CYP2B6, CYP2D6, and CYP3A4 Catalyze the Primary Oxidative Metabolism of Perhexiline Enantiomers by Human Liver Microsomes

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ABSTRACT:

The cytochrome P450 (P450)-mediated 4-monohydroxylations of the individual enantiomers of the racemic antiangiinal agent perhexiline (PHX) were investigated in human liver microsomes (HLMs) to identify stereoselective differences in metabolism and to determine the contribution of the polymorphic enzyme CYP2D6 and other P450s to the intrinsic clearance of each enantiomer. The cis-, trans1-, and trans2-4-monohydroxylation rates of (+)- and (−)-PHX by human liver microsomes from three extensive metabolizers (EMs), two intermediate metabolizers (IMs), and two poor metabolizers (PMs) of CYP2D6 were measured with a high-performance liquid chromatography assay. P450 isoform-specific inhibitors, monoclonal antibodies directed against P450 isoforms, and recombinantly expressed human P450 enzymes were used to define the P450 isoform profile of PHX 4-monohydroxylations. The total in vitro intrinsic clearance values (mean ± S.D.) of (+)- and (−)-PHX were 1376 ± 330 and 2475 ± 321, 230 ± 225 and 482 ± 437, and 63.4 ± 1.6 and 54.6 ± 1.2 μl/min/mg for the EM, IM, and PM HLMs, respectively. CYP2D6 catalyzes the formation of cis-OH- (+)-PHX and trans1-OH- (+)-PHX from (+)-PHX and cis-OH-(−)-PHX from (−)-PHX with high affinity. CYP2B6 and CYP3A4 each catalyze the trans1- and trans2-4-monohydroxylation of both (+)- and (−)-PHX with low affinity. Both enantiomers of PHX are subject to significant polymorphic metabolism by CYP2D6, although this enzyme exhibits distinct stereoselectivity with respect to the conformation of metabolites and the rate at which they are formed. CYP2B6 and CYP3A4 are minor contributors to the intrinsic P450-mediated hepatic clearance of both enantiomers of PHX, except in CYP2D6 PMs.

Perhexiline [2-(2,2-dicyclohexylethyl)piperidine, PHX] produces significant incremental antiangiinal effects in patients with intractable angina who are unsuitable for surgical treatment and intolerant of or refractory to maximal antiangiinal therapy (Cole et al., 1990). Its use is limited by the potential to cause severe hepatotoxicity and peripheral neuropathy associated with elevated plasma PHX concentrations (Shah et al., 1982; Morgan et al., 1984), although the risk of toxicity is significantly reduced by maintaining total plasma PHX concentrations between 0.15% and 0.60 mg/l (0.54–2.16 μM) (Cole et al., 1990). The confounding factor in treatment with PHX is its dependence on the polymorphic CYP2D6 isoform for hepatic cis-4-monohydroxylation (Cooper et al., 1984) as the principal determinant of clearance (Sallustio et al., 2002), resulting in extreme interindividual pharmacokinetic variability. CYP2D6 poor metabolizers (PMs) are typically maintained within the therapeutic range with a dose of approximately 100 mg of PHX maleate per week, whereas intermediate (IMs), extensive (EMs), and ultra-rapid metabolizers require doses of 100 to 500 mg per day to achieve similar concentrations in plasma (Sallustio et al., 2002).

An investigation of the in vitro enzyme kinetics of PHX 4-monohydroxylation in human liver microsomes (HLMs) determined that it is almost exclusively catalyzed with high affinity by CYP2D6 in EMs, with Km values within the range of therapeutic PHX concentrations in plasma (Sørensen et al., 2003); this is consistent with the nonlinear kinetics observed clinically in EMs (Cooper et al., 1985). The intrinsic clearance of PHX was approximately 100-fold lower in PMs, presumably mediated by P450 isoforms other than CYP2D6 with a much lower affinity for PHX (Sørensen et al., 2003), although these iso-

ABBREVIATIONS: PHX, perhexiline; HLM, human liver microsome; EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer; thio-TEPA, triethylenethiophosphoramide; MAB, monoclonal antibody; fumic, unbound fraction in the microsomal compartment; CDP, bound drug concentration; Cfp, free drug concentration; Bmax, maximal binding capacity; Kd, equilibrium dissociation constant; CCl, total drug concentration; Km, Michaelis-Menten constant; Vmax, maximal reaction rate; V, reaction rate; S, free substrate concentration; Ks, substrate inhibitor constant; OH, hydroxy; QC, quality control; CLint, intrinsic clearance.
forms and the 4-monohydroxy metabolites they produced were not identified.

PHX is formulated as a racemic mixture of (+)- and (−)-enantiomers (Pexsig; Sigma Pharmaceuticals, Clayton, VIC, Australia), and six 4-monohydroxy metabolites are possible, composed of one pair each of cis-, trans1-, and trans2-4-monohydroxy PHX enantiomers (Fig. 1) that coelute chromatographically (Davies et al., 2006b), although their absolute configurations are unknown. Gould et al. (1986) were the first to investigate the pharmacokinetics of the individual enantiomers of PHX. Single 300-mg oral doses of either (+)- or (−)-PHX were administered to eight EMs. The oral clearance of the PHX enantiomer and the area under the curve of the corresponding CYP2D6-dependent cis-4-monohydroxy metabolite were 2.5- and 28-fold greater, respectively, after administration of (−)- than of (+)-PHX. Whereas (+)-PHX was metabolized to cis-OH-(+)-PHX and trans1-1-OH-(+)-PHX at similar rates, (−)-PHX displayed distinct stereoselective metabolism to cis-OH-(−)-PHX. Thus, the authors suggested that (+)-PHX may display a smaller polymorphic effect in its metabolism. A recent investigation into the effect of CYP2D6 on the in vivo disposition of the enantiomers of PHX by this laboratory (Inglis et al., 2006) concluded that both enantiomers display significant polymorphic and saturable metabolism by CYP2D6. Among EM patients, the median oral clearance of (−)-PHX was 1.4-fold greater than that of (+)-PHX, attributable to enantioselective metabolism by CYP2D6. Interestingly, PM patients demonstrated greater enantioselectivity in the oral clearance of (−)- versus (+)-PHX, with a median ratio of 2.3, although the mechanism responsible is unknown.

To date, chiral analytical methods capable of resolving the 4-monohydroxy metabolites of racemic PHX with sufficient sensitivity for pharmacokinetic studies have not been reported. However, this laboratory recently developed an achiral method (Davies et al., 2006b) that is suitable for characterizing the 4-monohydroxy metabolites of the individual enantiomers of PHX. The current study was undertaken to determine the in vitro kinetics of the formation of the 4-monohydroxy metabolites of (+)- and (−)-PHX in HLMs from CYP2D6 EMs, IMs, and PMs, to characterize the P450 isoform profile of these metabolic pathways and to describe any stereochemical differences in metabolism.

**Materials and Methods**

**Materials.** (+)- and (−)-PHX HCl were prepared according to the method described by Davies et al. (2006c). The cis-OH-(±)-PHX reference compound was supplied by Marion Merrell Dow (Kansas City, KS). The trans1- and trans2-OH-(±)-PHX reference compounds were supplied by Sigma Pharmaceuticals. Furafylline and S-mephenytoin were purchased from Ultrafine Chemicals (Manchester, UK). Diethylthiocarbamate, (±)-isoorotic acid Na+, isocitrate dehydrogenase (NADP, type IV), sulfaphenazole, troleandomycin, and coumarin were purchased from Sigma Chemical Co. (St. Louis, MO). Quinidine sulfate and nicotinamide dinucleotide phosphate sodium salt were purchased from Merck (Darmstadt, Germany). Triethylene thiophosphoramide (thio-TEPA) was purchased from Sigma Pharmaceuticals. Monoclonal antibodies raised against human CYP1A2 (MAB-1A2), CYP2D6 (MAB-2D6), CYP2E1 (MAB-2E1), and CYP3A4 (MAB-3A4) and microsomes from human lymphoblastoid cells containing recombinantly expressed CYP2C19, CYP2D6, CYP3A4 or the expression vector without P450 cDNA (control microsomes) were purchased from BD Gentest (Woburn, MA). Recombinantly expressed and purified CYP2B6 from DH5α strain Escherichia coli (Notley et al., 2002) was a gift of Dr. Elizabeth Gillam (Department of Physiology and Pharmacology, School of Biomedical Sciences, University of Queensland, St Lucia, Australia). A monoclonal antibody raised against human CYP2B6 (MAB-2B6) was purchased from Invitrogen Corporation (Carlsbad, CA). All other reagents and chemicals were obtained from commercial sources and were of analytical grade.

**Human Liver Microsomes.** Ethics approval was obtained from the Human Ethics Committee of the Royal Adelaide Hospital to obtain human liver samples (n = 7) during partial hepatectomy from patients who had given written informed consent for their tissue to be used, as reported previously (Hutchinson et al., 2004). Samples were stored at −80°C. The donors all had normal clinical chemistry and hematology measurements before surgery, and all the tissue samples used were normal based on gross morphology. HLMs 18, 21, 24, 31, 36, 39, and 46 (internal code) were prepared by differential centrifugation of liver homogenates and were stored in buffer at −80°C until use (Zanger et al., 1988). The total protein concentration (Lowry et al., 1951) of the HLM preparations ranged from 17.0 to 32.7 mg/ml and the total P450 concentration (Omuraya and Sato, 1964) ranged from 202 to 437 pmol/mg microsomal protein.

Genomic DNA was isolated from liver tissue samples using a QIAamp DNA mini kit, according to the manufacturer’s protocol (QIAGEN Pty. Ltd., Clifton Hill, Australia). CYP2D6 genotyping was performed as previously reported (James et al., 2004; Davies et al., 2006a). HLMs 21, 31, and 46 each carried two functional CYP2D6 alleles (genotypes of CYP2D6*1/*1, *1/*41, and *4/*41 respectively).
and *1/*2D, respectively) and were classified as EMs. HLMs 18 and 36 each carried one functional CYP2D6 allele (genotypes of CYP2D6*1/*4 and *2/*4, respectively) and were classified as IMs. HLMs 24 and 39 carried no functional CYP2D6 alleles (genotypes of CYP2D6*4/*4) and were classified as PMs.

**Microsomal Incubations.** Microsomal incubations were linear with time up to 60 min and with protein concentrations up to 1.0 mg/ml with respect to cis-, trans-1-, and trans-2-4-monohydroxylation of (+)- or (-)-PHX at total concentrations of 0.1 to 100 μM. Accordingly, microsomal incubations were performed in triplicate at 37°C in a shaking water bath for 30 min in 0.1 M phosphate buffer (pH 7.4) containing HLM protein (0.25 mg/ml), (+)- or (-)-PHX HCl (0.1–100 μM), added as a stock dissolved in methanol to produce a final methanol concentration of 0.5% v/v, and an NADPH generating system composed of 1 mM NADP, 5 mM isocitrate, 1 U/ml isocitrate dehydrogenase type IV, and 5 mM MgCl₂. The incubations were stopped by rapid cooling on ice.

**High-Performance Liquid Chromatography Assay of the 4-Monohydroxy Metabolites of (+)- and (-)-PHX.** The formation of cis-, trans-1-, and trans-2-4-monohydroxylation of (+)- or (-)-PHX from (+)- or (-)-PHX in microsomal incubations was determined using a recently developed method (Davies et al., 2006b). The interassay (n = 31) CV and bias were <10% and <5%, respectively, for the 0.4 mg/ml QC samples, <15% and <10%, respectively, for the 0.075 mg/ml QC samples, and <15% for both CV and bias for the 0.015 mg/ml QC samples. There was no chromatographic interference with any of the peaks of interest from endogenous compounds in HLMs, the NADPH generating system, putative chemical inhibitors, monoclonal antibodies, or human lymphoblastoid cells containing recombinantly expressed P450s (data not shown).

**Non-specific Binding of (+)- or (-)-Perhexiline in Microsomal Incubations.** The non-specific binding of (+)- or (-)-PHX to HLMs was determined by equilibrium dialysis using dialysis cells of 4.5-m1 capacity per side and Spectra/Por #4 dialysis membrane with a molecular mass cutoff of 12 to 14 kDa, purchased from Spectrum Medical Industries Inc. (Los Angeles, CA). The sample volume on each side of the cell was 4 ml. The dialysis membrane was prepared according to the manufacturer’s instructions. Equilibrium dialysis was performed for 16 h at 37°C according to the method of McLure et al. (2000), except that the microsomal protein concentration was the same as that for the microsomal incubations (0.25 mg/ml), the final total (+)- or (-)-PHX concentrations were in the range of 1 to 100 μM (added as a stock dissolved in methanol to produce a final methanol concentration of 0.5% v/v), and high- and low-concentration controls, used to establish the 16-h dialysis time necessary to attain equilibrium, contained, respectively, 100 and 5 μM (+)- or (-)-PHX. After dialysis, total and free (+)- and (-)-PHX concentrations were determined by high-performance liquid chromatography (Davies et al., 2006c). Standard curves for microsomes and buffer were linear with r² values of 0.995 to 0.999. Triplicate determinations of three concentration points in the standard curves and of all samples produced coefficients of variation <10%.

For each dialysis cell, the unbound fraction of (+)- or (-)-PHX in the microsomal compartment ([f_unbound]) was calculated as the free drug concentration (concentration in the buffer compartment) divided by the total drug concentration (concentration in the microsomal compartment). These values were entered into iterative unweighted nonlinear least-squares regression analyses using GraphPad Prism v4.02 (GraphPad Software Inc., San Diego, CA) for

\[
C_u = (B_{max} + C_P)(K_D + C_P)
\]

where \(C_u\) is the concentration of drug bound, \(C_P\) is the free drug concentration, \(B_{max}\) is the maximal binding capacity, and \(K_D\) is the equilibrium dissociation constant, equivalent to the free drug concentration at which binding is half its maximum.

**Kinetic Analyses of (+)- and (-)-PHX Metabolism in Microsomal Incubations.** To simulate the effects of non-specific binding on in vitro kinetics, the free (+)- and (-)-PHX concentrations in the microsomal incubations were calculated using the \(B_{max}\) and \(K_D\) values derived from the non-specific binding study to solve eq. 1 for the free substrate concentration (\(C_P\)) corresponding to each total substrate concentration (\(C_u\)), where \(C_u = C_P + C_P\) (McLure et al., 2000).

The rates of cis-, trans-1-, and trans-2-4-monohydroxylation of (+)- and (-)-PHX were expressed as pmol/min/mg microsomal protein. Initial estimates of \(K_m\) and \(V_{max}\) were obtained from Eadie-Hofstee (\(V/\sqrt{S}\) versus \(V\)) plots. These values were entered into iterative unweighted nonlinear least-squares regression analyses using GraphPad Prism v4.02 for one- and two-enzyme Michaelis-Menten models, both with and without simple uncompetitive substrate inhibition (Tracy and Hummel, 2004), as follows:

**one-enzyme model**

\[
V = \frac{V_{max} \cdot S}{K_m + S} + S
\]

(2)

**two-enzyme model**

\[
V = \frac{V_{max} \cdot S}{(K_m + S) + (S/H)}
\]

(3)

**two-enzyme model with competitive substrate inhibition**

\[
V = \frac{V_{max} \cdot S}{(K_m + S) + (S/H)}
\]

(4)

**Chemical Inhibition Studies.** HLMs from three CYP2D6 EMs (numbers 21, 31, and 46) and one CYP2D6 IM (number 18) were incubated in triplicate with the NADPH regenerating system and a selection of P450 isofrom-specific chemical inhibitors. Free substrate concentrations approximated the apparent \(K_m\) values estimated for each pathway in each HLM. The P450 isofrom-specific chemical inhibitors (Baldwin et al., 1995; Newton et al., 1995; Tucker et al., 2001; Rae et al., 2002) used (P450 isoform and final microsomal incubation concentration) were quinidine (CYP2D6, 1 μM), coumarin (CYP2A6, 100 μM), diethyldithiocarbamate (CYP2E1, 10 μM), and thio-TEPA (CYP2B6, 50 μM), each dissolved in water; S-mephenytoin (CYP2C19, 100 μM) and tolledamycin (CYP3A4, 10 μM), each dissolved in 0.5% methanol; furafylline (CYP1A2, 100 μM) dissolved in 0.1% acetonitrile; and sulfaphenazole (CYP2C9, 100 μM) dissolved in 0.83% dimethyl sulfoxide. Where inhibitor stocks were made using organic solvents, incubations containing equivalent solvent concentrations were used as controls. The incubation conditions were not altered from the kinetic analyses, except that the mechanism-based inhibitors diethyldithiocarbamate, thio-TEPA, furafylline, and tolledamycin were preincubated for 20 min at 37°C with all incubation constituents before initiating the reaction by adding the substrate. Control incubations were similarly preincubated, but in the absence of the inhibitor.

The results of the IM and the three EM HLMs were grouped to assess the significance of inhibition using one-way paired t tests. Statistical significance was set at \(P < 0.05\) and all the data were expressed as the mean ± S.D. Changes in activity of 15% or less from the controls were not considered to be significant. Based upon the interpretation of these results, one PM HLM (number 24) was similarly incubated with the inhibitors quinidine, coumarin, tolledamycin, and thio-TEPA.

**Inhibition by Monoclonal Antibodies.** HLMs from a CYP2D6 EM (number 31) were incubated in duplicate with the NADPH regenerating system and a selection of monoclonal antibodies directed toward CYP1A2 (MAB-1A2), CYP2B6 (MAB-2B6), CYP2D6 (MAB-2D6), CYP2E1 (MAB-2E1), and CYP3A4 (MAB-3A4). The concentration of antibodies was in accordance with the manufacturers’ instructions. Free substrate concentrations approximated the apparent \(K_m\) values estimated for each pathway. The incubation conditions were not altered from the kinetic analyses, except that the HLMs and antibodies were preincubated for 15 min on ice, before addition of the remaining
incubation constituents. Control incubations were similarly preincubated, but without antibodies. Changes in activity of 15% or less from the controls were not considered to be significant.

**Metabolism by Recombinantly Expressed Human P450 Isoforms.** Microsomes containing recombinantly expressed human CYP2B6, CYP2D6, CYP3A4, or expression vector without P450 cDNA were incubated in duplicate with the NADPH regenerating system and 10 μM free (+)- or (-)-PHX to qualitatively determine metabolite formation. Incubation conditions were the same as in the kinetic study, except that the microsomes were kept on ice until added to the reaction mixture. The protein concentration used was the same as in the kinetic studies (0.25 mg/ml).

**Metabolism by Recombinantly Expressed Human CYP3A4 with 100 μM Coumarin.** Microsomes containing recombinantly expressed human CYP3A4 were incubated in duplicate with the NADPH regenerating system and 1 μM free (+)- or (-)-PHX, with or without coumarin (final coumarin concentration of 100 μM), to detect a change in metabolite formation caused by the presence of coumarin. Incubation conditions were the same as in the kinetic study, except that the microsomes were kept on ice until added to the reaction mixture. The protein concentration used was the same as in the kinetic studies (0.25 mg/ml).

**Results**

**Nonspecific Binding of (+)- and (-)-Perhexiline in Microsomal Incubations.** (+)- and (-)-PHX were extensively bound to the microsomal membrane. Binding was saturable, with $B_{max}$ and $K_D$ values (±S.E.) of 54.6 ± 3.5 and 3.6 ± 0.7 μM, respectively, for (+)-PHX, and 54.3 ± 5.0 and 4.8 ± 1.1 μM, respectively, for (-)-PHX (Fig. 2). Over the range of total (+)- and (-)-PHX concentrations used in the HLM studies (0.1–100 μM), the $f_{unbound}$ ranged from approximately 0.07 to 0.5.

**Kinetic Analyses.** The Eadie-Hofstee plots generally displayed an inflection in the upper quadrant (data not shown), indicative of substrate inhibition, and most of the kinetic data best fitted a model incorporating substrate inhibition (Table 1). All the data best fitted single-enzyme models, except for the formation of trans1-OH- (+)-PHX by the three EM HLMs and one of the IM HLMs (number 18).

In these instances, the Eadie-Hofstee plots were biphasic (data not shown), indicating the involvement of a high-affinity and a low-affinity process (Table 1, trans1 section). Representative cis-, trans1-, and trans2-4-monohydroxylation formation rates from (+)- or (-)-PHX versus free substrate concentration curves are shown for an EM, IM, and PM HLM (Fig. 3).

**cis-4-Monohydroxylation.** cis-OH- (+)-PHX and cis-OH- (-)-PHX concentrations were below the limit of quantification in incubations with PM HLMs. For the EM and IM HLMs, cis-4-monohydroxylation of both PHX enantiomers was mediated by a high-affinity enzyme [(+)-PHX: $K_m$ (mean ± S.D.) 0.060 ± 0.067 μM, range 0.024–0.160 μM; (-)-PHX: $K_m$ 0.057 ± 0.028 μM, range 0.032–0.104 μM], except for the formation of cis-OH- (+)-PHX by IM HLM 36, which was formed with low affinity ($K_m$ 2.5 μM) (Table 1, cis section).

**trans1-4-Monohydroxylation.** Formation of trans1-OH- (+)-PHX was mediated by a high-affinity reaction for the EM HLMs and the IM HLM18 ($K_m$ 0.020 ± 0.004 μM, range 0.015–0.023 μM). A low-affinity reaction also catalyzed this process in all the HLMs ($K_m$ 2.0 ± 0.4 μM, range 1.6–2.7 μM). A single low-affinity reaction was indicated in the formation of trans1-OH- (-)-PHX ($K_m$ 2.3 ± 0.6 μM, range 1.8–3.3 μM) (Table 1, trans1 section).

**trans2-4-Monohydroxylation.** A single low-affinity reaction was indicated in the formation of trans2-OH- (+)-PHX ($K_m$ 1.9 ± 0.5 μM, range 1.4–2.6 μM) and trans2-OH- (-)-PHX ($K_m$ 2.6 ± 0.7 μM, range 1.8–3.7 μM) (Table 1, trans2 section) for all HLMs.

**Intrinsic clearance.** The total CL_int (mean ± S.D.) of (+)-PHX was 1376 ± 330, 230 ± 225, and 63.4 ± 1.6 μl/min/mg for the EM, IM, and PM HLMs, respectively. Likewise, for (-)-PHX, the total CL_int was 2475 ± 321, 482 ± 437, and 54.6 ± 1.2 μl/min/mg, respectively. The mean involvement of each oxidative metabolic pathway for EM, IM, and PM HLMs is presented in Fig. 4. The primary metabolite of (+)-PHX for EM and IM HLMs was trans1-OH- (+)-PHX, whereas for (-)-PHX it was cis-OH- (-)-PHX. With respect to PM HLMs, the CL_int of (+)- and (-)-PHX by trans1- or trans2-4-monohydroxylation was similar.

**P450 Isoform-Specific Chemical Inhibition Studies.** EM and IM HLMs. cis-OH- (+)- and cis-OH- (-)-PHX formation was significantly inhibited by quinidine (91 ± 6%, $p = 0.002$ and 94 ± 6%, $p = 0.006$, respectively) (Fig. 5a). The high-affinity formation of trans1-OH- (+)-PHX was significantly inhibited by troleandomycin (43 ±
Inhibition by P450 Isoform-Specific Monoclonal Antibodies. cis-OH(+)-PHX and cis-OH(−)-PHX formation was inhibited 65% and 74%, respectively, by MAB-2D6 (Fig. 7a). The high-affinity formation of trans1-OH(+)-PHX was inhibited 34% by MAB-3A4 and 34% by MAB-2D6. The low-affinity formation of this metabolite was inhibited 21% by MAB-3A4, 19% by MAB-2D6, and 21% by MAB-2B6 (Fig. 7b). trans2-OH(+)-PHX formation was inhibited 41% by MAB-3A4 and 27% by MAB-2B6 (Fig. 7b). trans2-OH(−)-PHX formation was inhibited 35% by MAB-3A4 and 32% by MAB-2B6 (Fig. 7c).

Metabolism by Recombinantly Expressed Human P450 Isoforms. Recombinantly expressed CYP2B6 and CYP3A4 similarly catalyzed the formation of trans1-OH(+)-PHX, trans1-OH(−)-PHX, trans2-OH(+)-PHX, and trans2-OH(−)-PHX. Recombinantly expressed CYP2D6 catalyzed the formation of cis-OH(−)-PHX, cis-OH(−)-PHX, and trans1-OH(−)-PHX. Recombinantly expressed CYP2C19 or the expression vectors without P450 cDNA did not metabolize (+) or (−)-PHX to any of the 4-monohydroxy metabolites (data not shown).

Metabolism by Recombinantly Expressed Human CYP3A4 and 100 μM Coumarin. trans1-OH(+)-PHX, trans1-OH(−)-PHX, trans2-OH(+)-PHX, trans2-OH(−)-PHX, and trans2-OH(−)-PHX formation by recombinantly expressed CYP3A4 was increased 108%, 120%, 119%, and 124%, respectively, by coinubcation with coumarin (data not shown).

2%, p = 0.018) and quinidine (30 ± 22%, p = 0.034). The low-affinity formation of this metabolite was significantly inhibited by thio-TEPA (27 ± 9%, p = 0.047), troleandomycin (57 ± 2%, p = 0.005) and was significantly increased by coumarin (83 ± 35%, p = 0.019).

In Table 1, kinetic parameters for the cis-, trans1-, and trans2-4-monohydroxylation of (+) - and (−)-perhexiline by human liver microsomes from three CYP2D6 EMs, two CYP2D6 IMs, and two CYP2D6 PMs are presented.
Discussion

The microsomal data from this study unequivocally demonstrate that both enantiomers of PHX are subject to significant polymorphic metabolism by CYP2D6, although this enzyme exhibits distinct stereoselectivity with respect to the conformation of metabolites and the rate at which they are formed, consistent with the enantioselective pharmacokinetics observed in vivo for CYP2D6 EMs (Gould et al., 1986; Inglis et al., 2006). This study is the first
Fig. 5. Chemical inhibition of cis-4-mono-hydroxylation (a), trans1-4-mono-hydroxylation (b), and trans2-4-mono-hydroxylation (c) of (−)-PHX (white columns) and (+)-PHX (high-affinity site, black columns; low-affinity site, gray columns) by human liver microsomes from three CYP2D6 EMs and one CYP2D6 IM. Data are the mean of triplicate determinations. Error bars indicate S.D.
FIG. 6. Chemical inhibition of trans-1,4-monohydroxylation (a) and trans-2,4-mono-
hydroxylation (b) of (−)-PHX (white columns) and (+)-PHX (black columns) by
human liver microsomes from one CYP2D6 PM. Data are the mean of triplicate deter-
minations.

FIG. 7. Inhibition of cis-4-monohydroxylation (a), trans-1,4-monohydroxylation (b), and
trans-2,4-monohydroxylation (c) of (−)-PHX (white columns) and (+)-PHX (high-affinity site, black col-
umns; low-affinity site, gray columns) by monoclonal antibodies
directed toward human P450s in human liver microsomes from a
CYP2D6 EM. Data are the mean of duplicate determinations.
to identify the involvement of CYP2B6 and CYP3A4 in the metabolism of PHX. They are minor contributors to the intrinsic P450-mediated hepatic clearance of both enantiomers, except in CYP2D6 PMs. Metabolism by these two enzymes does not display any distinct stereoselectivity and is therefore unable to explain the significant enantioselectivity observed in vivo for PMs (Inglis et al., 2006).

PHX is a weak lipophilic base, and nonspecific binding in microsomal incubations can markedly influence the choice of kinetic model describing metabolite formation and interpretation (McLure et al., 2000). Binding of each PHX enantiomer was saturable across the range of substrate concentrations used and was described by a standard binding model, but it did not exhibit any distinct enantioselectivity. Free substrate concentrations in microsomal incubations were calculated to prevent erroneous sigmoidal transformations of reaction curves (McLure et al., 2000). For most of the kinetic data, better fits were obtained by incorporating substrate inhibition (Tracy and Hummel, 2004) in the Michaelis-Menten model. Uncompetitive substrate inhibition may be explained by a two-site binding model in which one site is favored for oxidation, but binding of substrate to the second site (at higher concentrations) produces a substrate-enzyme-substrate complex less capable of forming product than the enzyme-substrate complex (Hutzler and Tracy, 2002). The calculated $K_S$ values were greater than their corresponding $K_m$ values as the substrate preferentially bound to the productive site and were generally above the range of substrate concentrations used in the microsomal incubations, yet this model was used when it provided a statistically better solution to prevent the erroneous kinetic parameter estimations that can occur if a standard Michaelis-Menten hyperbolic curve is forced through the data (Houston and Kenworthy, 2000).

Formation of cis-OH-(-) and cis-OH-(+)-PHX was below the limit of quantification in PM incubations, consistent with CYP2D6-mediated catalysis. For EMs and IMs, high-affinity single-enzyme Michaelis-Menten models best described the kinetics of cis-4-mono-hydroxylation for each enantiomer of PHX, except for the formation of cis-OH-(+)-PHX by one IM (number 36). These $K_m$ values are within the range of unbound (+) and (-) PHX concentrations observed in clinical plasma specimens (Inglis et al., 2006), and are consistent with the saturaibility of cis-4-mono-hydroxylation metabolite formation at therapeutic concentrations in vivo (Sallustio et al., 2002). Sørensen et al. (2003) also reported a high-affinity 4-monohydroxylation reaction by EMs in vitro, although the $K_m$ values were larger because they reflected total rather than unbound microsomal PHX concentrations. The mean contribution of cis-4-monohydroxylation to mean total CL$_{int}$ for (+)-PHX was 43% in EMs and 12% in IMs, and for (-)-PHX it was 99% and 89%, respectively. The lower contribution of cis-4-monohydroxylation to total CL$_{int}$ in IMs can be explained by the lower expression of CYP2D6 in livers with only one functional CYP2D6 allele compared with EM subjects with two functional CYP2D6 alleles (Zanger et al., 2001), producing correspondingly smaller $V_{max}$ values. IM 36 was genotyped as having only one functional allele, CYP2D6*2J, which has been characterized as having impaired in vivo function by sparteine phenotyping in two individuals (Raimundo et al., 2004). The significantly larger $K_m$ observed in the current study suggests that the enzyme is defective, possibly as a result of an as yet unidentified post-transcriptional modification attributable to the 2939G>A single nucleotide polymorphism unique to the allele (Marez et al., 1997), producing a different protein to CYP2D6.2.

The cis-4-monohydroxylation of both enantiomers of PHX was effectively abolished by the CYP2D6-specific chemical inhibitor quinidine in EM and IM incubations, in agreement with Sørensen et al. (2003), and was inhibited by monoclonal antibodies directed against CYP2D6. Recombinantly expressed CYP2D6 cis-4-monohydroxylated (+) and (-)-PHX. When these findings are considered with the observation that this metabolite is effectively absent in PMs, it can be concluded that the cis-4-monohydroxylation of both PHX enantiomers is catalyzed exclusively by CYP2D6 (Fig. 8). The mean CL$_{int}$ of (-)-PHX by cis-4-monohydroxylation was approximately 4- and 16-fold higher in EMs and IMs, respectively, than for (+)-PHX and is consistent with the enantioselective metabolism of (-)-PHX to cis-OH-(+)-PHX reported in vivo (Gould et al., 1986).

The formation of trans1-OH-(+)-PHX by the EMs and by one IM (number 18) was catalyzed by a high- and a low-affinity reaction. For the other IM (number 36) and both PMs, only a single low-affinity reaction was indicated. This suggested that CYP2D6 was the high-affinity enzyme and was confirmed by the inhibition produced by quinidine and MAB-2D6 (Fig. 8). With a similar affinity to cis-4-monohydroxylation, formation of trans1-OH-(+)-PHX by CYP2D6 is also likely to be a saturable process in vivo. The high-affinity formation of trans1-OH-(+)-PHX was also inhibited by troleandomycin, indicating the involvement of CYP3A4, although the inhibition was greater with respect to the low-affinity process. Involvement of CYP2B6 in the low-affinity process was indicated by the inhibition produced by thio-TEPA. The roles of CYP3A4 and CYP2B6 in the low-affinity formation of trans1-OH-(+)-PHX (Fig. 8) were confirmed by the results of the chemical inhibitors in the PMs and by the incubation of monoclonal antibodies with an EM (number 31). Incubations of recombinantly expressed CYP2B6, CYP2D6, and CYP3A4 all produced trans1-OH-(+)-PHX. trans1-OH-(+)-PHX formation was an important contributor to the mean total CL$_{int}$ of (-)-PHX, accounting for 56% in EMs, 77% in IMs, and 48% in PMs. For EMs and IMs, the mean CL$_{int}$ of (+)-PHX by trans1-4-monohydroxylation was approximately 33- and 5-fold higher, respectively, than for (-)-PHX and is consistent with observations in vivo (Gould et al., 1986).

The formation of trans1-OH-(+)-PHX was catalyzed by a low-affinity process in all HLMs and, as indicated by the inhibition studies, was most likely mediated by CYP2B6 and CYP3A4 (Fig. 8). This was confirmed by incubations of the recombinantly expressed enzymes with (+)-PHX. Although the selective CYP2C19 inhibitor S-mephenytoin produced a small but statistically significant inhibition of trans1-OH-(+)-PHX formation, the role of CYP2C19 was subsequently excluded from involvement in this metabolic pathway because incubations of (-)-PHX with the recombinantly expressed human CYP2C19 enzyme did not produce any detectable metabolite. trans1-OH-(+)-PHX formation was a minor contributor to the mean total CL$_{int}$ of (-)-PHX in EMs and IMs, accounting for only 1 and 7%, respectively, because of the very high clearance of (-)-PHX by CYP2D6-mediated cis-4-monohydroxylation. In contrast, formation of trans1-OH-(+)-PHX was responsible for 64% of the mean total CL$_{int}$ of (-)-PHX in PMs.

The kinetics and P450 isoform profile of the formation of trans2-OH-(+)- and trans2-OH-(−)-PHX was similar to that of trans1-OH-(−)-PHX. A single low-affinity reaction was indicated for all HLMs, and inhibition studies determined that their formation was mediated by CYP2B6 and CYP3A4. This was confirmed by incubations of recombinantly expressed CYP2B6 and CYP3A4 with each PHX enantiomer. The formation of the trans2-4-monohydroxy metabolites from both enantiomers was similar in all HLMs and was only a significant contributor to mean total CL$_{int}$ in PMs because of the larger contributions of CYP2D6-mediated 4-monohydroxylation in EMs and IMs. With respect to the mean total CL$_{int}$ of (+)-PHX, trans2-OH-(+)-PHX formation accounted for 2% in EMs, 11% in
IMs, and 52% in PMs. For (+)-PHX, trans2-OH-(-)-PHX formation accounted for <1% in EMs, 4% in IMs, and 36% in PMs.

The data clearly demonstrate that CYP2D6 is the major enzyme catalyzing the metabolism of both (+)- and (-)-PHX by EMs and IMs, in agreement with the in vivo findings of Inglis et al. (2006). Formation of cis-OH-(-)-PHX by CYP2D6 accounts for 99% and 89% of the mean total CL_int of (-)-PHX by EMs and IMs, respectively, and formation of trans1-OH-(+)-PHX and cis-OH-(+)-PHX by CYP2D6 accounts for 97% and 76% of the mean total CL_int of (+)-PHX by EMs and IMs, respectively. Interestingly, whereas CYP2D6-catalyzed cis-4-monohydroxylation was enantioselective for (+)-PHX, CYP2D6-catalyzed trans1-4-monohydroxylation was enantiospecific for (+)-PHX. The importance of CYP3A4 and CYP2B6 in PHX metabolism increases as the contribution of CYP2D6 decreases, such that these two enzymes account for the majority of hepatic P450-mediated clearance in PMs (Fig. 8), with the inhibitor studies suggesting a proportionately larger role for CYP3A4 than CYP2B6. Neither of these P450 isoforms exhibits any clear enantioselectivity for PHX metabolism, as indicated by the kinetics in PMs.

The distinct enantioselectivity of PHX metabolism displayed by PMs in vivo (Inglis et al., 2006) may be due to extrahepatic metabolism, non-P450 metabolism, or transporters involved in absorption, renal, biliary, or intestinal excretion, and merits further investigation.

CYP3A is the most abundant P450 in the liver (Shimada et al., 1994) and is composed primarily of CYP3A4, except in approximately one quarter of Caucasians carrying at least one CYP3A5*1 allele, where CYP3A5 constitutes at least half of the total hepatic CYP3A (Lin et al., 2002). CYP3A5 may be involved in PHX metabolism because CYP3A4 and CYP3A5 have similar substrate specificity (Gillam et al., 1995), and the 3-fold range in the oral clearance of five PMs observed by Sallustio et al. (2002) may be due to individual differences in CYP3A expression (Lin et al., 2002). CYP2B6 shares regulatory features with CYP3A4 (Rodriguez-Antona et al., 2000), and its expression is also highly variable (Stresser and Kupfer, 1999). It constitutes approximately 6% of total hepatic P450 (Stresser and Kupfer, 1999), and its importance in drug metabolism is increasing as more substrates are recognized (Bertz and Granneman, 1997; Ekins and Wrighton, 1999; Lewis et al., 2002; Rendic, 2002), al-

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**Fig. 8. Formation of the 4-monohydroxy metabolites of (+)-PHX (a) and (-)-PHX (b) and the P450 isoforms responsible.**
though the functional consequences of polymorphisms of CYP2B6 have not yet been fully elucidated (Daly, 2004).

The CYP2A6-specific inhibitor coumarin enhanced the trans-1- and trans-2-hydroxylation of both enantiomers of PHX in all HLMs. This effect was not observed for the CYP2D6-dependent formation of cis- OH (+) - or cis-OH (−)-PHX. Heterotrophic cooperativity by CYP3A4 has been reported in the literature for several substrates in various in vitro systems (Hutlzer and Tracy, 2002), but not for CYP2B6. Because coumarin is a substrate of CYP3A4 (Born et al., 2002), the possibility that it is a positive effector of (+)- and (−)-PHX metabolism by CYP3A4 was investigated by co-inoculating coumarin, (+)- or (−)-PHX, and recombinantly expressed human CYP3A4.

trans-1- and trans-2-4-Hydroxylation of both enantiomers was increased over 100%. The heterotrophic cooperativity of CYP3A4 has been observed mainly in vitro. Corresponding in vivo data are rare (Tang and Starnes, 2001). Further studies need to be undertaken to determine whether enhanced metabolism of PHX occurs in vivo to assess its potential to cause drug-drug interactions, particularly in PMs, for whom CYP3A4-mediated metabolism is a primary determinant of PHX clearance. Importantly, the data demonstrate that the use of coumarin as a specific inhibitor of CYP2A6 may be confounded by possible heterotrophic cooperative interactions with other compounds that are also CYP3A4 substrates.

In conclusion, CYP2D6 is the major enzyme catalyzing the 4-monoxygenation of both (+)- and (−)-PHX in EMs and IMs. It displays Km values within the range of unbound concentrations in plasma attained clinically for both enantiomers of PHX. Thus, saturable and polymorphic CYP2D6 metabolism underlies the extreme variability in the pharmacokinetics of both PHX enantiomers in vivo. CYP3A4 and CYP2B6 are the major enzymes responsible for the 4-monohydroxylation of (+)- and (−)-PHX in PMs and did not show appreciable enantioselectivity. This is in contrast to the significant enantioselectivity in the pharmacokinetics of (+)- and (−)-PHX observed clinically in PMs.

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References


