The Revised Human Liver Cytochrome P450 “Pie”: Absolute Protein Quantification of CYP4F and CYP3A Enzymes Using Targeted Quantitative Proteomics

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ABSTRACT

The CYP4F subfamily of enzymes has been identified recently to be involved in the metabolism of endogenous compounds (arachidonic acid and leukotriene B₄), nutrients (vitamins K₁ and E), and xenobiotics (pafuramidine and fingolimod). CYP4F2 and CYP4F3B are reported to be expressed in the human liver. However, absolute concentrations of these enzymes in human liver microsomes (HLMs) and their interindividual variability have yet to be determined because of the lack of specific antibodies. Here, an liquid chromatography with tandem mass spectrometry (LC-MS/MS)-based targeted quantitative proteomic approach was employed to determine the absolute protein concentrations of CYP4F2 and CYP4F3B compared with CYP3A in two panels of HLMs (*n* = 31). As a result, the human hepatic cytochrome P450 (P450) “pie” has

been revised to include the contribution of CYP4F enzymes, which amounts to 15% of the total hepatic cytochrome P450 enzymes. CYP4F3B displayed low interindividual variability (3.3-fold) in the HLM panels whereas CYP4F2 displayed large variability (21-fold). However, CYP4F2 variability decreased to 3.4-fold if the two donors with the lowest expression were excluded. In contrast, CYP3A exhibited 29-fold interindividual variability in the same HLM panels. The proposed marker reaction for CYP4F enzymes pafuramidine/DB289 M1 formation did not correlate with CYP4F protein content, suggesting alternate metabolic pathways for DB289 M1 formation in HLMs. In conclusion, CYP4F enzymes are highly expressed in the human liver and their physiologic and pharmacologic roles warrant further investigation.

Introduction

Cytochrome P450 (P450) enzymes make up a superfamily of proteins involved in the oxidative metabolism of both endogenous and exogenous substrates. The human genome contains 57 functional P450 genes arranged into 18 families and 42 subfamilies (Guengerich, 2005). Members of the CYP1, CYP2, CYP3, and, to a lesser extent, CYP4 families metabolize exogenous substrates (e.g., drugs, herbs, and environmental pollutants) with little contribution from other P450 families (Nebert and Russell, 2002). The CYP3A subfamily has received the most attention of all drug-metabolizing enzymes because of its abundant expression in major organs of drug elimination (i.e., liver and intestine) (Shimada et al., 1994; Paine et al., 2006), its ability to metabolize a wide range of structurally diverse substrates, and its dominant role in drug metabolism (Wilkinson, 2005). Within the CYP3A subfamily, CYP3A4 is the most abundantly expressed form in the liver whereas CYP3A5 expression at the protein level is only about 10.6% of that of CYP3A4 (Wang et al., 2008). CYP3A7 is considered a fetal-specific P450 enzyme (Leeder et al., 2005), and CYP3A43 has extremely low expression in the liver and contributes little to drug metabolism (Domanski et al., 2001; Westlind et al., 2001).

The CYP4F subfamily of enzymes was discovered during efforts to identify enzymes involved in the ω -hydroxylation of eicosanoids (e.g., leukotriene B₄ [LTB₄], prostaglandins, and arachidonic acid) (Kalsotra and Strobel, 2006; Hsu et al., 2007). This subfamily is composed of five members: CYP4F2, CYP4F3, CYP4F8, CYP4F11, and CYP4F12. Of these, the *CYP4F3* gene produces two tissue-specific splice variants, *CYP4F3A* (myeloid form) and *CYP4F3B* (liver form); however, transcripts of both are present in other tissues (Christmas et al., 2001). CYP4F2 is the principal hepatic ω -hydroxylase of the proinflammatory agent LTB₄ (Jin et al., 1998) and arachidonic acid (Powell et al., 1998), resulting in deactivation of LTB₄ and formation of the potent vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE), respectively. In addition, CYP4F2 catalyzes the ω -hydroxylation of tocopherol (vitamin E) and phyloquinone (vitamin K₁) phytyl side chains, suggesting roles in the regulation of vitamin E status and synthesis of clotting factors in the liver (Sontag and Parker, 2002; McDonald et al., 2009). Both CYP4F3A and CYP4F3B catalyze the ω -hydroxylation of LTB₄ and arachidonic acid; however, CYP4F3A has 30-fold greater affinity (*K_m*) for LTB₄ and conversely 8.4-fold lower affinity for arachidonic acid than CYP4F3B (Christmas et al., 2001). Because of the involvement of CYP4F enzymes in the inactivation of LTB₄, it has been suggested that these enzymes play an important role in the resolution of inflammation (Hardwick, 2008; Sehgal et al., 2011).

Besides endogenous substrates, CYP4F enzymes have been implicated in the metabolism of xenobiotics. Notably, CYP4F11 metabolizes

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ABBREVIATIONS: CI, confidence interval; CV, coefficient of variation; CYP or P450, cytochrome P450; DB289, pafuramidine; DHB, 6',7'-dihydroxybergamottin; HET0016, *N*-hydroxy-*N'*-(4-butyl-2-methylphenyl)-formamidine; HLM, human liver microsome; HPLC, high-pressure liquid chromatography; IS, internal standard; LC-MS/MS, liquid chromatography with tandem mass spectrometry; LTB₄, leukotriene B₄; MRM, multiple reaction monitoring; MS, mass spectrometry.

erythromycin and ethylmorphine (Kalsotra et al., 2004), and CYP4F12 metabolizes ebastine (Hashizume et al., 2002). CYP4F2 and/or CYP4F3B are the major hepatic enzymes that catalyze the ω -hydroxylation of fingolimod (FTY720), an oral drug for relapsing multiple sclerosis (Jin et al., 2011), and *O*-demethylation of the antitrypanosomal methamidoxime prodrugs pafuramidine (DB289), DB844, and DB868 (Wang et al., 2006; Generaux et al., 2013; Ju et al., 2014). In addition, a CYP4F2 genetic polymorphism has been shown to significantly affect the required maintenance dose of warfarin (Caldwell et al., 2008). Taken together, CYP4F enzymes, particularly hepatic CYP4F2 and CYP4F3B, play an important role in drug metabolism as well as endogenous lipid metabolism. Therefore, it is important to quantify these enzymes at the protein level to define their interindividual variability in hepatic expression and function.

CYP4F2 and CYP4F3B have 93% amino acid sequence identity and both share a high degree of identity with other CYP4F enzymes, making it difficult to produce highly specific antibodies for semi-immunoquantification. Advances in liquid chromatography with tandem mass spectrometry (LC-MS/MS)-based targeted quantitative proteomics have allowed for the quantification of CYP3A4 and CYP3A5 (84% amino acid sequence identity) in human liver microsomes (HLMs) by employing specific proteotypic peptides (i.e., AQUA, signature or surrogate peptides) for the detection and quantification of proteins of interest after tryptic digestion of HLMs (Wang et al., 2008). Similar methodology also has been applied for the quantification of drug transporters and UDP-glucuronosyltransferases (UGTs) (Fallon et al., 2008; Kamiie et al., 2008; Li et al., 2008). We developed and employed a quantitative proteomic approach to quantify the major hepatic CYP4F enzymes, CYP4F2 and CYP4F3B, in a panel of individual donor HLMs and to compare them with CYP3A enzymes in terms of relative abundance and interindividual variability. Our secondary objective was to evaluate DB289 *O*-demethylation (DB289 M1 formation) as a marker reaction for CYP4F2 and CYP4F3B.

Materials and Methods

Chemicals. Pafuramidine (DB289), M1 (DB775), and deuterium-labeled DB289 (DB289-d₈; internal standard [IS]) were kindly provided by the Consortium for Parasitic Drug Development (CPDD; University of North Carolina at Chapel Hill, Chapel Hill, NC). HET0016 [*N*-hydroxy-*N'*-(4-butyl-2-methylphenyl)-formamidine] was purchased from Cayman Chemical (Ann Arbor, MI). Fluvoxamine was obtained from Toronto Research Chemicals (Toronto, ON, Canada). Midazolam and 6',7'-dihydroxybergamottin (DHB) were purchased from Cerilliant (Round Rock, TX). Isotope-labeled ¹³C₃-1'-hydroxymidazolam was obtained from BD Biosciences (San Jose, CA). Azamulin was purchased from Sigma-Aldrich (St. Louis, MO). High-pressure liquid chromatography (HPLC)-grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Analytic-grade acetic acid, ammonium acetate, ammonium bicarbonate, dimethylsulfoxide, dithiothreitol, formic acid, iodoacetamide, potassium phosphate, and other reagents were purchased from VWR (Bridgeport, NJ). Sequencing-grade modified trypsin and NADPH were purchased from Promega (Madison, WI).

Synthetic Proteotypic Peptides. Synthetic unlabeled and ¹³C-, ¹⁵N-labeled proteotypic peptides (Table 1) were ordered from Thermo Scientific (Ulm, Germany). Peptide purity (>94%), determined by reverse-phase HPLC UV (detection wavelength of 215 nm) and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS), was provided by the manufacturer. Peptide identity was confirmed from product ion spectra obtained via peptide infusion into an API 4000 QTrap (MDS Sciex, Framingham, MA) triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). The proteotypic peptide sequences selected for CYP4F2 and CYP4F3B contain amino acid sequences that are unique to the individual enzymes and do not overlap with other members of the human CYP4F family. The proteotypic peptide sequences selected for CYP3A4 and CYP3A5 contain unique amino acid sequences, whereas the CYP3A peptide contains an amino acid sequence present in CYP3A4, CYP3A5, and CYP3A7.

Human Liver Microsomes, Recombinant Human P450 Enzymes, and Antibodies. All nonfetal (age 6–69 years) individual donor and pooled HLMs were purchased from XenoTech, LLC (Lenexa, KS). Two panels of individual donor HLMs (Table 2) were obtained and characterized separately. The first panel consisted of 20 donors: 12 males and 8 females (H0422, H0425, H0428, H0430, H0431, H0432, H0441, H0444, H0450, H0451, H0452, H0453, H0459, H0472, H0482, H0715, H0728, H0737, H0743, and H0802). The second panel had 10 donors: 5 males and 5 females (H0024, H0079, H0081, H0134, H0199, H0215, H0232, H0246, H0292, and H0307). Two pooled HLMs (XTreme200, pool of 200, Lot 1010420; H0610, pool of 50, Lot 0510025) also were used in this study. Recombinant human CYP3A4, CYP3A5, CYP4F2, and CYP4F3B Supersomes, prepared from baculovirus-infected insect cells expressing human P450 enzymes and reconstituted with NADPH-cytochrome P450 reductase, were purchased from BD Gentest (Woburn, MA). CYP4F2 and CYP4F3B Supersomes also contained cytochrome *b₅*. The total P450 concentration (pmol/ml) of each Supersomes, determined spectrophotometrically (Omura and Sato, 1964), was provided by the supplier. A mouse monoclonal anti-CYP3A antibody for Western blot analysis (WB-MAB-3A, Lot 30494), which detects human CYP3A4, CYP3A5 and CYP3A7, was purchased from BD Gentest. A polyclonal antibody against CYP4F2 (1 mg IgG/ml), which reacts similarly with CYP4F3B (Wang et al., 2007), was obtained from Research Diagnostics (Concord, MA).

Optimization of Ionization Parameters for MS Quantification of Proteotypic Peptides. Solutions of synthetic proteotypic peptides were prepared in 50:50 (v/v) acetonitrile:1% (v/v) acetic acid to a final concentration of ~1 μ M. The solutions were infused (0.01 ml/min), with supplementary mobile phase (0.4 ml/min; 50% (v/v) acetonitrile containing 0.1% (v/v) formic acid), into an Applied Biosystems API4000 QTrap triple quadrupole mass spectrometer equipped with a Turbo IonSpray interface (MDS Sciex, Framingham, MA). Ion spray voltage and turbo heater temperature were maintained at 2500 V and 500°C, respectively. The product ion (MS/MS) spectrum for each proteotypic peptide was acquired on the doubly charged precursor ion. Ion fragments yielding the best sensitivity were selected as the basis for the multiple reaction monitoring (MRM) transitions used for peptide quantification. Compound specific instrument parameters were optimized for each MRM transition to obtain the most abundant signal. The MS instrument was operated under positive ion mode and unit resolution for both Q1 and Q3.

Protein Digestion and Quantification by LC-MS/MS Analysis. Recombinant P450 and HLM digestions were performed as previously described elsewhere (Wang et al., 2008) with slight modifications. Briefly, protein samples (10–30 μ g) were reduced in buffer (90 μ l) containing 50 mM ammonium bicarbonate (pH 8.0) and 4 mM dithiothreitol, followed by heating at 60°C for 60 minutes to further denature the proteins. After cooling to room temperature, the samples were alkylated with iodoacetamide (10 mM final concentration) for 20 minutes in the dark and then subjected to digestion with 1 μ g trypsin at 37°C for 4 hours. A half-volume of ice-cold acetonitrile containing ¹³C-, ¹⁵N-labeled proteotypic peptides as internal standards was added to terminate the reactions. After vortexing and centrifugation, the supernatants were transferred to 96-well microplates, and 8 μ l was injected for LC-MS/MS analysis. Chromatographic separation of the proteotypic peptides was performed on an Agilent HPLC column (Zorbax Poroshell 300SB-C18, 2.1 $\times$ 75 mm, 5 μ m; Agilent, Santa Clara, CA) as described previously elsewhere (Wang et al., 2008).

Preparation of Calibration Standards. Calibration standards were prepared by subjecting various concentrations of recombinant P450 (0.01–10 pmol) to digestion in parallel with HLMs. The proteotypic peptides resulting from protein trypsinolysis were used to generate calibration curves. Quantification was performed using the area ratio of proteotypic peptide peak to the corresponding isotope-labeled peptide peak (present at a constant level due to its use as an IS) with $1/x^2$ weighting. The accuracy and the coefficient of variation (CV) of the methods were determined by measuring triplicate preparations of the calibration standards.

Marker Activities and Western Blot Analysis. Testosterone 6 β -hydroxylation and midazolam 1'-hydroxylation activities for the two HLM panels were provided by XenoTech. Midazolam 1'-hydroxylation activity also was evaluated in-house as described previously elsewhere (Wang et al., 2007) to confirm the manufacturer-provided values and ensure sample integrity after delivery and storage. DB289 M1 formation, a proposed marker for CYP4F activity in HLMs, was measured as described previously elsewhere (Wang et al., 2006). Briefly, after preincubation at 37°C for 5 minutes, DB289 (3 μ M) or midazolam (3 μ M) was incubated with HLMs (0.2 mg/ml) in standard incubation mixtures (100 mM phosphate buffer [pH 7.4], 3.3 mM MgCl₂, and

TABLE 1.
Proteotypic peptides for CYP3A and CYP4F enzymes.

P450	Peptide Name	Proteotypic Peptide Sequence	M+H	MRM m/z
CYP3A ^a	3A_pep1	S ₁₃₁ LLSPTFTSGK ₁₄₁	1138	569.6 → 737.6
	3A_IS_pep1 ^b	S ₁₃₁ LLSPTF(9 ¹³ C, ¹⁵ N)TSGK ₁₄₁	1148	574.4 → 834.5
CYP3A4	3A4_pep1	E ₂₄₄ VTNFLR ₂₅₀	879	439.9 → 650.5
	3A4_IS_pep1	E ₂₄₄ VTNF(9 ¹³ C, ¹⁵ N)LR ₂₅₀	889	444.8 → 660.5
CYP3A5	3A5_pep1	D ₂₄₄ TINFLSK ₂₅₁	938	469.5 → 608.4
	3A5_IS_pep1	D ₂₄₄ TINF(9 ¹³ C, ¹⁵ N)LSK ₂₅₁	948	474.4 → 618.5
CYP4F2	4F2_pep1	S ₁₀₉ VINASAAIAPK ₁₂₀	1142	571.6 → 842.6
	4F2_IS_pep1	S ₁₀₉ VI(6 ¹³ C, ¹⁵ N)NASAAIAPK ₁₂₀	1149	575.0 → 962.6
	4F2_pep2	V ₄₈₀ VLALTLLR ₄₈₈	998	499.6 → 799.7
	4F2_IS_pep2	V ₄₈₀ VL(6 ¹³ C, ¹⁵ N)ALTLLR ₄₈₈	1005	503.0 → 806.7
CYP4F3B	4F3B_pep1	S ₁₀₉ VINASAAIVPK ₁₂₀	1170	585.6 → 870.6
	4F3B_IS_pep1	S ₁₀₉ VI(6 ¹³ C, ¹⁵ N)NASAAIVPK ₁₂₀	1177	589.0 → 870.6
	4F3B_pep2	V ₄₈₀ VLGLTLLR ₄₈₈	984	492.5 → 785.6
	4F3B_IS_pep2	V ₄₈₀ VL(6 ¹³ C, ¹⁵ N)GLTLLR ₄₈₈	991	496.0 → 792.6

^aThis peptide detects CYP3A4, CYP3A5, and CYP3A7, but not CYP3A43.

^bThe ¹³C- and ¹⁵N-labeled peptides served as internal standards during quantitation.

1 mM NADPH). Reactions were initiated with the addition of NADPH and performed for 5 minutes for DB289 or 2 minutes for midazolam. M1 formation had been demonstrated previously to be linear under these conditions (Wang et al., 2006). Reactions were terminated by the addition of an equal volume of ice-cold acetonitrile containing IS (20 nM DB289-d₈ or 1 μ M ¹³C₃-1'-hydroxymidazolam). Stock solutions of DB289 and DB289-d₈ were prepared in dimethylsulfoxide, and stock solutions of midazolam and ¹³C₃-1'-hydroxymidazolam were prepared in methanol. Final incubation mixtures contained less than 1% (v/v) organic solvent. The terminated reaction mixtures were vortexed, and precipitated proteins were removed by centrifugation at 4,000 rpm for 15 minutes. The resulting supernatants were analyzed for concentrations of M1 and 1'-hydroxymidazolam by LC-MS/MS as described herein.

TABLE 2.

Donor information for the two panels of individual human liver microsomes.

Sample	Gender	Age (yr)	Race	Cause of Death
H0422	Male	69	Caucasian	Cerebrovascular accident
H0425	Female	60	Caucasian	Anoxia
H0428	Female	57	Caucasian	Head trauma
H0430	Male	59	Caucasian	Cerebrovascular accident
H0431	Male	40	Hispanic	Head trauma
H0432	Male	60	African American	Cerebrovascular accident
H0441	Male	63	Caucasian	Cerebrovascular accident
H0444	Female	43	Caucasian	Cerebrovascular accident
H0450	Female	46	Caucasian	Head trauma
H0451	Male	58	Caucasian	Cerebrovascular accident
H0453	Female	25	Caucasian	Anoxia
H0459	Female	25	Caucasian	Head trauma
H0472	Male	56	Caucasian	Anoxia
H0482	Female	67	Caucasian	Cardiac arrest
H0715	Male	21	Caucasian	Head trauma
H0728	Male	39	Caucasian	Cerebrovascular accident
H0737	Female	49	African American	Anoxia
H0743	Male	35	Caucasian	Cerebrovascular accident
H0802	Male	33	Caucasian	Cerebrovascular accident
H0024	Male	47	Caucasian	Head trauma
H0079 ^a	Female	56	Caucasian	Cerebrovascular aneurysm
H0081 ^b	Female	67	Caucasian	Cerebrovascular aneurysm
H0134	Female	61	Caucasian	Cerebrovascular aneurysm
H0199	Female	48	African American	Cerebrovascular aneurysm
H0215	Male	6	Caucasian	Head trauma
H0232	Male	43	Caucasian	Cerebrovascular aneurysm
H0246	Female	40	Caucasian	Anoxia
H0292	Male	65	African American	Cerebrovascular aneurysm
H0307	Male	39	Caucasian	Anoxia

^a5–6 cigarettes/day for 40 years.

^b1 pack/day for 40 years and 1 beer/month.

HLMs (10 μ g; single determinations for the first and triplicate determinations for the second panel) and recombinant CYP4F2 and CYP3A4 proteins (0.02–1.0 pmol; calibration standards) were separated by 10% SDS-PAGE and immunoblotted as described previously elsewhere (Wang et al., 2008).

Chemical Inhibition Assays. P450-selective chemical inhibitors were added to standard incubation mixtures (200 μ L) containing 3 μ M DB289 and either 0.2 mg/ml HLM or 10 pmol/ml recombinant P450 enzyme. The substrate concentration was selected to be consistent with the concentration used earlier to determine DB289 M1 formation rates in the panels of HLMs and because it was previously observed that the inhibition pattern was the same whether the concentration of DB289 was 0.3 μ M or 3 μ M (Wang et al., 2006). Reactions were performed for 5 minutes, and samples were processed as described earlier. The chemical inhibitors used were fluvoxamine (3 μ M; CYP1A2 inhibitor), HET0016 (0.1 μ M; an arachidonic acid ω -hydroxylase inhibitor that strongly inhibits CYP4F2 and CYP4F3B), DHB (30 μ M; CYP3A inhibitor), and azamulin (3 μ M; CYP3A inhibitor). A double inhibition experiment was performed with both fluvoxamine (3 μ M) and azamulin (3 μ M) in the same incubation. Control incubations contained methanol in place of chemical inhibitor.

LC-MS/MS Analysis for Marker Activities. Substrates and primary metabolites from marker activity reactions were quantified with an API 4000 QTrap triple quadrupole mass spectrometer equipped with Turbo IonSpray interface and Analyst software (version 1.4.2; Applied Biosystems). Samples were introduced through a Shimadzu LC-20 HPLC system (Shimadzu, Columbia, MD) and Valco solvent divert valve (Valco, Houston, TX) from a cooled (10°C) CTC PAL LEAP autosampler (LEAP Technologies, Carrboro, NC). Chromatographic separation was obtained using an Agilent HPLC column (Zorbax SB-C8, 2.1 $\times$ 50 mm, 3.5 μ m) and 4-minute gradient elution method. HPLC mobile phase (A) consisted of 95:5 (v/v) water:methanol with 2 mM ammonium acetate and 0.1% (v/v) formic acid; (B) consisted of 5:95 (v/v) water:methanol with 2 mM ammonium acetate and 0.1% (v/v) formic acid. The initial condition of 40% B was held for 0.5 minutes. From 0.5 to 3 minutes, B was increased linearly to 95% and then held for an additional 2 minutes. At 5 minutes, the column was re-equilibrated with 40% B for 0.5 minutes before the next injection.

Data Analysis. Data for the quantitation of enzyme protein content are presented as the mean and standard deviation (S.D.) of at least triplicate determinations unless indicated otherwise. The final CYP4F2 and CYP4F3B protein content was calculated as the average values determined using two proteotypic peptides, "pep1" and "pep2." The total CYP4F content was determined as the sum of CYP4F2 and CYP4F3B protein content for each individual donor HLM. The contribution of CYP4F to the human liver P450 "pie" was determined by assuming the total CYP3A enzyme content measured in this study represented the same percentage of the original P450 pie reported for CYP3A (i.e., 28.8%) by Shimada et al. (1994). This permitted direct extrapolation of the observed total CYP4F enzyme content to a percentage of the pie, which was assumed to make up a portion of the previously unknown

P450 content. Data for the quantitation of enzyme activity are presented as the mean of duplicate determinations as there was little variability (difference <10% of the mean). For correlation with marker activities, metabolite formation rates were plotted versus enzyme protein content and the Pearson r value was determined using GraphPad Prism Software (version 6.0; San Diego, CA). Relative activity factor-adjusted protein contents were calculated by scaling the CYP4F2 and CYP4F3B enzyme content individually using the intrinsic clearance values for DB289 determined previously elsewhere (Wang et al., 2006). For inhibition studies, the results are expressed as percentage of the control, in which the amount of metabolite formed in incubations without inhibitor was set to 100%.

Results

Performance of Protein Quantification Assay. Representative MRM chromatograms of CYP4F2 and CYP4F3B proteotypic peptides and internal standards are shown in Fig. 1. A minimum of 7 P450 concentration levels (0.01–10 pmol) were included in each calibration curve. A strong correlation ($r^2 \geq 0.982$) was observed for all proteotypic peptides of interest. The observed lower limit of quantitation, using 30 μ g of HLM, was 1.7 pmol/mg HLM for CYP4F2 and CYP4F3B, and 0.3 pmol/mg HLM for CYP3A4, CYP3A5 and total CYP3A. For CYP4F2, the accuracy and the average CV of the analytic method were 83%–108% and 13% using 4F2_pep1, and 86.4%–108% and 20% using 4F2_pep2, respectively. For CYP4F3B, the accuracy and the average CV of the analytical method were 87.3%–113% and 19% using 4F3B_pep1, and 83.7%–118% and 9% using 4F3B_pep2, respectively. In addition, the accuracy and the average CV of the analytical method for CYP3A, CYP3A4 and CYP3A5 were 77%–124% and 19% using 3A_pep1, 85.3%–108% and 18% using 3A4_pep1, and 81.3%–114% and 22% using 3A5_pep1, respectively, similar to previously reported values (Wang et al., 2008).

Absolute Quantification of CYP4F2 and CYP4F3B in HLMs and Correlation to Immunoquantification. The absolute protein expression levels of CYP4F2 and CYP4F3B were determined in two individual donor HLM panels. Two unique proteotypic peptides, designated “_pep1” and “_pep2,” were identified for each isoform and

used for the quantification of protein content. Similar protein quantification results were obtained from the pairs of proteotypic peptides (4F2_pep1 versus 4F2_pep2; 4F3B_pep1 versus 4F3B_pep2), as evidenced by strong correlations ($r^2 = 0.89$ and 0.81), near-unity slopes (0.89 and 1.05), and near-zero Y-intercepts (0.9 and -3.8) (Fig. 2). As such, final protein concentrations were calculated as the average of the two quantification results based on _pep1 and _pep2. The final average (range and 95% confidence interval [CI]) CYP4F2 protein content in the first and second HLM panels ($n = 20$ and $n = 11$, respectively) was 16.1 (10.7–27.1 and 13.8–18.3) and 11.0 (1.3–19.2 and 7.3–14.8) pmol/mg HLM protein, respectively (Fig. 3A). The final average (range and 95% CI) CYP4F3B protein content was 10.4 (6.7–13.9 and 9.4–11.4) and 12.8 (6.4–20.9 and 10–15.8) pmol/mg HLM protein, respectively (Fig. 3B). Overall, the average (range and 95% CI) CYP4F2 and CYP4F3B protein contents in both HLM panels ($n = 31$) were 14.3 (1.3–27.1 and 12.1–16.3) and 11.3 (6.4–20.9 and 10.1–12.5) pmol/mg HLM protein, respectively.

To validate the MS-based quantification of CYP4F2 and CYP4F3B, these results were compared with the immunoquantification of CYP4F enzymes in the same HLM panels. The polyclonal antibody against CYP4F2 used in this study was shown previously elsewhere to cross-react with CYP4F3B in a similar manner but not with CYP3A4, CYP3A5, or CYP4A11 (Wang et al., 2007). Hence, immunoquantification results represent the sum of CYP4F2 and CYP4F3B expression. Immunoblots of the two HLM panels are shown in Fig. 4, A and B. The average (range and 95% CI) CYP4F content in the first and second panels was 39.6 (22.7–57.9 and 35.2–44.0) and 42.0 (16.0–69.3 and 32.6–51.4) pmol/mg HLM protein, respectively. The total CYP4F content (the sum of CYP4F2 and CYP4F3B) determined by MS-based methods correlated with the immunoquantification results ($r^2 = 0.696$, $P < 0.0001$) (Fig. 4C).

Absolute Quantification of CYP3A4, CYP3A5, and total CYP3A in HLMs and Correlation to Immunoquantification. The absolute protein expression levels of CYP3A4, CYP3A5, and total CYP3A were determined in the two individual donor HLM panels. The results for the second HLM panel, and a third HLM panel from the Clinical Pharmacology Liver Bank, were reported previously elsewhere (Wang et al., 2008); here, we present the results from the first HLM panel (Fig. 5). Total CYP3A was quantified using the proteotypic peptide 3A_pep1 (Table 1), which detects CYP3A4, CYP3A5, and CYP3A7 but not CYP3A43. The average (range and 95% CI) protein contents for CYP3A, CYP3A4, and CYP3A5 in the first HLM panel were 52.5 (10.8–124 and 33.8–70), 42.2 (14.6–102 and 28.2–55.9), and 4.0 (0.89–18 and 1.5–6.5) pmol/mg HLM protein, respectively. The method was determined to be reproducible, as shown by average CVs for CYP3A, CYP3A4, and CYP3A5 of 12%, 8%, and 14%, respectively. The average (range and 95% CI) protein contents for CYP3A, CYP3A4, and CYP3A5 in the second HLM panel were reported previously to be 57 (9–257 and 10–104), 67 (9–322 and 8.6–125) and 4 (0.3–20 and 0–8.3) pmol/mg HLM protein (Wang et al., 2008). The average (range and 95% CI) protein contents for CYP3A, CYP3A4, and CYP3A5 in the third HLM panel were reported previously to be 32 (6–85 and 22–42), 30 (9.5–70 and 22–39), and 4.7 (0.2–34 and 1.5–8.0) pmol/mg HLM protein (Wang et al., 2008).

Overall, CYP3A5 was expressed at much lower levels than CYP3A4 (~10% of CYP3A4), and exhibited a 170-fold interindividual variability within the 52 individual donor HLMs studied. CYP3A4 also exhibited significant interindividual variability, 36-fold overall or 11-fold if H079 was excluded. Total CYP3A showed 43-fold interindividual variability, unless H079 was excluded, at which it was only 21-fold. When total CYP3A (based on 3A_pep1) was plotted against the sum of CYP3A4 and CYP3A5 (based on 3A4_pep1 and 3A5_pep1, respectively) for the first

Fig. 1. LC-MS/MS MRM analyses of the in-solution tryptic digestion of a pooled HLM. Representative MRM chromatograms (30 μ g HLM loaded) are for proteotypic peptides and isotopically-labeled proteotypic peptides for CYP4F2 (A) and CYP4F3B (B).

Fig. 2. Coherence of absolute expression levels determined using different proteotypic peptides. Correlation analyses of CYP4F2 (A) and CYP4F3B (B) protein concentrations, determined using peptides designated “_pep1” and “_pep2,” were performed to evaluate the coherence of the proteotypic peptides.

HLM panel, a strong correlation ($r^2 = 0.996$) and near-unity slope (slope = 1.27) were observed (data not shown), similar to those previously reported for the second and third HLM panels (Wang et al., 2008). These results confirm concordance of the LC-MS/MS methodology and suggest that CYP3A4 and CYP3A5 account for the majority of CYP3A protein in the nonfetal human liver.

Correlation of Protein Content and Marker Activity. Our previous studies (Wang et al., 2006, 2007) suggested that CYP4F enzymes, primarily CYP4F2 and CYP4F3B, are the major hepatic and enteric enzymes responsible for the initial *O*-demethylation of the antitrypanosomal DB289 (DB289 M1 formation). Here, we determined the M1 formation rates for the first HLM panel. When the total CYP4F protein content versus M1 formation rates were plotted (Fig. 6A), no correlation was observed ($r^2 = 0.022$ and $P = 0.53$ for MS-based method; $r^2 = 0.044$

and $P = 0.37$ for immunoquantification). Taking into account differing CYP4F2 and CYP4F3B intrinsic clearances (CL_{int}) toward M1 formation ($CL_{int} = 11$ and $3.1 \mu\text{L}/\text{min}/\text{pmol}$ P450, respectively) (Wang et al., 2006), we also attempted to use a relative activity factor (RAF)-adjusted CYP4F protein content for correlation analysis, but no improvement in correlation with M1 formation rates was seen ($r^2 = 0.026$ and $P = 0.49$; data not shown).

In contrast to CYP4F protein content, strong correlations ($r^2 \geq 0.751$, $P < 0.0001$) were observed between CYP3A marker activities, testosterone 6β -hydroxylation and midazolam $1'$ -hydroxylation, and CYP3A protein content, determined by either the MS-based method or immunoquantification (Fig. 6, B and C). To confirm the manufacturer's activity values and ensure that our sample handling (i.e., storage and freeze/thaw) did not alter the P450 activities, a confirmatory midazolam $1'$ -hydroxylation

Fig. 3. Absolute expression levels of CYP4F2 and CYP4F3B proteins in individual HLM samples. Expression levels of CYP4F2 (A) and CYP4F3B (B) enzymes were determined in liver microsomal samples obtained from two individual donor panels. Bars and error bars represent the mean and standard deviation of at least triplicate determinations.

Fig. 4. Cross-validation of MS-based protein quantification with quantitative Western blot analysis. Recombinant CYP4F2 was used for calibration standards for the immunoquantification of CYP4F enzymes in the first (A) and second (B) HLM panels (10 μ g loaded). Correlation analyses between the MS-based approach and quantitative Western blot approach are shown for CYP4F (C) and CYP3A (D).

assay was performed in parallel with MS-based protein quantification. A strong correlation with the manufacturer's values was observed ($r^2 = 0.87$, $P < 0.0001$; data not shown).

Chemical Inhibition and Correlation using Residual Activities.

Several recombinant human P450s, notably CYPs 1A2, 2J2, and 3A4, were implicated previously in catalyzing M1 formation, though these were shown to have a minor contribution to overall M1 formation in pooled HLMs (Wang et al., 2006). However, it is possible that interindividual variability in P450 expression could result in some individual donor HLMs having more-than-average contributions from these non-CYP4F enzymes, thus complicating the correlation analyses described earlier. To assess this possibility, we determined residual M1 formation activities upon inhibition of non-CYP4F enzymes by P450-selective chemical inhibitors. Because of the low expression of CYP2J2 in HLMs (<1.7 pmol/mg HLM proteins; unpublished data) (Girault et al., 2005), no experiments were performed with CYP2J2 inhibitors. Fluvoxamine was chosen as a CYP1A2 inhibitor (Pastrakuljic et al., 1997), and azamulin (Stresser et al., 2004) and dihydroxybergamottin (DHB) (Araki et al., 2012) as CYP3A inhibitors. We chose not to use ketoconazole as a CYP3A inhibitor because it is known to inhibit CYP4F2-mediated reactions (e.g., DB289 M1 formation (Wang et al., 2006) and fingolimod ω -hydroxylation (Jin et al., 2011)). In addition, we chose HET0016, an arachidonic acid P450-mediated metabolism inhibitor (Wang et al., 2007), as a CYP4F inhibitor to assess the contribution of CYP4F enzymes to M1 formation in our panel of individual donor HLMs.

Before incubation with HLMs, the inhibitors were incubated with recombinant P450s and DB289 to evaluate their specificity with respect to M1 formation (Fig. 7A). HET0016 (0.1 μ M) was a potent inhibitor of CYP4F2 (92% inhibition) and CYP4F3B (99% inhibition), and to

a lesser extent, CYP2J2 (71% inhibition). HET0016 marginally inhibited CYP1A2 (27% inhibition) and CYP3A4 (4% inhibition). Fluvoxamine (3 μ M) was a potent inhibitor of CYP1A2 (98% inhibition), but marginally inhibited CYP3A4 (21% inhibition), CYP4F2 (12% inhibition), CYP4F3B (24% inhibition), and CYP2J2 (27% inhibition). Azamulin (3 μ M) was a moderate inhibitor of both CYP3A4 (54% inhibition) and CYP2J2 (46% inhibition), and did not significantly inhibit CYP1A2 (no inhibition), CYP4F2 (21% inhibition), or CYP4F3B (20% inhibition). Unexpectedly, DHB (30 μ M) was a potent inhibitor of CYP4F2 (95% inhibition), CYP1A2 (84% inhibition), and CYP2J2 (93% inhibition), and to a lesser extent, CYP4F3B (59% inhibition). Interestingly, DHB, which potently inhibited CYP3A4-mediated midazolam 1'-hydroxylation (93% inhibition; data not shown), did not inhibit CYP3A4-mediated DB289 M1 formation (Fig. 7A).

Based on the observed specificity of the chemical inhibitors, HET0016, fluvoxamine, and azamulin were selected and incubated with the first individual donor HLM panel to determine the residual DB289 M1 formation activities. HET0016 markedly inhibited M1 formation with an average percentage inhibition (range) of 51% (34–66%) (Fig. 7B). Fluvoxamine exhibited marginal inhibition at 19% (12%–27%), and azamulin showed moderate inhibition at 37% (26%–47%). When azamulin and fluvoxamine were coinubated (double inhibition; 3 μ M each) with the first individual donor HLM panel, slightly more inhibition of M1 formation was observed with an average percentage inhibition (range) of 42% (30%–51%). To determine whether inhibition of non-CYP4F-mediated M1 formation would improve correlations, the residual activities for DB289 M1 formation in the presence of both fluvoxamine and azamulin were plotted against the total CYP4F protein content determined by the MS-based method or immunoquantification (Fig. 7C). Unfortunately, poor correlations were observed with double inhibition ($r^2 = 0.004$, $P = 0.79$).

Fig. 5. Absolute expression levels of CYP3A proteins in individual HLM samples. Expression levels of total CYP3A (A), CYP3A4 (B), and CYP3A5 (C) proteins were determined in liver microsomal samples obtained from two individual human donor panels. Bars and error bars represent the mean and standard deviation of at least triplicate determinations.

Revised Human Liver Cytochrome P450 Pie. Based on our MS-based quantification of CYP4F2, CYP4F3B and total CYP3A in the two individual donor HLM panels ($n = 31$ total), CYP4F enzymes (i.e., sum of CYP4F2 and CYP4F3B) are expressed at 25.5 pmol/mg HLM protein, whereas CYP3A enzymes are expressed at 54.1 pmol/mg HLM protein (or 47.5 pmol/mg HLM protein excluding H079). CYP4F enzymes are expressed at a level equal to 47%–54% of CYP3A enzymes. Because Shimada et al. (1994) had shown previously that CYP3A enzymes represent approximately 28.8% of the total P450s in human liver, it then can be estimated that CYP4F enzymes represent approximately 15% of the total P450s in human liver (Fig. 8).

Discussion

Our study describes a rapid and robust LC-MS/MS analytic method to determine the absolute protein content of various CYP4F and CYP3A enzymes in HLMs. This method was cross-validated with the orthogonal technique of quantitative Western blot analysis, and good

correlations ($r^2 \geq 0.60$) were observed for all enzymes of interest. The ability to quantitate absolute protein content without the need for antibody production is highly attractive. The cost and time associated with developing antibodies specific for the accurate quantitation of homologous proteins are significant. In addition, antibody-based assays are not always amenable to high-throughput screening, which is desired from a drug-development perspective. Therefore, the use of LC-MS/MS techniques for quantitative proteomics has received significant attention in recent years.

The currently described LC-MS/MS method was applied to a panel of 20 individual donor HLMs to evaluate absolute protein content and interindividual variability of specific enzymes. A second panel of 11 individual donor HLMs that were characterized previously for CYP4F content using similar methodology also was included. Overall, CYP4F2 exhibited considerable interindividual variability (21-fold) in the two HLM panels (Fig. 3A). Conversely, CYP4F3B displayed much less interindividual variability (3.3-fold) (Fig. 3B). However, it should be noted that two HLM samples contained very low levels of CYP4F2 (H0215 and

Fig. 6. Correlation analyses between protein content and marker activities. Protein content, determined by the MS-based method (left y-axis) and immunoquantification (right y-axis), was plotted against individual marker activities. CYP4F activity was evaluated using DB289 M1 formation (A). CYP3A activity was evaluated in-house using midazolam 1'-OH formation (B), in addition to using the manufacturer provided values for testosterone 6 β -OH formation (C).

H0246; ≤ 2 pmol/mg HLM protein) and were the major contributors underlying the high CYP4F2 variability. The remaining samples demonstrated a more limited interindividual variability (3.4-fold), which is consistent with a previous estimate based on the observed rate of leukotriene B₄ 20-hydroxylation (approximately 3-fold) (Jin et al., 1998). Total CYP3A variability observed in the two HLM panels was about 29-fold (Fig. 5A). It has been estimated that the constitutive variability in CYP3A enzymes is about 5-fold, but that this variability can be increased as much as 400-fold due to various factors, including coadministration of inhibitors or inducers, environmental factors, and various disease states (Wilkinson, 2005).

The results presented herein suggest that the CYP4F family of enzymes represents a significant portion of the overall human liver P450 pie. It was shown previously that CYP1A2, CYP2A6, CYP2B6, CYP2C, CYP2D6, CYP2E1, and CYP3A accounted for approximately 70% of the total human liver P450 content; the remaining 30% consisted of then unknown enzymes (Shimada et al., 1994). Based on our results, we propose that a significant portion ($\sim 15\%$) of the previously unknown enzyme content is accounted for by CYP4F enzymes, making them the third largest piece of the human liver P450 pie (Fig. 8). This supports the hypothesis that CYP4F enzymes may play an important role in the metabolism of endogenous and exogenous substances in the human body.

Compared with the previously reported average CYP4F2 content in the HLM (Edson et al., 2013), an identical result (14.3 pmol/mg HLM protein) was obtained in our study consisting of 31 individual donor HLMs. In addition, Edson et al. (2013) reported that another member of CYP4F subfamily, CYP4F11, was also expressed in the human liver

at a level of 8.4 pmol/mg HLM protein. This would suggest that CYP4F enzymes make up an even larger portion of the P450 pie than that reported herein, approximately 18%–21% when CYP4F11 is also taken into consideration. It is not known whether DB289 is a substrate of CYP4F11. If true, it may have contributed to the lack of correlation between DB289 M1 formation and CYP4F protein content (sum of CYP4F2 and CYP4F3B) observed in our study (Fig. 6A).

In addition to the liver, CYP4F enzymes have been identified as the major enteric enzymes responsible for DB289 M1 formation, indicating there is significant expression of these enzymes in the intestine (Wang et al., 2007). However, quantitative analysis has yet to be performed to indicate how CYP4F content in the intestine contributes to the overall enteric P450 pie. Further study is required to understand the significance of CYP4F expression in the intestine and how it may affect processes such as the first-pass metabolism of therapeutic agents or the maintenance of intestinal homeostasis through the metabolism of various endogenous substances.

The correlation of protein content and enzyme activity was evaluated for CYP4F and CYP3A enzymes. No correlation was observed between CYP4F protein content (or RAF-adjusted CYP4F protein content) and DB289 M1 formation (Fig. 6A), though it has been shown previously that CYP4F2 and CYP4F3B are the major hepatic enzymes responsible for this biotransformation (Wang et al., 2006). The good coherence between LC-MS/MS protein content and immunoquantified values suggests that the lack of correlation with activity is unlikely due to incorrectly measured protein content. The chemical inhibition assays (Fig. 7B) suggest that while CYP4F enzymes are responsible for

Fig. 7. Chemical inhibition of DB289 M1 formation in recombinant P450s and HLM samples. A panel of chemical inhibitors was evaluated for the ability to inhibit recombinant P450s known to participate in DB289 M1 formation (A; duplicate determinations) and the first panel of HLM samples (B). Bars and error bars represent the mean and standard deviation of 20 individual donor HLM samples. Residual activity after double inhibition by azamulin (3 μ M) and fluvoxamine (3 μ M) was plotted against CYP4F protein content (C).

a major portion of the observed DB289 M1 formation, additional metabolic pathways likely exist. Our previous work (Wang et al., 2006) implicated CYP1A2, CYP2J2, and CYP3A4 as enzymes capable of catalyzing DB289 M1 formation, though these were shown to have a minor contribution in the overall M1 formation in pooled HLMs. Nonetheless, it is possible that interindividual variability in P450 expression could result in some individual donor HLMs having more-than-average contributions from these non-CYP4F enzymes, thus complicating the correlation analyses. To assess this possibility, residual activities of DB289 M1 formation were determined upon inhibition of non-CYP4F enzymes using P450-selective chemical inhibitors (Fig. 7C). No correlation was observed between CYP4F protein content and the residual M1 formation rates. The lack of an improved correlation after chemical inhibition suggests that DB289 M1 formation is not a good marker activity for CYP4F2 and/or CYP4F3B in HLMs. However, due to the ease of this assay compared with other CYP4F lipid substrates (e.g., LTB4 and arachidonic acid), DB289 M1 formation still may be a useful functional activity assay for CYP4F2 or CYP4F3B when other contributing pathways are absent (e.g., recombinant enzymes and drug induction studies with complementary assays).

Strong correlations ($r^2 \geq 0.78$) were observed for all CYP3A marker activities. This was not unexpected as CYP3A continues to be one of the most studied P450 subfamilies. As such, the metabolic pathways that are used to evaluate CYP3A enzymatic activity have been well-characterized over decades of use. However, surprising results were obtained with respect to the inhibition of CYP3A4-mediated DB289 M1 formation by DHB. This compound has been considered to be a

substrate-independent reversible and mechanism-based inhibitor of CYP3A (Paine et al., 2004), yet negligible inhibition of CYP3A4-mediated DB289 M1 formation was observed (Fig. 7A). This underlines previous observations that inhibition of CYP3A4 catalytic activity is substrate dependent (Stresser et al., 2000; Wang et al., 2000) and that DHB may not be substrate independent as previously thought.

Another potential explanation for the lack of correlation between CYP4F protein content and the observed DB289 M1 formation rate may be altered catalytic activity due to CYP4F2 genetic polymorphisms. Two functional variants, W12G and V433M, were identified recently (Bardowell et al., 2010). The V433M variant has been implicated in interindividual variability associated with warfarin maintenance dose due to effects on vitamin K₁ metabolism (McDonald et al., 2009). Both variants exhibit altered vitamin E metabolism (Bardowell et al., 2010). Interestingly, the variants have been linked to both increased and decreased catalytic activity, with the effects appearing to be substrate dependent. In addition, it has been suggested that the effects on metabolic activity may occur through reduction of the amount of enzyme present, either by inhibiting translation or inducing degradation, or by attenuation of catalytic activity itself. However, it was not possible to examine the effect of genetic polymorphisms on CYP4F expression and catalytic activity in this study due to the lack of genetic polymorphism information for the HLM panels.

In summary, a rapid and reliable LC-MS/MS method was applied for the absolute quantitation of CYP4F and CYP3A protein expression. The method was cross-validated with immunoquantification and evaluation of marker activities with probe substrates. The underlying

Fig. 8. Revised human liver P450 pie. The inset figure depicts the percentage contributions of individual P450 enzymes based on the immunoquantification performed by Shimada et al. (1994). The larger figure shows the revised P450 pie with CYP4F contributing to 15% of the total hepatic P450s.

cause for the lack of correlation between CYP4F protein content and DB289 M1 formation remains unclear at this time, though the presence of functional CYP4F2 genetic variants in the HLMs used may play a role in altering catalytic activity. CYP4F enzymes constitute the third largest piece of the human liver P450 pie, suggesting that they may play an important role in the metabolism of endogenous and exogenous substances in the human body.

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Authorship Contributions

Participated in research design: Wang.
 Conducted experiments: Michaels, Wang.
 Performed data analysis: Michaels, Wang.
 Wrote or contributed to the writing of the manuscript: Michaels, Wang.

References

- Araki N, Tsuruoka S, Hasegawa G, Yanagihara H, Omasa T, Enosawa S, Yamazoe Y, and Fujimura A (2012) Inhibition of CYP3A4 by 6',7'-dihydroxybergamottin in human CYP3A4 over-expressed hepG2 cells. *J Pharm Pharmacol* **64**:1715–1721.
- Bardowell SA, Stec DE, and Parker RS (2010) Common variants of cytochrome P450 4F2 exhibit altered vitamin E-omega-hydroxylase specific activity. *J Nutr* **140**:1901–1906.
- Caldwell MD, Awad T, Johnson JA, Gage BF, Falkowski M, Gardina P, Hubbard J, Turpaz Y, Langae TY, and Eby C, et al. (2008) CYP4F2 genetic variant alters required warfarin dose. *Blood* **111**:4106–4112.

- Christmas P, Jones JP, Patten CJ, Rock DA, Zheng Y, Cheng SM, Weber BM, Carlesso N, Scadden DT, and Rettie AE, et al. (2001) Alternative splicing determines the function of CYP4F3 by switching substrate specificity. *J Biol Chem* **276**:38166–38172.
- Domanski TL, Finta C, Halpert JR, and Zaphiropoulos PG (2001) cDNA cloning and initial characterization of CYP3A43, a novel human cytochrome P450. *Mol Pharmacol* **59**:386–392.
- Edson KZ, Prasad B, Unadkat JD, Suhara Y, Okano T, Guengerich FP, and Rettie AE (2013) Cytochrome P450-dependent catabolism of vitamin K: ω -hydroxylation catalyzed by human CYP4F2 and CYP4F11. *Biochemistry* **52**:8276–8285.
- Fallon JK, Harbourt DE, Maleki SH, Kessler FK, Ritter JK, and Smith PC (2008) Absolute quantification of human uridine-diphosphate glucuronosyl transferase (UGT) enzyme isoforms 1A1 and 1A6 by tandem LC-MS. *Drug Metab Lett* **2**:210–222.
- Generaux CN, Ainslie GR, Bridges AS, Ismail MA, Boykin DW, Tidwell RR, Thakker DR, and Paine MF (2013) Compartmental and enzyme kinetic modeling to elucidate the biotransformation pathway of a centrally acting antitrypanosomal prodrug. *Drug Metab Dispos* **41**:518–528.
- Girault I, Rougier N, Chesné C, Lidereau R, Beaune P, Bieche I, and de Waziers I (2005) Simultaneous measurement of 23 isoforms from the human cytochrome P450 families 1 to 3 by quantitative reverse transcriptase-polymerase chain reaction. *Drug Metab Dispos* **33**:1803–1810.
- Guengerich FP (2005) Human cytochrome P450 enzymes, in: *Cytochrome P450 Structure, Mechanism, and Biochemistry* (Ortiz de Montellano PR ed), pp 377–530. Kluwer Academic/Plenum, New York.
- Hardwick JP (2008) Cytochrome P450 omega hydroxylase (CYP4) function in fatty acid metabolism and metabolic diseases. *Biochem Pharmacol* **75**:2263–2275.
- Hashizume T, Imaoka S, Mise M, Terauchi Y, Fujii T, Miyazaki H, Kamataki T, and Funae Y (2002) Involvement of CYP2J2 and CYP4F12 in the metabolism of ebastine in human intestinal microsomes. *J Pharmacol Exp Ther* **300**:298–304.
- Hsu MH, Savas U, Griffin KJ, and Johnson EF (2007) Human cytochrome p450 family 4 enzymes: function, genetic variation and regulation. *Drug Metab Rev* **39**:515–538.
- Jin R, Koop DR, Raucy JL, and Lasker JM (1998) Role of human CYP4F2 in hepatic catabolism of the proinflammatory agent leukotriene B4. *Arch Biochem Biophys* **359**:89–98.
- Jin Y, Zollinger M, Borell H, Zimmerlin A, and Patten CJ (2011) CYP4F enzymes are responsible for the elimination of fingolimod (FTY720), a novel treatment of relapsing multiple sclerosis. *Drug Metab Dispos* **39**:191–198.
- Ju W, Yang S, Ansedé JH, Stephens CE, Bridges AS, Voyksner RD, Ismail MA, Boykin DW, Tidwell RR, and Hall JE, et al. (2014) CYP1A1 and CYP1B1-mediated biotransformation of the antitrypanosomal methamidoxime prodrug DB844 forms novel metabolites through intramolecular rearrangement. *J Pharm Sci* **103**:337–349.

- Kalsotra A and Strobel HW (2006) Cytochrome P450 4F subfamily: at the crossroads of eicosanoid and drug metabolism. *Pharmacol Ther* **112**:589–611.
- Kalsotra A, Turman CM, Kikuta Y, and Strobel HW (2004) Expression and characterization of human cytochrome P450 4F11: Putative role in the metabolism of therapeutic drugs and eicosanoids. *Toxicol Appl Pharmacol* **199**:295–304.
- Kamiie J, Ohtsuki S, Iwase R, Ohmine K, Katsukura Y, Yanai K, Sekine Y, Uchida Y, Ito S, and Terasaki T (2008) Quantitative atlas of membrane transporter proteins: development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. *Pharm Res* **25**:1469–1483.
- Leeder JS, Gaedigk R, Marcucci KA, Gaedigk A, Vyhldal CA, Schindel BP, and Pearce RE (2005) Variability of CYP3A7 expression in human fetal liver. *J Pharmacol Exp Ther* **314**:626–635.
- Li N, Nemirovskiy OV, Zhang Y, Yuan H, Mo J, Ji C, Zhang B, Brayman TG, Lepsy C, and Heath TG, et al. (2008) Absolute quantification of multidrug resistance-associated protein 2 (MRP2/ABCC2) using liquid chromatography tandem mass spectrometry. *Anal Biochem* **380**:211–222.
- McDonald MG, Rieder MJ, Nakano M, Hsia CK, and Rettie AE (2009) CYP4F2 is a vitamin K1 oxidase: An explanation for altered warfarin dose in carriers of the V433M variant. *Mol Pharmacol* **75**:1337–1346.
- Nebert DW and Russell DW (2002) Clinical importance of the cytochromes P450. *Lancet* **360**:1155–1162.
- Omura T and Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**:2370–2378.
- Paine MF, Criss AB, and Watkins PB (2004) Two major grapefruit juice components differ in intestinal CYP3A4 inhibition kinetic and binding properties. *Drug Metab Dispos* **32**:1146–1153.
- Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, and Zeldin DC (2006) The human intestinal cytochrome P450 “pie”. *Drug Metab Dispos* **34**:880–886.
- Pastrakuljic A, Tang BK, Roberts EA, and Kalow W (1997) Distinction of CYP1A1 and CYP1A2 activity by selective inhibition using fluvoxamine and isosafrole. *Biochem Pharmacol* **53**:531–538.
- Powell PK, Wolf I, Jin R, and Lasker JM (1998) Metabolism of arachidonic acid to 20-hydroxy-5,8,11, 14-eicosatetraenoic acid by P450 enzymes in human liver: involvement of CYP4F2 and CYP4A11. *J Pharmacol Exp Ther* **285**:1327–1336.
- Sehgal N, Agarwal V, Valli RK, Joshi SD, Antonovic L, Strobel HW, and Ravindranath V (2011) Cytochrome P4504f, a potential therapeutic target limiting neuroinflammation. *Biochem Pharmacol* **82**:53–64.
- Shimada T, Yamazaki H, Mimura M, Inui Y, and Guengerich FP (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* **270**:414–423.
- Sontag TJ and Parker RS (2002) Cytochrome P450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin E status. *J Biol Chem* **277**:25290–25296.
- Stresser DM, Blanchard AP, Turner SD, Erve JC, Dandeneau AA, Miller VP, and Crespi CL (2000) Substrate-dependent modulation of CYP3A4 catalytic activity: analysis of 27 test compounds with four fluorometric substrates. *Drug Metab Dispos* **28**:1440–1448.
- Stresser DM, Broudy MI, Ho T, Cargill CE, Blanchard AP, Sharma R, Dandeneau AA, Goodwin JJ, Turner SD, and Erve JC, et al. (2004) Highly selective inhibition of human CYP3Aa in vitro by azamulin and evidence that inhibition is irreversible. *Drug Metab Dispos* **32**:105–112.
- Wang MZ, Sauter JY, Usuki E, Cheung YL, Hall M, Bridges AS, Loewen G, Parkinson OT, Stephens CE, and Allen JL, et al. (2006) CYP4F enzymes are the major enzymes in human liver microsomes that catalyze the O-demethylation of the antiparasitic prodrug DB289 [2,5-bis (4-amidinophenyl)furan-bis-O-methylamidoxime]. 2,5-bis 4-amidinophenyl furan-bis-O-methylamidoxime *Drug Metab Dispos* **34**:1985–1994.
- Wang MZ, Wu JQ, Bridges AS, Zeldin DC, Kombluth S, Tidwell RR, Hall JE, and Paine MF (2007) Human enteric microsomal CYP4F enzymes O-demethylate the antiparasitic prodrug pafuramidine. *Drug Metab Dispos* **35**:2067–2075.
- Wang MZ, Wu JQ, Dennison JB, Bridges AS, Hall SD, Kombluth S, Tidwell RR, Smith PC, Voyksner RD, and Paine MF, et al. (2008) A gel-free MS-based quantitative proteomic approach accurately measures cytochrome P450 protein concentrations in human liver microsomes. *Proteomics* **8**:4186–4196.
- Wang RW, Newton DJ, Liu N, Atkins WM, and Lu AY (2000) Human cytochrome P-450 3A4: in vitro drug-drug interaction patterns are substrate-dependent. *Drug Metab Dispos* **28**:360–366.
- Westlind A, Malmbo S, Johansson I, Otter C, Andersson TB, Ingelman-Sundberg M, and Oscarson M (2001) Cloning and tissue distribution of a novel human cytochrome p450 of the CYP3A subfamily, CYP3A43. *Biochem Biophys Res Commun* **281**:1349–1355.
- Wilkinson GR (2005) Drug metabolism and variability among patients in drug response. *N Engl J Med* **352**:2211–2221.

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