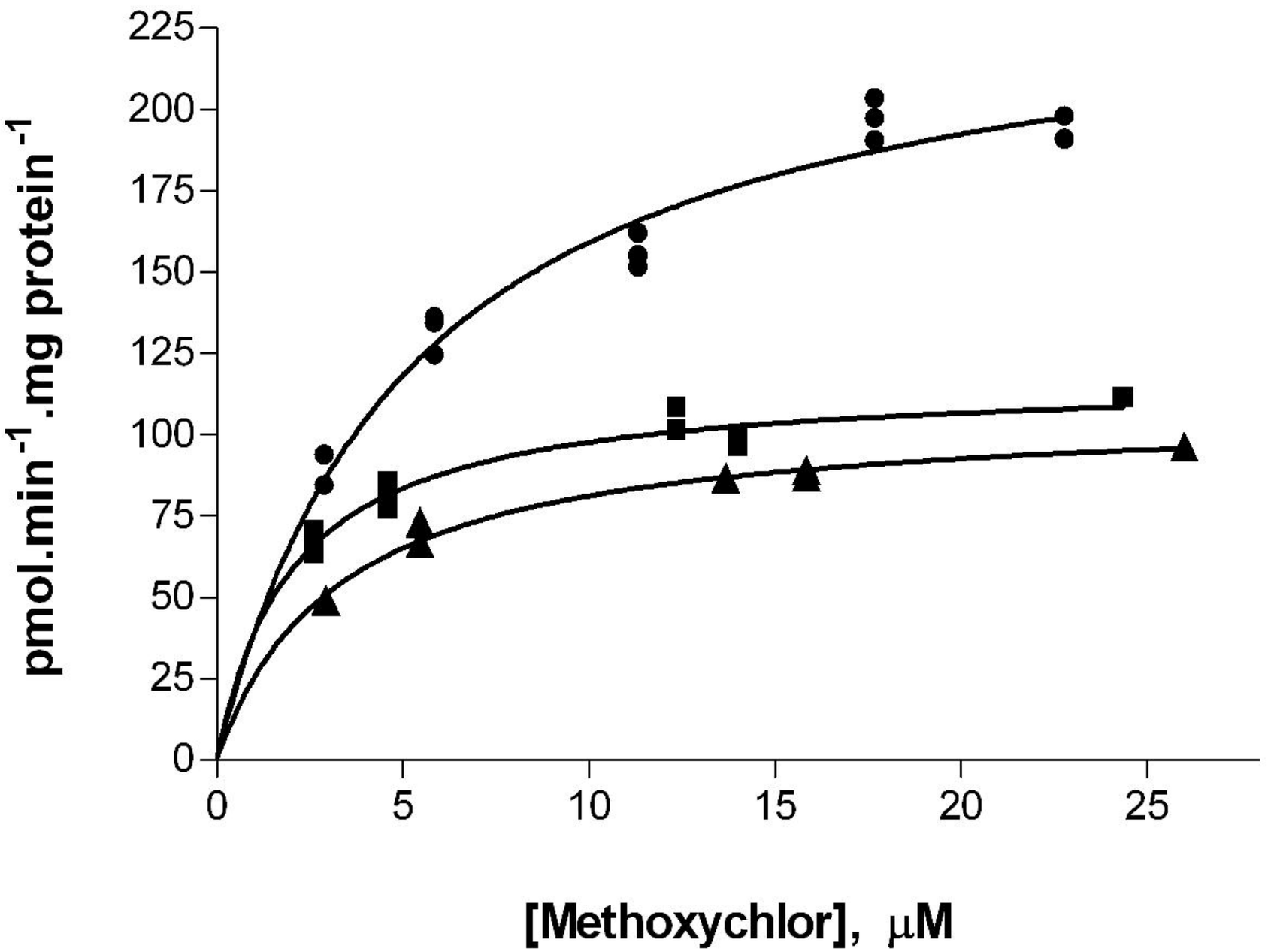
H3CO
Cl
Cl
Cl
P450
OH-MXC
OH
H3CO
Cl
Cl
Cl
P450
HPTE
OH
HO
Cl
Cl
Cl

Figure 2


225
200
175
150
125
100
75
50
25
0
pmol.min-1.mg protein-1
0
5
10
15
20
25
[Methoxychlor], μM

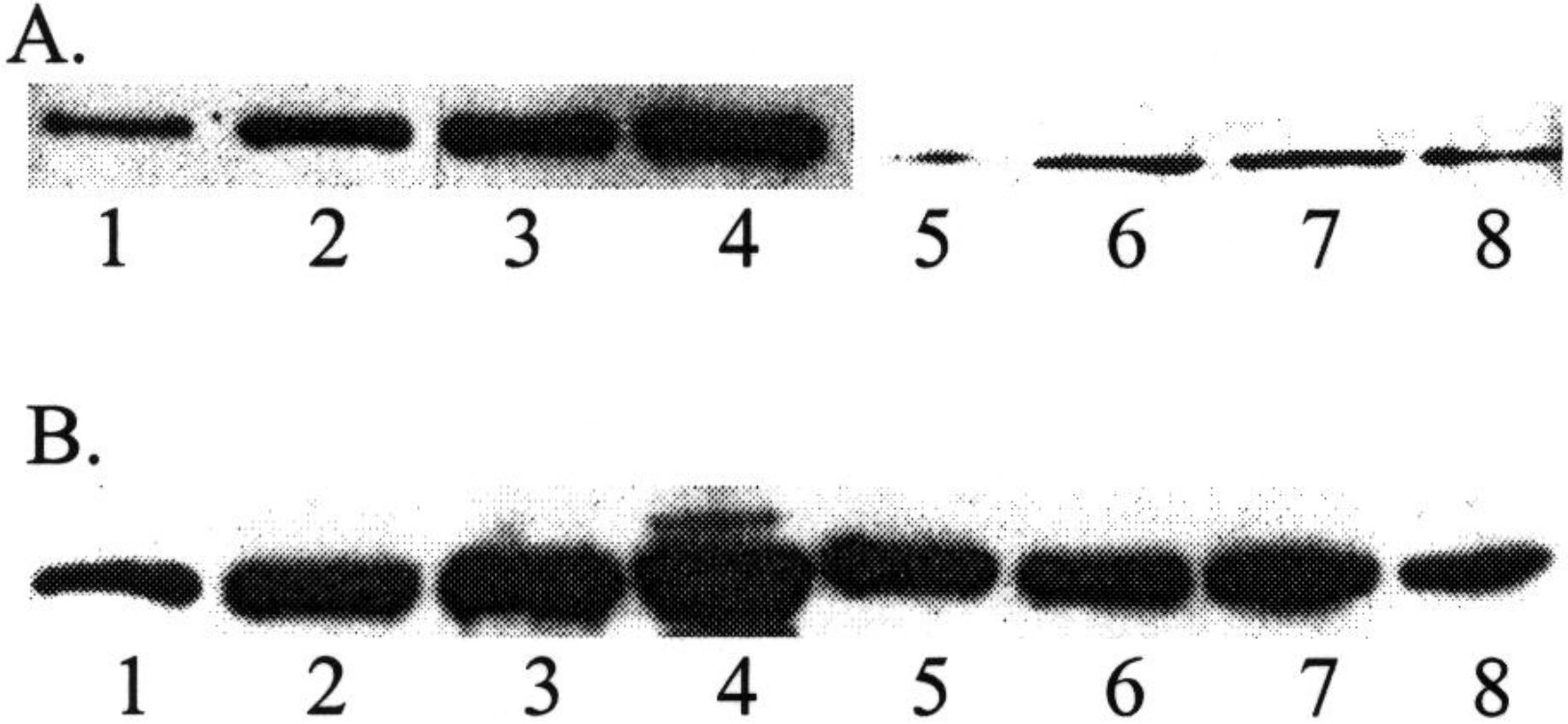
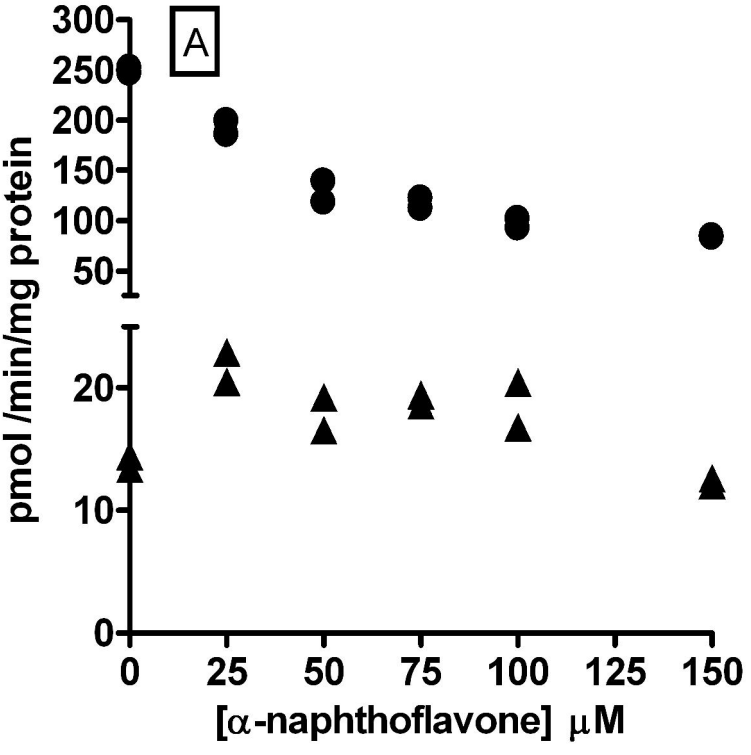
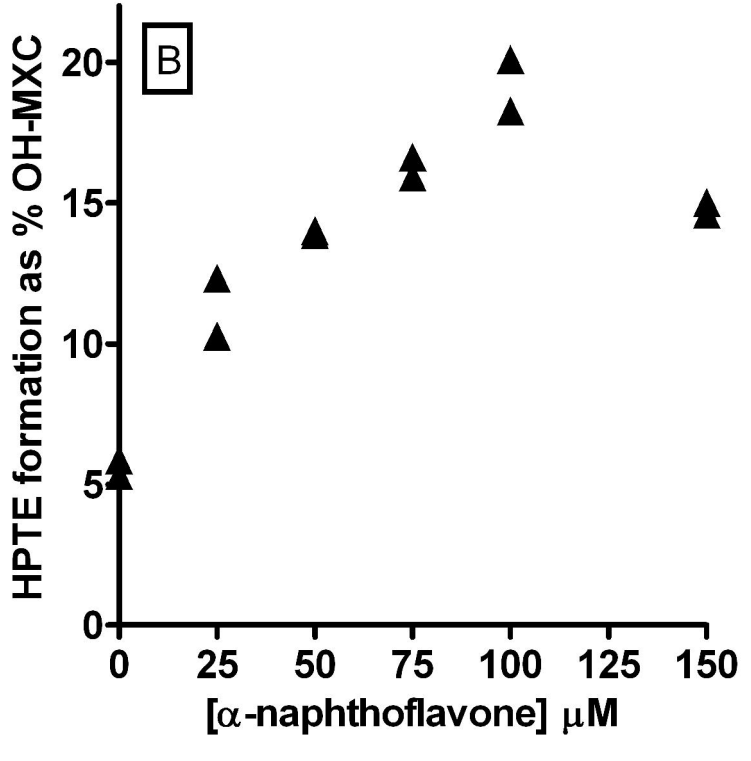


Figure 3

Figure 4


A
300
250
200
150
100
50
20
10
0
pmol /min/mg protein
0
25
50
75
100
125
150
[α-naphthoflavone] μM


B
20
15
10
5
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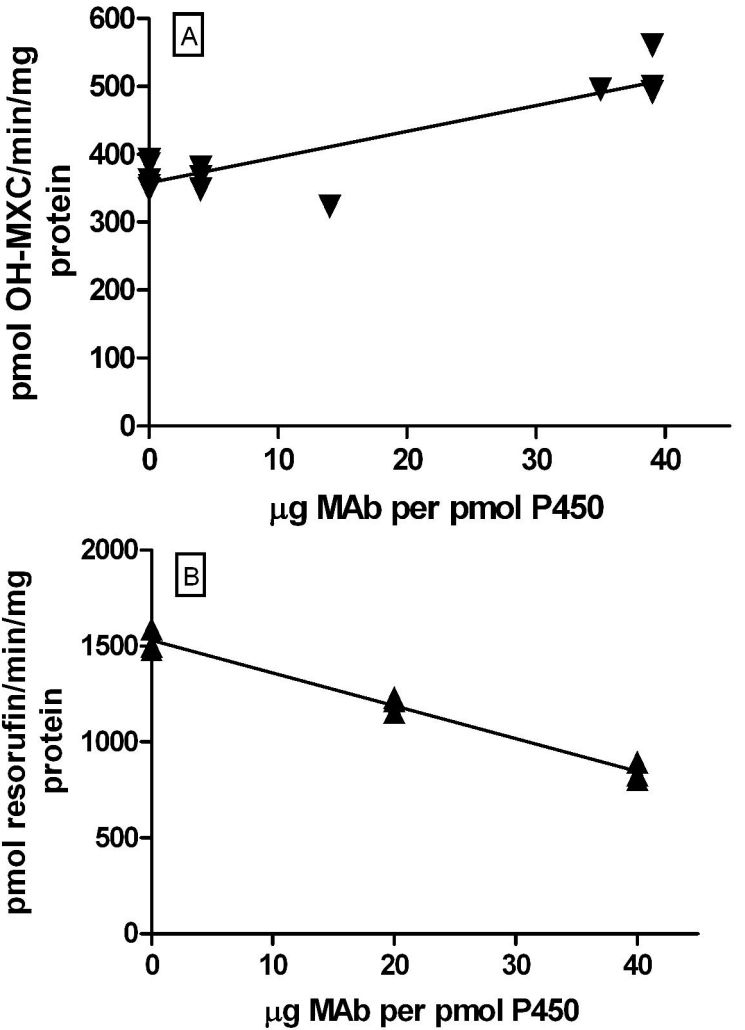
HPTE formation as % OH-MXC
0
25
50
75
100
125
150
[α-naphthoflavone] μM

Figure 5


A
pmol OH-MXC/min/mg protein
600
500
400
300
200
100
0
0
10
20
30
40
μg MAb per pmol P450
B
pmol resorufin/min/mg protein
2000
1500
1000
500
0
0
10
20
30
40
μg MAb per pmol P450