Catalytic Characteristics of CYP3A4: Requirement for a Phenolic Function in *ortho* Hydroxylation of Estradiol and Mono-*O*-demethylated Methoxychlor[†]

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ABSTRACT: CYP3A4 is the major human cytochrome P-450 in a superfamily of heme-thiolate proteins that catalyze the oxidation of numerous lipophilic compounds. In this investigation, we report that CYP3A4 requires a phenolic function for *ortho* hydroxylation of estradiol and mono-*O*-demethylated methoxychlor and that CYP3A4 aromatic hydroxylation in general may be dependent on the presence of a free phenolic group. Indeed, when methoxyls were present instead of phenolic hydroxyls, CYP3A4 essentially failed to catalyze *ortho* hydroxylation. By contrast, of eight additional cDNA-expressed P-450s (CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2D6, and 2E1) examined, only CYP1A2 and CYP2B6 could catalyze *ortho* hydroxylation of [*o*-^{3}H]methoxychlor (7.2 and 14.6 pmol/90 min/pmol P-450, respectively), indicating that these isoforms do not require a phenolic hydroxyl for aromatic hydroxylation and that methoxyls do not sterically hinder catalysis by these CYPs. However, with [*o*-^{3}H]mono-*O*-demethylated methoxychlor, containing a phenolic group, five isoforms (CYP1A2, 2B6, 2D6, 2E1, and 3A4) supported *ortho* hydroxylation. Of these, CYP3A4 exhibited by far the highest rate of hydroxylation at 87.8 pmol/90 min/pmol P-450. Further studies with [2-^{3}H]estradiol 3-methyl ether and with [2-^{3}H]estradiol revealed a similar and dramatic augmentation of CYP3A4-mediated C_2 hydroxylase activity of approximately 75-fold by the presence of the phenolic group in the 3-position. The mechanism of augmentation by the phenolic hydroxyl does not appear to involve the acidic proton of estradiol, since CYP3A4-catalyzed estradiol 2-hydroxylation and testosterone 6-β-hydroxylation were diminished to an equal extent when incubations were performed at increasing buffer pH values from 7 to 9. Both estradiol and its 3-methoxy derivative bound with similar affinity to cDNA-expressed, microsomal CYP3A4: spectral dissociation constants were 270 and 370 μM, respectively, and both compounds exhibited type I spectra. Thus, the disparities in aromatic hydroxylation rates between compounds containing phenolic hydroxyls and those with methoxyls cannot be explained by differences in their binding affinities. To explain the mode via which the phenolic hydroxyl facilitates *ortho* hydroxylation, a mechanism in which the phenolic moiety attacks the iron−oxo double bond of CYP3A4, resulting in oxygen transfer to the *ortho* position, is proposed. It is anticipated that these findings will assist in forecasting the CYP-mediated metabolic fate of phenolic compounds.

The P-450 cytochromes (EC 1.14.14.1, CYP[1]) are membrane-bound heme-thiolate proteins that catalyze the oxidation of numerous, structurally diverse compounds. In mammals, there are 14 CYP gene families and 26 subfamilies currently known (Nelson et al., 1996). The enzymes CYP1A2, 2A6, 2B6, 2C, 2D6, 2E1, and 3A4 are believed responsible for the majority of endo- and xenobiotic oxidations in livers of adult humans (Shimada et al., 1994). In recent years, there has been an explosion of information describing human CYP isoform catalyzed metabolism of various compounds. This has permitted the assignment of characteristic substrates and reactions to certain P-450s. For example, most substrates of CYP2C9 and 2D6 appear to be restricted to a certain size range (Jones et al., 1996; Mancy et al., 1995; Smith & Jones, 1992) suggesting that there is a spatial component to their substrate specificity. By contrast, CYP3A4, the major human P-450, can accommodate substrates that are highly diverse in structure and size, such as cyclosporin A (a cyclic undecapeptide) and acetaminophen, with molecular weights of 1203 and 151, respectively. A flexible binding site (Koley et al., 1995) and hydrophobic interactions (Smith & Jones, 1992) have been suggested to dictate access to the sites of CYP3A4 catalysis. Upon examination of the reactions catalyzed by CYP3A4 (Table 1), it appears that certain types of *reactions* (e.g., *N*-demethylation, allylic hydroxylation, etc), rather than *substrates* (e.g., size or charge requirements), are the overriding themes of CYP3A4 catalysis (Smith & Jones, 1992).

Previously, this laboratory identified CYP2B1/2-mediated ring-hydroxylation of methoxychlor *ortho* to the methoxyls followed by *O*-demethylation as an alternate metabolic pathway leading to catechol formation in liver microsomes from phenobarbital-treated rats (Dehal & Kupfer, 1994). This finding allowed the development of a convenient tritium

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[1] Abbreviations and common names used: CYP, cytochrome P-450; OR, NADPH oxidoreductase (P-450 reductase); COMT, catechol-*O*-methyl transferase; SAM, (*S*)-adenosyl-L-methionine; methoxychlor, 1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane; bis-OH-M, 1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane; mono-OH-M, 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane; TLC, thin layer chromatography.

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Table 1: Selected Reactions Catalyzed by CYP3A4

reaction	substrate	reference
N-dealkylation	lidocaine, erythromycin, verapamil, tamoxifen	Imaoka et al., 1990; Smith & Jones, 1992; Mani et al., 1993
methyl hydroxylation	midazolam	Smith & Jones, 1992
aromatic hydroxylation (catechol formation)	ethynyl estradiol, estradiol	Smith & Jones, 1992; Aoyama et al., 1990
hydroxylation of proline ring	dihydroergotamine	Smith & Jones, 1992
C-hydroxylation	diazepam, ifosfamide	Chang et al., 1993; Walker et al., 1994
N-oxidation	quinidine, senecionine, acetaminophen	Guengerich et al., 1986; Miranda et al., 1991; Thummel et al., 1993
O-demethylation	FK-506, rapamycin, epipodophyllotoxins	Vincent et al., 1992; Sattler et al., 1992; Relling et al., 1994
aromatic hydroxylation	paclitaxel (Taxol)	Sonnichsen et al., 1995
nitroreduction	clonazepam	Seree et al., 1993
N-hydroxylation	dapsone	Fleming et al., 1992
benzylic hydroxylation	salmetrol	Manchee et al., 1996
hydroxylation α to carbonyl	(*R*)-warfarin	Brian et al., 1990
aromatization, 4-dealkylation	substituted diydropyridines	Bocker & Guengerich, 1986
allylic hydroxylation	cyclosporin, quinidine, tetrahydrocannabinol, lovastatin, steroids	Smith & Jones, 1992

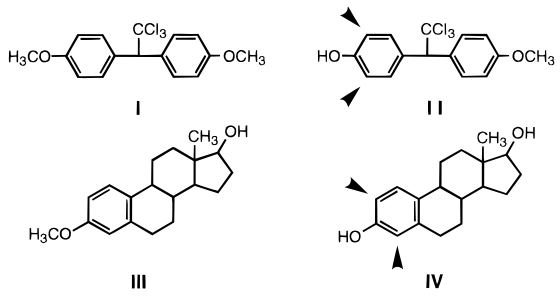


FIGURE 1: Structures of methoxychlor (**I**), mono-OH-M (**II**), estradiol 3-methyl ether (**III**), and estradiol (**IV**). The sites of CYP3A4-catalyzed metabolism are indicated by arrowheads.

release assay for CYP2B1/2 hydroxylation activity employing [*o*-^{3}H]methoxychlor as a substrate (Stresser et al., 1996). Further exploration with [*o*-^{3}H]methoxychlor and [*o*-^{3}H]-mono-demethylated methoxychlor (mono-OH-M) as substrates revealed that only the latter was a substrate for cDNA-expressed CYP3A4. Subsequent investigation along these lines demonstrated that a similar "substrate activation" by a phenolic group occurred with estradiol, when compared with its 3-methoxy derivative. The structures of these four compounds are shown in Figure 1. In this investigation, we report that the presence of a phenolic hydroxyl imparts *o*-hydroxylase capability to CYP3A4, and in fact, may be required for CYP3A4 aromatic hydroxylation.

MATERIALS AND METHODS

Materials. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADPH, EDTA disodium salt, estradiol, estradiol 3-methyl ether, COMT, SAM iodide salt, and activated charcoal (untreated powder) were purchased from Sigma Chemical Co., (St. Louis, MO). Dextran was from Schwarz/Mann (Orangeberg, NY). Dithiothreitol (DTT) was purchased from Calbiochem (La Jolla, CA). Ultima-Gold, a biodegradable scintillation cocktail, was obtained from Packard Instrument Co., (Meriden, CT). Bis-OH-M was kindly provided by Dr. T. Fujita (Kyoto University, Japan) and Dr. J. Sanborn (Illinois Natural History Survey, Urbana, IL). Dimethylsulfate was from Aldrich Chemical Co. (Milwaukee, WI). Methoxychlor was purchased from Chem Service (West Chester, PA). [*ring*-UL-^{14}C]methoxychlor was obtained from Sigma (5.85 mCi/mmol) or California Bionuclear (1.8 mCi/mmol), La Brea, CA. [2-^{3}H]estradiol (22.0 Ci/mmol), [4-^{14}C]estradiol (55.5 mCi/mmol), [^{14}C]-testosterone (57.3 mCi/mmol), and [methyl-^{3}H]-SAM (15 Ci/mmol) were obtained from Du Pont NEN Research Products (Boston, MA). 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 Chemsym Science Laboratories (Lenexa, KS). 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 (Stresser et al., 1996). Final specific activities of the tritium-labeled substrates were 15 000−40 000 dpm/nmol, whereas those of ^{14}C-labeled substrates were 3200−4000 dpm/nmol. Microsomes from human lymphoblastoid cell

lines engineered to express single human CYP isoforms, or from the same cell line transfected with the vector lacking human CYP cDNA (used as controls), were purchased from Gentest, Inc. (Woburn, MA). Microsomes containing CYP2C9 that have 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 that overexpress human CYP3A4 cDNA were purchased from Gentest. Reversed-phase (C_{18}) and normal-phase TLC plates were purchased from Whatman, Inc. (Clifton, NJ).

Preparation of [2-^{3}H]estradiol 3-Methyl Ether, [^{14}C]-estradiol 3-Methyl Ether, [o-^{3}H]mono-OH-M and Radioinert Mono-OH-M. [2-^{3}H]estradiol 3-methyl ether and [4-^{14}C]-estradiol 3-methyl ether were prepared by a phenolic methylation procedure (Mitscher et al., 1985). To carrier free [2-^{3}H]estradiol (20−40 μCi) or [^{14}C]estradiol in 500 μL of anhydrous acetone was added 20−30 mg of potassium carbonate and dimethylsulfate [67 mg (50 μL)]. The mixture was then stirred overnight at ambient temperature. The reaction was quenched by the addition of 1.0 mL of water, and the resulting mixture was extracted with dichloromethane (3 × 1.0 mL). The organic phase was evaporated at ambient temperature under a stream of nitrogen, and the residue was dissolved in ethanol and stored at 4 °C until TLC analysis (normal phase, 9:1 chloroform:acetone, v/v). The abatement of a peak representing [2-^{3}H]estradiol coincided with the appearance of a radiolabeled peak having identical R_f as authentic estradiol 3-methyl ether as determined by radioscanning using a System 200 Imaging Scanner (Bioscan, Inc., Washington, DC). The methylated product was resolved from unreacted [2-^{3}H]estradiol and from a product less polar than estradiol 3-methyl ether by TLC and collected by ethanol elution of the appropriate zone. The identity of [2-^{3}H]- and [4-^{14}C]estradiol 3-methyl ether was confirmed using both normal (9:1 chloroform:acetone) and reversed phase (90:9:1 methanol:water:acetic acid) TLC by comparison with R_f values of authentic, radioinert estradiol 3-methyl ether. [*o*-^{3}H]mono-OH-M was prepared from approximately 50 μCi [*o*-^{3}H]bis-OH-M (sp act. 40 000 dpm/nmol) in a similar manner, except the reaction was terminated after 2 h to preclude complete methylation. [*o*-^{3}H]mono-OH-M was separated from unreacted [*o*-^{3}H]bis-OH-M and [*o*-^{3}H]-methoxychlor by TLC (normal phase, 9:1 chloroform:acetone v/v) and isolated by ethanol elution of the appropriate zone. Radioinert mono-OH-M was prepared in a similar manner starting with 17.9 mg of bis-OH-M dissolved in 1 mL of acetone. Structural identity was confirmed by comparison with R_f values of authentic mono-OH-M as previously described (Dehal & Kupfer, 1994).

Incubation of Microsomes Containing cDNA Expressed Human P-450s with [o-^{3}H]mono-OH-M, [o-^{3}H]methoxychlor, [2-^{3}H]estradiol, and [2-^{3}H]estradiol 3-Methyl Ether. To determine *ortho* hydroxylation, incubations containing 125 μg of microsomal protein were conducted for 20 or 90 min in the presence of NADPH in a volume of 250 μL as described previously (Kupfer et al., 1981), with minor modifications (Stresser et al., 1996). Incubations were terminated by the addition of 250 μL of 0.016 M $CaCl_2$ to aggregate and precipitate microsomes and 250 μL of dextran coated charcoal solution to adsorb unreacted substrate and organic metabolites followed by centrifugation. The extent of hydroxylation was then calculated by quantifying the release of ^3H_2O into the medium using scintillation spectrometry (Tri-carb 2250CA scintillation counter, Packard, Meriden, CT). The programmed transformed index of the external standard (tSIE) method was used to correct for sample quenching and disintegrations per minute determination. When CYP2A6 or 2C9 was used, phosphate was replaced with Tris in the incubation buffer, as recommended by the supplier.

Incubation of Microsomes Containing cDNA Expressed Human P-450s with [ring-^{14}C]methoxychlor and [4-^{14}C]-estradiol 3-methyl ether. To determine demethylation, incubations were performed by a modification of a method previously described (Dehal & Kupfer, 1994). Incubations were conducted in 1 dram glass vials containing the following constituents: 150 μL (15 μmol) of sodium phosphate (or Tris, when CYP2A6 or 2C9 was used) buffer (pH 7.4); 25 μL (2.5 μmol) of magnesium chloride; microsomal suspension [125 to 250 μg of protein in 12.5−25 μL of 0.1 M phosphate (or Tris) buffer]; [^{14}C]substrate (2.5−6.25 nmol, 8000−25000 dpm) added in 2.5 μl of ethanol; 25−37.5 μl of 1.15% potassium chloride; and EDTA (1 μmol) in a final volume of 250 μL. After a preincubation period of 2 min at 37 °C, the reaction was initiated by the addition of an NADPH regenerating system [glucose 6-phosphate (2.5 μmol), NADPH (0.125 μmol), and glucose-6-phosphate dehydrogenase (0.5 IU) in 25 μL H_2O] and the vials were incubated at 37 °C in a water bath for 20, 90, or 120 min. The reaction was terminated by the addition of 1 mL of ice-cold ethanol, and the contents were centrifuged at full speed in a tabletop centrifuge to pellet the precipitate. The supernatant (containing metabolites) was transferred to glass test tubes. The pellet was washed with an additional of 1 mL of ethanol, which was combined with the supernatant from the centrifugation step. The ethanolic mixture was evaporated under a stream of nitrogen and the residue was resuspended in 50 μL of ethanol. A portion of the resuspended metabolites was analyzed by TLC (normal phase, 9:1, v/v, chloroform:acetone, or in addition, for methxoychlor metabolites, reverse phase, 75:24:1, v/v, methanol, H_2O, acetic acid). After developing and drying the plates, the chromatogram was subjected to quantitation by radioscanning, as described above.

Incubation of Microsomes Containing cDNA Expressed Human P-450s with [^{14}C]testosterone. The specific activity of [^{14}C]testosterone was adjusted to 2080 dpm/nmol. Incubations were performed with the testosterone preparation at 120 μM in 1.5 mL polypropylene tubes containing identical incubation mixture as in the estradiol 2-hydroxylation assay. After 20 min at 37 °C, the reaction was terminated by placing the vials on ice and by addition of 1 mL of methylene chloride. Vials were shaken vigorously (Vortexer II genie, VWR) and centrifuged to separate the phases. The organic layer was transferred to glass test tubes and an additional 1 mL of methylene chloride was added to the aqueous layer and the extraction process repeated. The combined extracts were evaporated under a stream of nitrogen and the residue was resuspended in 50 μL of ethanol. A portion of the resuspended material was applied to a normal-phase TLC plate and 6-β-hydroxylation of testosterone was determined as described previously (Li et al., 1993).

Determination of Catechol Formation. Incubations to determine catechol formation from mono-OH-M or estradiol

contained the following constituents: 125 μL (12.5 μmol) of sodium phosphate buffer (pH 7.4); 25 μL (2.5 μmol) of magnesium chloride; microsomal suspension (125 μg of protein in 12.5 μL of 0.1 M 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 7.7 μL of 20% ethanol); EDTA (1 μmol); 6.25 units of COMT; 17.5 nmol of DTT, 12.5 nmol of ascorbic acid. After a preincubation period of 2 min at 37 °C, the reaction was initiated by the addition of an NADPH regenerating system [glucose 6-phosphate (2.5 μmol), NADPH (0.125 μmol); and glucose-6-phosphate dehydrogenase (0.5 IU)] in 25 μL of H_2O and the vials were incubated at 37 °C in a water bath for 90 min. The reaction was terminated by placing vials on ice and adding 1 mL of hexane. The contents of the vials were mixed briefly (Vortexer II genie, VWR), and the phases were allowed to separate. The hexane layer containing mono-[^{3}H]methylated catechol was transferred to separate vials. The samples were extracted with hexane a second time, the combined extracts were evaporated under a stream of nitrogen, and the residue was suspended in 100 μL of ethanol. Fifty microliters of the ethanolic suspension was mixed with 4 mL of Ultima-gold scintillation cocktail (Packard), and the radioactivity (disintegrations per minute) was determined by scintillation spectroscopy.

Determination of Spectral Dissociation Constant. The spectral interactions of estradiol or estradiol 3-methyl ether with microsomes from baculovirus infected insect cells that overexpress human CYP3A4 cDNA was determined by the method of Schenkman et al. (Schenkman et al., 1967). Ethanol and a ligand dissolved in ethanol were added in increasing amounts to the reference and sample cuvettes, respectively, that contained 500 pmol/mL CYP3A4 in 0.1 M Tris buffer, pH 7.4. After subtracting the absorbance contributed by the reference cuvette, the absorbance difference between 390 and 420 nm in the sample cuvette was determined and used to calculate a spectral dissociation constant (K_s).

RESULTS AND DISCUSSION

ortho Hydroxylation of [o-^{3}H]methoxychlor and [o-^{3}H]-mono-OH-M. Incubations of [*o*-^{3}H]methoxychlor in the presence of microsomes from lymphoblastoid cells engineered to express single human CYP isoforms demonstrated that only CYP1A2 and 2B6 of the nine isoforms examined, exhibited substantial (at least 2-fold higher)[2] *o*-hydroxylase activity compared with that native to the cell line transfected with the vector only (Table 2). However, when [*o*-^{3}H]mono-OH-M was employed as a substrate, *ortho* hydroxylation was observed with five isoforms. By far, the highest activity was exhibited by microsomes from cells expressing cDNAs for both CYP3A4 and NADPH P-450 oxidoreductase (OR). This exceeded by approximately 4-fold the activity of CYP2B6 and by 8-fold the activity of CYP3A4, each of which contained endogenous OR but lacked the supplemented, coexpressed OR. Whether CYP2B6 (and 1A2) would exhibit comparable enhancement in activity as a result of coexpresion of OR could not be determined as such cell lines, to our knowledge, have not been hitherto engineered.

[2] The activity of at least 2-fold higher than in control microsomes (containing the vector but lacking CYP transfects) was arbitrarily assumed as being significant, because of the occasional variability in activity of up to 150% observed in control microsomes.

Table 2: *O*-ring Hydroxylase Activity of Various cDNA-Expressed Human CYPs

	substrate			
	Methoxychlor (pmol/90 min/pmol P450)		Mono-OH-M (pmol/90 min/pmol P450)	
microsomes[a]	mean	SD	mean	SD
1A2	7.2	0.7	9.4[b]	1.6
2B6	14.6	2.2	21.0[b]	4.8
2D6 + OR[c]	−	−	1.2	0.1
2E1 + OR	−	−	1.4	0.2
3A4	−	−	11.4	1.9
3A4 + OR	−	−	87.8	2.4

[a] In addition to those indicated by dash marks, other microsomes tested that also *failed* to exhibit at least 2-fold higher activity than control microsomes were from cell lines engineered to express CYP1A1, CYP2A6, CYP2A6 + OR, CYP2C8, CYP2C9−Cys + OR, or OR only. [b] CYP1A2 and 2B6 can hydroxylate *ortho* to methoxyl groups (as shown with methoxychlor as substrate) and *ortho* to hydroxyl groups as shown with bis-OH-M as substrate (D. M. Stresser and D. Kupfer, unpublished data). These values were calculated assuming both reactions are equally contributory to total release of tritium. [c] OR = Co-expressed NADPH P450 oxidoreductase.

Does Ring Hydroxylation of [o-^{3}H]mono-OH-M Occur ortho to the Hydroxyl or ortho to the Methoxyl? Since [*o*-^{3}H]-mono-OH-M is tritiated in the two positions *ortho* to the methoxyl and *ortho* to the phenolic hydroxyl group (four total), tritium release could indicate *ortho* hydroxylation on either side of the molecule (Stresser et al., 1996). It was anticipated that if hydroxylation of the radioinert mono-OH-M occurs *ortho* to the free hydroxyl, thus yielding a catechol, then it could be detected radiometrically by COMT-mediated methylation using [^{3}H-methyl]SAM as the methylating agent. Indeed, utilizing this method, we demonstrated that CYP3A4-mediated hydroxylation occurs *ortho* to the phenolic hydroxyl (Table 3). Moreover, *ortho* hydroxylation of mono-OH-M (catechol formation), shown to occur by this method, accounted for 101% of the values obtained by the tritium release assay. These data demonstrate that CYP3A4 catalyzed-ring hydroxylation does not occur significantly, *ortho* to the methoxyl group. Support for this conclusion is derived from our previous observations that CYP3A4 *does not* catalyze hydroxylation *ortho* to methoxyls in methoxychlor (Table 2). As expected, using this method, radioinert estradiol gave rise to marked catechol formation (Table 3), apparently via both 2- and 4-hydroxylation (Kerlan et al., 1992), 2-hydroxylation being the major pathway (Aoyama et al., 1990).

2-Hydroxylation of Estradiol and Estradiol 3-Methyl Ether. To determine whether there is also a requirement for a phenolic hydroxyl in CYP3A4-mediated *ortho* hydroxylation of other substrates, we compared the hydroxylation of estradiol and estradiol 3-methyl ether. In a 90 min incubation with nine different microsomal preparations containing expressed CYPs or allelic variants, only CYP1A2, 2C9-Cys + OR, 2C9-Arg + OR, and CYP3A4 + OR exhibited low or minimal estradiol 3-methyl ether 2-hydroxylase activity that exceeded twice the activity of the control (vector only) microsomes (Table 4). However, in a 90 min incubation with estradiol as substrate, these active preparations, as well as CYP2B6 and CYP3A4, were found to catalyze 2-hydroxylation. With microsomes containing CYPs that catalyze *ortho* hydroxylation of both substrates, the rate of 2-hydroxylation of estradiol exceeded 2-hydrox-

Table 3: CYP3A4-Mediated Catechol Formation from Mono-OH-M or Estradiol

substrate	microsomes[a]	*n*	NADPH	pmol product/90 min mean (SD or range)	pmol product/90 min/pmol P450 mean (SD or range)
mono-OH-M	CYP3A4	4	+	544 (74)	89 (12)
mono-OH-M	Control	4	+	18 (4)	*b*
mono-OH-M	CYP3A4	1	−	13	2
mono-OH-M	Control	1	−	7	*b*
estradiol	CYP3A4	4	+	2061 (244)	336 (40)
estradiol	CYP3A4	2	−	67 (8)	11 (1)
none	CYP3A4	1	+	7[c]	1[c]

[a] CYP3A4 microsomes contained supplemented, co-expressed NADPH P450 oxidoreductase. [b] Contains vector only. Control microsomes do not possess spectrally or immunochemically quantifiable P450, and therefore activity was not normalized to P450 content. [c] Presumably due to contaminating radioactivity from [^{3}H]SAM.

Table 4: 2-Hydroxylation of Estradiol or Estradiol 3-Methyl Ether by Human CYPs

microsomes[a]	estradiol pmol/20 min/pmol P450 mean	estradiol pmol/20 min/pmol P450 range	estradiol pmol/90 min/pmol P450 mean	estradiol pmol/90 min/pmol P450 range	estradiol 3-methyl ether pmol/90 min/pmol P450 mean	estradiol 3-methyl ether pmol/90 min/pmol P450 range	fold stimulation[c]
CYP1A2	0.96	0.03	6.00	0.15	0.85	0.05	7.1
CYP2B6	−[b]	−	1.99	0.03	−	−	
CYP2C9−Cys + OR	−	−	8.03	0.42	0.22	0.04	36.5
CYP2C9−Arg + OR	4.39	0.75	22.50	1.33	2.02	0.27	11.1
CYP3A4	8.61	0.54	27.10	0.76	ND[d]	ND	
CYP3A4 + OR	35.15	0.15	76.56	0.47	1.01	0.03	75.8

[a] Microsomes from cell lines engineered to express CYP1A1, CYP2A6, CYP2A6 + OR, CYP2D6 + OR, and CYP2E1 + OR *did not* exhibit activity of at least 2-fold higher than control (vector only) microsomes, with either substrate. [b] Dash marks (−) indicate that activity with this isoform did not exceed at least twice that observed with control microsomes. [c] Ratio of 2-hydroxylated product formed from estradiol and product formed with estradiol 3-methyl ether in a 90 min incubation. [d] ND = not done.

ylation of estradiol 3-methyl ether by at least 7-fold, and with CYP3A4 + OR, by *75-fold*. The rate of estradiol 2-hydroxylation by CYP3A4 + OR is sublinear over the 90 min, as indicated by the extent of 2-hydroxylation in a 20 min incubation (Table 4). Thus, a 75-fold potentiation in the 2-hydroxylase activity due to an adjacent hydroxyl instead of methoxyl substituent represents only submaximal value of enhancement.

The above observation that CYP3A4 has some activity, although minor, toward estradiol 3-methyl ether suggests the possibility that during a 90 min incubation with estradiol 3-methyl ether, some *O*-demethylation has occurred, resulting in estradiol formation. This was examined using [4-^{14}C]-estradiol 3-methyl ether as a substrate. It was found that among microsomal preparations containing nine different human CYPs or allelic variants, only those with CYP1A2 and CYP2C9-Arg exhibited detectable demethylase activity (yielding 21.9 ± 0.7 and 113 ± 31 pmol/90 min/pmol P-450, respectively). Nevertheless, the possibility that CYP3A4 catalyzes a low level of demethylation, below limits of detection, resulting in formation of estradiol and subsequent *ortho* hydroxylation, has not been excluded. Although, the enhancement conferred by the presence of the phenolic hydroxyl is dramatic with CYP3A4, this activity does not appear limited to this isoform, since both CYP2C9 allelic variants and CYP1A2 activities are augmented, although to a markedly lesser extent. In addition, 2-hydroxylation of estradiol by CYP2B6 is observed, whereas with the methyl ether derivative, it cannot be detected.

Is There a Role for the Acidic Proton and/or Ionization of the Phenolic Function in the Activation of Substrate for Aromatic Hydroxylation? Phenolic hydroxyls are strong *ortho/para* directors of electrophilic substitutions; hence, it appeared conceivable that a suitable oxygen species generated by CYP3A4 could be incorporated into the *ortho* position and that phenoxide ions will facilitate such a reaction. To explore the mechanism via which a phenolic group may activate a substrate for CYP3A4-mediated *ortho* hydroxylation, we compared the effect of increasing buffer pH values, from 7.0 to 9.0 on estradiol 2-hydroxylation and on testosterone 6-β-hydroxylation. The pK_a of estradiol is approximately 9.2, while the K_m of human liver microsomal 2-hydroxylation, catalyzed primarily by CYP3A4, has been estimated to be 15−22 μM (Kerlan et al., 1992). Thus, at 25 μM estradiol, the concentration of the phenoxide would approach the K_m near pH 9. If the phenolic hydroxyl or phenoxide ion was intimately involved in substrate-binding or catalysis, it might be expected that rates of estradiol 2-hydroxylation would diverge from the rates of testosterone 6-β-hydroxylation as pH is altered. However, our data demonstrated that estradiol 2-hydroxylation diminished with increasing pH and that a similar extent of diminution occurred with testosterone 6-β-hydroxylation (Figure 2). If hydrogen abstraction was involved in the rate-determining step of 6-β-hydroxylation of testosterone, a somewhat higher rate of hydroxylation at higher pH would have been expected; however, the opposite was the case. Furthermore, since the allylic position of testosterone is not readily ionizable in this pH range, the data indicate that the diminution in activity of CYP3A4-catalyzed reactions is most probably due to pH effects on the enzyme *per se* rather than due to effects on the substrate. Our observations that methoxyl groups, in estradiol 3-methyl ether, mono-OH-M, and methoxychlor, do not significantly support the *ortho* hydroxylation catalysis by CYP3A4, indicate that the electronic effects of these *ortho* directing groups *per se* are not contributory to substrate activation and that anchoring hydrogen bonding of the phenolic hydroxyl may be necessary. Indeed, the participa-

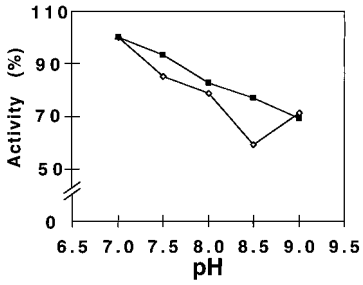


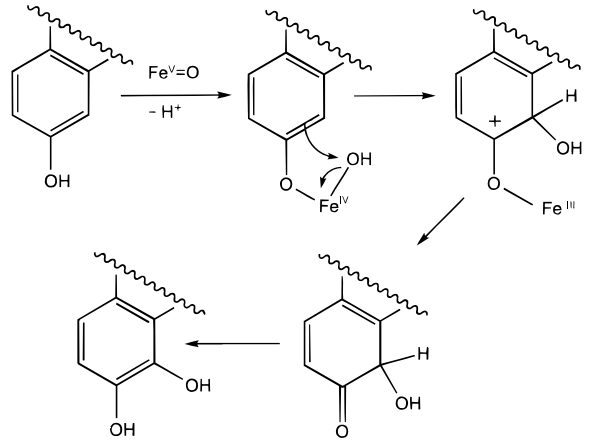
FIGURE 2: Effect of pH on the 2-hydroxylation of estradiol (open diamonds) and on the 6-β-hydroxylation of testosterone (filled squares). The values represent the average activity of duplicate incubations (the value at pH 7.0 was taken as 100%) conducted in 0.1 M phosphate, at the pH indicated. The absolute activity values at pH 7.0 were 44 $\pm$ 3.1 and 247 $\pm$ 28 pmol/20 min/pmol P450, with estradiol and testosterone, respectively.

tion of hydrogen bonding has been suggested in the catalysis of certain other P-450s (Jones et al., 1996; Mancy et al., 1995). Numerous studies have been performed to explain the mechanism of P-450 catalyzed hydroxylations of aliphatic carbons (Atkinson et al., 1994; Newcomb et al., 1995a, 1995b). Some of those studies propose that aliphatic hydroxylations involve hydrogen abstraction by $[FeO]^{3+}$ followed by homolytic substitution by the alkyl radical in the so-called "oxygen rebound" step (Newcomb et al., 1995a). A concerted mechanism has also been proposed (Newcomb et al., 1995b).

By contrast, only little is known about the mechanism of aromatic hydroxylation by P-450s (Guengerich & MacDonald, 1990). There is evidence that naphthalene hydroxylation at C_1 occurs by an initial 1,2-epoxidation, however, this is apparently not the case with most aromatic hydroxylations (Guengerich & MacDonald, 1990). On the basis of the lack of isotope effect between 2-^{3}H and 2-^{1}H in the 2-hydroxylation of estradiol (Kupfer et al., 1981) and in the *ortho* hydroxylation of methoxychlor (Stresser et al., 1996), we propose that hydrogen abstraction in the *ortho* position is not the rate-determining step and hence most probably is not an important step in the *ortho* hydroxylation of phenolic or methoxyl-containing compounds. Additionally, there was no NIH shift (Guroff et al., 1967) in the 2-hydroxylation of estradiol by rat liver microsomes (Kupfer et al., 1981), indicating that the hydroxylation *ortho* to the hydroxyl does not involve a concerted mechanism of insertion of the hydroxyl concomitant with a shift of the C_2-hydrogen to the neighboring C_1-position.

It appears that oxidation of aromatic compounds is highly sensitive to physical factors (substrate-binding and/or orientation) (Guengerich & MacDonald, 1990). To determine whether substrate-binding could explain the dramatic differences in catalytic activity of CYP3A4 toward estradiol and estradiol 3-methyl ether, we examined the spectral interactions (Schenkman et al., 1967) of these compounds with microsomes containing cDNA expressed CYP3A4; both compounds formed type I spectra and the estimated K_s values for estradiol and estradiol 3-methyl ether 270 and 370 μM, respectively. By contrast, human liver microsomes were found to exhibit a low apparent K_m (15−22 μM) toward CYP3A4-mediated 2-hydroxylation of estradiol, suggesting that the spectral and kinetic parameters are highly dissimilar and possibly reflect binding to different sites on the enzyme. Moreover, the relatively small difference in affinities of binding of estradiol and estradiol 3-methyl ether, does not

Scheme 1[a]



[a] Proposed mechanism of phenolic hydroxyl assisted and directed *ortho* hydroxylation by CYP3A4.

explain the "all or none" observations in CYP3A4 catalysis of *ortho* hydroxylation. As CYP3A4 is efficient at catalyzing *N*-dealkylation reactions (Table 1), it is conceivable that in those substrates the amine function is interacting with active site ionic character, allowing approach to the reactive iron−oxo species that results in catalysis. Apparently, hydrophobic arenes may not be readily accessible to this site. However, a phenolic group may interact with the active site akin to an amine or alternatively, and more likely, it may participate in the catalysis by initiating a nucleophilic attack on the iron−oxo double bond, similar to the nucleophilic addition to carbonyls. We speculate that the latter interaction may result in oxygen transfer to the arene via a five-membered cyclic transition state producing a hydroxyketo intermediate that tautomerizes into the catechol (Scheme 1). The ability of CYP3A4 to catalyze either 2- or 4-hydroxylation of estradiol is supportive of the cyclic transition state without invoking a dual mode of orientation of estradiol binding to the heme iron. By contrast, methoxyl groups could not generate such a cyclic transition state and thus would not support the *ortho* hydroxylation.

Whether the hydroxyl moiety *per se* is passively or intimately involved in permitting *ortho* hydroxylation (as speculated mechanisticly in Scheme 1) or whether this reaction is repressed by the presence of the methoxyl group cannot be ascertained from the present data. A number of additional possibilities, beyond that which is presented in Scheme 1, might explain phenolic hydroxyl-assisted or methoxyl-repressed *ortho* hydroxylation. For example, it is conceivable that the methoxyl group may sterically hinder *o*-ring hydroxylation. This possibility seems remote because CYP3A4 is able to accommodate compounds of unusually large size differences (see Table 1). Additionally, although methoxychlor is not metabolized by CYP3A4, it nevertheless binds to the enzyme and inhibits competitively the 6-β-hydroxylation of testosterone (Li et al., 1993). Interestingly, steric hindrance by methoxyls was not indicated in the aroclor-induced rat liver microsomal P-450-catalyzed *ortho* hydroxylation of anisole relative to (unhindered) *para* hydroxylation (Hanzlik et al., 1984); however, in that study no assignment of specific P-450 isoform catalyzing those reactions was undertaken. Additionally, most compounds *lacking* substituents on aromatic rings, and hence having little or no steric hindrance, are not substrates for CYP3A4 aromatic hydroxylation (Smith & Jones, 1992; Dehal et al.,

1996; Nielsen et al., 1996; Imaoka et al., 1990). For instance, the 4-hydroxylation of unsubstituted tamoxifen is not catalyzed by CYP3A4 (Dehal et al., 1996), and only after forming 4-hydroxytamoxifen by another P-450, CYP3A4 can then *ortho* hydroxylate the monohydroxy metabolite to form the 3,4-tamoxifen catechol (S. S. Dehal and D. Kupfer, unpublished results).

The greater lipophilicity conferred by the methoxyl substituent relative to the hydroxyl substituent might be expected to affect aromatic hydroxylation. However, substrates of CYP3A4 tend to be relatively lipophilic (log $D_{7.4}$ values for some CYP3A4 substrates range from 0.4 to 8) compared with substrates for other CYP isoforms (Smith, 1991).[3] Thus, the methoxy compounds might be expected to undergo enhance *ortho* hydroxylation, in contrast to the data. It is possible that the methoxyl group invokes a preferred *O*-demethylation pathway at the expense of *o*-ring hydroxylation. However, lymphoblast microsomes containing expressed CYP3A4 + OR failed to catalyze detectably estradiol 3-methyl ether *O*-demethylation (*vide supra*) or [^{14}C]-methoxychlor *O*-demethylation (data not shown), demonstrating that competition between these two pathways does not occur.

Our cyclic model for *ortho* hydroxylation does not implicate mechanism for potential *para* hydroxylation. However, the compounds we studied are not suitable for exploration of *para* hydroxylation. Thus, to examine whether CYP3A4 could catalyze both *ortho* and *para* hydroxylation, [^{14}C]phenol (that, unlike estradiol or mono-OH-M, has a free *para* position) was incubated with lymphoblast microsomes containing CYP3A4 or CYP2E1. Neither the *p*-hydroxy nor the *o*-hydroxy metabolite was formed by CYP3A4, but, as expected, both were produced by CYP2E1 (data not shown) [also see Guengerich and Shimada (1991)]. The finding that CYP3A4 did not yield the *o*-hydroxy metabolite (catechol) indicates that not all phenolic compounds are hydroxylated in the *ortho* position by CYP3A4. Addition of 100 μM α-naphthoflavone, an activator of CYP3A4, did not facilitate phenol metabolism by this enzyme. It appears that enzyme−substrate interaction at sites distal to the phenolic group (that might be expected to occur with larger substrates) is an additional requirement for CYP3A4 *ortho* hydroxylation of phenolic compounds. An example of CYP3A4 *ortho* hydroxylation, but not of *para* hydroxylation catalysis, is the case with droloxifene, a 3-hydroxy derivative of tamoxifen. This compound, which possesses free *ortho* and *para* positions, was found to be hydroxylated by CYP3A4 in the position *ortho* to the phenolic group (S. S. Dehal and D. Kupfer, unpublished observations), but formation of the *p*-hydroxy metabolite has not been detected or reported, to our knowledge. This indicates that our cyclic model is applicable to *ortho* hydroxylation of another compound as well.

Earlier studies with reconstituted rabbit CYP2B4 (LM_2), NADPH P-450 reductase, and dilauryl phosphatidylcholine demonstrated an almost absolute requirement for the presence of holo-cytochrome b_5 for catalysis of prostaglandin hydroxylation (Vatsis et al., 1982). An essential requirement for cytochrome b_5 for optimal oxidation of testosterone and nifedipine by CYP3A4 was also amply demonstrated (Yamazaki et al., 1996). These findings raised the possibility that the lack of catalysis of *ortho* hydroxylation of methoxy aromatic compounds by CYP3A4 may be due to the absolute requirement for cytochrome b_5, rather than due to the intrinsic inability of CYP3A4 to catalyze that activity. However, microsomes of CYP3A4 cDNA expressed lymphoblastoid cells contain approximately 80 pmol of cytochrome b_5/mg protein (Vaughn Miller, Gentest Corporation, personal communication), indicating that the inability by CYP3A4 to *ortho* hydroxylate methoxy aromatic compounds is not due to a requirement for cytochrome b_5.

CONCLUSION

Earlier studies and our current observations demonstrate the idiosyncratic nature of CYP3A4 catalysis. Several human P-450s, notably 2C9 and 2D6 can hydroxylate "unactivated" carbons, in substrates of relatively restricted size (Jones et al., 1996; Mancy et al., 1995; Smith & Jones, 1992) suggesting that catalysis may involve the "lock and key" phenomenon that is dictated by the fit of the substrate into the active site. By contrast, the preponderance of C-hydroxylations by CYP3A4 occur at *activated* positions, such as allylic and benzylic sites,[4] with little or no restriction by the type of structure and size of the substrate (Smith & Jones, 1992). Recently, it has been demonstrated that CYP3A4 can exist in at least three distinct conformers in baculovirus infected insect cells, each possessing distinct substrate specificities (Koley et al., 1995). This suggests one mechanism that might explain the ability of 3A4 to accept numerous and diversified substrates. Interestingly, whereas certain P-450s (e.g., 2C9 and 2D6) can hydroxylate *aromatic* carbons, CYP3A4 generally cannot catalyze such reactions (Smith & Jones, 1992; Dehal et al., 1996; Nielsen et al., 1996; Imaoka et al. 1990) [note one exception in case of paclitaxel aromatic hydroxylation (Table 1)]. However, 3A4 catalyzes effectively aromatic hydroxylation of larger compounds (e.g., MW > 300) that contain phenolic functions and it is suggested that hydroxylation occurs solely *ortho* to the hydroxyl (Aoyama et al., 1990, and this study). We have proposed a speculative mechanism via which the phenolic groups assist and direct the CYP3A4 hydroxylation toward *ortho* positions. In turn, we believe that the above findings should be helpful in future studies on the mechanism of P-450-mediated aromatic hydroxylation in general.

It is noteworthy that CYP3A4 constitutes approximately 30% of human liver P-450 (Shimada et al., 1994) and is involved in the metabolism of the majority of the drugs in use. Therefore, the knowledge of whether a compound (potential drug) is metabolized by CYP3A4 is important for the development of that drug. When confronted with a series of chemical compounds with similar pharmacological/toxicological profiles, the pharmacologist and the chemist often must select a single compound for clinical development. An important criterion in the selection of a compound involves its rate and mode of metabolism. For instance, in a series of aromatic compounds, it might be advantageous or disadvantageous to select a compound with phenolic function. If the aim is to facilitate the disposition of the

[3] Log $D_{7.4}$ describes the partioning of the compound between octanol and buffer at the specified pH of 7.4, uncorrected for the degree of ionization (Manners et al., 1988).

[4] The energy required for breaking of a simple carbon−hydrogen bond is approximately 420 kJ/mol and only 340−360 kJ/mol for equivalent allylic and benzylic bond (Smith & Jones, 1992).

compound by increasing the polarity of its metabolite, then a compound with a phenolic hydroxyl would be advantageous; alternatively, if it is important to prolong the compound's action by blocking its vulnerability to metabolism by CYP3A, then compounds with phenolic groups should be avoided. Thus, our observations that aromatic hydroxylation by CYP3A4 is markedly facilitated by the presence of a phenolic hydroxyl, if proven universal, may be useful to the development of novel drug entities.

NOTE ADDED IN PROOF

While this manuscript was in review, it was reported that CYP3A4 catalyzes hydroxylation *ortho* to the phenolic group of acetaminophen (Chen et al., 1996).

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