Biochemical Pharmacology, Vol. 55, pp. 1861–1871, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00060-4

*Pro*substrates of CYP3A4, the Major Human Hepatic Cytochrome P450

TRANSFORMATION INTO SUBSTRATES BY OTHER P450 ISOFORMS

*David M. Stresser and David Kupfer**

DEPARTMENT OF PHARMACOLOGY AND MOLECULAR TOXICOLOGY, UNIVERSITY OF MASSACHUSETTS MEDICAL CENTER, SHREWSBURY, MA 01545, U.S.A.

ABSTRACT. This study demonstrates interplay among human hepatic cytochrome P450 (CYP) isoforms in transforming aromatic compounds from being *pro*substrates of CYP3A4 into phenolic substrates. Incubation of methoxychlor with CYP2C19 yields the phenolic monodemethylated derivative (mono-OH-M). Additionally, CYP2C19 catalyzes the *ortho*-hydroxylation of mono-OH-M and of residual methoxychlor. CYP3A4 does not catalyze the *O*-demethylation or hydroxylation of methoxychlor, but does hydroxylate mono-OH-M (*ortho* to the phenolic hydroxyl) (Stresser DM and Kupfer D, *Biochemistry* **36:** 2203–2210, 1997). A combination of reconstituted CYP2C19 and 3A4 in the same vessel elicits stimulation of the *ortho*-hydroxylation of mono-OH-M compared with 2C19 alone. It is unlikely that stimulation of hydroxylation was due to protein–protein interactions, generating more active P450(s), because progression of the stimulation was time-dependent. When reconstituted CYP3A4 was added to an ongoing incubation containing reconstituted 2C19, stimulation of catechol formation occurred. In another experiment, stimulatory activity was similar when 2C19 and 3A4 were reconstituted together in the same vesicles or separately. Cumulative evidence demonstrates that the stimulation of catechol formation resulted from CYP3A4-mediated *ortho*-hydroxylation of the phenolic metabolite(s) generated by CYP2C19. Similarly, estradiol 3-methyl ether is demethylated by CYP2C19 into estradiol, a CYP3A4 substrate for *ortho*-hydroxylation; there was significant stimulation of hydroxylation by combined 2C19 and 3A4. These findings demonstrate that *pro*-phenolic compounds (methoxychlor and estradiol 3-methyl ether) are *pro*substrates of CYP3A4. Because catalysis may become evident only after *pro*substrate conversion (by a different P450) into a substrate, caution is warranted when concluding a lack of catalytic involvement by a particular P450 isoform, based solely on data from the use of individual cDNA-expressed P450s. BIOCHEM PHARMACOL **55**;11:1861–1871, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cytochrome P450; CYP2D6; CYP2C9; CYP2C19; CYP3A4; *pro*substrate; aromatic hydroxylation; *ortho*-hydroxylation; methoxychlor; estradiol 3-methyl ether; 2-hydroxyestradiol

CYP† is a superfamily of heme proteins that function primarily in the oxidative catalysis of endogenous [1] and xenobiotic [2] compounds. Based on sequence homologies, CYPs have been categorized into families and subfamilies [3]. These enzymes largely serve to detoxify or inactivate excessive residues of xenobiotic and endogenous compounds, among which are drugs, pesticides, other environmental pollutants, steroid hormones, and fatty acids, including eicosanoids [4]. CYP3A4 is the most abundant P450 in human liver and gut, and it can metabolize many structurally diverse compounds, including numerous drugs [5].

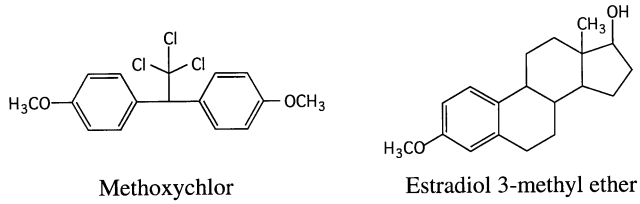
Whereas several P450s (e.g. CYP2C9 and 2D6) can readily catalyze hydroxylation of unsubstituted aromatic rings [6, 7], such catalysis by CYP3A4 appears to be rare [8]. Additionally, CYP3A4 does not usually hydroxylate unactivated alicyclic carbons [9]. Most recently, we demonstrated that aromatic rings can be hydroxylated effectively by CYP3A4, if a phenolic group is present, and that the hydroxylation occurs *ortho* to the phenolic hydroxyl [10]. However, the mere substitution of the phenolic hydroxyl with a methoxyl, in an analogous compound, abolishes or markedly diminishes the ability of CYP3A4 (but not of certain other P450s) to hydroxylate the aromatic ring [10].

In a biosystem, such as human liver microsomes, that contains a full complement of P450s, aromatic compounds may undergo hydroxylation or *O*-demethylation to form phenols by certain P450s, such as 2D6 [7, 11] or members of

* Corresponding author: David Kupfer, Ph.D., University of Massachusetts Medical Center, Shrewsbury Campus, 222 Maple Ave., Shrewsbury, MA 01545. Tel. (508) 842-8921, Ext. 129; FAX (508) 842-9632; E-mail: Kupfer@sci.wfbr.edu.

† *Abbreviations and common names:* COMT, catechol *O*-methyl transferase; CYP, cytochrome P450; E_2, estradiol, estradiol-17β; methoxychlor, [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane]; mono-OH-M, [1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane]; bis-OH-M, [1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane]; catechol-M, [1,1,1-trichloro-2-(3,4-dihydroxyphenyl)-2-(4-methoxyphenyl)ethane]; tris-OH-M, [1,1,1-trichloro-2-(3,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)ethane]; *ortho*-OH-M, [1,1,1-trichloro-2-(3-hydroxy-4-methoxyphenyl)-2-(4-methoxyphenyl)ethane]; 2-OH-E_2, 2-hydroxyestradiol; OR, NADPH-cytochrome P450 reductase; PB-microsomes, liver microsomes from phenobarbital-treated rats; and SAM, *S*-adenosylmethionine.

Received 15 August 1997; accepted 5 January 1998.



Methoxychlor Estradiol 3-methyl ether

FIG. 1. Structures of *pro*substrates of CYP3A4.

the 2C subfamily [12]. In turn, resulting phenolic derivatives are expected to be suitable substrates for CYP3A4. These considerations led us to propose the *pro*substrate concept for P450s that can be depicted in the conversion of compound A (a *pro*substrate of CYP3A) into compound B (a substrate of CYP3A) that is converted into compound C (final product), as follows:

$$\begin{array}{ccccc} & \xrightarrow{CYP2C\ or\ 2D} & & \xrightarrow{CYP3A} & \\ Compound\ A & & Compound\ B & & Compound\ C \\ (CH_3O\text{-}AR) & [demethylation] & (HO\text{-}AR) & & (HO\text{-}AR\text{-}OH) \\ (AR) & [hydroxylation] & (HO\text{-}AR) & & (HO\text{-}AR\text{-}OH) \end{array}$$

Where **AR** represents an aryl compound; **CH_3O**-AR represents a methoxylated aryl derivative; **HO**-AR represents a phenolic compound; and **HO**-AR-**OH** represents an *ortho*-dihydroxy phenyl derivative (i.e. catechol).

In these studies, we utilized two model compounds with methoxylated aromatic rings: (i) methoxychlor, a biodegradable DDT analog, that is metabolized into phenolic estrogenic derivatives [13], and (ii) estradiol 3-methyl ether, which is demethylated into E_2. Evidence is provided that methoxychlor and estradiol 3-methyl ether (Fig. 1) are *pro*substrates of CYP3A that are demethylated by other P450s into substrates of CYP3A. Additionally, we propose that these findings suggest a cautionary note that the potentially harmful side-effects of drug–drug interaction in multiple drug therapy involving a single P450 (e.g. CYP3A4)‡ may not be due necessarily to the interaction of the parent compounds, but also could result from interaction of one drug with a metabolite of the second drug (the latter prior to metabolism being merely a *pro*substrate).

Furthermore, our data illustrate that, when exploring the biotransformation of a given substrate, caution should be exercised in concluding lack of catalytic involvement by a particular P450 isoform based solely on data from the (now routine) use of individual cDNA-expressed P450 isoforms. This is because catalysis may become evident in a biosystem only after conversion of the *pro*substrate by a different P450 into the substrate.

‡ Fatal drug–drug interactions have been noted in combined treatment of the antihistamine Seldane™ (terfenadine) with ketoconazole, involving CYP3A4 [14].

MATERIALS AND METHODS

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADPH, EDTA disodium salt, E_2, estradiol 3-methyl ether, COMT, SAM iodide salt, tranylcypromine hydrochloride, glutathione, sodium cholate, potassium HEPES, L-α-dioleoyl-*sn*-glycero-3-phosphocholine, L-α-dilauroyl-phosphotidylserine, L-α-dilauroyl-phosphatidylcholine, and activated charcoal (untreated powder) were purchased from the Sigma Chemical Co. Ketoconazole was obtained from Research Biochemicals Inc. Methoxychlor was purchased from Chem Service. [*Ring*-UL-^{14}C]Methoxychlor was obtained from Sigma (5.85 mCi/mmol) or California Bionuclear (1.8 mCi/mmol). [2-^{3}H]Estradiol (22.0 Ci/mmol), [4-^{14}C]estradiol (55.5 mCi/mmol), and [*methyl*-^{3}H]SAM (15 Ci/mmol) were obtained from DuPont/NEN Research Products. 1,1,1-Trichloro-2,2-bis-([3-^{3}H]-4-hydroxyphenyl) ethane ([*o*-^{3}H]bis-OH-M) (2.47 Ci/mmol) was custom-synthesized by Chemsyn Science Laboratories. The purity of [*o*-^{3}H]bis-OH-M was 95% by radio-HPLC and radio-TLC. The tritium labeling *ortho* to the methoxyls was confirmed by ^{3}H-NMR. [2-^{3}H]Estradiol 3-methyl ether, [4-^{14}C]estradiol 3-methyl ether, [*o*-^{3}H]methoxychlor, [*o*-^{3}H] mono-OH-M, and radioinert mono-OH-M were prepared by methylation of [2-^{3}H]estradiol, [4-^{14}C]estradiol, [*o*-^{3}H] bis-OH-M, or bis-OH-M as described below or as described previously for conversion of [*o*-^{3}H]bis-OH-M into methoxychlor [15]. Final specific activities of the tritium-labeled substrates were 15,000–40,000 dpm/nmol, whereas those of ^{14}C-labeled substrates were 3200–5000 dpm/nmol. Reversed-phase (C_{18}) and normal-phase TLC plates were purchased from Whatman, Inc. A CYP3A-immunoinhibition kit was purchased from Gentest Inc. The antiserum in this kit selectively inhibits CYP3A4 catalytic activity in human liver microsomes.

Microsome Preparation

Human livers were acquired from the National Disease Research Interchange. Microsomes were prepared as previously described [16]. Additional human liver microsomes were purchased from the International Institute for the Advancement of Medicine or from Human Biologics, Inc. Microsomes from human lymphoblastoid cell lines engineered to express single human CYP isoforms, or from the same cell line transfected with the vector only (controls), were from Gentest, Inc. Microsomes containing CYP2C9

with a Cys or Arg residue at position 144 are referred to as CYP2C9-Cys or CYP2C9-Arg, respectively (the latter is the more common allele). Microsomes prepared from baculovirus-infected insect cells ("Supersomes") expressing either CYP2C19 or CYP3A4 were purchased from Gentest, Inc. Recombinant CYP2C9, 2C19, 2D6, and human NADPH cytochrome P450 reductase, purified from *Escherichia coli*, were provided by Eric Johnson. Purified, recombinant (*E. coli*) CYP1A2, 3A4, and human cytochrome b_5 were purchased from the Panvera Corp.

Enzyme Assays

Demethylase activity was determined by a modification of a method previously described [17]. Incubation mixtures contained human liver microsomes (1 mg/mL), sodium phosphate buffer (60 mM, pH 7.4), EDTA (1 mM), magnesium chloride (10 mM), and [*ring*-^{14}C]methoxychlor (25 μM) or [4-^{14}C]estradiol 3-methyl ether (25 μM) in a volume of 250 μL. The reaction was initiated by an NADPH-regenerating system [glucose-6-phosphate (2.5 μmol), NADPH (0.125 μmol), and glucose-6-phosphate dehydrogenase (0.5 IU)]. With incubations containing methoxychlor, the reaction was terminated after 10 min by the addition of 1 mL of ice-cold ethanol, and the contents were subjected to centrifugation to pellet the protein. The supernatant and a subsequent 1-mL ethanol wash of the pellet were combined in a test tube. The ethanolic mixture containing metabolites was evaporated under a stream of nitrogen at ambient temperature, the residue was resuspended in 50 μL of ethanol, and a portion was analyzed by TLC [normal phase, 9:1 (v/v) chloroform:acetone]. Radioquantitation of the chromatogram was performed with a Bioscan Imaging Scanner (Bioscan, Inc.). Structural identity of metabolites was confirmed by co-chromatography with authentic mono-OH-M and bis-OH-M [17]. For incubations containing estradiol 3-methyl ether, 125 μL was withdrawn after 10 min and dispensed into tubes containing 1 mL of ice-cold methylene chloride. After mixing vigorously for 1 min, the bottom organic layer was withdrawn, and the aqueous phase was extracted again with 1 mL ethyl acetate. The organic phases were combined and evaporated to dryness. The residue was resuspended in 50 μL of ethanol and analyzed by TLC, and the demethylated product (E_2) was quantified radiometrically as described above for methoxychlor metabolism. In liver microsomes, there was a linear relationship between time and metabolite production with both methoxychlor and estradiol 3-methyl ether for at least 10 min. Incubation mixtures with lymphoblast microsomes containing single P450s were conducted as with liver microsomes, but occasionally required longer incubation periods (up to 30 min with methoxychlor and 90 min with estradiol 3-methyl ether) in order to obtain sufficiently quantifiable product. In incubations of CYP2A6, 2C9-Cys, or 2C9-Arg, 60 mM of Tris was the buffer.

[*o*-^{3}H]Methoxychlor *ortho*-hydroxylase activity in human liver microsomes was determined as described previously [15]. Incubation components and conditions were similar to those stated for [^{14}C]methoxychlor, but the reactions were terminated by the addition of an equal volume of ice-cold 0.016 M of $CaCl_2$ followed by the same volume of dextran-coated charcoal solution [15, 18]. When chemical inhibitors or antibodies were added (see below), the incubation period was extended from 10 to 20 and 60 min, respectively.

Incubations with Chemical Inhibitors or Polyclonal Antisera

The concentration of ketoconazole used (1 μM) to maximize selectivity for CYP3A4 was based on the data of Newton *et al.* [19]. Tranylcypromine, an inhibitor of multiple P450 isoforms,§ was used at a concentration of 50 μM. To examine inhibition of [*o*-^{3}H]methoxychlor *ortho*-hydroxylase activity, polyclonal antiserum and nonimmune serum in ratios yielding equivalent amounts of protein were added to microsomes and incubated on ice for 20 min. The remaining components of the incubation were added, and the reaction was carried out as described above. Additional processing required of samples that contained antiserum has been described previously [15].

Reconstitution of Purified P450 Isoforms

Incubation mixtures with purified P450 isoforms consisted of 0.04 μM of P450, 1.2 U/mL of NADPH CYP reductase‖ (OR), 0.08 μM of cytochrome b_5, 0.5 mM of sodium cholate, 20 μg/mL of a phospholipid mixture [L-α-dioleoyl-*sn*-glycero-3-phosphocholine, L-α-dilauroyl-phosphatidylserine, L-α-dilauroyl-phosphatidylcholine, 1:1:1 ratio (w/w)] in 50 mM of potassium HEPES buffer (pH 7.4) containing 30 mM of $MgCl_2$, 3.0 mM of GSH, and substrate added in ethanol (ethanol final concentration was 1%). To examine stimulatory activity in a time-course reaction with [*o*-^{3}H]methoxychlor as the substrate, components were similar except that one or two P450 isoforms were present and the reductase level was increased twofold.¶ The final volume was 200–250 μL. P450, NADPH cytochrome P450 reductase, cytochrome b_5, phospholipid mix, and sodium cholate were preincubated on ice for 25

§ Although often purported to be a selective inhibitor of CYP2C19, tranylcypromine has been shown recently to inhibit CYP2C9 [20] and CYP2A6 [21]. Additionally, we have observed that 50 μM of tranylcypromine inhibited CYP3A4-catalyzed 6β-hydroxylation of testosterone in human liver microsomes (Stresser DM and Kupfer D, unpublished results).

‖ The ratio of P450 to reductase used (10 pmol:0.3 U reductase) was based on the recommendations of Drs. Toby Richardson and Eric Johnson, and our own observations that this ratio was optimal.

¶ The ratio of *total* P450 to reductase in incubation mixtures that contained two P450 isoforms was thus maintained at 10 pmol:0.3 U (saturating). However, because there are different isoforms in the mixture, the ratio of each isoform is 10 pmol:0.6 U. Therefore, with methoxychlor, when one isoform only was used, in order to maintain equivalent ratios of P450 to reductase, and to make a more valid comparison to assess stimulation of *o*-hydroxylase activity, the ratio of 10 pmol P450:0.6 U reductase was used.

min prior to the addition of buffer and other components. The mixtures were then incubated at 37° for 2 min, prior to initiating the reaction with an NADPH-regenerating system. When [2-^{3}H]estradiol 3-methyl ether was the substrate, the reaction was terminated by the addition of methylene chloride as described above. To terminate stages of the time-course reaction with [*o*-^{3}H]methoxychlor as substrate, one-third of the incubation was mixed with an equal volume of ice-cold 0.016 M of $CaCl_2$, followed by an equal volume of dextran-coated charcoal [15, 18]. The extent of hydroxylation was calculated by quantifying the release of ^3H_2O, using scintillation spectrometry as previously described [15].

Exploration of Protein–Protein Interactions

To examine the potential for protein–protein (i.e. P450–P450) interactions in a reconstituted system and its effect on [*o*-^{3}H]methoxychlor *ortho*-hydroxylase activity, two experiments were carried out. In the first experiment, CYP2C19 and CYP3A4 were each reconstituted with lipid, cholate, cytochrome b_5 and OR in separate vessels and kept on ice. After 25 min, buffer, $MgCl_2$, and GSH were added to the vessel containing CYP2C19 only. Then reconstituted CYP3A4 was added to this vessel followed by the addition of substrate. After the 2-min preincubation period at 37°, NADPH was added to initiate the reaction, which was terminated after 20 min. The extent of [*o*-^{3}H]methoxychlor *ortho*-hydroxylation was compared with that catalyzed in a 20-min reaction by a *ternary* mixture, where CYP2C19 and CYP3A4 were reconstituted together in one vessel with lipid, cholate, cytochrome b_5, and OR and kept on ice for 25 min prior to the addition of the remaining components. The various ratios of total P450, lipid, cytochrome b_5, and OR were kept constant. In the second experiment, CYP2C19 alone was reconstituted with or without OR, and the reaction with [*o*-^{3}H]methoxychlor was allowed to proceed for 30 min. At that time, additional P450 protein (either CYP3A4 or CYP2D6) in a reconstituted system with or without OR was added, and the reaction was allowed to continue for an additional 30 min. Then the reaction was terminated by sequential addition of $CaCl_2$ and dextran-coated charcoal solution, and *ortho*-hydroxylase activity was determined as described above.

Occasionally, methoxychlor catechol formation was quantified, using an assay employing COMT-mediated methyl group transfer from [^{3}H]-SAM to catechol. Incubations contained the following: 125 μL (12.5 μmol) of sodium phosphate buffer (pH 7.4); 25 μL (2.5 μmol) of $MgCl_2$; CYP2C19 in a microsomal suspension of lymphoblasts (125 μg of protein in 12.5 μL of 0.1 M of phosphate buffer); radioinert estradiol or mono-OH-M (6.25 nmol in 1.25 μL of ethanol); [^{3}H]-SAM (50 nmol, 0.5 μCi in 3.4 μL of H_2O); EDTA (1 μmol); 6.25 U of COMT; 12.5 nmol of dithiothreitol; and 12.5 nmol of ascorbic acid. After a 2-min preincubation at 37°, the reaction was initiated by an NADPH-regenerating system in 25 μL of H_2O to bring the volume to 250 μL, and the vials were incubated at 37°, for 90 min. The reaction was terminated by placing the vials on ice, adding 1 mL hexane, and mixing briefly (Vortexer II genie, VWR). The hexane layer containing mono-[^{3}H]methylated catechol was transferred to separate vials. The samples were extracted with fresh hexane, the combined hexane extracts were evaporated under a stream of nitrogen, and the residue was suspended in 100 μL of ethanol. A 50-μL aliquot of the ethanolic suspension was mixed with 4 mL of Ultima-gold scintillation fluid (Packard), and the radioactivity (dpm) was determined by scintillation spectrometry.

Statistical Analysis

With experiments involving chemical inhibitors, analysis of variance was used to determine significant differences within groups of three. When significant differences were indicated ($P < 0.05$), Dunnett's multiple comparison post test was employed to identify groups differing significantly from control. Differences between two groups were determined by Student's *t*-test.

RESULTS AND DISCUSSION

Previous studies demonstrated that the incubation of methoxychlor with liver microsomes from untreated rats (control microsomes) generated primarily the mono- and bis-demethylated products, mono OH-M and bis-OH-M, respectively (for structures, see Fig. 2), and formed only little or no catechol, tris-OH-M [22, 23]. By contrast, PB-microsomes yielded both of the demethylated products, as well as the hydroxylated products, *ortho*-OH-M†† and tris-OH-M [17, 23]. This finding indicated that the hydroxylated metabolites were formed by ring-hydroxylation of methoxychlor *ortho* to the methoxyls followed by *O*-demethylation or by an initial *O*-demethylation followed by *ortho*-hydroxylation (Fig. 2). There was a relatively low level of accumulation of *ortho*-OH-M after a 10-min incubation of methoxychlor with PB-microsomes without a significant amount of tris-OH-M‡‡; however, after a 30- or 60-min incubation, there was a significant level of the tris-OH-M. This indicated that the major portion of the catechol is derived from the *ortho*-hydroxylation of the mono-OH-M and bis-OH-M. The level of the demethylated methoxychlor products in incubations with PB-microsomes was similar to that observed with control microsomes, indicating that demethylation is catalyzed primarily by constitutive P450s [17]. By contrast, induced levels of CYP2B and 3A in the PB-microsomes appear to be responsible for the enhanced *ortho*-hydroxylating activity [17].

The current study with human liver microsomes demonstrated that *ortho*-OH-M (compound **IV**) is formed by

†† This metabolite was termed previously, ring-OH-methoxychlor [15, 17].

‡‡ Dehal SS and Kupfer D, unpublished observations.

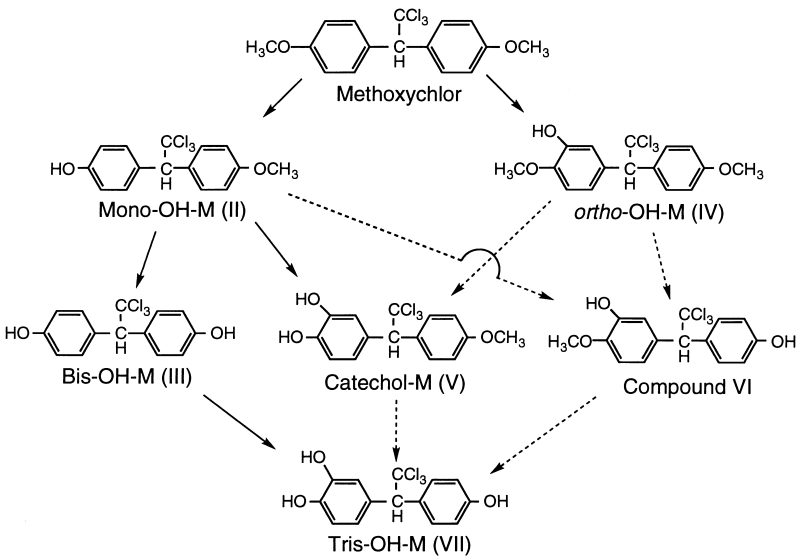


FIG. 2. Proposed metabolic pathways of P450-mediated demethylation and ring-hydroxylation of methoxychlor. Pathways indicated by dotted arrows are speculative (see Ref. 17).

hydroxylation of methoxychlor, catalyzed by CYP2B6 and CYP2C19, and that catechol-M (compound **V**) and compound **VI** are generated by demethylation of the *ortho*-OH-M or, alternatively, by an initial demethylation of methoxychlor into mono-OH-M (compound **II**), catalyzed primarily by CYP2C isoforms, followed by *ortho*-hydroxylation by CYP3A enzymes (Fig. 2).§§

Metabolism of Methoxychlor and Estradiol 3-Methyl Ether by Human Liver Microsomes

Incubation of methoxychlor with human liver microsomes resulted in the formation of mono-OH-M and a minimal amount of bis-OH-M (Table 1). Additionally, *ortho*-hydroxylated metabolites that are formed at a concentration too low for detection on TLC (Fig. 3A) were demonstrated by tritium release (^3H_2O assay) from the *ortho* position (Table 1). Because heterologously expressed CYP3A4 does not catalyze the *O*-demethylation (data not shown) and exhibits negligible hydroxylation of methoxychlor [10], those reactions appeared to involve P450s other than 3A4. Indeed, the demethylation was attributed to catalysis primarily by CYP2C enzymes (*vide infra*). Similar results were obtained with estradiol 3-methyl ether, i.e. demethylation yielded E_2 (Fig. 3B, Table 1), followed by *ortho*-hydroxylation, resulting in 2-hydroxyestradiol (Table 1) and also probably a low amount of 4-hydroxyestradiol [24]. The effects of two inhibitors of liver microsomal methoxychlor *O*-demethylation or *ortho*-hydroxylation are shown in Fig 4.

To probe further the P450 catalysis of methoxychlor *ortho*-hydroxylation (i.e. formation of *ortho*-OH-M, catechol-M, compound **VI** and tris-OH-M) and *O*-demethylation, we examined the effects of chemical inhibitors or immunoinhibitory antibodies on these reactions. Ketoconazole, a CYP3A-selective inhibitor, significantly inhibited ortho-hydroxylation, but not *O*-demethylation, indicating CYP3A involvement only in the former reaction. In contrast, tranylcypromine, an inhibitor of CYP2C19 and

§§ Current study, and Stresser DM and Kupfer D, manuscript in preparation.

TABLE 1. Demethylation and hydroxylation of methoxychlor and estradiol 3-methyl ether by human liver microsomes

		Metabolite (pmol/min/mg protein)	
Substrate	**Metabolite**	**Mean**	**(SEM)**
[*ring*-^{14}C]Methoxychlor	Mono-OH-M	343	(30)
[*ring*-^{14}C]Methoxychlor	Bis-OH-M	20	(3)
[*o*-^{3}H]Methoxychlor	*ortho*-OH-M, [catechol-M and compound **VI**]*, Tris-OH-M	16	(3)
[4-^{14}C]Estradiol 3-methyl ether	Estradiol	18	(3)
[2-^{3}H]Estradiol 3-methyl ether	2-Hydroxyestradiol + 2-hydroxyestradiol 3-methyl ether	14	(3)

Microsomes (0.5 to 1 mg/mL) prepared from individual human livers (N = 8 or 9) were incubated in duplicate or triplicate for 10 min in the presence of substrate (25 μM of [*ring*-^{14}C]methoxychlor, 25 μM of [*o*-^{3}H]methoxychlor, 25 μM of [4-^{14}C]estradiol 3-methyl ether, or 25 μM of [2-^{3}H]estradiol 3-methyl ether) and an NADPH-regenerating system. [^{14}C]Mono-OH-M and [^{14}C]bis-OH-M were resolved by TLC and quantified by radioscanning. The sum of *ortho*-OH-M, catechol-M, compound **VI**, and tris-OH-M was quantified by tritium release (as ^3H_2O). E_2 metabolite was resolved by TLC and quantified by radioscanning. 2-Hydroxyestradiol and 2-hydroxyestradiol 3-methyl ether formation were determined by quantifying the extent of tritium release (as ^3H_2O).

*Inferred.

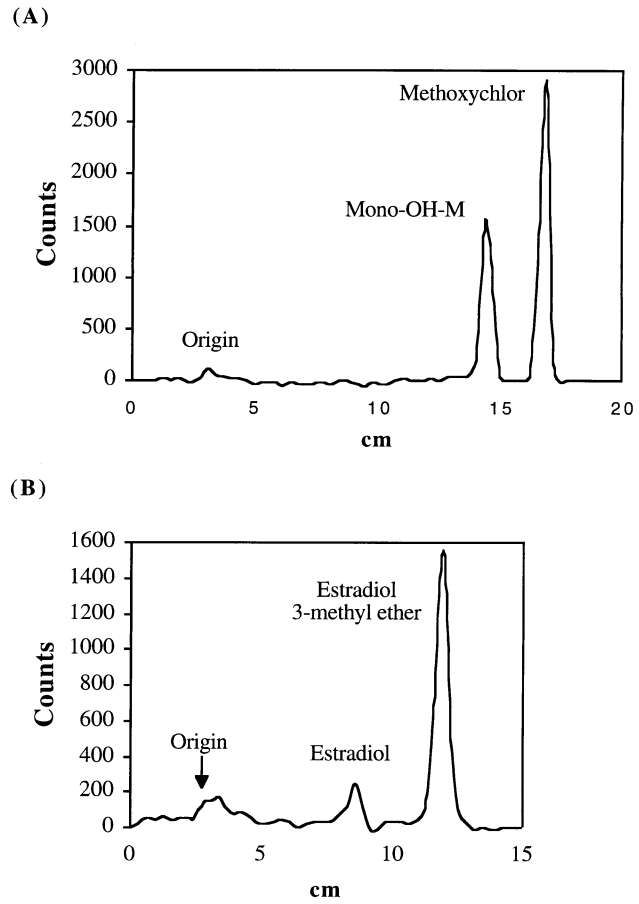


FIG. 3. (A) Representative TLC chromatogram of an extract of a 20-min incubation of [^{14}C]methoxychlor (50 μM) with purified recombinant CYP2C19 (0.04 μM) and CYP3A4 (0.04 μM). (B) Representative TLC chromatogram of an extract of a 90-min incubation of estradiol 3-methyl ether (25 μM) with purified recombinant CYP2C9 (0.04 μM).

several other isoforms (see Materials and Methods), significantly inhibited both reactions. Inhibition of O-demethylation is consistent with the observation of high methoxychlor O-demethylase activity by recombinant 2C19 (Table 2). Diminution of *ortho*-hydroxylase activity may have occurred directly by inhibition of P450 isoforms catalyzing this reaction, or indirectly via reduced formation of CYP3A4 substrate (i.e. mono-OH-M) for catechol-M formation. Additional evidence for CYP3A4 involvement in liver microsomal catechol-M formation is provided in Fig. 5, which shows that rabbit anti-rat CYP3A2 polyclonal antiserum that selectively inhibits CYP3A4 in human liver microsomes can inhibit this activity.

Demethylation of Methoxychlor and Estradiol 3-Methyl Ether by Individual cDNA Expressed Human P450s

Microsomes prepared from human lymphoblast cell lines engineered to express single human P450s were tested for their capacity to demethylate methoxychlor and estradiol 3-methyl ether. Out of 12 isoforms of P450 examined, CYP1A2, 2A6, 2B6, 2C8, 2C9-Cys, 2C9-Arg, and 2C19 catalyzed the demethylation of methoxychlor. By far, CYP2C9 (both Arg_{144} and Cys_{144} variants) and 2C19 isoforms were the most active in catalyzing the mono-demethylation of methoxychlor (Table 2). Also the demethylation of estradiol 3-methyl ether was catalyzed by CYP1A1, 1A2, and 2C9-Arg (Table 2); the demethylation rate of estradiol 3-methyl ether was markedly lower than that of methoxychlor. Similarly, reconstituted recombinant CYP1A2, 2C9, and 2C19 catalyzed at a low rate estradiol 3-methyl ether demethylation (Table 2). These observations indicated that methoxychlor and estradiol 3-methyl ether are *pro*substrates of CYP3A4; however, to demonstrate that demethylation was necessary for the subsequent

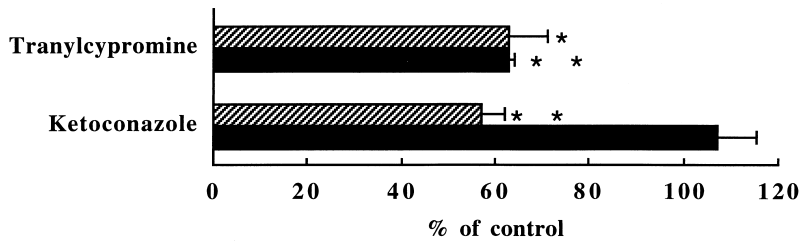


FIG. 4. Effects of chemical inhibitors on the mono-demethylation (filled bars) and *ortho*-hydroxylation (striped bars) of methoxychlor catalyzed by pooled (N = 3) human liver microsomes. [^{14}C]Methoxychlor (25 μM) or [*o*-^{3}H]methoxychlor (25 μM) was incubated with liver microsomes and an NADPH-regenerating system in the presence of 50 μM of tranylcypromine or 1 μM of ketoconazole. Mono-OH-M was resolved by TLC and quantified by radioscanning. *Ortho*-hydroxylated product was quantified indirectly by determining the extent of $^{3}H_2O$ release into the medium, as described in Materials and Methods. Statistical significance is indicated by asterisks (*P < 0.01, **P < 0.001). Values represent the means ± SEM of quadruplicate incubations. Control values for *ortho*-hydroxylation were 13.8 pmol/min/mg of protein (ketoconazole control) and 18.1 pmol/min/mg of protein (tranylcypromine control). Control values for mono-demethylation were 155 pmol/min/mg of protein (ketoconazole control) and 335 pmol/min/mg of protein (tranylcypromine control). The differences in control activities are likely attributable to different preparations of the pooled microsomes.

TABLE 2. Demethylation of methoxychlor and estradiol 3-methyl ether by human P450s

		Substrate			
		Methoxychlor (pmol product/min/pmol P450)		Estradiol 3-methyl ether (pmol product/min/pmol P450)*	
P450 isoform	Source	Mean	(range)	Mean	(range)
CYP1A1	Lymphoblast microsomes	—†		0.07	(0.01)
CYP1A2	"	0.3		0.02	(0.01)
CYP1B1 + OR§	"	—	(0.0)‡	—	
CYP2A6 + OR	"	0.3	(0.0)	—	
CYP2B6	"	0.3	(0.0)	—	
CYP2C8 + OR	"	0.1	(0.0)	—	
CYP2C9-Cys + OR	"	1.7	(0.1)	—	
CYP2C9-Arg + OR	"	2.5	(0.0)	0.10	(0.03)
CYP2C19	"	11.3	(0.4)	—‖	
CYP2D6 + OR	"	—		—	
CYP2E1 + OR	"	—		—	
CYP3A4 + OR	"	—		—	
CYP1A2	purified and reconstituted	ND¶		0.35	(0.04)
CYP2C9	"	ND		0.52	(0.07)
CYP2C18	"	ND		—	
CYP2C19	"	ND		0.64	(0.08)
CYP3A4	"	ND		0.09	(0.03)

Microsomes (1 mg/mL) prepared from human lymphoblasts were incubated in duplicate in the presence of 25 μM of [*ring*-^{14}C]methoxychlor (10 or 30 min) or 25 μM of [4-^{14}C]estradiol 3-methyl ether (90 min) and an NADPH-regenerating system. Mono-OH-M and estradiol were resolved by TLC and quantified by radioscanning. Values given for microsomes are normalized to P450 content of the microsomes. Purified P450 isoforms (0.04 μM) were incubated for 90 min in the presence of 25 μM of [4-^{14}C]estradiol 3-methyl ether and an NADPH-regenerating system.

*Because of the low rate of demethylation, incubations were conducted for 90 min.

†Dash marks (—) indicate that no metabolite was detected.

‡0.0 = <0.05.

§OR = co-expressed NADPH P450 reductase. The P450 in microsomes without co-expressed OR is supported by reductase native to the cell line.

‖The lack of activity observed using lymphoblast microsomes containing CYP2C19 was unexpected. This was most likely due to the low P450 content of these microsomes compared with most other lymphoblast microsomes used.

¶ND = not done.

hydroxylation, a system involving an interplay of the appropriate P450s was required. This was achieved in an incubation of methoxychlor with a combination of reconstituted CYP2C19 and 3A4 (*vide infra*), and in an incubation of estradiol 3-methyl ether with a combination of two sets of microsomes from baculovirus-infected insect cells overexpressing either CYP2C19 or CYP3A4.

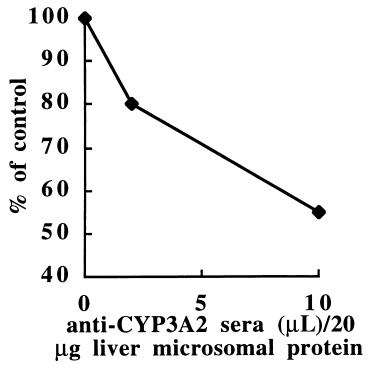


FIG. 5. Effect of polyclonal anti-rat CYP3A2 sera on *ortho*-hydroxylation of methoxychlor by human liver microsomes. [*o*-^{3}H]Methoxychlor (25 μM) was incubated with human liver microsomes (0.09 mg/0.25 mL) and an NADPH-regenerating system for 60 min in the presence of increasing amounts of serum from rabbits immunized with rat CYP3A2. The control (100%), which contained nonimmune serum only, catalyzed this reaction at a rate of 2550 pmol/60 min/mg of protein. The range of duplicate determinations for all points was <5% of the mean.

Metabolism of Methoxychlor by a Combination of Reconstituted cDNA-Expressed CYP2C19 and 3A4

The above finding demonstrated that CYP2C19 catalyzes effectively the demethylation of methoxychlor. Indeed, CYP2C19 was found to perform the *ortho*-hydroxylation as well (Fig. 6).‖‖ By contrast, as expected, CYP3A4 essentially did not catalyze the *ortho*-hydroxylation of methoxychlor. However, when both CYP2C19 and CYP3A4 were reconstituted together and incubated with methoxychlor, a significant stimulation in hydroxylation was observed, suggesting that 2C19 converted methoxychlor into a substrate(s) of CYP3A4-mediated hydroxylation (Fig. 6). In that experiment, the extent of mono-demethylation of methoxychlor was also determined. A chromatogram depicting mono-OH-M formation in a 20-min incubation of

‖‖We incubated radioinert methoxychlor in the presence of COMT, [^{3}H]SAM, and microsomes from lymphoblasts engineered to express CYP2C19. CYP2C19 catalyzed the formation of a product that was a substrate for COMT (1.18 ± 0.24 pmol of methylated product/90 min/pmol of P450), demonstrating that a catechol is one of the end-products of CYP2C19 metabolism of methoxychlor.

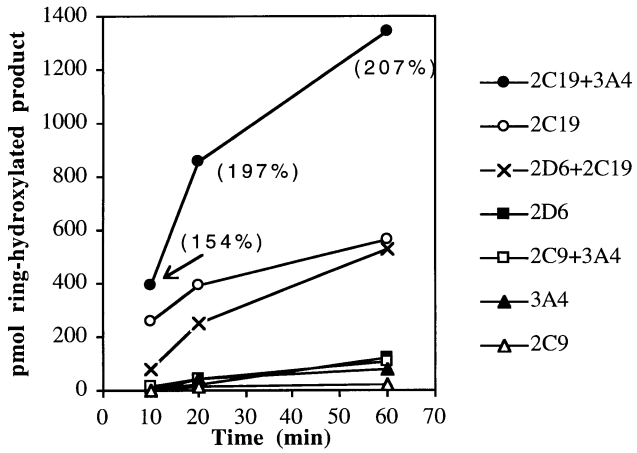


FIG. 6. Time course of the metabolism of [*o*-^{3}H]methoxychlor by single P450 isoforms or a combination of two isoforms. P450 isoforms (0.04 μM) were reconstituted with OR, cytochrome b_5, lipid mixture and sodium cholate, as described in Materials and Methods. When two P450s were combined, each was present at 0.04 μM. After a 25-min incubation on ice, samples were brought to 450 μL with buffer, solutions of magnesium chloride and glutathione, and [*o*-^{3}H]methoxychlor (25 nmol) dissolved in 5 μL of ethanol. After a preincubation period of 2 min at 37°, the reaction was initiated by an NADPH-regenerating system. The extent of ring-hydroxylation was determined at 10, 20, and 60 min in aliquots. Values in parentheses represent the product formation in the sample containing both CYP2C19 and 3A4, expressed as a percentage of the combined total of product formed by CYP2C19 and 3A4 when each was incubated alone, at 10, 20, and 60 min, respectively. Values represent the mean of product formation from duplicate incubations.

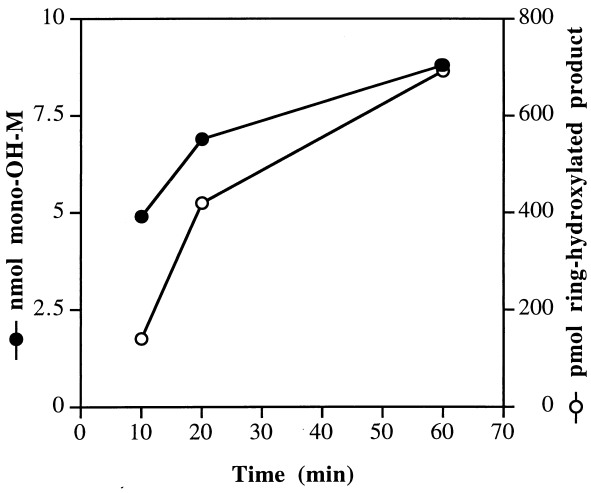


FIG. 7. Correlation of methoxychlor mono-demethylated product formed with ring-hydroxylated product formed. Left Y axis, filled circles: Mono-OH-M formed in the incubation that contained both CYP2C19 and 3A4. Activated charcoal, used to adsorb unmetabolized methoxychlor and mono-OH-M metabolite in the ring-hydroxylase assay, was extracted twice with ethanol and once each with methylene chloride and hexane to recover these compounds. Right Y-axis, open circles: Ring-hydroxylated product attributable to CYP3A4 in the sample that contained both CYP2C19 and CYP3A4. That catalytic activity was calculated by subtracting the total formation of ring-hydroxylated product formed by CYP3A4 and CYP2C19, when each was incubated individually.

methoxychlor with purified CYP2C19 and 3A4 is shown in Fig. 3A. No mono-OH-M was found in incubations of methoxychlor with CYP3A4.

Is the Stimulatory Effect in ortho-Hydroxylation of Methoxychlor by 2C19 and 3A4 due to Protein–Protein Interaction or to Conversion of Prosubstrate to Substrate?

Recently, it was demonstrated that both CYP1A2 and CYP1A1 could stimulate the 6β-hydroxylation of testosterone catalyzed by CYP3A4 in a reconstituted system [25]. Thus, although our interpretation represents the most likely explanation for the observed stimulatory effect of *ortho*-hydroxylation, the possibility should be considered that the stimulation was a function of a positive effector mechanism involving protein–protein interaction of 2C19 and 3A4, i.e. that the interaction of 2C19 with 3A4 augmented the ability of one or both of the P450s to catalyze the *ortho*-hydroxylation (*vide infra*). Therefore, the potential protein–protein interaction between CYP2C19 and 3A4 was examined. It was assumed that the protein–protein interaction would constitute a relatively rapid event and, if indeed it occurred, then the stimulatory effect by the two P450s would be elicited within a brief period of incubation. By contrast, if the conversion of a *pro*substrate into a substrate were the crucial requirement, then the stimulation should proceed with augmentation in a time-dependent fashion and become evident only after sufficient accumulation of the phenolic substrate had been achieved. The observation that the stimulatory effect increased with time by the combined presence of CYP3A4 and 2C19 as compared with 2C19 or 3A4 alone (stimulation of 154, 197, and 207% at 10, 20, and 60 min, respectively) (Fig. 6) pointed to an increase in substrate availability, i.e. accumulation of demethylated methoxychlor, as being the primary cause of the stimulatory effect. The lack of stimulation by CYP2D6 of the 2C19 activity and by CYP2C9 of the 3A4 activity (Fig. 6) further demonstrated that the stimulation was not due to non-specific protein–protein interactions and supported our contention that conversion of the *pro*substrate (methoxychlor) into the substrate (mono-OH-M) was the necessary event for the stimulation to be elicited. Additionally, the direct relationship between the time course of demethylation and ring-hydroxylation (Fig. 7) supports that conclusion.

It has been demonstrated previously that individual P450s can interact when reconstituted within the same lipid vesicles [25]. However, when individual P450s are reconstituted and only then brought together, interaction between individual P450s would not be expected to occur, unless there is leakage between the vesicles. Indeed, demonstration of P450 containment in vesicles has been reported previously [26]. Thus, we compared the [*o*-^{3}H]methoxychlor *ortho*-hydroxylase activity in an incubation

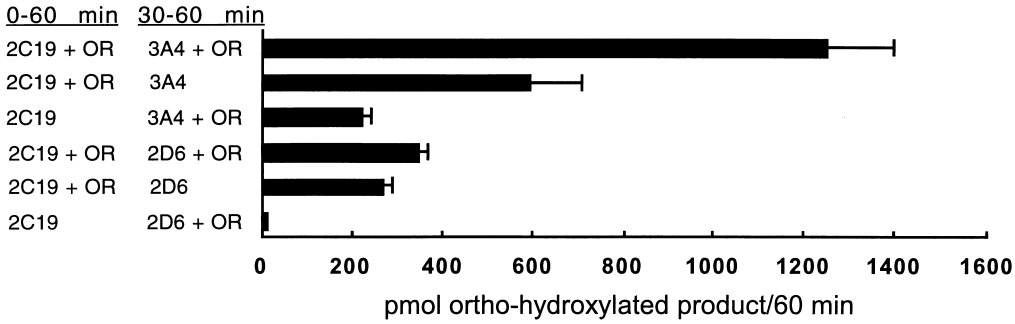


FIG. 8. Effect of delayed addition of complete or partially reconstituted CYP3A4 or 2D6 to a partial or complete CYP2C19 reconstituted system on *ortho*-hydroxylation of methoxychlor. CYP2C19 (0.04 μM) was reconstituted with or without OR, cytochrome b_5, lipid mixture, and sodium cholate, and kept on ice for 25 min. The remaining components of the incubations were then added, and the reaction was initiated by NADPH. After 30 min, either CYP3A4 or CYP2D6, with or without OR, in a similarly prepared reconstituted system was added in a small volume, and the reaction was allowed to continue for another 30 min. Error bars represent the range of duplicate incubations.

containing CYP2C19 and CYP3A4, which were reconstituted together in a single mixture of lipid, cytochrome b_5, OR, and cholate (ternary mixture), to the activity in an incubation containing CYP2C19 and CYP3A4 which, in this case, were each reconstituted with lipid and other components separately (binary mixture). We observed that there was no significant difference in the rates of catalysis (27.6 ± 6.4 pmol/20 min/pmol of P450, ternary mixture vs 25.3 ± 4.1 pmol/20 min/pmol of P450, binary mixture). Therefore, these data represent additional evidence that the augmentation in *ortho*-hydroxylase activity provided to CYP2C19 by CYP3A4, shown in Fig. 6, is not a consequence of protein–protein interactions.

To further address the possibility of protein–protein interactions, we incubated reconstituted CYP2C19, in the presence or absence of OR, with [*o*-^{3}H]methoxychlor for 30 min. At that time, another P450 (either CYP3A4 or CYP2D6, reconstituted with or without OR) was added, and the reaction was allowed to continue for an additional 30 min (Fig. 8). When included in the reaction, OR was provided to saturate the enzyme to attain maximal catalytic activity. As expected, the highest activity was observed in the incubation containing 2C19 + OR and CYP3A4 + OR. When OR was omitted from reconstituted CYP3A4, the amount of product formed declined by more than 50%. This suggests that catalytically competent reconstituted CYP3A4, and not the mere presence of the protein was essential for maximal activity. Moreover, maximal activity was also dependent on catalytically competent CYP2C19 as the incubation containing 2C19 lacking OR and CYP3A4 plus OR exhibited < 25% apparent maximal activity. This activity is likely catalyzed by CYP2C19, since CYP3A4 exhibits only negligible [*o*-^{3}H]methoxychlor *ortho*-hydroxylase activity. It appears that the OR within the reconstituted CYP3A4, added to the incubation at 30 min, is positioned in the lipid vesicles in such a way as to be available as a reductant of CYP2C19 lacking OR in its reconstitution system. CYP2D6, with or without OR, added at 30 min failed to augment catalytically competent CYP2C19 activity. Interestingly, whereas CYP3A4 + OR could stimulate CYP2C19 that lacked OR, CYP2D6 + OR could not. CYP2D6 apparently diminishes OR interaction with CYP2C19. Additionally, CYP2D6 itself may be inhibiting CYP2C19 catalytic activity (*ortho*-hydroxylation or *O*-demethylation). Indeed global comparison of the data in Fig. 8, as well as that presented in Fig. 6, seems to support that possibility.

Metabolism of Estradiol 3-Methyl Ether by a "Combination" of cDNA-Expressed CYP2C9 or 2C19 and 3A4

We examined the possible stimulation of *ortho*-hydroxylation by purified CYP2C9 (an estradiol 3-methyl ether demethylase) and CYP3A4 (an efficient estradiol *ortho*-hydroxylase) by incubating each of these P450 isoforms alone, or in combination in the presence of [2-^{3}H]estradiol 3-methyl ether. In these experiments, we could not demonstrate stimulation conclusively (data not shown). Apparently, the low demethylase activity (i.e. minimal formation of the substrate from the *pro*substrate) precluded formation of a sufficient concentration of E_2 for CYP3A4-mediated E_2 2-hydroxylase activity. However, a similar experiment with microsomes from insect cells designed to overexpress either CYP2C19 (an estradiol 3-methyl ether demethylase) or CYP3A4 exhibited significant stimulation of 144% (Fig. 9) in a 90-min incubation. This occurred apparently due to higher catalytic activity of these enzyme preparations, compared with the reconstituted, purified P450 isoforms. It is unlikely that the presence of overexpressed cytochrome b_5 in microsomes containing CYP3A4 stimulated CYP2C19 demethylation of estradiol 3-methyl ether, thus providing CYP3A4 with more substrate culminating in apparent stimulation. This is because cytochrome b_5 exhibits no significant effect on methoxychlor mono-demethylation catalyzed by purified CYP2C19 in a reconstituted

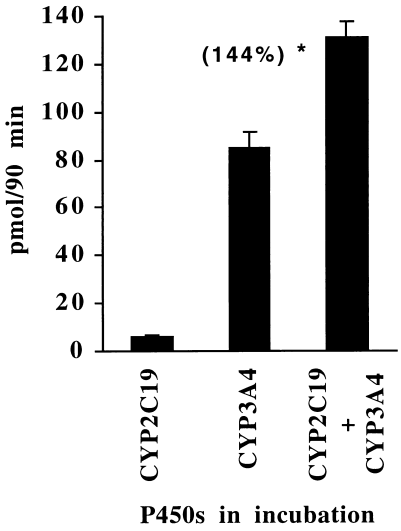


FIG. 9. 2-Hydroxylation of [2-^{3}H]estradiol 3-methyl ether in an incubation containing microsomes prepared from insect cells that overexpress P450. Incubations contained either CYP2C19 (0.06 μM) or CYP3A4 (0.06 μM) or both isoforms in combination (0.06 plus 0.06 μM). The value in parentheses represents the product formation in the sample containing both CYP2C19 and 3A4, expressed as a percentage of the combined total of product formed by CYP2C19 and 3A4 when each was incubated alone. The number of picomoles formed in the incubation containing both isoforms was significantly different ($P < 0.01$, Student's *t*-test) than the sum of the number of picomoles formed by the isoforms when each isoform was incubated individually [indicated by asterisk (*)]. Values represent the means ± SEM of quadruplicate incubations.

system.[¶¶] The less potent stimulation observed with estradiol 3-methyl ether compared with methoxychlor as the *pro*substrate is in accord with the minimal estradiol 3-methyl ether O-demethylase activity exhibited by CYP2C19 (Table 2).

Conclusion

These studies describe that certain human P450s are required to convert *pro*substrates of CYP3A4 into substrates of that enzyme. Collectively, these findings, in conjunction with our previous observations [10], illustrate definitively that the presence of phenolic hydroxyls facilitates effective aromatic hydroxylation by CYP3A4 and should yield information for further studies of the mechanism of CYP3A4-mediated catalysis. These results herein can be distinguished from simply a case of sequential metabolism, where the same P450 might catalyze the first and subsequent biotransformations [27, 28]. In the present study, the initial biotransformation of the *pro*substrate is catalyzed by an enzyme that is distinct from the enzyme responsible for catalysis of the newly formed substrate. Moreover, the data demonstrate directly, in a defined system, that substrates are released from the catalytic site of one P450 and proceed to the catalytic site of another, distinct P450. Usually, sequential metabolism is demonstrated in the heterogeneous mixture where the isoform-specific catalysis is not defined. Also, protein–protein interaction as the cause for stimulation was ruled out.

This study underscores the need for prudent interpretation of data obtained by routine use of cDNA-expressed P450 isoforms, whose catalysis can be studied advantageously in the absence of other P450s. For example, concluding lack of catalytic involvement by a particular P450 isoform may be in error because catalysis may become evident only after conversion of the *pro*substrate into the substrate by a different P450. Additionally, it is conceivable that the compound under investigation may be a *pro*-inhibitor, which may not inhibit a given P450 isoform until converted to an inhibitor by a different isoform. Thus, although valuable information can be obtained from studies of individual P450s, it would need corroboration from experiments in the more native biosystem (liver microsomes, hepatocytes, liver slices) and thus may require an oscillating approach between studies of the complex biosystem and experiments with individual or combined P450s.

Sincere thanks are due to Drs. Eric Johnson and Toby Richardson for providing the purified recombinant P450s. The project described was supported by Grants ES00834 and ES05737 from the National Institute of Environmental Health Sciences, NIH. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH. Thanks are due to one of the reviewers for suggesting experiments to unequivocally rule out the possibility of protein–protein interactions as being involved in the P450 activation and stimulation of ortho-hydroxylation.

References

1. Kupfer D, Endogenous substrates of monooxygenases: Fatty acids and prostaglandins. In: *Hepatic Cytochrome P-450 Monooxygenase System* (Eds. Schenkman JB and Kupfer D), pp. 157–187. Pergamon Press, New York, 1982.
2. Guengerich FP, Reactions and significance of cytochrome P-450 enzymes. *J Biol Chem* **266:** 10019–10022, 1991.
3. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC and Nebert DW, P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6:** 1–42, 1996.
4. Porter TD and Coon MJ, Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J Biol Chem* **266:** 13469–13472, 1991.
5. Maurel P, The CYP3 family. In: *Cytochromes P450* (Ed. Ioannides C), pp. 241–270. CRC Press, Boca Raton, 1996.
6. Veronese ME, Mackenzie PI, Doecke CJ, McManus ME, Miners JO and Birkett DJ, Tolbutamide and phenytoin hydroxylations by cDNA-expressed human liver cytochrome P4502C9. *Biochem Biophys Res Commun* **175:** 1112–1118, 1991.
7. Dehal SS and Kupfer D, CYP2D6 catalyzes tamoxifen 4-hydroxylation in human liver. *Cancer Res* **57:** 3402–3406, 1997.
8. Sonnichsen DS, Liu Q, Schuetz EG, Schuetz JD, Pappo A and Relling MV. Variability in human cytochrome P450 paclitaxel metabolism. *J Pharmacol Exp Ther* **275:** 566–575, 1995.
9. Smith DA and Jones BC, Speculations on the substrate

[¶¶] Stresser DM and Kupfer D, unpublished observations.

structure–activity relationship (SSAR) of cytochrome P450 enzymes. *Biochem Pharmacol* **44:** 2089–2098, 1992.
10. Stresser DM and Kupfer D, Catalytic characteristics of CYP3A4: Requirement for a phenolic function in *ortho* hydroxylation of estradiol and mono-*O*-demethylated methoxychlor. *Biochemistry* **36:** 2203–2210, 1997.
11. Rodrigues AD, Kukulka MJ, Surber BW, Thomas SB, Uchic JT, Rotert GA, Michel G, Thome-Kromer B and Machinist JM, Measurement of liver microsomal cytochrome P450 (CYP2D6) activity using [*O-methyl*-^{14}C]dextromethorphan. *Anal Biochem* **219:** 309–320, 1994.
12. Goldstein JA and de Morais SMF, Biochemistry and molecular biology of the human CYP2C subfamily. *Pharmacogenetics* **4:** 285–299, 1994.
13. Bulger WH, Feil VJ and Kupfer D, Role of hepatic monooxygenases in generating estrogenic metabolites from methoxychlor and from its identified contaminants. *Mol Pharmacol* **27:** 115–124, 1985.
14. Food and Drug Administration, *Adverse Reaction Report*. June 5, 1992.
15. Stresser DM, Dehal SS and Kupfer D, Ring hydroxylation of [*o*-^{3}H]methoxychlor as a probe for liver microsomal CYP2B activity: Potential for *in vivo* CYP2B assay. *Anal Biochem* **233:** 100–107, 1996.
16. Burstein SH and Kupfer D, Hydroxylation of *trans*-Δ^{1}-tetrahydrocannabinol by hepatic microsomal oxygenase. *Ann NY Acad Sci* **191:** 61–67, 1971.
17. Dehal SS and Kupfer D, Metabolism of the proestrogenic pesticide methoxychlor by hepatic P450 monooxygenases in rats and humans. Dual pathways involving novel *ortho* ring-hydroxylation by CYP2B. *Drug Metab Dispos* **22:** 937–946, 1994.
18. Kupfer D, Miranda GK and Bulger WH, A facile assay for 2-hydroxylation of estradiol by liver microsomes. *Anal Biochem* **116:** 27–34, 1981.
19. Newton DJ, Wang RW and Lu AY, Cytochrome P450 inhibitors. Evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metab Dispos* **23:** 154–158, 1995.
20. Crespi CL, Miller VP and Penman BW, Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. *Anal Biochem* **248:** 188–190, 1997.
21. Draper AJ, Madan A and Parkinson A, Inhibition of coumarin 7-hydroxylase activity in human liver microsomes. *Arch Biochem Biophys* **341:** 47–61, 1997.
22. Bulger WH, Muccitelli RM and Kupfer D, Studies on the *in vivo* and *in vitro* estrogenic activities of methoxychlor and its metabolites. Role of hepatic mono-oxygenase in methoxychlor activation. *Biochem Pharmacol* **27:** 2417–2423, 1978.
23. Kupfer D, Bulger WH and Theoharides AD, Metabolism of methoxychlor by hepatic P-450 monooxygenases in rat and human. 1. Characterization of a novel catechol metabolite. *Chem Res Toxicol* **3:** 8–16, 1990.
24. Kerlan V, Dreano Y, Bercovici JP, Beaune PH, Floch HH and Berthou F, Nature of cytochromes P450 involved in the 2-/4-hydroxylations of estradiol in human liver microsomes. *Biochem Pharmacol* **44:** 1745–1756, 1992.
25. Yamazaki H, Gillam EMJ, Dong M-S, Johnson WW, Guengerich FP and Shimada T, Reconstitution of recombinant cytochrome P450 2C10(2C9) and comparison with cytochrome P450 3A4 and other forms: Effects of cytochrome P450–P450 and cytochrome P450-b_5 interactions. *Arch Biochem Biophys* **342:** 329–337, 1997.
26. Backes WL and Eyer CS, Cytochrome P-450 LM2 reduction. Substrate effects on the rate of reductase-LM2 association. *J Biol Chem* **264:** 6252–6259, 1989.
27. Sugiyama K, Nagata K, Gillette JR and Darbyshire JF, Theoretical kinetics of sequential metabolism *in vitro*. Study of the formation of 16α-hydroxyandrostenedione from testosterone by purified rat P450 2C11. *Drug Metab Dispos* **22:** 584–591, 1994.
28. Xu BQ, Aasmundstad TA, Christophersen AS, Mørland J and Bjørneboe A, Evidence for CYP2D1-mediated primary and secondary *O*-dealkylation of ethylmorphine and codeine in rat liver microsomes. *Biochem Pharmacol* **53:** 603–609, 1997.