Development of an In Vitro System with Human Liver Microsomes for Phenotyping of CYP2C9 Genetic Polymorphisms with a Mechanism-Based Inactivator

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ABSTRACT:

Polymorphisms in cytochrome P450 enzymes can significantly alter the rate of drug metabolism, as well as the extent of drug-drug interactions. Individuals who homozygotically express the *CYP2C9**3 allele (I359L) of *CYP2C9* exhibit ~70 to 80% reductions in the oral clearance of drugs metabolized through this pathway; the reduction in clearance is ~40 to 50% for heterozygotic individuals. Although these polymorphisms result in a decrease in the activity of individual enzyme molecules, we hypothesized that decreasing the total number of active enzyme molecules in an in vitro system (*CYP2C9**1/*1 human liver microsomes) by an equivalent percentage could produce the same net change in overall metabolic capacity. To this end, the selective *CYP2C9* mechanism-based inactivator tienilic acid was used to reduce irreversibly the total *CYP2C9* activity in human liver microsomes. Tienilic acid concentrations were effectively titrated to produce microsomal

preparations with 43 and 73% less activity, mimicking the *CYP2C9**1/*3 and *CYP2C9**3/*3 genotypes, respectively. With probe substrates specific for other major cytochrome P450 enzymes (*CYP1A2*, *CYP2B6*, *CYP2C8*, *CYP2C19*, *CYP2D6*, *CYP2E1*, and *CYP3A4*), no apparent changes in the rate of metabolism were noted for these enzymes after the addition of tienilic acid, which suggests that this model is selective for *CYP2C9*. In lieu of using rare human liver microsomes from *CYP2C9**1/*3 and *CYP2C9**3/*3 individuals, a tienilic acid-created knockdown in human liver microsomes may be an appropriate in vitro model to determine *CYP2C9*-mediated metabolism of a given substrate, to determine whether other drug-metabolizing enzymes may compensate for reduced *CYP2C9* activity, and to predict the extent of genotype-dependent drug-drug interactions.

Introduction

The cytochrome P450 (P450) superfamily of enzymes is responsible for the oxidative metabolism of more than 90% of current therapeutic xenobiotics (Wang et al., 2009). *CYP2C9* is one of the most abundant P450 enzymes in the human liver and accounts for up to 20% of the total hepatic P450 protein content and 15 to 20% of P450-mediated xenobiotic metabolism, which includes agents such as losartan, many nonsteroidal anti-inflammatory drugs, phenytoin, tolbutamide, and (*S*)-warfarin (Shimada et al., 1994; Rendic, 2002). The widely prescribed drugs phenytoin and (*S*)-warfarin, with narrow therapeutic windows, are of particular interest because impairment of the metabolic activity of *CYP2C9* can lead to difficulties in dose adjustment for these agents and increased frequencies of adverse events (Lee et al., 2002).

CYP2C9 is highly polymorphic. To date, at least 35 variants and a series of subvariants of the *CYP2C9* allele within the coding region have been reported (www.imm.ki.se/CYPalleles). The most common allele, which is considered to be the wild-type allele, is designated

*CYP2C9**1. *CYP2C9**3, which results from a missense mutation in exon 7 that leads to an I359L substitution, is one of the most well studied and clinically significant *CYP2C9* allelic variants. In the white population, 0.4% of individuals are homozygous carriers of *CYP2C9**3 and 15% heterozygous (Wang et al., 2009). Both in vitro and in vivo studies of the *CYP2C9**3 allele have consistently demonstrated significant loss of enzymatic activity, compared with the wild-type allele (Haining et al., 1996; Sullivan-Klose et al., 1996; Steward et al., 1997; Takanashi et al., 2000). Among individuals with *CYP2C9**1/*3 and *CYP2C9**3/*3, oral clearance of commonly used drugs is reduced by 40 to 50% and 70 to 80%, respectively (Kumar et al., 2008).

Polymorphisms in *CYP2C9* significantly alter not only the rate of drug metabolism but also the extent of drug-drug interactions (Hummel et al., 2005; Kumar et al., 2008). Because of the scarcity of human liver preparations from individuals with *CYP2C9**1/*3 and *CYP2C9**3/*3, in vitro studies to date have relied heavily on expressed enzyme systems for evaluation of *CYP2C9* polymorphisms. These systems make it relatively easy to characterize allelic variants, both functionally and structurally, but lack a true physiological environment and do not allow study of the contributions of other enzymes to total metabolism. Pooled human liver microsomes (HLMs), which contain oxidative P450 enzymes and are less expensive and more readily available than human hepatocytes, are a

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ABBREVIATIONS: HLMs, human liver microsomes; P450, cytochrome P450.

valuable in vitro tool for simultaneous examination of drug effects on multiple P450 pathways and rapid predictions of potential drug-drug interactions (Bjornsson et al., 2003). As alluded to above, however, only a limited supply of human microsomes that express CYP2C9 allelic variants exist for genotype-dependent studies. A reliable, practical, in vitro model system is needed to enhance our understanding of CYP2C9 polymorphisms in P450-mediated drug metabolism and drug interactions and ultimately to improve patient safety.

In mechanism-based inactivation, a compound that is catalytically transformed by a P450 enzyme to yield a reactive electrophilic intermediate can inactivate the enzyme irreversibly (Hollenberg et al., 2008). The thiophene derivative tienilic acid is an uricosuric diuretic drug that was withdrawn from the market after it led to rare cases of immunoallergic hepatitis (Homberg et al., 1984). Further investigation revealed that tienilic acid is oxidized by CYP2C9 into a reactive thiophene sulfoxide that covalently binds to CYP2C9, which results in enzyme inactivation (Dansette et al., 1991; López-García et al., 1994; Koenigs et al., 1999). We demonstrated that defined percentages of reduction of CYP2C9 enzymatic activity could be obtained by titrating tienilic acid and recombinant CYP2C9 concentrations (Hutzler et al., 2009).

On the basis of these findings, we hypothesized that a mechanism-based inactivator could be titrated to decrease the total number of active CYP2C9 enzymes in pooled HLMs, such that the net metabolic activity would resemble the reduced metabolic activity of an individual with *CYP2C9**1/*3 or *CYP2C9**3/*3. In the current study, pooled HLMs were exposed to varying tienilic acid concentrations, to mimic the reported net enzymatic activity of the *CYP2C9**1/*3 and *CYP2C9**3/*3 genotypes. To assess the selectivity of this manipulation for CYP2C9, the effects of tienilic acid on other major P450 enzymes also were explored.

Materials and Methods

Chemicals. Tienilic acid was purchased from Cayman Chemical (Ann Arbor, MI). NADPH was obtained from Calbiochem (La Jolla, CA). (S)-Flurbiprofen, 4'-hydroxyflurbiprofen, and 2-fluoro-4-biphenyl acetic acid were gifts from the former Pharmacia (Kalamazoo, MI). Amodiaquine, chlorzoxazone, dextromethorphan, dextrorphan, ethoxyresorufin, levallorphan, omeprazole, resorufin, 6-hydroxychlorzoxazone, 6 β -hydroxytestosterone, and 7-hydroxycoumarin were purchased from Sigma-Aldrich (St. Louis, MO). Bufuralol, bupropion, hydroxybupropion, hydroxybupropion-*d*₆, *N*-desethylamodiaquine, *N*-desethylamodiaquine-*d*₅, testosterone, 1'-hydroxybufuralol, 5-hydroxyomeprazole, and 5-hydroxyomeprazole-*d*₃ were obtained from Toronto Research Chemicals (North York, ON, Canada). 6 β -Hydroxytestosterone-*d*₃ was obtained from Cerilliant (Round Rock, TX). Acetonitrile, ammonium formate, phosphoric acid, and methanol were of high-performance liquid chromatography grade; all other chemicals were of American Chemical Society grade and were obtained from standard commercial sources.

Human Liver Microsomes. The microsomal fraction from frozen human liver tissue, which was obtained through the Liver Tissue Procurement and Distribution System, was prepared through differential centrifugation according to standard methods (Tracy et al., 1993). All procedures were performed at 4°C, and microsomes were stored at -80°C. A bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA) was used to determine total protein concentrations. HLMs from a *CYP2C9**3/*3 liver (HH519) were purchased from BD Biosciences (San Jose, CA).

Determination of CYP2C9 Genotype. A DNeasy tissue kit (QIAGEN, Valencia, CA) was used to isolate DNA from human liver HL9310. DNA was quantitated with a NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). *CYP2C9**3 (rs1057910) was determined with a TaqMan probe (Roche Diagnostics, Indianapolis, IN)-based allelic discrimination assay at the University of Minnesota BioMedical Genomics Center (Minneapolis, MN) (Kumar et al., 2008).

Inactivation of CYP2C9. HLMs (2 mg/ml) were incubated in 50 mM potassium phosphate or Tris buffer (pH 7.4) with tienilic acid. To mimic

*CYP2C9**1/*3 and *CYP2C9**3/*3 genotypes, 4 and 14 μ M tienilic acid was used, respectively. After a 5-min preincubation at 37°C, the reaction was initiated with NADPH (final concentration, 1 mM). Thirty minutes later, the reaction was used to start a second incubation (final HLM concentration, 0.1 mg/ml) with either flurbiprofen, ethoxyresorufin, bupropion, amodiaquine, omeprazole, bufuralol, dextromethorphan, chlorzoxazone, or testosterone, to probe for P450 2C9, 1A2, 2B6, 2C8, 2C19, 2D6, 2D6, 2E1, or 3A4 activity, respectively.

P450 Activity Measurements. *CYP1A2.* Ten-microliter aliquots of HLMs (2 mg/ml) were placed in a 96-well plate. The reaction was initiated with the addition of 190 μ l of prewarmed solution, which contained ethoxyresorufin (final concentration, 0.05–20 μ M) and 50 μ l of NADPH (4 mM) in 50 mM potassium phosphate (pH 7.4). The reaction proceeded for 10 min at 37°C and was stopped with the addition of 50 μ l of ice-cold methanol. Resorufin formation was detected on the basis of fluorescence with a Synergy HT multimode microplate reader (BioTek Instruments, Winooski, VT), with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

P450s 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. Specific incubation conditions for each assay are defined in Tables 1 and 2. In general, potassium phosphate or Tris buffer (50 mM, pH 7.4) containing substrate and 50 μ l of NADPH (4 mM) was preincubated at 37°C for 5 min. To start the reaction, HLMs (final concentration, 0.1 mg/ml) were added, for a total incubation volume of 200 μ l. The reaction was quenched after a specified time period (Tables 1 and 2).

Metabolite Analysis. Analytical conditions for each P450 assay are defined in Tables 1 and 2. P450 assays described in Table 1 used a high-performance liquid chromatography system (Waters, Milford, MA) consisting of an Alliance 2695 autosampler/pump and either a 474 scanning fluorescence detector (for detection of 4'-hydroxyflurbiprofen, 1'-hydroxybufuralol, and dextrorphan) or a 2487 dual-wavelength absorbance detector (for detection of 6-hydroxychlorzoxazone). Analytical methods described in Table 2 were conducted with liquid chromatography-tandem mass spectrometry with a system consisting of an Acquity ultra-performance liquid chromatography autosampler/pump and Micromass Quattro Ultima triple-quadrupole mass spectrometer (Waters) (for detection of hydroxybupropion, *N*-desethylamodiaquine, and 5-hydroxyomeprazole) or an Agilent 1200 series autosampler/pump (Agilent Technologies, Santa Clara, CA) and Thermo Finnigan TSQ Quantum triple-quadrupole mass spectrometer (Thermo Fisher Scientific) (for detection of 6 β -hydroxytestosterone). For all analyses, the mass spectrometers were operated in the positive-ion electrospray mode.

Data Analysis. Unless noted otherwise, all data consisted of three independent experiments and are represented as mean $\pm$ S.D. Kinetic parameters for the substrates were estimated through nonlinear regression analysis with SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA). Data were fit either to a typical Michaelis-Menten equation, $v = (V_{\max} \cdot S)/(K_m + S)$, or to the biphasic equation, which describes a substrate binding to two sites within a single enzyme molecule, $v = [(V_{\max 1} \cdot S) + (CL_{\text{int}} \cdot S^2)]/(K_m + S)$, where CL_{int} is the intrinsic clearance and represents the linear portion of the biphasic kinetic curve ($V_{\max 2}/K_{m2}$). Goodness of fit was determined through visual examination of the resulting curves, comparison of the coefficients of determination, and inspection of Eadie-Hofstee plots. All statistical analyses were conducted with SigmaStat 3.1 (Systat Software, Inc.), by using either Student's *t* test or analysis of variance. Statistical significance was accepted at $p < 0.05$.

Results

CYP2C9 Activity in *CYP2C93/*3 Human Liver Microsomes.** Formation of 4'-hydroxyflurbiprofen, an indication of CYP2C9-mediated activity, was measured in microsomes prepared from five different human livers (data not shown). Four of the HLM preparations demonstrated similar kinetic parameters and were pooled. For the remaining microsomes, the $V_{\max}$ was reduced nearly 95% and the K_m was increased approximately 5-fold, compared with the pooled HLMs. Genotyping confirmed that these microsomes were from a *CYP2C9**3/*3 liver (HL9310). CYP2C9-mediated activity was also measured in commercially available microsomes from a *CYP2C9**3/*3 human liver (HH519). These microsomes (HH519) are the only known commercially available

TABLE 1
Incubation conditions and analytical parameters for CYP2C9, CYP2D6, and CYP2E1 metabolism assays

P450	CYP2C9	CYP2D6	CYP2D6	CYP2E1
Substrate	Flurbiprofen	Bufuralol	Dextromethorphan	Chlorzoxazone
Metabolite	4'-Hydroxyflurbiprofen	1'-Hydroxybufuralol	Dextrophan	6-Hydrochlorzoxazone
Internal standard	2-Fluoro-4-biphenyl acetic acid	Levallorphan	Levallorphan	7-Hydroxycoumarin
Incubation conditions				
Substrate concentrations, μM	2–300	0.5–50	1–200	5–1000
Incubation time, min	20	10	20	20
Quench solvent	90% Acetonitrile/10% phosphoric acid, 100 μl	Acetonitrile, 100 μl	50% Phosphoric acid/50% water, 50 μl	Acetonitrile, 100 μl
Analytical parameters				
Injection volume, μl	30	20	30	50
Column	Brownlee Spheri-5 C18, 4.6 $\times$ 100 mm (PerkinElmer Life and Analytical Sciences, Waltham, MA)	Aqua C18, 2 $\times$ 150 mm (Phenomenex, Torrance, CA)	Cyano, 4.6 $\times$ 150 mm (Regis Technologies, Morton Grove, IL)	YMC-Pack Pro C8, 4.6 $\times$ 150 mm (YMC America, Allentown, PA)
Mobile phase A	90% 5 mM Potassium phosphate (pH 3)/10% acetonitrile	100 mM Ammonium acetate (pH 3)	10% Acetonitrile/0.25% triethylamine (pH 3)	10 mM Ammonium acetate
Mobile phase B	Acetonitrile	Acetonitrile	Methanol	Acetonitrile
Flow rate, ml/min	1	0.45	1	1
Program, % B	Isocratic, 33	20 (0 min) to 60 (8 min) to 20 (9 min) to 20 (10.5 min)	Isocratic, 10	Isocratic, 20
λ (nm), excitation/emission	260/320	252/302	280/310	287
Analyte retention time, min	2.5	2.1	3.5	5.4
Source	Tracy et al., 2002	Crespi et al., 2006	Subramanian et al., 2009	Chittur and Tracy, 1997

CYP2C9*3/*3 HLMs. 4'-Hydroxyflurbiprofen formation was substantially decreased with both CYP2C9*3/*3 liver-derived microsomes (HH519 and HL9310), compared with the pooled HLMs (Fig. 1). Table 3

shows that the kinetic parameters for flurbiprofen 4'-hydroxylation with the pooled HLMs (control) were significantly different from those for the CYP2C9*3/*3 HLMs.

TABLE 2
Incubation conditions and analytical parameters for CYP2B6, CYP2C8, CYP2C19, and CYP3A4 metabolism assays

P450	CYP2B6	CYP2C8	CYP2C19	CYP3A4
Substrate	Bupropion	Amodiaquine	Omeprazole	Testosterone
Metabolite	Hydroxybupropion	N-Desethylamodiaquine	5-Hydroxyomeprazole	6 β -Hydroxytestosterone
Internal standard	Hydroxybupropion- d_6	N-Desethylamodiaquine- d_5	5-Hydroxyomeprazole- d_3	6 β -Hydroxytestosterone- d_3
Incubation conditions				
Substrate concentrations, μM	5–900	0.1–100	1–400	25–500
Incubation time, min	20	10	20	7.5
Quench solvent	Acetonitrile, 100 μl	Acetonitrile, 100 μl	Acetonitrile, 100 μl	Acetonitrile, 100 μl
Analytical parameters				
Injection volume, μl	5	5	2.5	5
Column	Zorbax Eclipse XDB-C18, 4.6 $\times$ 50 mm (Agilent)	YMC Pro C4, 2 $\times$ 50 mm (YMC America)	Zorbax Eclipse XDB-C18, 4.6 $\times$ 50 mm (Agilent)	Brownlee Spheri-5 C18, 4.6 $\times$ 100 mm (PerkinElmer Life and Analytical Sciences)
Mobile phase A	0.3% Formic acid in 5 mM ammonium formate	0.3% Formic acid in 5 mM ammonium formate	0.1% Formic acid in water	90% 10 mM Ammonium formate/10% methanol
Mobile phase B	0.3% Formic acid in methanol	0.3% Formic acid in methanol	0.3% Formic acid in methanol	Methanol
Flow, ml/min	0.5	0.27	0.5	0.5
Program, % B	40 (0 min) to 40 (1 min) to 100 (2.5 min) to 100 (3 min) to 40 (3.1 min) to 40 (5 min)	5 (0 min) to 5 (0.3 min) to 15 (0.6 min) to 30 (2.5 min) to 95 (2.6 min) to 95 (3 min) to 5 (3.1 min) to 5 (3.5 min)	40 (0 min) to 40 (1 min) to 100 (2.5 min) to 100 (3 min) to 40 (3.1 min) to 40 (5.75 min)	50 (0 min) to 50 (2.5 min) to 95 (4 min) to 95 (10 min) to 50 (11 min) to 50 (16 min)
Metabolite retention time, min	2.5	1.7	2.6	5.0
Mass spectrometry parameters				
Metabolite m/z transition	256 $\rightarrow$ 139	328 $\rightarrow$ 283	362 $\rightarrow$ 214	305 $\rightarrow$ 269
Internal standard m/z transition	262 $\rightarrow$ 139	333 $\rightarrow$ 283	365 $\rightarrow$ 214	308 $\rightarrow$ 272
Source	Walsky et al., 2006; Coles and Kharasch, 2008		Li et al., 2004	Subramanian et al., 2010

FIG. 1. CYP2C9-mediated 4'-hydroxyflurbiprofen formation with *CYP2C9*1/*1* (pooled) (●) and *CYP2C9*3/*3* (○, HH519; ▼, HL9310) HLMs. Data are expressed as mean $\pm$ S.D. and were fit to the Michaelis-Menten equation.

Inactivation of CYP2C9 with Tienilic Acid. The mechanism-based inactivator tienilic acid was used to inactivate CYP2C9. Various tienilic acid and total protein concentrations were incubated in the presence of NADPH for 30 min before the addition of 200 μ M flurbiprofen, for determination of the proportion of CYP2C9-mediated flurbiprofen activity remaining with respect to no tienilic acid exposure. Loss of CYP2C9-mediated activity was dependent on both tienilic acid and microsomal protein concentrations (Fig. 2). Increasing the tienilic acid concentration increased the inactivation of CYP2C9, whereas increasing the protein concentration tended to decrease CYP2C9 inactivation at a given tienilic acid concentration. Of the three total protein concentrations, 0.1 mg/ml resulted in the most-rapid loss of CYP2C9-mediated activity. A 5 μ M tienilic acid concentration resulted in a loss of approximately half of the activity in the 0.1 mg/ml HLM group; for a similar resulting loss in activity, 15 or 40 μ M tienilic acid was necessary for the 0.2 and 0.5 mg/ml HLM groups, respectively. To limit tienilic acid exposure, all subsequent experiments used a 0.1 mg/ml HLM concentration.

Tienilic Acid-Created CYP2C9 Genotype Equivalents. The tienilic acid titration curve (Fig. 2) was used to estimate that 4 and 14 μ M tienilic acid concentrations would be required to generate 40 to 50% and 70 to 80% losses of CYP2C9 activity and therefore to mimic the *CYP2C9*1/*3* and *CYP2C9*3/*3* genotypes, respectively. Figure 3 depicts 4'-hydroxyflurbiprofen formation in the tienilic acid-created *CYP2C9*1/*3* and *CYP2C9*3/*3* equivalents. The $V_{\max}$ was reduced by 43% with the *CYP2C9*1/*3* chemical equivalent ($p = 0.009$) and by 73% with the *CYP2C9*3/*3* chemical equivalent ($p < 0.001$), compared with *CYP2C9*1/*1* (Table 4). Differences in K_m between *CYP2C9*1/*1* and the chemical equivalent genotypes were not statistically significant.

TABLE 3

Kinetic parameters of 4'-hydroxyflurbiprofen formation with pooled, HH519, and HL9310 human liver microsomes

Data were fit to the Michaelis-Menten equation and are presented as mean $\pm$ S.E. of the fit.

HLM Sample	CYP2C9 Genotype	$V_{\max}$	K_m	$V_{\max}/K_m$
		$\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$	μM	
Pooled	*1/*1	856 ± 10	5 ± 0.3	171
HH519	*3/*3	$167 \pm 4^{***}$	$17 \pm 2^*$	10
HL9310	*3/*3	$49 \pm 3^{***}$	$24 \pm 6^*$	2

* $p < 0.05$.

*** $p < 0.001$.

FIG. 2. Proportions of remaining CYP2C9-mediated flurbiprofen activity, expressed as mean $\pm$ S.D., with 0.1 (▼), 0.2 (○), or 0.5 (●) mg/ml total human liver microsomal protein after treatment with 0 to 100 μ M tienilic acid. The 0.2 and 0.5 mg/ml microsomal protein data were collected once in triplicate.

Effects of Tienilic Acid on Non-CYP2C9 P450s. Ethoxyresorufin *O*-deethylation, bupropion hydroxylation, amodiaquine *N*-deethylation, omeprazole 5-hydroxylation, bufuralol 1'-hydroxylation, dextromethorphan *O*-demethylation, chlorzoxazone 6-hydroxylation, and testosterone 6 β -hydroxylation were studied after an initial incubation in the absence or presence of tienilic acid, to determine the effect of tienilic acid on the enzymatic activity of P450s 1A2, 2B6, 2C8, 2C19, 2D6 (bufuralol and dextromethorphan), 2E1, and 3A4, respectively. Resorufin formation was best fit with biphasic kinetics, as indicated by the Eadie-Hofstee plot (data not shown), and the $V_{\max}$ ($p = 0.851$) and K_m ($p = 0.682$) values were statistically similar among the three groups (Table 5). The formation of hydroxybupropion, *N*-desethylamodiaquine, 5-hydroxyomeprazole, 1'-hydroxybufuralol, 6-hydroxy-chlorzoxazone, and 6 β -hydroxytestosterone all displayed simple Michaelis-Menten kinetics. Statistical comparisons (analysis of variance) demonstrated no statistically significant differences ($p > 0.05$) in the kinetic parameter estimates for each of these probe substrates in the *CYP2C9*1/*3* and *CYP2C9*3/*3* chemical equivalent HLMs, compared with the pooled control HLMs (*CYP2C9*1/*1*) (Table 5). These data suggest that tienilic acid is selective for CYP2C9.

FIG. 3. CYP2C9-mediated flurbiprofen metabolism in *CYP2C9*1/*1* pooled HLMs (●), compared with tienilic acid-created *CYP2C9*1/*3* (4 μ M tienilic acid) (○) and *CYP2C9*3/*3* (14 μ M tienilic acid) (▼) enzymatic activity equivalents. Data are expressed as mean $\pm$ S.D. and were fit to the Michaelis-Menten equation.

TABLE 4

Kinetic parameters of 4'-hydroxyflurbiprofen formation with tienilic acid-created CYP2C9 equivalent genotypes

Data were fit to the Michaelis-Menten equation and are expressed as mean $\pm$ S.E. of the fit.

Chemical Genotype Equivalent	$V_{\max}$	K_m	$V_{\max}/K_m$
	$\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$	μM	
CYP2C9*1/*1	858 $\pm$ 13	7 $\pm$ 1	123
CYP2C9*1/*3	490 $\pm$ 6**	7 $\pm$ 0.4	70
CYP2C9*3/*3	232 $\pm$ 5***	17 $\pm$ 2	14

** $p < 0.01$.

*** $p < 0.001$.

The generation of dextromethorphan from dextromethorphan was different in the absence and presence of tienilic acid. Michaelis-Menten kinetics were observed for the CYP2C9*1/*3 and CYP2C9*3/*3 groups, whereas the CYP2C9*1/*1 group data were best fit to a biphasic kinetic profile (Table 5). These differences in kinetic profiles were confirmed visually with an Eadie-Hofstee plot (data not shown). The formation rates in the three groups were similar up to $\sim 20 \mu\text{M}$ dextromethorphan but began to diverge after the $50 \mu\text{M}$ dextromethorphan concentration. Statistical analyses were performed with the formation rates with the various genotype equivalents at each substrate (dextromethorphan) concentration. The formation rates were observed to be statistically different, such that the p value was < 0.001 at substrate concentrations above $50 \mu\text{M}$ (CYP2C9*1/*1 versus CYP2C9*1/*3 or CYP2C9*3/*3 genotype equivalents). There was no statistical difference in the $V_{\max}$ or K_m values between the CYP2C9*3 chemical equivalents.

Discussion

Considering genetics when choosing a drug regimen and dosage for an individual patient is becoming increasingly common. Therefore, understanding how genotypes affect the metabolism of specific substrates and concomitant administration of drugs is important both for

the optimization and individualization of drug-based patient treatment and for the development of safer and more cost-effective drug therapies. Our ability to study the impact of these genetic differences in vitro in the presence of other drug-metabolizing enzymes is restricted because of the limited availability of genotype-determined HLMs and hepatocytes. Here, the possibility of using a CYP2C9-selective, mechanism-based inactivator to mimic the net loss of catalytic activity observed in CYP2C9 polymorphisms in pooled HLMs is explored.

The CYP2C9*3 allele leads to significant reductions in CYP2C9-mediated metabolism. In the present study, CYP2C9*3/*3 HLMs HH519 and HL9310 exhibited 3.4- and 4.8-fold increases in K_m and 5.1- and 17.5-fold decreases in $V_{\max}$, respectively, compared with pooled HLMs (CYP2C9*1/*1). The CYP2C9*3/*3 chemical equivalent exhibited a 2.4-fold increase in K_m and a 3.7-fold decrease in $V_{\max}$. These trends in the kinetic parameter estimates are consistent with previous in vitro reports with other CYP2C9 substrates (Haining et al., 1996; Bhasker et al., 1997; Coller et al., 2002; Tracy et al., 2002). Takanashi et al. (2000) examined the effect of the CYP2C9*3 allele on CYP2C9-mediated metabolism in an expressed system. Compared with the wild-type allele, the CYP2C9*3 allele increased the K_m 1.5- to 11.4-fold across seven substrates and decreased the $V_{\max}$ 2.2- to 21.5-fold with five of the seven substrates. Although the catalytic activity is reduced with the CYP2C9*3 allele, the magnitude of impairment in CYP2C9-mediated metabolism seems to be substrate-dependent. Data from the current study are within these ranges.

Six substrate recognition sites within CYP2C9, which constitute $\sim 16\%$ of the total residues, have been identified; the I359L substitution responsible for the CYP2C9*3 allele falls within substrate recognition site 5 (Gotoh, 1992). Crystallographic studies suggest that the CYP2C9*3 amino acid substitution, which is located in the interior of the enzyme, is not in the vicinity of the substrate binding site and/or active site around the heme, which implies that the loss of CYP2C9-mediated activity is not caused by alterations in substrate binding (Wester et al., 2004). Our laboratory reported that neither altered substrate binding affinity nor altered coupling affinity with the redox

TABLE 5

Kinetic parameters of the effect of tienilic acid on non-CYP2C9 P450 enzymatic activity

Data were fit to the Michaelis-Menten equation or the biphasic equation and are presented as mean $\pm$ S.E. of the best fit.

P450	Substrate	CYP2C9 Chemical Equivalent	$V_{\max}$	K_m	$V_{\max}^a$	K_m^a	CL_{int}^a
			$\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$	μM	$\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$	μM	$\mu\text{l} \cdot \text{min}^{-1} \cdot \text{pmol P450}^{-1}$
1A2	Ethoxyresorufin	*1/*1			997 $\pm$ 57	0.2	32 $\pm$ 4
		*1/*3			992 $\pm$ 68	0.3 $\pm$ 0.1	25 $\pm$ 5
		*3/*3			1074 $\pm$ 79	0.3 $\pm$ 0.1	24 $\pm$ 6
2B6	Bupropion	*1/*1	340 $\pm$ 3	153 $\pm$ 4			
		*1/*3	331 $\pm$ 3	150 $\pm$ 3			
		*3/*3	321 $\pm$ 2	138 $\pm$ 3			
2C8	Amodiaquine	*1/*1	1180 $\pm$ 22	3 $\pm$ 0.3			
		*1/*3	1164 $\pm$ 22	3 $\pm$ 0.3			
		*3/*3	1184 $\pm$ 18	3 $\pm$ 0.2			
2C19	Omeprazole	*1/*1	76 $\pm$ 2	9 $\pm$ 1			
		*1/*3	71 $\pm$ 1	9 $\pm$ 0.4			
		*3/*3	71 $\pm$ 2	9 $\pm$ 1			
2D6	Bufuralol	*1/*1	146 $\pm$ 7	13 $\pm$ 2			
		*1/*3	147 $\pm$ 6	14 $\pm$ 1			
		*3/*3	148 $\pm$ 6	15 $\pm$ 1			
2D6	Dextromethorphan	*1/*1			480 $\pm$ 31	10 $\pm$ 2	1 $\pm$ 0.2
		*1/*3	453 $\pm$ 5	10 $\pm$ 0.5			
		*3/*3	429 $\pm$ 5	9 $\pm$ 0.5			
2E1	Chlorzoxazone	*1/*1	2789 $\pm$ 261	48 $\pm$ 18			
		*1/*3	3192 $\pm$ 190	58 $\pm$ 13			
		*3/*3	2996 $\pm$ 289	61 $\pm$ 22			
3A4	Testosterone	*1/*1	640 $\pm$ 14	106 $\pm$ 6			
		*1/*3	597 $\pm$ 21	97 $\pm$ 9			
		*3/*3	629 $\pm$ 17	115 $\pm$ 8			

^a Fit to the biphasic equation.

partner protein could explain the difference in CYP2C9 catalytic activity with the *CYP2C9*3* allele, and we considered disruption of the water network to be a key factor in the decreased catalytic activity (Wei et al., 2007). On the basis of the substrate-free CYP2C9 crystal structure, molecular dynamic simulations indicated that the I359L substitution creates a ~30% expansion in the binding pocket near the F' helix (Sano et al., 2010). This expansion of space increases the fluctuation of residues in the F-G helices, which are responsible for substrate binding, which may explain why the magnitude of impairment is substrate-dependent.

Each individual CYP2C9.3 molecule exhibits reduced enzymatic activity, and the total population of such molecules exhibits a net reduction in enzymatic activity. Tienilic acid, a CYP2C9 mechanism-based inactivator, is hydroxylated by CYP2C9 on the thiophene ring to form the major metabolite, 5-hydroxytienilic acid (Mansuy et al., 1984). A highly reactive, electrophilic intermediate is generated during the metabolism of tienilic acid, which covalently binds to and inactivates the CYP2C9 molecule (López-García et al., 1994; Koenigs et al., 1999). In contrast to CYP2C9.3 enzyme molecules that exhibit reduced function, tienilic acid completely inactivates each enzyme molecule. Tienilic acid can be incubated with CYP2C9 enzymes under specific conditions such that only a portion of the total enzyme pool is inactivated and therefore a net reduction in total enzymatic activity is observed (McGinnity et al., 2006; Hutzler et al., 2009) (Fig. 2). On the basis of the logic that the total reduction in catalytic activity would be the same regardless of whether it was derived from the presence of a *CYP2C9*3* allele or tienilic acid-exposed CYP2C9 enzymes, the catalytic activity of CYP2C9 polymorphisms was mimicked by using tienilic acid in pooled HLMs (Fig. 3).

With an approach of this type, it must be ensured that other P450 enzymes are not affected during the process. Metabolism of probe substrates ethoxyresorufin, bupropion, amodiaquine, omeprazole, bufuralol, chlorzoxazone, and testosterone by P450s 1A2, 2B6, 2C8, 2C19, 2D6, 2E1, and 3A4, respectively, were unaffected by tienilic acid in pooled HLMs, which suggests that the tienilic acid-based model is selective for CYP2C9 (Table 5). Various human P450s expressed independently in yeast were examined for estimation of the role of CYP2C9 as a producer of tienilic acid metabolites and as a target of covalent binding in HLMs (Lecoeur et al., 1994). Of all of the P450s tested (1A1, 1A2, 2C8, 2C9, 2C18, 2D6, and 3A4), CYP2C9 produced more than 92% of the total reactive metabolites. The selectivity of tienilic acid for CYP2C9 in earlier recombinant enzyme models is in agreement with the results presented here with endogenous human P450s expressed in HLMs.

In contrast to CYP2D6-mediated hydroxylation of bufuralol, *O*-demethylation of the CYP2D6 probe substrate dextromethorphan was altered in the presence of tienilic acid. Dextromethorphan concentrations greater than 50 μ M exhibited increased dextrophan formation in the absence of tienilic acid. It is unclear whether the biphasic kinetics observed in the absence of tienilic acid may be explained by multiple binding sites within the CYP2D6 active site that exhibit different affinities and turnover rates or whether dextromethorphan metabolism involves more than one enzyme. If the involvement of more than one enzyme is responsible for the biphasic kinetics, then it is likely that, in addition to CYP2D6, CYP3A4 is participating. CYP2D6 is reported to contribute to at least 80% of dextrophan formation, with a K_m of 3.7 μ M, and CYP3A4 is predicted to contribute ~15% of dextrophan formation, with a K_m of 157 μ M (Yu and Haining, 2001). The phenomenon seems to be specific for the probe dextromethorphan, because neither bufuralol metabolism by CYP2D6 nor testosterone metabolism by CYP3A4 was altered by tienilic acid.

The impact of CYP2C9 genotype-dependent drug interactions has been gaining more attention (Hummel et al., 2005; Kumar et al., 2006). Individuals expressing no, one, or two *CYP2C9*3* alleles exhibited gene dose-dependent effects, such that essentially no change in flurbiprofen clearance occurred in *CYP2C9*3/*3* individuals when flurbiprofen and fluconazole were coadministered (Kumar et al., 2008), in contrast to the significant interaction observed for *CYP2C9*1/*1* individuals. The effect of CYP2C9 inhibition was minimal for *CYP2C9*3/*3* individuals, because such a small proportion of the total flurbiprofen clearance was mediated through a CYP2C9 pathway. In vivo flurbiprofen clearance and the magnitude of drug interaction predictions from corresponding in vitro studies with recombinant CYP2C9 variants were consistent with the in vivo noncompartmental results, which indicates that in vitro genotype-dependent drug interaction studies can be used to predict in vivo results.

Although the in vitro model described herein does not create the true genotype but only mimics the activity of the CYP2C9 variant, it offers several advantages, compared with current systems. HLMs and hepatocytes with known CYP2C9 variants are rare, but the system described herein provides an abundant inexpensive model that eliminates individual variability. The mechanism-based inactivator can be titrated to decrease the total activity to various levels that represent various genotypes and therefore is not restricted to mimicking only the *CYP2C9*3* genotype. The model also has numerous potential in vitro applications, such as the determination of CYP2C9-mediated metabolism of a given substrate, the determination of whether other drug-metabolizing enzymes may compensate for reduced CYP2C9 activity, and the prediction of the extent of genotype-dependent drug interactions. It should be noted that, to maintain consistency, tienilic acid concentrations need to be adjusted for each new HLM pool (data not shown). Tienilic acid-treated microsomes should be diluted and/or washed to minimize unbound molecules, because tienilic acid has been reported to react with protein nucleophiles, to deplete glutathione levels, and to up-regulate genes involved in oxidative stress responses and phase II drug metabolism (Belghazi et al., 2001; López-García et al., 2005; Nishiya et al., 2008); this is most applicable to hepatocyte preparations.

In conclusion, tienilic acid can be used to decrease selectively total CYP2C9 catalytic activity, such that it resembles the net CYP2C9 catalytic activity observed in CYP2C9 polymorphic variants. This demonstration of feasibility of an in vitro human liver microsomal model of CYP2C9 variants provides a potentially valuable tool for drug metabolism and drug interaction studies, particularly as pharmacological therapies become increasingly tailored toward individualized medicine.

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Authorship Contributions

Participated in research design: Flora and Tracy.

Conducted experiments: Flora.

Performed data analysis: Flora.

Wrote or contributed to the writing of the manuscript: Flora and Tracy.

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