ENANTIOSELECTIVE METABOLISM OF THE ENDOCRINE DISRUPTOR PESTICIDE METHOXYCHLOR BY HUMAN CYTOCHROMES P450 (P450s): MAJOR DIFFERENCES IN SELECTIVE ENANTIOMER FORMATION BY VARIOUS P450 ISOFORMS

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ABSTRACT:

Methoxychlor, a currently used pesticide that in mammals elicits proestrogenic/estrogenic activity and reproductive toxicity, has been classified as a prototype endocrine disruptor. Methoxychlor is prochiral, and its metabolites 1,1,1-trichloro-2,2-bis(4-methoxyphenyl)-2-(4-methoxyphenyl)ethane (mono-OH-M); 1,1,1-trichloro-2-(4,4-dihydroxyphenyl)ethane (catechol-M); and 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(3, 4-dihydroxyphenyl)ethane (tris-OH-M) are chiral; whereas 1,1,1-trichloro-2-(4-hydroxyphenyl)ethane (bis-OH-M) is achiral. These metabolites are formed during methoxychlor incubation with liver microsomes or recombinant cytochrome P450s (rP450s). Since methoxychlor-metabolite enantiomers may have different estrogenic/antiestrogenic/antiandrogenic activities than corresponding racemates, the possibility that P450s preferentially generate or use R or S enantiomers, was examined. Indeed, rCYP1A2 and r2A6 mono-demethylated methoxychlor primarily into (R)-mono-OH-M at 91 and 75%, respectively, whereas rCYP1A1, 2B6, 2C8, 2C9, 2C19, and 2D6 formed the (S)-enantiomer at 69, 76, 75, 95, 96, and 80%, respectively. However, rCYP3A4, 3A5, and 2B1(rat) weakly demethylated methoxychlor without enantioselectivity. Human liver microsomes generated (S)-mono-OH-M (77–87%), suggesting that CYP1A2 and 2A6 display only minor catalytic contribution. P450 inhibitors demonstrated that CYP2C9 and possibly 2C19 are major hepatic catalysts forming (S)-mono-OH-M, and CYP1A2 is primarily involved in forming the (R)-mono-OH-M. Demethylation rate of (S)-mono-OH-M versus (R)-mono-OH-M forming achiral bis-OH-M by rCYP1A2 was 97/3, compared with 15/85 and 17/83 for rCYP2C9 and 2C19, respectively, indicating opposite substrate enantioselectivity of rCYP1A2 versus 2C9 and 2C19. Also, rCYP1A2 preferentially O-demethylated (R)-catechol-M into (R)-tris-OH-M (at 80%), contrasting r2C9 and r2C19 that yielded (S)-tris-OH-M at 80 and 77%, respectively. Ortho-hydroxylation of mono-OH-M into catechol-M and bis-OH-M into tris-OH-M was primarily by 3A4 and was not enantioselective. In conclusion, enantiomeric abundance of methoxychlor metabolites depends on the relative catalytic activity of the hepatic P450 isoforms.

Methoxychlor is a chlorinated hydrocarbon pesticide currently used as a substitute for the banned DDT in the industrially developed countries (Metcalfe et al., 1971; Gardner and Bailey, 1975; Metcalfe, 1976). However, despite the relatively low toxicity and short half-life of methoxychlor as compared with DDT, there is considerable concern over exposure to methoxychlor. This concern stems from observations that methoxychlor is a proestrogen (Kupfer and Bulger, 1979; Bulger et al., 1978a,b; Kupfer and Bulger, 1979) that elicits marked reproductive toxicity in animals (Gray et al., 1988; Cummings and Gray, 1989; Cummings and Laskey, 1993); consequently methoxychlor has been classified as a prototype endocrine disruptor, and its toxicity is being extensively investigated (Chapin et al., 1997; Cummings, 1997; Bigsby et al., 1999; You et al., 2002).

Studies of methoxychlor metabolism in vitro with liver preparations and in vivo in mammalian species have characterized several metabolites, some of which are estrogenic (Kupfer and Bulger, 1979; Oosterhout et al., 1981; Bulger et al., 1985). The most potent estrogenic methoxychlor metabolites are the mono- and bis-demethylated compounds (mono-OH-M and bis-OH-M, respectively), both being phenolic derivatives. Recent studies demonstrated that the bis-OH-M mediated “estrogenic” 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 reproductive tissues following exposure of animals to estradiol-17β and methoxychlor. Several studies of methoxychlor metabolism, including a most recent study that identified a novel catechol metabolite (Bulger et al., 1978a,b; Bulger et al., 1985; Kupfer et al., 1990; Dehal and Kupfer, 1994; Hu and Kupfer, 2002), provided a metabolic scheme that involves P450-isoform-specific demethylation...
tions and aromatic hydroxylations, leading to the formation of tris-OH-M (Fig. 1).

Investigations of the estrogenic activity of methoxychlor metabolites demonstrated that the phenolic methoxychlor metabolites mono-OH-M and bis-OH-M exhibit dual albeit opposing effects, being agonists of the estrogen receptor $\alpha$ (ER$\alpha$) but being antagonists of both ER$\beta$ and of the androgen receptor (Gaido et al., 2000). Since ER$\alpha$ and ER$\beta$ may have similar or different biological functions and these receptor isoforms reside preferentially in different animal tissues, ER$\alpha$ being primarily in the uterus, bone, and testis and ER$\beta$ in the ovaries, prostate, and mammary gland (Couse et al., 1997; Kuiper et al., 1997; Denger et al., 2001; Saji et al., 2001), it is conceivable that the various methoxychlor metabolites could exhibit similar or opposing biological activities in the different tissues.

Methoxychlor per se is prochiral, and consequently some of its metabolites are chiral (Fig. 1), and thus the above-mentioned estrogenic/antiestrogenic/antiandrogenic activities and reproductive toxicity may reflect the resultant actions of the enantiomers present in the racemates of the synthetic preparations of these compounds. Additionally, it appears reasonable that the qualitative and quantitative biological/pharmacological activities of the methoxychlor metabolite enantiomers $R$ and $S$ would depend on the relative abundance of each enantiomer that has been generated in vivo or in vitro through the enantiotopic selectivity of the P450 isomers catalyzing those reactions. Moreover, since it is not ethically appropriate to test this notion in humans, studies have been initiated to examine the activities of individual enantiomers toward human ER$\alpha$, ER$\beta$, and androgen receptor in vitro.

The mono-O-demethylation of methoxychlor by several rat cytochrome P450s showed that CYP2A1 and 2C6 selectively O-demethylated methoxychlor to the ($S$)-mono-OH-M, whereas 2B1 and 2B2 were not enantioselective (Kishimoto et al., 1995; Kishimoto and Kurihara, 1996). However, the questions whether human P450s exhibit enantiotopic selectivity of methoxychlor metabolism and whether the subsequent transformation of the metabolites to secondary and tertiary products was enantioselective have not been hitherto studied. The current investigation is designed to examine the enantiotopic selectivity of the five reactions in the pathway of methoxychlor metabolism (Fig. 1) catalyzed by human liver microsomes (HLM) and by various human cDNA-expressed P450s.

Materials and Methods

Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Methoxychlor (laboratory grade = 99+ % pure) was purchased from Chem Service Inc. (West Chester, PA). Mono-OH-M and catechol-M were synthesized in our laboratory (Hu and Kupfer, 2002). Bis-OH-M was kindly provided by Dr. Sanborn (Illinois State Natural History Survey, Urbana, IL). Tris-OH-M was custom synthesized by Chemsyn Science Laboratories (Lenexa, KS). HPLC grade acetoniitrile was purchased from J. T. Baker, Mallinckrodt Baker Inc. (Phillipsburg, NJ). HPLC columns were obtained from Phenomenex Prodigy ODS3 250 $\times$ 4.6 mm (Phenomenex, Torrance, CA), chiral Ultron ES-OVM column (150 $\times$ 4.6 mm; Shinwa Chemical Industries, Ltd., Tokyo, Japan) was obtained from Agilent Technologies (Wilmington, DE) and chiral cyclobond I 2000 column (250 $\times$ 4.6 mm) was from Astec (Whippany, NJ), Chiral-AGP column was obtained from Chromtech (Apple Valley, MN). cDNA-expressed recombinant human P450 enzymes (P450s) coexpressed with NADPH-P450 reductase and, in certain preparations, containing coexpressed cytochrome b$_6$ (in insect cell line, referred as supersomes) provided as microsomal preparations and human liver microsomes, were purchased from BD Gentest (Woburn, MA). Furafylline, sulfaphenazole, and lansoprazole were obtained from Sigma-Aldrich; delavirdine was obtained from Biomol Research Labs (Plymouth Meeting, PA).

Incubations. Incubations were conducted in 20-ml vials under an atmosphere of air in a Dubnoff metabolic shaking incubator. The incubation contained the following constituents in 1 ml of final volume: 50 nM cDNA-expressed P450 enzymes (supersomes) or human liver microsomes (2 mg/ml); the substrate methoxychlor or methoxychlor metabolite (25 $\mu$M) added in 10 $\mu$l ethanol; MgCl$_2$ (10 mM) added in 60 mM sodium phosphate buffer (pH 7.4). After 3 min preincubation at 37°C (for temperature equilibration), the reaction was initiated by NADPH and regenerating system (0.5 mM NADPH, 10 mM glucose 6-phosphate, 2 IU glucose-6-phosphate dehydrogenase). These reaction mixtures were usually incubated at 37°C for 60 min; however, when ($R$)- or ($S$)-mono-OH-M (primary methoxychlor metabolites) were the substrates, incubations were conducted for 10, 20, 30, 40, and 60 min to obtain a time course of product formation. Reactions were terminated by the addition of 3 ml of diethyl ether (ether), followed by 1 ml of water and the products were extracted with ether (2 $\times$ 3 ml). In certain experiments, the reactions were terminated by placing the vials in a $-78$°C acetone/dry-ice bath and kept frozen until work-up. In the latter case, after thawing, 1 ml of water was added.
and the metabolites were extracted with ether (2 × 3 ml). In all work-up procedures, the ether phase containing the incubation extracts was evaporated to dryness at room temperature under a stream of nitrogen gas. The residues were dissolved in 0.5 ml of methanol, passed through 0.20 of μm PTFE filter, and the filtrate was evaporated to dryness at room temperature under a stream of nitrogen. The resulting residues were dissolved in 40 μl of methanol and subjected to HPLC analysis to identify the metabolites and to determine the rates of product formation.

**Metabolic Studies with Inhibitors of P450 Isoforms.** Incubations with selective chemical inhibitors of CYP1A2, 2C9, and 2C19 were conducted with human liver microsomes (several donors) or with cDNA-expressed CYP1A2, 2C9, and 2C19, using procedures similar to the incubations described above. The concentrations of inhibitors were 10 μM furafylline, 30 μM sulfa-

phena-zole, 100 μM delavirdine, and 10 and 100 μM lansoprazole. Procedure for incubations containing furafylline (a mechanism-based inhibitor of 1A2): after a 3-min preincubation at 37°C, in the absence of methoxychlor but containing furafylline, there was a 10-min incubation in the presence of the NADPH-generating system, then methoxychlor was added, and the mixture was incubated for an additional 20 or 60 min (controls lacked furafylline). However, when sulfaphena-zole (inhibitor of CYP2C9) or delavirdine and lansoprazole (both inhibitors of CYP2C9 and 2C19) were employed, these compounds were added essentially simultaneously with the substrate (without employing the prior 10-min incubation), 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.

**Metabolite Analysis and Purification.** Metabolites were identified by HPLC, comparing their retention time with authentic compounds, using Waters 6000 pump with a 5-μm Phenomenex Prodigy ODS3 column (250 × 4.6 mm) at a flow rate of 1 ml/min under the following conditions: 1) isocratic mobile phase acetonitrile/water (70:30) used for analysis of extracts from methoxychlor incubation; retention times were 15.2 min for methoxychlor, 7.1 min for mono-OH-M, 5.2 min for catechol-M, 4.1 min for bis-OH-M, and 3.5 min for tris-OH-M. 2) Acetonitrile/water (60:40) was used for analysis of extracts from mono-OH-M incubation; retention times were 11.8 min for mono-OH-M, 7.8 min for catechol-M, and 5.6 for bis-OH-M. 3) Acetonitrile/ water (50:50) was used for analysis of extracts from bis-OH-M or catechol-M incubation; retention times were 16.2 min for catechol-M, 10.4 min for bis-OH-M and 7.0 min for tris-OH-M. The enantiomers of mono-OH-M, catechol-M, and tris-OH-M were collected off the HPLC (using λ = 230 nm), and the solvent was evaporated under a stream of nitrogen gas at 40–45°C. The R and S mono-OH-M enantiomers were separated on semi-preparative chiral-AQF column, 100 × 10.0 mm, 5 μm, at λ = 230 nm, with acetonitrile/ water (20:80), at flow rate = 3 ml/min. The isolated metabolites were stored dry 80°C for analysis and for subsequent use in metabolic studies.

**Enantiotopic Analysis of Metabolites.** The metabolites were dissolved in 50 μl of methanol and aliquots were injected onto chiral HPLC column for enantiomer analysis. The enantiomers of mono-OH-M were resolved on a chiral Ultron ES-OVM column (150 × 4.6 mm; Shinwa Chemical Industries, Ltd.), using acetonitrile/water (17:83) at 1 ml/min. The enantiomers of catechol-M and tris-OH-M were resolved on a chiral Astec cyclobond I 2000 column (250 × 4.6 mm) at λ = 230 nm, with acetonitrile/water (40:60) at flow rate of 1 ml/min.

**Results and Discussion.**

Five enantioselective reactions in the methoxychlor metabolic pathway catalyzed by cDNA-expressed P450s (rP450s) and by HLM, have been investigated. The enantiomers of methoxychlor metabolites have been resolved, identified and quantified by employing a relatively facile method involving chiral HPLC. The enantiomers of mono-

OH-M (the primary methoxychlor metabolite) were separated and identified based on chromatographic characteristics of an authentic standard of (S)-mono-OH-M. Sequential enzymatic reactions were applied to R-and S- mono-OH-M and to racemic catechol to determine the absolute configuration of other methoxychlor metabolites. It is noteworthy that the absolute configuration of tris-OH-M enantiomers was determined by an enzymatic reaction of a racemic catechol-M substrate, in a controlled experiment of 40 to 60% substrate conversion (demethylation), followed by the chiral HPLC analysis of both residual substrate and the product formed. Consequently the latter procedure did not require the separation of enantiomers to be used as substrates.

**Resolution of the Enantiomers of Methoxychlor Metabolites.** To determine the enantiotopic selectivity of P450-catalyzed reactions in the methoxychlor metabolic pathway, we established the following procedures by which the enantiomers of these compounds could be resolved and identified. 1) To resolve the enantiomers of mono-OH-M, a previously reported HPLC method was employed (Kurihara and Oku, 1991) with a minor modification (i.e., the mobile phase contained water instead of phosphate buffer); the absolute configuration of the mono-OH-M enantiomers was determined using an authentic (S)-mono-OH-M as a chromatographic reference (kindly provided by Dr. N. Kurihara, Japan); and the first peak on the chiral Shinwa Ultron ES-OVM column was the (R)-mono-OH-M with retention time of 18.2 min and the second peak was the S-enantiomer having retention time of 20.3 min (coeluting with the authentic standard). 2) The enantiomers of the catechol and tris-OH-M were resolved on an Astec cyclobond I 2000 column. The retention times were 9.4 and 8.1 min for the catechol-M enantiomers and 10.0 and 9.0 min for the tris-OH-M enantiomers (see below for assignment of their absolute configuration).

**Determination of the Absolute Configuration of Catechol-M Enantiomers.** Purified (S)-mono-OH-M (containing ~3% of the (R)-enantiomer) was incubated with CYP3A4 using the same procedures as described above. The (S)-mono-OH-M was expected to be converted by ortho-hydroxylation to the (R)-catechol-M (Fig. 2A); the R/S switching from the (S)-mono-OH-M to the R-configuration of the catechol-M was merely due to conventional nomenclature usage. The catechol-M was also analyzed by chiral HPLC (cyclobond I 2000). The presence of the minor enantiomer (derived from the conversion of the small impurity of (R)-mono-OH-M) served as a reference to distinguish the configuration of the two enantiomers. The first HPLC peak (the major enantiomer) was identified as the (R)-catechol-M (retention time 8.1 min) and the second peak as the (S)-enantiomer (retention time 9.4 min).

**Determination of the Absolute Configuration of Tris-OH-M Enantiomers.** The racemic catechol-M (25 μM) was incubated with 2C19 (conditions as described above) yielding tris-OH-M, at 40 to 60% substrate conversion. In this reaction, the (R)-catechol-M was converted to the (R)-tris-OH-M and the (S)-catechol-M to the (S)-tris-OH-M (see Figs. 1 and 2B). Both the remaining substrate residues and the metabolites were analyzed with a chiral HPLC. The residual substrate, after the incubation, contained substantially more of the (R)-catechol-M than of the S-enantiomer, indicating that CYP2C19 preferred the (S)-catechol-M as the substrate rather than the (R)-catechol-M. Consequently, it was expected that the products will contain more (S)-tris-OH-M than (R)-tris-OH-M. Thus, the major enantiomeric product was identified as the (S)-tris-OH-M, being the second HPLC peak (retention time 10.0 min), and from that finding we deduced that the first peak (minor component) was the (R)-tris-OH-M (retention time 9.0 min).

**Enantiotopic Selectivity of O-Demethylation of the Prochiral Methoxychlor by Individual P450s and by HLM.** In the O-demethylation of prochiral methoxychlor to mono-OH-M, the O-demethylation occurs at either pro-R or pro-S side of the substrate, yielding (R)- or (S)-mono-OH-M. Ten human rP450s, one rat P450 (CYP2B1), and HLM from four individual donors and one from a pooled sample (from 21 livers) were examined in this reaction (Fig. 3). CYP1A2 and 2A6 preferentially formed the (R)-mono-OH-M, whereas 1A1, 2B6,
2C8, 2C9, 2C19, and 2D6 preferentially formed the (S)-enantiomer. In panel A, (S)-Mono-OH-M (containing ~3% of R-) was incubated with rCYP3A4 for 60 min. For incubation conditions and procedures of isolation of products see Materials and Methods. HPLC Ultron ES-OVM column, acetonitrile/water (17:83) was used for separating enantiomers of catechol-M. In panel B, racemic catechol-M was incubated with rCYP2C19 for 30 min. HPLC cyclobond I 2000 column, acetonitrile/water (40:60) was used for separating enantiomers of catechol-M and tris-OH-M.

2C8, 2C9, 2C19, and 2D6 preferentially formed the (S)-enantiomer. CYP1A2, 2C9, and 2C19 were the most enantioselective P450 isoforms, each yielding 91, 95, and 96% of a single enantiomer, respectively. CYP2D6, 2A6, 2C8, 1A1, and 2B6 exhibited moderate enantiotopic selectivity at 80, 75, 75, 69, and 66%, respectively. CYP3A4, 3A5, and the rat 2B1 were not enantioselective. The observation that 2B1 was not enantioselective in the above reaction is in accordance with an earlier report (Kishimoto et al., 1995). Additionally, despite the fact that HLM HK27 and H103 samples were allelic for 2C9, as compared with H030 and probably HK25 (see Legend to Fig. 3), the enantioselectivities of the four HLM samples were essentially identical, suggesting that in methoxychlor demethylation, the 2C9 allelic variants (with substantially lower enzymatic activity toward 2C9 substrates than H030 and HK25) retained the enantioselectivity of the wild-type 2C9. Finally, the finding that the various HLM samples predominately formed the (S)-mono-OH-M, at 77 – 87%, is particularly noteworthy (see below for discussion).

Effects of P450 Inhibitors on Enantiotopic Selectivity of HLM in Catalysis of Mono-O-Demethylation of Methoxychlor. In a previous study, we observed that CYP1A2 and 2C19 were the most active P450s in catalysis of methoxychlor O-demethylation (Hu and Kupfer, 2002). The current investigation demonstrates that CYP1A2, 2C9, and 2C19 are the most enantioselective P450s in catalysis of that reaction (Fig. 3). Furthermore, experiments were conducted with inhibitors of CYP1A2, 2C9, and 2C19, to evaluate the contribution of these P450 enzymes to methoxychlor O-demethylation (see Table 3 in Hu and Kupfer, 2002) and of their effects on enantioselectivity in HLM (Table 1). In the absence of inhibitors (without preincubation), HLM (donor H030) generated 16 to 17% of (R)-mono-OH-M (Table 1, experiments 1 and 2) and 83 to 84% of the (S)-enantiomers. Preincubation in the presence of NADPH that markedly inactivated rCYP1A2 (12% activity remaining) only slightly inhibited methoxychlor demethylation in the various HLM preparations (not shown), and there was no significant effect on the enantioselectivity of these HLM (Table 1). Furafylline at 10 μM (a mechanism-based inhibitor of 1A2) increased the (S)-enantiomer (in H030) from 83% (preincubation control) to 90 and 94%, see Table 1, experiments 1 and 2, respectively. Similarly, furafylline increased the (S)-enantiomer in the other HLM samples tested. Additionally, in parallel experiments with rP450s, furafylline inhibited CYP1A2-mediated catalysis by 94%, whereas 2C9 and 2C19 were only minimally affected. Sulfaphenazole (specific inhibitor of CYP2C9) elevated the (R)-enantiomer in all the HLM tested (Table 1), however, it also lowered the total O-demethylation by 33 to 45% (not shown), indicative of major involvement of 2C9 in that catalysis. In individual rP450s incubation, sulfaphenazole suppressed rCYP2C9 O-demethylation by 80%, but only minimally affected the catalytic activity of 1A2 and 2C9 (not shown). Our attempts to specifically inhibit CYP2C9 (without inhibiting 2C9 and 1A2) by delavirdine (Voormann et al., 2001) and lansoprazole were not successful due to the relatively high inhibition of 2C9 and 1A2, being 47 to 83% and 34 to 39%, respectively. The conversion of methoxychlor to the mono-OH-M by HLM was greatly reduced (by 51 and 45%) by delavirdine and lansoprazole, respectively (not shown). However, lansoprazole (at either 10 or 100 μM) only slightly altered the enantiotopic selectivity of HLM; by contrast, delavirdine markedly increased the formation of (R)-mono-OH-M from 16 to 17 to 42% (Table 1). Because there are no apparent selective inhibitors of CYP2C9, the question of whether 2C19 is a major catalyst in the enantioselective metabolism of methoxychlor could not be ascertained. For instance, in examining the catalysis of mono-OH-M demethylation by HLM, both delavirdine and lansoprazole were found to be only moderate inhibitors of CYP2C9 but were strong inhibitors of CYP1A2 and 2C9, at the 100 μM levels tested (Hu and Kupfer, 2002). Additionally, although previous studies suggested that at 10 μM, lansoprazole would preferentially inhibit 2C9 over 2C9 (Ko et al., 1997), that concentration of lansoprazole did not provide better selectivity in inhibiting 2C9 in methoxychlor O-demethylation (Hu and Kupfer, 2002).

O-Demethylation of Individual (R) or (S)-Mono-OH-M in Forming Bis-OH-M [Relative Activities (Rates) as an Indication of Enantioselectivity]. The enantiotopic selectivity of P450s could not be determined in the O-demethylation of racemic mono-OH-M, since the product, bis-OH-M, is achiral. Therefore, to determine the enantioselectivity of demethylation of mono-OH-M, we resorted to employing single individual enantiomers as substrates and used their respective relative rates of metabolism as an indication of enantioselectivity. In this study, the enantiotopic selectivity by P450s was estimated from the ratio of the reaction rates of conversion of the individual enantiomeric substrates into bis-OH-M [i.e., using the (R)-versus the (S)-mono-OH-M (Fig. 4)]. The total mono-OH-M O-demethylase activity (from both R and S mono-OH-M) was in the order of CYP2C19 > 1A2 > 2D6 >> 2A6 and 2C9, well in accordance with the earlier study with racemic mono-OH-M as substrate (Hu and Kupfer, 2002). Interestingly, CYP1A2 overwhelmingly de-
methylated the (S)-mono-OH-M over the (R)-enantiomer, at a ratio of 97:3. By contrast, both CYP2C9 and 2C19 favored the opposite enantiomer the (R)-mono-OH-M at 85 and 83%, respectively. CYP2D6 slightly favored the (R)-mono-OH-M at 63%, whereas 2A6 was essentially not enantioselective in this reaction.

**Ortho-hydroxylation of Racemic Mono-OH-M Forming the Catechol-M (Enantiotopic Selectivity by P450s).** In the interaction of the various ortho-hydroxylation P450 enzyme(s), with the mono-OH-M (in the racemic mixture), the enantiomers represent two substrate entities that could bind to the enzymes with different affinities; in fact a competition would be expected between the (R)- and (S)-mono-OH-M enantiomers in catalysis of their metabolism. Indeed, there was a preferential hydroxylation of one enantiomer over the other. Thus, CYP2A6 and 2B6 appeared to slightly prefer the (R)-mono-OH-M in forming the (S)-catechol-M, and CYP2C19 slightly favored the (S)-mono-OH-M to form the (R)-catechol-M at 64 to 67%. Interestingly, the CYP2B1 (rat) preferentially formed the (R)-catechol-M at 81%, indicating that the enzyme favored the (S)-mono-OH-M. By contrast, CYP1A2, 2C8, 2D6, 3A4, and 3A5 were not appreciably enantioselective in catalyzing the ortho-hydroxylation reaction (Fig. 5).

**Ortho-hydroxylation of Prochiral Bis-OH-M (Enantiotopic Selectivity by P450s).** Generally, the P450s examined demonstrated...

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**FIG. 3.** Enantiotopic selectivity in the mono-O-demethylation of methoxychlor by cDNA-expressed P450s (rP450s) and HLM; HLM pooled represents samples from 21 livers.

Incubations contained rP450s (50 nM) or individual human liver microsomes (2.0 mg of protein/ml) 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). HLM from individual donors were generally used; HLM (pooled) represents combined aliquot samples from 21 human livers. Genotyped HLM are as follows (see Gentest.com for genotyping and for extensive description of various enzymatic activities): H030 = *1/*1 for both 2C9 and 2C19; H103 = *2/*2 (2C9), *1/*1 (2C19); HK27 = *3/*3 (2C9), *1/*1 (2C19); and HK25 (genotyping not available). Enzymatic activities of these HLM are as follows: H030 had highest activity for both 2C9 and 2C19; HK25 had similar 2C9 activity and somewhat lower 2C19 activity compared with H030; HK103 = ~1/5ed (2C9) and ~1/15th (2C19) activities compared with H030; HK27 = ~1/10th (2C9) of H030 and similar (2C19) activities as H030.

**FIG. 4.** Relative P450 activities (rates) in O-demethylation of individual (R)- or (S)-mono-OH-M; formation of bis-OH-M.

Incubation conditions as in Fig. 3. The ordinate represents the relative consumption (in percent) of individual enantiomers of mono-OH-M being transformed into bis-OH-M during incubation with the various P450s for 30 min. Rate of consumption of total mono-OH-M (R + S) in nmol/30 min/nmol rP450 for CYP1A2, 2A6, 2C9, 2C19, and 2D6 was 62, 6.1, 6.8, 193, and 48, respectively.

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**TABLE 1** Enantioselectivity of methoxychlor mono-demethylation by HLM in the presence of selective inhibitors of P450 isoforms

<table>
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<tr>
<th>HLM</th>
<th>pooled</th>
<th>H030</th>
<th>HK25</th>
<th>expt. 1</th>
<th>expt. 2</th>
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<tr>
<td>Control (pre-incubation with NADPH)</td>
<td>24:76</td>
<td>17:83</td>
<td>17:83</td>
<td>14:86</td>
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<tr>
<td>Furafylline (10 μM)</td>
<td>17:83</td>
<td>10:90</td>
<td>9:4</td>
<td>10:90</td>
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<tr>
<td>Control (no pre-incubation)</td>
<td>23:77</td>
<td>17:83</td>
<td>16:84</td>
<td>13:87</td>
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<tr>
<td>Sulfaphenazole (30 μM)</td>
<td>39:61</td>
<td>41:59</td>
<td>37:63</td>
<td>31:69</td>
<td></td>
</tr>
<tr>
<td>Lansoprazole (10 μM)</td>
<td>26:74</td>
<td>21:79</td>
<td>N.D.</td>
<td>15:85</td>
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<tr>
<td>Lansoprazole (100 μM)</td>
<td>N.D.</td>
<td>25:75</td>
<td>18:82</td>
<td>19:81</td>
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<tr>
<td>Delavirdine (100 μM)</td>
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<td>N.D.</td>
<td>42:58</td>
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</tr>
</tbody>
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N.D., not determined.

* a HLM from various donors. For quantification of the P450s activities in the HLM samples see Table 1 in Hu and Kupfer (2002).
* b Control (preincubation with NADPH-10 min) for incubations containing furafylline.
* c Whereas incubations in expt. 2 (HLM-H030) were conducted for 60 min, all others were for 20 min.
* d Control for incubations containing sulfaphenazole, lansoprazole, and delavirdine did not undergo preincubation.
only moderate activities in catalysis of this reaction. Additionally, CYP2B6-, 3A4-, and 3A5-catalyzed ortho-hydroxylation of the prochiral bis-OH-M exhibited no significant enantioselectivity in forming tris-OH-M yielding 58, 57, and 46% of the (R)-enantiomer, respectively. Moreover, CYP1A2, 2C9, and 2C19 did not produce sufficient tris-OH-M for an accurate enantiotopic analysis.

O-Demethylation of Racemic Catechol-M to form Tris-OH-M (Enantiotopic Selectivity by P450s). CYP1A2 preferably used the (R)-catechol-M as a substrate to form (R)-tris-OH-M at 80%, whereas CYP2C8, 2C9, and 2C19 preferred the (S)-catechol-M to form the (S)-tris-OH-M enantiomer at 72, 80, and 77%, respectively. Interestingly, CYP1A2 also exhibited opposite enantioselectivity to that of CYP2C9 and 2C19 in the O-demethylation of methoxychlor to mono-OH-M. By contrast, in the O-demethylation of racemic catechol-M to tris-OH-M, CYP2B6 and 2D6 only minimally preferred formation of (S)-tris-OH-M at approximately 62 to 64%. However, CYP3A4 was not enantioselective (Fig. 6), and CYP2A6 and 3A5 did not generate sufficient tris-OH-M for performing enantiotopic analysis.

Relationship between Extent of Conversion of a Racemic Substrate and Enantioselectivity (Changes in Enantioselectivity). It is anticipated that the extent of substrate conversion could affect the enzymatic-enantioselectivity when a racemic substrate is employed. That is, if a given P450 favors one enantiomeric substrate over the other, then as the reaction progresses with time, the concentration of the less favored residual substrate would tend to increase, and hence a change would occur in the relative reaction rate of the two enantiomeric substrates (with the less favored substrate becoming preferentially utilized); consequently, the result of such a situation will reflect an apparent alteration in the enantiotopic selectivity over the reaction time course. To examine such a possibility, we explored the potential changes in enantioselectivity versus the progress of substrate conversion in the CYP2C19-catalyzed O-demethylation of racemic catechol-M reaction to form tris-OH-M. Results demonstrated that CYP2C19 uses 23% of (S)-catechol-M and 77% of the (R)-enantiomer as substrates when substrate conversion was limited to less than 15%. However, with increased substrate conversion, the percentage of the (S)-enantiomer (the originally less favored substrate) gradually increased (Fig. 7), causing a loss of enantioselectivity. Overall, the enantioselectivity was not significantly affected up to ~30% of substrate conversion. In summary, these findings indicate that to demonstrate potentially preferred enantioselectivity in catalysis involving a racemic mixture, it is important that metabolism does not proceed beyond a substantial substrate conversion (with catechol demethyl-
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OH-M, to the active P450 isoforms in methoxychlor demethylation. Additionally, we observed that the (S)-mono-OH-M was the major enantiomer (77–87%) produced by human liver microsomes from the several donors (including a pooled sample from 21 livers). The question whether this remarkable enantioselective preference reflects the catalytic characteristics of the livers of the general human population or merely that of the liver sampling inadvertently selected by us cannot be unequivocally answered with a relatively small sampling of human livers. The observation that HLM samples, of two different 2C9 allelic variants, display similar enantioselectivity in methoxychlor demethylation as HLM samples containing wild-type 2C9 (Legend to Fig. 3) tempts a speculation that generally human liver samples would exhibit similar enantioselectivity to methoxychlor metabolism. Additionally, the above finding and the preferred enantiotopic selectivity of the individual P450s is of interest to the description of the catalytic characteristics of the various P450 isoforms. Moreover, if studies on biological activities of these enantiomers demonstrate that there are marked differences in the estrogenic/antiestrogenic activities of the two enantiomers and/or that there are major differences in their respective interactions with ERα and/or that there are major differences in their respective interactions with ERα and ERβ, then the considerable differences in the enantioselectivity by P450s observed in vitro in our studies may provide useful information for interpreting estrogenic/antiestrogenic effects of methoxychlor in different tissues in vivo.

Interestingly, CYP2C8, 2C9, 2C19 preferred the (R)-mono-OH-M, to the S-enantiomer, as a substrate for demethylation. However, by contrast, CYP1A2 favored the (S)-mono-OH-M. The same preferences for enantiomers have been consistently observed in all three P450-mediated O-demethylation reactions (i.e., CYP1A2 always exhibited the opposite enantioselectivity to that of 2C9 and 2C19); this was independent of whether the reaction was carried out with an achiral substrate (methoxychlor) or chiral substrates (mono-OH-M and catechol-M). This finding suggests that in the O-demethylation reaction, the orientation of the substrate in the binding pocket of CYP1A2 is in the opposite direction than in CYP2C9 and 2C19. However, a conclusive substantiation of this suggestion would require crystal structure analysis of these enzymes in the presence and absence of the above substrates.

The five enantioselective reactions examined above represent the combination of two kinds of enzymatic catalysis, O-demethylation and ortho-hydroxylation involving two types of substrates [i.e., prochiral (methoxychlor and bis-OH-M) and racemic (mono-OH-M, catechol-M and tris-OH-M)]. It is clearly apparent for most of the P450s that the O-demethylation path is much more enantioselective than the ortho-hydroxylation. It was found that CYP1A2, 2C9, and 2C19 have greater enantiotopic selectivity than other P450s involved in the O-demethylation of methoxychlor, mono-OH-M and catechol-M. Overall, ortho-hydroxylation by human P450s using the methoxychlor metabolites was not enantioselective, whether the reactions were from the racemic mixture of mono-OH-M to form the catechol-M or from the prochiral bis-OH-M to form the tris-OH-M.

In comparing the enantioselectivity of P450s among species, it was found that human CYP2C9, 2C19, and rat CYP2C6 preferentially demethylate methoxychlor to the (S)-mono-OH-M; however, by contrast, human CYP2B6 and rat 2B1 were not enantioselective in that catalysis. It is of interest that certain P450 isoforms belonging to a similar subclass, but from different animal species [e.g., 2A6 (human) and 2A1 (rat)] exhibit opposing enantioselectivities, that is, CYP2A6 preferentially formed the (R)-mono-OH-M, whereas 2A1 primarily produced the (S)-enantiomer.

In conclusion, the question of whether the biological, pharmacological, or endocrinological activities of enantiomers in a racemic mixture of a given methoxychlor metabolite are similar or not is of interest. Consequently, a study has been initiated to determine whether the (R)- and (S)-enantiomers of methoxychlor metabolites (mono-OH-M, catechol-M, and tris-OH-M) exhibit similar or different interactions with the ERα and ERβ isoforms and androgen receptor. If these studies demonstrate marked differences in the interactions of the (R)- and (S)-enantiomers with the ERα and ERβ and androgen receptor, then it is conceivable that in vivo exposure to methoxychlor or to methoxychlor metabolites, particularly in species that preferentially metabolize methoxychlor into different enantiotopic metabolites, could display different hormonal activities (quantitative and possibly qualitative). Thus, it would be of interest to resolve the questions of whether P450s of various animal species demonstrate enantioselectivity of methoxychlor metabolism and whether the ratios of the enantiomers of the methoxychlor metabolites are similar to those in the human or to other species.

References
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