METABOLISM OF THE ENDOCRINE DISRUPTOR PESTICIDE-METHOXYCHLOR BY HUMAN P450S: PATHWAYS INVOLVING A NOVEL CATECHOL METABOLITE

YIDING HU AND DAVID KUPFER

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts

(Received April 4, 2002; accepted June 11, 2002)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:
The metabolism of methoxychlor, a proestrogenic pesticide (endocrine disruptor), was investigated with cDNA expressed human cytochrome P450s and liver microsomes (HLM). In addition to 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane (mono-OH-M), 1,1,1-trichloro-2, 2-bis(4-hydroxyphenyl)ethane (bis-OH-M), and 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(3, 4-dihydroxyphenyl)ethane (tris-OH-M), a new metabolite was identified as 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane (catechol-M; previously assumed to be ring-OH-M) and as a key metabolic intermediate. A novel metabolic route was proposed involving methoxychlor O-demethylation to mono-OH-M, followed by bifurcation of the pathway, both leading to the same final product tris-OH-M: pathway a, mono-OH-M is demethylated to bis-OH-M, followed by ortho-hydroxylation forming tris-OH-M and pathway b, mono-OH-M is ortho-hydroxylated forming catechol-M that is O-demethylated forming tris-OH-M. Among the human cDNA-expressed P450s examined, CYP1A2, 2A6, 2C8, 2C9, 2C19, and 2D6 exhibited mainly O-demethylation, with CYP2C19 being the most catalytically competent. CYP3A4, 3A5, and rat 2B1 catalyzed primarily ortho-hydroxylation of mono-OH-M (CYP3A4 being catalytically the most active) but were weak in O-demethylation. CYP1A1, 1B1, 2E1, and 4A11 demonstrated little or no catalytic activity. CYP2B6 appeared unique, catalyzing effectively both O-demethylation and ortho-hydroxylation. Thus, CYP2B6 demethylated methoxychlor to mono-OH-M and ortho-hydroxylated the mono-OH-M forming catechol-M; however, 2B6 did not appreciably demethylate mono-OH-M or ortho-hydroxylation bis-OH-M, suggesting a narrow substrate specificity. CYP2C19 catalyzed demethylation of methoxychlor, mono-OH-M and catechol-M, demonstrating relatively good substrate affinity (Km = 0.23 – 0.41 μM). However, the 3A4 ortho-hydroxylation of mono-OH-M and bis-OH-M exhibited lower affinity, Km = 12 and 25 μM, respectively. Thus, a phenolic group seems essential for efficient ortho-hydroxylation, forming catechol-M and tris-OH-M. Inhibition studies with HLM and P450s indicate that CYP2C9 and likely 2C19 are catalysts of methoxychlor-mono-demethylation.

Methoxychlor, a biodegradable pesticide and a substitute for the banned DDT, has a relatively low toxicity and short half-life (Metcalf et al., 1971; Gardner and Bailey, 1975; Metcalf, 1976). Despite these favorable features of methoxychlor, there is considerable concern for exposure to methoxychlor because of its estrogenic activity (Bulger et al., 1978b; Ousterhout et al., 1981). Whereas in vitro methoxychlor has little or no affinity for the estrogen receptor (ER), in vivo methoxychlor exhibits pronounced estrogenic activity, indicating that methoxychlor per se is a proestrogen (Bulger et al., 1978a,b; Kupfer and Bulger, 1979; Bulger et al., 1985). Indeed, methoxychlor undergoes oxidative metabolism by hepatic cytochrome P450 forming metabolites with pronounced estrogenic activity (Bulger et al., 1978a,b; Bulger et al., 1985). Additionally, methoxychlor was found to elicit considerable endocrine and reproductive toxicity (Gray et al., 1988; Cummings and Gray, 1989; Cummings and Laskey, 1993). However, hitherto it has not been established whether the endocrine and reproductive toxicities are associated with methoxychlor per se or are primarily due to its metabolites. Based on such and other findings, methoxychlor was categorized as a prototype endocrine disruptor, and the nature of its toxicity has been under protracted intensive investigation (Chapin et al., 1997; Cummings, 1997; You et al., 2002). Interestingly, the mono- and bis-demethylated methoxychlor metabolites (mono-OH-M and bis-OH-M) exhibit opposing activities toward the ERα and ERβ isoforms [i.e., both metabolites are agonists of ERα, but antagonists of ERβ, as well as antagonists of the androgen receptor (Gaio et al., 2000)]. Additionally, it was reported that bis-OH-M activity differs considerably from that of estradiol-17β in affecting certain gene expression in mice ovaries (Waters et al., 2001), possibly explaining the distinctive but overlapping pathologies in

Address correspondence to: Dr. David Kupfer, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation St., Room 815, Worcester, MA 01605-2324.
reproductive tissues following exposure of animals to estradiol-17β and methoxychlor.

In unrelated studies, methoxychlor was found to be an inducer of CYP2B and CYP3A in rats in vivo (Li et al., 1995; Li and Kupfer, 1998). The question of whether the induction was caused by methoxychlor or by its metabolites and whether induction would be expected in humans exposed to methoxychlor was recently addressed (Blizard et al., 2001). We observed that both methoxychlor and its metabolites activate the human constitutive androstane receptor system (Blizard et al., 2001), known to participate in CYP2B and in CYP3A induction (Moore et al., 2000; Sueyoshi and Negishi, 2001), indicating that both methoxychlor and its metabolites are active P450 inducers. The findings that methoxychlor and its metabolites activate human constitutive androstane receptor suggested that methoxychlor and/or its metabolites could induce CYP2B6 (Sueyoshi et al., 1999), and possibly 3A4 in humans, and thus may cause undue interactions with therapeutic agents and endogenous compounds that are metabolized by these P450s. Indeed, since 3A4 inactivates certain steroid hormones (e.g., by 6β-hydroxylating cortisol into inactive metabolite) (Kupfer, 1969; Kupfer and Partridge, 1970; Kinirons et al., 1993) and catalyzes estradiol hydroxilation (Badawi et al., 2001; Lee et al., 2001), it is conceivable that certain aspects of methoxychlor endocrine disruptor activity could be due to induction of CYP3A4. However, the question of whether these compounds could also activate the pregnane X receptor, primarily involved in CYP3A induction (Lehmann et al., 1998; Goodwin et al., 2002), has not been hitherto determined, and this aspect is currently under investigation in our laboratory.

These and other findings have made the elucidation of the methoxychlor metabolic pathway of prime interest. It has been known for some time that methoxychlor is O-demethylated to the mono-OH-M and bis-OH-M, followed by ortho-hydroxylation to form the tris-OH-M (Kupfer et al., 1990; Dehal and Kupfer, 1994; Stresser and Kupfer, 1997). Additionally, another metabolite, assumed to be the ortho-hydroxylated methoxychlor and referred to as ring-OH-M, was previously reported; however, because of paucity of availability of this substance and the lack of authentic standard, its structure has not been unequivocally elucidated (Dehal and Kupfer, 1994). Consequently, it was erroneously speculated that ring-OH-M undergoes O-demethylation to form tris-OH-M as the final methoxychlor metabolite.

The current study was undertaken to reinvestigate methoxychlor metabolism through exploration of its metabolic pathways and identification of the human P450 isoforms catalyzing those reactions. The study involved the synthesis of compounds for the unequivocal characterization of the metabolites and identification of a novel catechol (catechol-M), as being the previously miss-assigned ring-OH-M.

Materials and Methods

Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Methoxychlor was purchased from Chem Service (West Chester, PA). Bis-OH-M was kindly provided by Dr. Sanborn (Illinois State Natural History Survey, Urbana, IL). Tris-OH-M and tris-MeO-M were obtained from Chemsyn Science Laboratories (Lenexa, KS). Boron tribromide and silica-gel (Merck, 230–400 mesh, 60 Å) were purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade acetonitrile was purchased from Mallinkrodt (St. Louis, MO). cDNA-expressed P450s (supersomes) and human liver microsomes were purchased from Gentest Corp. (Woburn, MA).

Synthesis of Mono-OH-M. Boron tribromide (1.4 equivalents as 1 M in dichloromethane) was slowly added to a solution of methoxychlor in dichloromethane at –78°C (Fig. 1). The mixture was slowly warmed up to room temperature and stirred for 2 h under nitrogen gas. The reaction was monitored by TLC until the reaction was complete. The mixture was extracted twice with methylene chloride. The combined methylene chloride extracts were dried with MgSO₄, and the solvent was evaporated under a stream of nitrogen gas. The residue was purified by silica-gel chromatography using a gradient of hexane/ethyl acetate from 1:2 to 1:4 ratio (RF = 0.29 for mono-OH-M on silica gel TLC, using methanol/water/ethyl acetate 75:24:1).

Synthesis of Ring-OH-M and Catechol-M. Boron tribromide (0.8 equivalents as 1 M in dichloromethane) was slowly added to a solution of tris-MeO-M in dichloromethane at –78°C (Fig. 1). The mixture was stirred for 2 h at –78°C under nitrogen gas. The reaction was monitored by TLC until the reaction was complete. The mixture was extracted twice with methylene chloride. The combined methylene chloride extracts were dried with MgSO₄, and the solvent was evaporated under a stream of nitrogen gas. The residue was purified by silica-gel chromatography using a gradient of hexane/ethyl acetate from 1:2 to 1:4 ratio (RF = 0.29 for mono-OH-M on silica gel TLC, using methanol/water/ethyl acetate 75:24:1).
starting material disappeared; equal volume of water was added to the mixture and extracted twice with methylene chloride. The combined methylene chloride extracts were dried with MgSO\textsubscript{4}, and the solvent was evaporated under a stream of nitrogen gas. The crude products were purified on silica-gel column with gradient diethyl ether/hexane 1:10 to 1:1. Pure catechol-M and the mixture of ring-OH-M and catechol-M were obtained. Further purification of the mixture of the two compounds was performed on HPLC using a chiral cyclobond I 2000 column with acetonitrile/water 40:60 to yield ring-OH-M (retention time 11.10 and 12.43 min for two enantiomers) and ring-OH-M-mono-OH-M (retention time 7.10 min for unresolved racemic mixture).

Identification of the Compounds Synthesized.

Catechol-M, \( R_\text{f} \) 0.42 on TLC (silica gel, methanol/water/acetic acid 75:24:1).

1\textsuperscript{H}-NMR (400 MHz, CDCl\textsubscript{3}) \( \delta \) 7.50 (dd, 2H, \( J = 6.8, 2.0 \) Hz), 7.17 (d, 1H, \( J = 2.0 \) Hz), 7.03 (dd, 1H, \( J = 8.8, 2.0 \) Hz), 6.87 (dd, 1H, \( J = 6.8, 2.0 \) Hz), 6.82 (d, 1H, \( J = 8.8 \) Hz), 5.58 (s, 1H), 4.91 (s, 1H), 3.87 (s, 3H), 3.79 (s, 3H). ES-MS/MS (ion-trap) [M-H]- 349, 347, 345, daughter ion (of 345), 309.

Ring-OH-M, \( R_\text{f} \) 0.28 on TLC (silica gel, methanol/water/acetic acid 75:24:1).

1\textsuperscript{H}-NMR (400 MHz, CDCl\textsubscript{3}) \( \delta \) 7.52 (dd, 2H, \( J = 6.8, 2.0 \) Hz), 7.23 (d, 1H, \( J = 2.0 \) Hz), 7.07 (dd, 1H, \( J = 8.8, 2.0 \) Hz), 6.86 (dd, 1H, \( J = 6.8, 2.0 \) Hz), 6.80 (d, 1H, \( J = 8.8 \) Hz), 5.54 (s, 1H), 4.89 (s, 1H), 3.80 (s, 3H).

Ring-OH-M-mono-OH-M, \( R_\text{f} \) 0.28 on TLC (silica gel, methanol/water/acetic acid 75:24:1).

1\textsuperscript{H}-NMR (400 MHz, CDCl\textsubscript{3}) \( \delta \) 7.52 (dd, 2H, \( J = 6.8, 2.0 \) Hz), 7.13 (dd, 1H, \( J = 8.8, 2.0 \) Hz), 7.09 (d, 1H, \( J = 2.0 \) Hz), 6.89–6.86 (m, 3H), 5.59 (s, 1H), 4.93 (s, 1H), 3.88 (s, 3H), 3.80 (s, 3H).

Incubations.

Incubations were conducted in a 1-ml volume in 20-m1 vials in a Dubnoff metabolic shaker incubator under an atmosphere of air, containing the following: cDNA expressed P450s (supersomes) (50 nM) or human liver microsomes (2 mg or 1 mg of protein/ml); substrate 25 \( \mu \)M furafylline required prior metabolic activation for inhibition whereas the other inhibitors did not require prior incubation because of being intrinsically active. Procedure for incubations containing furafylline (a mechanism-based inhibitor of 1A2) was as follows. After 3 min of preincubation at 37°C in the absence of methoxychlor, there was a 10-min preincubation in the presence of the NADPH-generating system, then methoxychlor was added, and the mixture was incubated for an additional 20 min. Procedure for incubations containing other inhibitors was as follows. When sulfaphenazole (an inhibitor of CYP2C9) or delavirdine and lansoprazole were used, the 10-min preincubation procedure (with NADPH) was deleted, and the inhibitory compounds were added simultaneously with the substrate and after the 3-min preincubation (to equilibrate to 37°C); the reaction was initiated with the NADPH-generating system and conducted for 20 or 60 min.

Enzyme kinetics. CYP2C19 O-demethylase and 3AA ortho-hydroxylase activities were measured at enzyme concentration of 5 to 25 nM and substrate concentration of 0.25 to 50 \( \mu \)M and incubation times of 2 to 15 min. Incubations contained the same constituents as above, except that MgCl\textsubscript{2} was deleted, because in presence of MgCl\textsubscript{2} there was a spurious formation of an unidentified additional product derived from catechol-M (the rates of formation of the identified products were similar in the presence or absence of MgCl\textsubscript{2} in the O-demethylation of catechol-M by CYP2C19). The kinetic parameters were determined by fitting velocity and substrate concentration data into Michaelis-Menten equation via nonlinear regression program using SlideWrite Plus 5.01 (Advanced Graphics Software, Inc., Carlsbad, CA).

Results

Methoxychlor Metabolism and Its Metabolic Pathways.

Metabolites generated by incubations of methoxychlor with human liver microsomes or human cDNA-expressed P450s were analyzed by HPLC and identified by comparison with authentic standard compounds used as HPLC markers. In addition to formation of the mono-OH-M, bis-OH-M, and tri-OH-M, a novel catechol metabolite (catechol-M) was identified. Surprisingly, ring-OH-M, a metabolite previously characterized from incubation of \([1\text{H}]\)methoxychlor with human CYP2B6, rat CYP2B1, and phenobarbital-treated rat liver microsomes were not detected (using authentic ring-OH-M as a chromatographic marker). In the current investigation, the reverse-phase TLC (based on \( R_\text{f} \)) showed that catechol-M was localized in the same region that was previously attributed to ring-OH-M. This finding suggested that in the earlier studies, the catechol-M formed was misidentified as being ring-OH-M. That error was primarily due to the unavailability of authentic ring-OH-M as a standard. Also, there was insufficient amount of the metabolite for unequivocal NMR and mass spectrometry identification, because the putative ring-OH-M was rapidly transformed into other products. Indeed in the current study, incubation of synthetic ring-OH-M with CYP2B6 did not yield the expected catechol-M, but only formed a small amount of an unidentified bis-OH-M-like metabolite, detected by its retention time on HPLC (not shown). Based on these findings, ring-OH-M was ruled out as a metabolite of methoxychlor and consequently a new metabolic route is proposed (Fig. 2); methoxychlor is first O-demethylated to mono-OH-M, which is then either ortho-hydroxylated to form catechol-M or is further O-demethylated to yield bis-OH-M. The final product tri-OH-M is then generated from bis-OH-M via ortho-hydroxylation and from catechol-M by O-demethylation. To verify these pathways, each reaction step was investigated with human cDNA expressed P450s (see below).

O-Demethylation of Methoxychlor and Subsequent Metabolic Reactions Catalyzed by cDNA-Expressed P450s.

Incubation of methoxychlor with various P450 isoforms yielded the mono-OH-M (a one step reaction) and the bis-OH-M and catechol-M metabolites (two step reactions) (Fig. 3). Among the 14 P450 isoforms examined, CYP1A2 and 2C19 were the most active enzymes toward O-demethylation, producing mono-OH-M (\( 340 \) nmol/nmol P450/60min and \( \sim 20 \) and \( \sim 50 \) nmol/nmol P450 of bis-OH-M by 1A2 and 2C19, respectively); in another experiment involving shorter incubation (20
Materials and Methods

and were conducted for 60 min at 37°C (for incubation conditions and for procedure and 2C19 forming mono-OH-M (20 nmol/nmol P450/60min), demonstrating approximately one-tenth (or less) of the activities of 1A2, 3A4, 3A5, and rat CYP2B1 were relatively inactive with each demethylation. By contrast, CYP2A6, 2B6, and 2C9 were only one-third to one-half as active yielding mono-OH-M (110–160 nmol/nmol P450/60min), CYP1A2 and 2C19 yielded mono-OH-M at 284 and 425 nmol/nmol P450/60min) and bis-OH-M was formed via O-demethylation by 2C19 catalysis (54 nmol product/nmol P450/60min). The inhibition of 2C9-mediated

Fig. 2. Proposed metabolic pathway of methoxychlor catalyzed by human P450s.

Incubations contained cDNA expressed P450s (50 nM) and substrate (25 μM) and were conducted for 60 min at 37°C (for incubation conditions and for procedure of isolation of products see Materials and Methods).

Fig. 3. O-demethylation of methoxychlor and subsequent reactions catalyzed by cDNA-expressed P450s.

Methoxychlor was incubated with HLM preparations (H030, H161, and HK25) or with cDNA-expressed P450 isoforms (CYP1A2, 2C9, and 2C19), in the presence of chemical inhibitors of P450s, and the degree of inhibition was determined. The inhibitors employed were furafylline (a mechanism-based inhibitor of CYP1A2) and sulfaphenazole (an inhibitor of CYP2C9). Additionally, for lack of a selective inhibitor of 2C9, we examined 2C9 inhibitors that also inhibit CYP2C19; these were delavirdine, an inhibitor of CYP2C9, 2C19, 2D6, and 3A4 but not of CYP1A2 and 2E1 (Voorman et al., 2001) and lansoprazole, a competitive inhibitor of CYP2C9 and 2C19 and a mixed inhibitor of 2D6 (Ko et al., 1997). Delavirdine appeared to be a better inhibitor of 2C9 (apparent \( K_{i} = 2.6 \pm 0.4 \mu M \)) than of 2C19 (apparent \( K_{i} = 24 \pm 3 \mu M \)) (Voorman et al., 2001), hence we did not anticipate that delavirdine would selectively inhibit 2C9 without primarily inhibiting 2C9.

Furafylline (10 μM) inhibited ~90% methoxychlor O-demethylation by CYP1A2, with only minimal inhibition of the 2C9 and 2C19 activity. The preincubation of 1A2 and 2C19 with NADPH (as control for furafylline activation), substantially inactivated these enzymes (Table 3), consequently the additional inhibition of these enzymes by furafylline (beyond the inhibition due to preincubation) represented an approximate quantification. Surprisingly, furafylline did not significantly inhibit methoxychlor demethylation by the HLM, suggesting that 1A2 does not appreciably catalyze O-demethylation in these liver preparations. Sulfaphenazole (30 μM) inhibited both the cDNA-expressed 2C9 by 80% and HLM (H030, HK25, and H161) by 33 to 45% (Table 3), suggesting considerable involvement of hepatic 2C9 in methoxychlor hepatic O-demethylation. Delavirdine (100 μM) inhibited 1A2, 2C9, and 2C19 activity by 34, 83, and 13%, respectively (not shown). The inhibition of 1A2 in our studies was surprising, since earlier studies with delavirdine (up to 100 μM) demonstrated no inhibition of 1A2 (Voorman et al., 2001). Delavirdine (100 μM) inhibited methoxychlor O-demethylation (1-h incubation) in liver microsomes (H030) (2 mg of protein/ml) by 50% (not shown), suggesting involvement of CYP2C9 and possibly of 1A2 and 2C9 in O-demethylation. Lansoprazole (100 μM) inhibited methoxychlor demethylation by cDNA-expressed CYP1A2, 2C9, and 2C19 by 39, 47, and 31%, respectively. Similarly, even at the lower concentration, lansoprazole (10 μM) inhibited CYP1A2, 2C9, and 2C19 by 23, 28, and 18%, respectively (Table 3). The inhibition of 2C9-mediated
methoxychlor demethylation by 10 μM lansoprazole was both surprising and disappointing, since it precluded the unequivocal determination of whether 2C19 in HLM is a significant catalyst of methoxychlor mono-demethylation. Lansoprazole (at 10 μM) inhibited methoxychlor O-demethylation by HLM (H030, H103, and HK25) by 29, 17, and 16%, respectively, and lansoprazole (at 100 μM) inhibited H030 and HK27 by 29, 17, and 16%, respectively, and lansoprazole (at 100 μM) inhibited H030 and HK25 by 40%, suggesting that 1A2 and 2C9 and possibly 2C19 were more active in this O-demethylation. The inhibition of cDNA-expressed 1A2-mediated O-demethylation was surprising, since it was reported that 1A2 catalysis (phenacetin conversion to acetaminophen) was not inhibited by lansoprazole (Ko et al., 1997). Our findings that both delavirdine and lansoprazole inhibited the O-demethylation by CYP1A2 suggest the possibility that the inhibition of 1A2 was due to a higher affinity of delavirdine and lansoprazole than of methoxychlor for that enzyme. The possible inhibition by delavirdine and lansoprazole of the CYP2D6-catalyzed methoxychlor O-demethylation was not explored, since cDNA-expressed 2D6 exhibited only minimal methoxychlor O-demethylation (see Fig. 3) demonstrating that 2D6 catalysis is not relevant to that reaction; additionally, HLM (donor H030) contained extremely low 2D6 activity (Table 1). These inhibition studies conclusively demonstrate the involvement of CYP2C9 in catalysis of methoxychlor O-demethylation in HLM. However, the evidence for that catalysis by 1A2 and 2C19 is only tenuous. The lack of inhibition of methoxychlor demethylation by furafylline provides evidence against 1A2 participation. The similarity between the extent of inhibition (~40%) by lansoprazole (100 μM) and sulfaphenazole (a prime inhibitor of 2C9) provides support for the involvement of 2C9 but does not conclusively preclude the possibility of 2C19 participation. An availability of a selective inhibitor of 2C9 will be required for the unequivocal resolution of the question concerning involvement of 2C19 in HLM in these demethylation reactions.

**O-Demethylation and ortho-Hydroxylation of Mono-OH-M.** Subsequent to the formation of mono-OH-M from methoxychlor, the pathway branches out into either demethylation or hydroxylation of mono-OH-M, essentially depending on the P450s involved. Namely, CYP1A2, 2C8, 2C19, and 2D6 significantly catalyzed demethylation of mono-OH-M, with CYP2C19 being the most cataclysmically competent enzyme. Indeed, CYP2C19, 1A2, 2C8, and 2D6 yielded 183, 83, 55, and 66 nmol bis-OH-M/nmol P450/60 min, respectively (Fig. 5). By contrast, CYP2B6, 3A4, 3A5, and rat 2B1 exhibited significant hydroxylation activity yielding 161, 252, 68, and 92 nmol catechol-M/nmol P450/60 min, respectively. However, CYP2A6, 2B6, and 2C9 (competent methoxychlor O-demethylases) were not effective at demethylation of mono-OH-M, indicating the fastidious substrate specificities of these three enzymes. It is noteworthy that CYP2B6 is unique in that it demethylates methoxychlor well but does not demethylate the mono-OH-M. Instead, this enzyme ortho-hydroxylates the mono-OH-M to form the catechol-M.

**O-Demethylation of the Catechol-M to form Tris-OH-M.** CYP2C19 was found to be the only isoform that exhibits relatively high demethylation activity toward the catechol, yielding 481 nmol tris-OH-M/nmol P450/60 min, whereas 2C9 showed only moderate activity yielding 78 nmol tris-OH-M (Fig. 6). However, CYP1A1, 1A2, 2A6, 2B6, 2C8, 2D6, 3A4, and 3A5 were relatively poor at O-demethylation of the catechol-M.

**Ortho-Hydroxylation of Bis-OH-M to form Tri-OH-M.** Among the P450s examined, only CYP3A4 showed significant, but only moderate, hydroxylation activity of bis-OH-M forming tri-OH-M at 68 nmol/nmol P450/60 min (Fig. 7). Interestingly, both CYP2B6 (active in certain ortho-hydroxylation reactions) and 3A5 (often resembling catalysis and substrate specificity of 3A4) exhibited only minimal hydroxylation activity toward bis-OH-M. Similarly CYP1A2, 2C9, and 2C19 were not active in this hydroxylation.

**Enzyme Kinetics.** Since CYP2C19 and 3A4 were the only two human P450 enzymes that have high to moderate activities in O-demethylations and ortho-hydroxylations, respectively. The enzyme kinetics of the three O-demethylation reactions catalyzed by 2C19 and the two ortho-hydroxylation reactions catalyzed by 3A4 were examined; these kinetic values were processed through Michaelis-Menten equation with the SlideWrite software. All three demethylation reac-

---

**Table 1**

<table>
<thead>
<tr>
<th>Assay</th>
<th>H030</th>
<th>HK27</th>
<th>H103</th>
<th>HK25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total P450</td>
<td>535</td>
<td>300</td>
<td>345</td>
<td>240</td>
</tr>
<tr>
<td>Cyt b5</td>
<td>734</td>
<td>730</td>
<td>561</td>
<td>740</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>2290</td>
<td>1320</td>
<td>340</td>
<td>1100</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>1590</td>
<td>1320</td>
<td>560</td>
<td>210</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>12</td>
<td>31</td>
<td>44</td>
<td>43</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>69</td>
<td>480</td>
<td>2200</td>
<td>5600</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>315</td>
<td>460</td>
<td>190</td>
<td>82</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>9</td>
<td>130</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>1140</td>
<td>3000</td>
<td>1300</td>
<td>2700</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>8870</td>
<td>4910</td>
<td>2770</td>
<td>3700</td>
</tr>
</tbody>
</table>

*Total P450 and cytochrome b5 given in pmol/mg of protein.

Values rounded off.

Sample H161 (new catalog no. 452161) represents pooled aliquots from 21 liver samples.

---

**Figure 4.** O-demethylation of methoxychlor and subsequent reactions catalyzed by individual human liver microsomes (H030, H103, and HK27).

Incubations contained microsomes (2.0 mg of protein/ml) and methoxychlor (25 μM), conducted for 60 min at 37°C (for more explicit conditions see Materials and Methods).
The demethylation of methoxychlor, mono-OH-M, and catechol-M by 2C19, demonstrated reasonably good substrate affinity ($K_m$ values were between 0.23–0.41 μM). The $K_m$ values of ortho-hydroxylations catalyzed by 3A4 were 12 and 25 μM for the hydroxylation of mono-OH-M and bis-OH-M, respectively. From the intrinsic clearance values ($V_{max}/K_m$), the O-demethylations by CYP2C19 seem to predominate over the ortho-hydroxylations catalyzed by 3A4 (Table 2).

**Discussion**

Methoxychlor, a currently used pesticide, has been extensively studied regarding its endocrine and reproductive toxicity in animals. However, hitherto it has not been established whether these manifestations are due to methoxychlor per se or reflect the action of its estrogenic/antiestrogenic metabolites, nor have there been studies on whether humans are susceptible to these endocrine and reproductive toxicities. Additionally, although investigations in animals have provided considerable information on the P450 enzymes involved in methoxychlor metabolism, there has been limited data on the catalysis of methoxychlor metabolism by human enzymes. It was thus deemed important to establish the nature of the major methoxychlor metabolites generated by human liver preparations and by individual human isoforms and to define the pathways of metabolic conversions.

The availability of authentic compounds synthesized in our laboratory has permitted the unequivocal identification of methoxychlor metabolites. In turn, the information on the sequence of formation of these metabolites by human P450s and by human liver microsomes has provided the metabolic pathways of conversion of methoxychlor to its phenolic products.

In previous studies, it was proposed that ring-OH-M was a significant methoxychlor metabolite of CYP2B-catalysis. The structural assignment of this metabolite was based primarily on the following: 1) the mass spectrum of the methylated derivative of this metabolite was identical to that of the authentic standard tris-MeO-M; 2) there was a release of tritium (as $^3$H$_2$O) during the incubation of [ortho-$^3$H]methoxychlor with cDNA-expressed human P450s; and 3) this metabolite, the putative ring-OH-M, disappeared in incubations longer than 10 min, generating tris-OH-M. Additionally, this metabolite was detected only in incubations with low microsomal protein concentration. Consequently, there was insufficient amount of isolated pure metabolite for unequivocal structure identification. However, in the current study, the availability of synthetic standards of both ring-OH-M and catechol-M, and using both HPLC and NMR analyses, made it possible to unequivocally characterize this metabolite as the catechol-M.

The discovery of catechol-M, derived from methoxychlor via a two-step reaction involving O-demethylation and ortho-hydroxylation, provided a novel metabolic route. This novel pathway consists of four metabolites generated in five reactions of two types, the O-demethylation and ortho-hydroxylation. Methoxychlor is O-demeth-
yalted by several human P450 isoforms solely to the primary metabolite, the mono-OH-M. The subsequent enzymatic transformation from mono-OH-M to tris-OH-M proceeds along two pathways involving O-demethylation and ortho-hydroxylation. The hydroxylation reaction demonstrates that a phenolic group is essential for the efficient introduction of the second hydroxyl at the ortho position. Indeed, in earlier studies we observed a similar requirement for ortho-hydroxylation and proposed a mechanism for the enzymatic introduction of the second hydroxyl into phenolic substrates (a mechanism kindly proposed by Ronald White of Schering-Plough Co.) (Stresser and Kupfer, 1997). We suggest that these findings may be applicable to the exploration of the formation of catechol compounds in metabolism of various drugs and hormones catalyzed by P450s.

In the current study, it was observed that the cDNA-expressed P450s displayed catalytic activities as demethylases and/or hydroxylases. Interestingly, CYP2C19 was the only P450 that demonstrated relatively high activity in all three O-demethylation reactions involving both methoxychlor and its metabolites, indicating a lack of fastidious substrate specificity for 2C19. The catalytic potency of the P450-mediated O-demethylation activities of methoxychlor was in the order of CYP2C19 > 1A2, 2C9 > 2A6, 2B6, 2C8, 2D6 > 1A1, 3A4, 3A5, rat 2B1. The catalytic activity of ortho-hydroxylation was in the order of CYP3A4 > 2B6, 3A5. However, CYP1B1, 2E1, and 4A11 were essentially inert in both reactions. In contrast, 2B6 demonstrated remarkable dual catalytic activities, involving O-demethylation and ortho-hydroxylation. This surprising activity of 2B6 has apparently contributed to misidentifying ring-OH-M as the primary metabolite of methoxychlor, since upon incubation of [ortho-3H]methoxychlor with 2B6, the tritium from the ortho position was released as 3 H2O (Stresser et al., 1996), most probably due to an initial demethylation of methoxychlor to mono-OH-M, followed instantly by ortho-hydroxylation resulting in the observed 3 H2O.

Generally, the rates of metabolic transformations by human P450s were much higher with methoxychlor and with the less polar metabolites. Whereas the initial demethylation of methoxychlor by several P450s was pronounced (forming the mono-OH-M), the demethylation and hydroxylation of metabolites was substantially diminished, except for the remarkable catalysis by CYP2C19 (Fig. 6–8). Namely, lesser activities were observed in the rate of O-demethylation of mono-OH-M and catechol-M, as compared with that of methoxychlor. Similarly, there was much lower activity in the ortho-hydroxylation of bis-OH-M as compared with mono-OH-M. Also, incubation of tris-OH-M with CYP3A4 did not demonstrate hydroxylation, failing to produce detectable amount of tetra-OH-M (unpublished observation), suggesting that tris-OH-M is the final product of in vitro methoxychlor metabolism. However, the question of whether in vivo tris-OH-M is the final methoxychlor metabolite and whether catalysis by phase II enzymes could influence methoxychlor metabolism needs further exploration.

In analyzing the above reactions, it became evident that O-demethylation is the predominant reaction in the metabolic pathway of methoxychlor, since mono-OH-M was found to be the only primary metabolite. Intriguingly, O-demethylation also predominates over ortho-hydroxylation in the formation of the secondary metabolites in human liver microsomes. Namely, substantially more bis-OH-M was generated than catechol-M in the incubations of methoxychlor with human liver microsomes, indicating that O-demethylation enzymes, mainly CYP1A2, 2C9, and 2C19 were likely the major enzymes contributing to methoxychlor metabolism. Interestingly, although methoxychlor O-demethylation by cDNA-expressed CYP2C19 was ~2-fold more active than 2C9 in one experiment (Fig. 3) and ~6-fold in another experiment (Table 3), inhibition studies in human liver microsomes suggest that 2C9 may contribute more than 2C19 to me-

![Figure 8](https://example.com/figure8.png)

**Fig. 8.** Substrate selectivity of the P450 isoforms catalyzing O-demethylation and ortho-hydroxylation of methoxychlor metabolism.

The involvement of P450 isoforms in the above pathways was determined with cDNA-expressed P450s, and it does not constitute definitive evidence for their catalytic participation in HLM. However, using cDNA expressed P450s and chemical inhibitors, evidence was obtained for the involvement of 2C9 and possibly participation of 2C19 in catalysis of methoxychlor mono-demethylation by human liver microsomes (see Results and Discussion).

### TABLE 3

<table>
<thead>
<tr>
<th>Inhibitor, µM</th>
<th>HLM H161</th>
<th>HLM H030</th>
<th>HLM HK25</th>
<th>CYP1A2</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol mono-OH-M/mg microsomal protein/20 min</td>
<td>nmol mono-OH-M/mmol enzyme/20 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preincubation$^{a,b}$ + NADPH</td>
<td>6.1 ± 0.3</td>
<td>9.9 ± 0.1</td>
<td>7.2 ± 0.2</td>
<td>283.7 ± 27.5</td>
<td>67.0 ± 10.1</td>
<td>425.4 ± 18.3</td>
</tr>
<tr>
<td>Preincubation$^{a}$ + NADPH + Furafylline, 10 µM</td>
<td>5.2 ± 0.4 (15%)</td>
<td>8.7 ± 0.6 (12%)</td>
<td>7.1 ± 0.4 (1%)</td>
<td>34.5 ± 2.5 (88%)</td>
<td>55.5 ± 1.5 (17%)</td>
<td>234.0 ± 15.6 (45%)</td>
</tr>
<tr>
<td>Sulfaphenazole*, 30 µM</td>
<td>4.1 ± 0.3 (33%)</td>
<td>5.6 ± 0.2 (43%)</td>
<td>3.9 ± 0.3 (45%)</td>
<td>239.6 ± 11.1 (15%)</td>
<td>13.2 ± 0.3 (80%)</td>
<td>370.1 ± 29.7 (13%)</td>
</tr>
<tr>
<td>Lansoprazole*, 10 µM</td>
<td>4.3 ± 0.0 (29%)</td>
<td>8.2 ± 0.2 (17%)</td>
<td>6.0 ± 0.0 (16%)</td>
<td>218.7 ± 4.7 (23%)</td>
<td>48.5 ± 2.3 (28%)</td>
<td>349.2 ± 8.9 (18%)</td>
</tr>
<tr>
<td>Lansoprazole*, 100 µM</td>
<td>N.D.</td>
<td>5.4 ± 0.4 (46%)</td>
<td>4.2 ± 0.1 (41%)</td>
<td>173.6 ± 8.6 (39%)</td>
<td>35.8 ± 0.2 (47%)</td>
<td>293.5 ± 5.1 (31%)</td>
</tr>
</tbody>
</table>

N.D. Not determined.

$^{a}$ Preincubations were for 10 min in the presence of NADPH without or with furafylline (see Materials and Methods for details).

$^{b}$ Values served as control for incubations containing furafylline, consequently percent inhibition by furafylline was based on the respective control as being 100%.

$^{c}$ Incubations containing sulfaphenazole or lansoprazole did not undergo the 10 min preincubation with NADPH, similarly the controls (lacking inhibitors) did not undergo the preincubation, e.g., in the Table above (see H161), the 100% control without preincubation was 6.1 ± 0.3 nmol mono-OH-M/mg of protein/20 min.

$^{d}$ Represents pooled aliquots from 21 human livers.
thoxychlor demethylation. This finding is not surprising, since the activity of 2C9 in the human liver microsomes from donors employed in the experiments with inhibitors was relatively high except for HK27 (Table 1). Additionally, although neither the concentrations of 2C9 nor of 2C19 were quantified in the liver microsomes used in our studies, it is highly likely that 2C9 was present in substantially higher concentration than 2C19; indeed support for that assumption is derived from the observation that in the general human population, the relative hepatic abundance of 2C9 is approximately 4-fold higher than 2C19 (Rodrigues, 1999; Clarke and Jones, 2002).

Conclusion and Future Directions. It is apparent from stereochemical considerations that some of the methoxychlor metabolites (i.e., the mono-OH-M, catechol-M, and tris-OH-M) are chiral. Consequently, it would be of paramount interest to determine 1) whether there is enantiotopic selectivity in the demethylation of the prochiral methoxychlor by the human enzymes, and whether there is enantio- meric selectivity of the subsequent demethylation and hydroxylation reactions to yield both achiral and chiral products; 2) whether similar chiral products are generated in vivo; and 3) whether the estrogenic/ antiestrogenic and antiandrogenic activities and the P450-inducing activities of the individual enantiomers differ qualitatively and quantitatively from those of their respective racemic mixtures. Last, although only of peripheral interest to the above, the question of the reasons why phenolic hydroxyls are supportive or even essential for catalysis of the aromatic hydroxylation of methoxychlor and its metabolites needs resolution.

Acknowledgments. We are highly indebted to Drs. Lei Zhang and Piotr Dobrowolski of our institute for their assistance with the NMR studies.

References
