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

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Running Title: Tienilic Acid-Created CYP2C9 Genotype Equivalents

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Abbreviations Used: HLM, human liver microsome; P450, cytochrome P450
Abstract

Polymorphisms in cytochrome P450 enzymes can significantly alter the rate of drug metabolism as well as the extent of drug-drug interactions. Individuals homozygotically expressing the *3 allele (I359L) of CYP2C9 exhibit an ~70-80% reduction in oral clearance of drugs metabolized by this pathway, and in heterozygotic individuals the reduction in clearance is ~40-50%. Although these polymorphisms result in a decrease in the activity of an individual enzyme molecule, 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 mimic the same net change in overall metabolic capacity. To this end, the selective CYP2C9 mechanism-based inactivator tienilic acid was used to irreversibly reduce total CYP2C9 activity in human liver microsomes. Tienilic acid concentrations were effectively titrated to produce microsomal preparations with 43% and 73% less activity, respectively mimicking the CYP2C9*1/*3 and CYP2C9*3/*3 genotypes. Using probe substrates specific for other major P450 enzymes (CYP1A2, CYP2B6, CYP2C8, CYP2C19, CYP2D6, CYP2E1, and CYP3A4), no apparent changes in rate of metabolism were noted for these enzymes following the addition of tienilic acid, suggesting 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 are 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 non-steroidal anti-inflammatory drugs, phenytoin, tolbutamide, and S-warfarin (Shimada et al., 1994; Rendic, 2002). The widely prescribed narrow therapeutic drugs phenytoin and S-warfarin are of particular interest because impairment in the metabolic activity of CYP2C9 can lead to difficulties in dose adjustment for these agents as well as increased frequency 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 have been reported within the coding region (http://www.imm.ki.se/CYPalleles). The most common allele, also considered to be the wild-type allele, is designated CYP2C9*1. CYP2C9*3, which results from a missense mutation in exon 7 leading to an Ile359Leu substitution, is one of the most studied and clinically significant CYP2C9 allelic variants. Among the Caucasian population, 0.4% are homozygous carriers of CYP2C9*3 and 15% heterozygous (Wang et al., 2009). Both in vitro and in vivo studies of the *3 allele have consistently demonstrated significant loss of enzymatic activity compared to the wild-type allele (Haining et al., 1996; Sullivan-Klose et al., 1996; Steward et al., 1997; Takanashi et al., 2000). In CYP2C9*1/*3 and CYP2C9*3/*3 individuals oral clearance of commonly used drugs is reduced by 40-50% and 70-80%, 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 CYP2C9*1/*3 and CYP2C9*3/*3 individuals, in vitro studies to date have relied heavily on expressed enzyme systems to evaluate 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 contribution 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 valuable in vitro tool for examining the effect of a drug on multiple P450 pathways simultaneously and for rapidly predicting potential drug-drug interactions (Bjornsson et al., 2003). But as alluded to above, for genotype-dependent studies only a limited supply of human microsomes exist that express CYP2C9 allelic variants. Thus, a reliable and practical in vitro model system is needed to enhance our understanding of CYP2C9 polymorphisms on P450-mediated drug metabolism and drug interactions, and ultimately 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 leading 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 resulting in enzyme inactivation (Dansette et al., 1991; Lopez-Garcia et al., 1994; Koenigs et al., 1999). Recently, we demonstrated that a defined percent reduction of CYP2C9 enzymatic activity could be obtained by titrating tienilic acid and recombinant CYP2C9 concentrations (Hutzler et al., 2009).

Based on these findings, we hypothesized that a mechanism-based inactivator could be titrated in such a manner as to decrease the total number of active CYP2C9 enzymes in pooled HLMs such that the net metabolic activity resembled the reduced metabolic activity of a CYP2C9*1/*3 or CYP2C9*3/*3 individual. 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 effect of tienilic acid on other major P450 enzymes was also 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, Inc. (Kalamazoo, MI). Amodiaquine, chlorzoxazone, dextromethorphan, dextrophan, ethoxyresorufin, levallorphan, omeprazole, resorufin, 6-hydroxychlorzoxazone, 6β-hydroxytestosterone, and 7-hydroxycoumarin were purchased from Sigma-Aldrich in St. Louis, MO. Bufuralol, bupropion, hydroxybupropion, hydroxybupropion-d6, N-desethylamodiaquine, N-desethylamodiaquine-d5, testosterone, 1’-hydroxybufuralol, 5-hydroxyomeprazole, and 5-hydroxyomeprazole-d3 were obtained from Toronto Research Chemicals, Inc. (North York, ON, Canada). 6β-Hydroxytestosterone-d3 was from Cerilliant (Round Rock, TX). Acetonitrile, ammonium formate, phosphoric acid, and methanol were of HPLC-grade and all other chemicals were of ACS-grade and were obtained from standard commercial sources.

Human Liver Microsomes. The microsomal fraction from frozen human liver tissue obtained through the Liver Tissue Procurement and Distribution System (LTPADS) was prepared by differential centrifugation according to standard methods (Tracy et al., 1993). All procedures were carried out at 4ºC and microsomes were stored at -80ºC. A BCA protein assay kit (Pierce Biotechnology, Rockford, IL) was used to determine total protein concentrations. Human liver microsomes (HLMs) from a CYP2C9*3/*3 liver (HH519) were purchased from BD Biosciences (Woburn, MA).

Determination of CYP2C9 Genotype. A DNeasy tissue kit (Qiagen, Valencia, CA) was used to isolate DNA from human liver HL9310. DNA was quantitated on a NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). CYP2C9*3 (rs1057910) was determined by a TaqMan-based allelic discrimination assay at the University of Minnesota BioMedical Genomics Center (Minneapolis, MN) (Kumar et al., 2008).
Inactivation of CYP2C9. Human liver microsomes (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 µM tienilic acid were used, respectively. After a 5 min preincubation at 37ºC, the reaction was initiated with NADPH (1 mM final concentration). Thirty minutes later, the reaction was used to start a second incubation (0.1 mg/ml final HLM concentration) 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 microliters of 2 mg/ml HLMs were aliquoted into a 96-well plate. The reaction was initiated with the addition of 190 µl of prewarmed solution, which contained ethoxyresorufin (0.05 to 20 µM final concentration) and 50 µl 4 mM NADPH in 50 mM potassium phosphate (pH 7.4). The reaction proceeded for 10 min at 37ºC and was stopped with the addition of 50 µl ice-cold methanol. Resorufin formation was detected by fluorescence on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT) with an excitation wavelength of 530 nm and an emission of 590 nm.

CYP2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. Specific incubation conditions for each assay are defined in Tables 1 and 2. Generally, potassium phosphate or TRIS buffer (50 mM, pH 7.4) containing substrate and 50 µl 4 mM NADPH was preincubated at 37ºC for 5 min. To start the reaction, HLMs (0.1 mg/ml final concentration) were added for a total incubation volume of 200 µl. 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 an HPLC 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 λ Absorbance Detector (for detection of 6-hydroxychlorzoxazone). Analytical methods in Table 2
were conducted by LC-MS/MS on either a system consisting of an ACQUITY UPLC 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 (Santa Clara, CA) and Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (Thermo Fisher Scientific) system (for detection of 6β-hydroxytestosterone). For all analyses, the mass spectrometers were operated in the positive ionization electrospray mode.

**Data Analysis.** Unless noted, all data consist of three independent experiments and are represented as the mean ± standard deviation (S.D.). Kinetic parameters for the substrates were estimated by nonlinear regression analysis using SigmaPlot 10.0 (Systat Software, Inc., Chicago, IL). Data were fit to either a typical Michaelis-Menten equation (eq. 1), \( v = \frac{V_{\text{max}} \cdot S}{K_m + S} \), or to the biphasic equation (eq. 2), which describes a substrate binding to two sites within a single enzyme molecule: \( v = \frac{[(V_{m1} \cdot S) + (CL_{\text{int}} \cdot S^2)]}{K_m + S} \), where \( CL_{\text{int}} \) is referred to as intrinsic clearance and represents the linear portion of the biphasic kinetic curve \( \frac{V_{\text{max2}}}{K_m2} \). Goodness of fit was determined by 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.) using either a Student’s t-test or ANOVA. Statistical significance was accepted at \( p < 0.05 \).
Results

CYP2C9 Activity in CYP2C9*3/*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 human liver microsomes (HLMs) demonstrated similar kinetic parameters and were pooled. In the remaining microsomes, the $V_{\text{max}}$ was reduced nearly 95% and the $K_m$ increased approximately 5-fold compared to 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 (HH519) are the only known commercially available CYP2C9*3/*3 HLMs. 4'-Hydroxyflurbiprofen formation was substantially diminished in both CYP2C9*3/*3 liver-derived microsomes, HH519 and HL9310, compared to the pooled HLMs (Fig. 1). Table 3 shows that the kinetic parameters for flurbiprofen 4'-hydroxylation in the pooled HLMs (control) were significantly different than that of the CYP2C9*3/*3 HLMs.

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 prior to the addition of 200 µM flurbiprofen to determine the percent 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 tienilic acid concentration increased inactivation of CYP2C9, whereas increasing 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 µM tienilic acid concentration resulted in a loss of approximately half of the activity in the 0.1 mg/ml HLM group,
while for a similar resulting loss in activity 15 or 40 µM tienilic acid were necessary in 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 utilized to estimate that a 4 and 14 µM tienilic acid concentration would be required to generate a 40-50% and 70-80% loss of CYP2C9 activity and, therefore, 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% in the CYP2C9*1/*3 chemical equivalent (p = 0.009) and 73% in the CYP2C9*3/*3 chemical equivalent (p < 0.001) compared to CYP2C9*1/*1 (Table 4). Changes in K_m between CYP2C9*1/*1 and the chemical equivalent genotypes were not statistically significant.

**Effect 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 6β-hydroxylation were studied following an initial incubation in the absence and 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 an Eadie-Hofstee plot (data not shown), and the V_max (p = 0.851) and K_m (p = 0.682) were statistically similar among the three groups (Table 5). The formation of hydroxybupropion, N-desethylamodiaquine, 5-hydroxyomeprazole, 1'-hydroxybufuralol, 6-hydroxychlorzoxazone, and 6β-hydroxytestosterone all displayed simple Michaelis-Menten kinetics. Statistical comparisons (ANOVA) demonstrated no statistically significant difference (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 as...
compared to the pooled control HLMs (CYP2C9*1/*1) (Table 5). These data suggest that tienilic acid is selective for CYP2C9.

The generation of dextrorphan from dextromethorphan was different in the absence and presence of tienilic acid. Michaelis-Menten kinetics were observed with the CYP2C9*1/*3 and CYP2C9*3/*3 groups, while the CYP2C9*1/*1 group was best fit to a biphasic kinetic profile (Table 5). These differences in kinetic profiles were also confirmed visually with an Eadie-Hofstee plot (data not shown). The formation rates in the three groups were similar up to ~20 µM dextromethorphan, but began to diverge after the 50 µM dextromethorphan concentration. Statistical analyses were performed on the formation rates in the various genotype equivalents at each substrate (dextromethorphan) concentration. The formation rates were observed to be statistically different, such that the p value was less than 0.001, at substrate concentrations above 50 µM (CYP2C9*1/*1 vs. CYP2C9*1/*3 or CYP2C9*3/*3 genotype equivalents). Meanwhile there was no statistical difference in the V_{max} or K_{m} between the CYP2C9*3 chemical equivalents.
Consideration of genetics when choosing a drug regimen and dose for an individual patient is becoming increasingly more common. Therefore, understanding how genotypes affect the metabolism of specific substrates and, furthermore, concomitant administration of drugs is important both for the optimization and individualization of drug-based patient treatment as well as 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 due to the limited availability of genotype-determined human liver microsomes (HLMs) and hepatocytes. Here, the possibility of utilizing a CYP2C9-selective mechanism-based inactivator to mimic the net loss of catalytic activity observed in CYP2C9 polymorphisms in pooled human liver microsomes 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 a 3.4- and 4.8-fold increase in K_m and a 5.1- and 17.5-fold decrease in V_max, respectively, compared to pooled HLMs (CYP2C9*1/*1). The CYP2C9*3/*3 chemical equivalent exhibited a 2.4-fold increase in K_m and 3.7-fold decrease in V_max. These trends in the kinetic parameter estimates are consistent with previous in vitro reports using 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 *3 allele on CYP2C9-mediated metabolism in an expressed system. Compared to the wild-type allele, the *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 in five of the seven substrates. Thus, although the catalytic activity is reduced in the *3 allele, the magnitude of impairment in CYP2C9-mediated metabolism appears to be substrate-dependent. Data from the current study are within these ranges.
Six substrate recognition sites (SRS) within CYP2C9, which constitute about 16% of the total residues, have been identified; the Ile359Leu substitution responsible for the *3 allele falls within SRS5 (Gotoh, 1992). Crystallography studies suggest that the *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 and, therefore, 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 partner protein could explain the difference in CYP2C9 catalytic activity in the *3 allele, and attributed disruption of the water network to be a key factor in the decreased catalytic activity (Wei et al., 2007). Based on the substrate-free CYP2C9 crystal structure, molecular dynamic simulations indicate that the Ile359Leu substitution creates an approximately 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, and may explain why the magnitude of impairment is substrate-dependent.

Each individual CYP2C9.3 molecule exhibits reduced enzymatic activity and thus, the total population of such molecules also 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). During the metabolism of tienilic acid, a highly reactive electrophilic intermediate is generated which covalently binds to and inactivates the CYP2C9 molecule (Lopez-Garcia 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. Nevertheless, 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). Through the logic that the total reduction in catalytic activity is the same, regardless whether it is derived from the presence of a CYP2C9*3 allele or
tienilic acid-exposed CYP2C9 enzymes, the catalytic activity of CYP2C9 polymorphisms were mimicked using tienilic acid in pooled HLMs (Fig. 3).

With an approach of this type one must assure that other CYP enzymes are not affected in 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 suggesting that the tienilic acid-based model is selective for CYP2C9 (Table 5). Previously, various human P450s expressed independently in yeast were examined to estimate the role of CYP2C9 as a producer of tienilic acid metabolites and as a target of covalent binding in human liver microsomes (Lecoeur et al., 1994). Of all the P450s tested (1A1, 1A2, 2C8, 2C9, 2C18, 2D6, and 3A4), CYP2C9 produced more than 92% of the total reactive metabolites. Thus, the selectivity of tienilic acid for CYP2C9 in earlier recombinant enzyme models is in agreement with the results presented here using endogenous human P450s expressed in HLMs.

Interestingly, 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 µM exhibited increased dextrorphan formation in the absence of tienilic acid. It is unclear as to whether the biphasic kinetics observed in the absence of tienilic acid may be explained by multiple binding sites within the CYP2D6 active site, which exhibit different affinities and turnover rates, or if dextromethorphan metabolism involves more than one enzyme. If the involvement of more than one enzyme is responsible for the biphasic kinetics, it is likely that in addition to CYP2D6, CYP3A4 is participating. CYP2D6 is reported to contribute to at least 80% of dextrorphan formation with a $K_m$ of 3.7 µM, and CYP3A4 is predicted to contribute to approximately 15% of dextrorphan formation with a $K_m$ of 157 µM (Yu and Haining, 2001). Regardless, the
phenomenon appears to be specific to the probe dextromethorphan, as neither bufuralol metabolism by CYP2D6 nor testosterone metabolism by CYP3A4 were altered by tienilic acid.

The impact of CYP2C9 genotype-dependent drug interactions has recently been gaining more attention (Hummel et al., 2005; Kumar et al., 2006). Individuals expressing none, one, or two *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 in CYP2C9*1/*1 individuals. The effect of CYP2C9 inhibition was minimal in CYP2C9*3/*3 individuals since such a small proportion of the total flurbiprofen clearance was mediated through a CYP2C9 pathway. In vivo flurbiprofen clearance and magnitude of drug interaction predictions from corresponding in vitro studies using recombinant CYP2C9 variants were consistent with the in vivo noncompartmental results, indicating 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 to current systems. Considering that human liver microsomes and hepatocytes with known CYP2C9 variants are rare, the system herein provides an abundant and inexpensive model that eliminates individual variability. The mechanism-based inactivator can be titrated to knock down 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 in 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). Additionally, tienilic acid-treated microsomes should be diluted and/or washed to minimize unbound molecules, as tienilic acid
has been reported to react with protein nucleophiles, deplete glutathione levels, and upregulate
genes involved in oxidative stress response and phase II drug metabolism (Belghazi et al.,
2001; Lopez-Garcia et al., 2005; Nishiya et al., 2008); this is most applicable to hepatocyte
preparations.

In conclusion, tienilic acid can be used to selectively knock down 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 more 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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Footnotes

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Figure Legends

Fig. 1. CYP2C9-mediated 4'-hydroxyflurbiprofen formation in CYP2C9*1/*1 (pooled, ●) and CYP2C9*3/*3 (HH519, ○ and HL9310, ▼) human liver microsomes. Data were expressed as mean ± S.D. and fit to the Michaelis-Menten equation (eq. 1).

Fig. 2. Percent remaining CYP2C9-mediated flurbiprofen activity, expressed as mean ± S.D., in 0.1 (▼), 0.2 (○), or 0.5 (●) mg/ml of total human liver microsomal protein following treatment with 0 to 100 µM tienilic acid. The 0.2 and 0.5 mg/ml microsomal protein data were collected once in triplicate.

Fig. 3. CYP2C9-mediated flurbiprofen metabolism in CYP2C9*1/*1 pooled HLMs (●) compared to tienilic acid-created CYP2C9*1/*3 (4 µM tienilic acid, ○) and CYP2C9*3/*3 (14 µM tienilic acid, ▼) enzymatic activity equivalents. Data were expressed as mean ± S.D. and fit to the Michaelis-Menten equation (eq. 1).
### TABLE 1

**Incubation conditions and analytical parameters for CYP2C9, 2D6, and 2E1 metabolism assays**

<table>
<thead>
<tr>
<th>P450</th>
<th>CYP2C9</th>
<th>CYP2D6</th>
<th>CYP2D6</th>
<th>CYP2E1</th>
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<tr>
<td>Substrate</td>
<td>Flurbiprofen</td>
<td>Bufuralol</td>
<td>Dextromethorphan</td>
<td>Chloroxazone</td>
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<td>Metabolite</td>
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<td>1'-Hydroxybufuralol</td>
<td>Dextrorphan</td>
<td>6-Hydrochloroxazone</td>
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<tr>
<td>Internal Standard</td>
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<td>Levallorphan</td>
<td>Levallorphan</td>
<td>7-Hydroxycoumarin</td>
</tr>
</tbody>
</table>

**Incubation Conditions:**
- Substrate conc. range (µM): 2 – 300, 0.5 – 50, 1 – 200, 5 – 1000
- Incubation Time (min): 20, 10, 20, 20
- Quench solvent (volume in µl):
  - 90% Acetonitrile, 10% Phosphoric Acid (100)
  - Acetonitrile (100)
  - 50% Phosphoric Acid, 50% Water (50)
  - Acetonitrile (100)

**Analytical Parameters:**
- Injection volume (µl): 30, 20, 30, 50
- Column:
  - Brownlee Spheri-5 C18, 4.6x100mm (PerkinElmer, Waltham, MA)
  - Aqua C18, 2x150mm (Phenomenex, Torrance, CA)
  - Cyano, 4.6x150mm (Regis Technologies, Inc., Morton Grove, IL)
  - YMC-Pack Pro C8, 4.6x150mm (YMC America, Inc., Allentown, PA)
- Mobile phase A: 90% 5 mM potassium phosphate (pH 3), 10% acetonitrile
- Mobile phase B: 100 mM ammonium acetate (pH 3)
- Flow (ml/min): 1, 0.45, 1
- Program, %B(min):
  - Isocratic, 33%
  - 20(0) → 60(8) → 20(9) → 20(10.5)
  - Isocratic, 10%
  - 280, 310
  - 3.5
- λ (nm): excitation, emission:
  - 260, 320
  - 252, 302
  - 2.5
  - 2.1
- Analyte retention time (min):
  - 2.5
  - 2.1
- Modified From:
  - (Tracy et al., 2002)
  - (Crespi et al., 2006)
  - (Subramanian et al., 2009)
  - (Chittur and Tracy, 1997)
<table>
<thead>
<tr>
<th>P450</th>
<th>CYP2B6</th>
<th>CYP2C8</th>
<th>CYP2C19</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Bupropion</td>
<td>Amodiaquine</td>
<td>N-Desethylamodiaquine</td>
<td>Omeprazole</td>
</tr>
<tr>
<td>Metabolite</td>
<td>Hydroxybupropion</td>
<td>5-Hydroxyomeprazole</td>
<td>5-Hydroxyomeprazole-d3</td>
<td>Testosterone</td>
</tr>
<tr>
<td>Internal Standard</td>
<td>Hydroxybupropion-d6</td>
<td>N-Desethylamodiaquine-d5</td>
<td>5-Hydroxyomeprazole-d3</td>
<td>6β-Hydroxytestosterone</td>
</tr>
</tbody>
</table>

**Incubation Conditions:**
- **Substrate conc. range (µM):** 5 – 900, 0.1 – 100, 1 – 400, 25 – 500
- **Incubation time (min):** 20, 10, 20, 7.5
- **Quench solvent (volume in µl):** Acetonitrile (100)
- **Analytical Parameters:**
  - **Injection volume (µl):** 5, 5, 2.5, 5
  - **Column:** ZORBAX Eclipse XDB-C18 4.6x50mm (Agilent), YMC Pro C4 2x50mm (YMC America, Inc.), Brownlee Spheri-5 C18 4.6x100mm (PerkinElmer)
  - **Mobile phase A:** 0.3% formic acid in 5 mM ammonium formate
  - **Mobile phase B:** 0.3% formic acid in methanol
  - **Flow (ml/min):** 0.5
  - **Program, %B(min):** 40(0) → 40(1) → 100(2.5) → 95(3) → 40(5) → 2.5
  - **Metabolite retention time (min):** 2.5

**Mass Spectrometer Parameters:**
- **Metabolite m/z transition:** 256 → 139 → 328 → 283 → 362 → 214 → 305 → 269
- **Internal standard m/z transition:** 262 → 139 → 333 → 283 → 365 → 214 → 308 → 272
- **Modified From:** (Walsky et al., 2006; Coles and Kharasch, 2008; Li et al., 2004; Subramanian et al., 2010)
TABLE 3

Kinetic parameters of 4'-hydroxyflurbiprofen formation in Pooled, HH519, and HL9310 human liver microsomes

Data were fit to the Michaelis-Menten equation (eq. 1) and presented as a mean ± S.E. of the fit. * and *** indicate $p < 0.05$ and $p < 0.001$, respectively.

<table>
<thead>
<tr>
<th>HLM Sample</th>
<th>CYP2C9 Genotype</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
<th>$V_{\text{max}}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmol/min/mg protein</td>
<td>$\mu$M</td>
<td></td>
</tr>
<tr>
<td>Pooled</td>
<td>*1/*1</td>
<td>856 ± 10</td>
<td>5 ± 0.3</td>
<td>171</td>
</tr>
<tr>
<td>HH519</td>
<td>*3/*3</td>
<td>167 ± 4***</td>
<td>17 ± 2*</td>
<td>10</td>
</tr>
<tr>
<td>HL9310</td>
<td>*3/*3</td>
<td>49 ± 3***</td>
<td>24 ± 6*</td>
<td>2</td>
</tr>
</tbody>
</table>
TABLE 4

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

Data were fit to the Michaelis-Menten equation (eq. 1) and expressed as a mean ± S.E. of the fit. ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively.

<table>
<thead>
<tr>
<th>Chemical Genotype Equivalent</th>
<th>$V_{\text{max}}$ (pmol/min/mg protein)</th>
<th>$K_m$ (µM)</th>
<th>$V_{\text{max}}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C9*1/*1</td>
<td>858 ± 13</td>
<td>7 ± 1</td>
<td>123</td>
</tr>
<tr>
<td>CYP2C9*1/*3</td>
<td>490 ± 6**</td>
<td>7 ± 0.4</td>
<td>70</td>
</tr>
<tr>
<td>CYP2C9*3/*3</td>
<td>232 ± 5***</td>
<td>17 ± 2</td>
<td>14</td>
</tr>
</tbody>
</table>
TABLE 5

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

Data were fit to the Michaelis-Menten (eq. 1) or biphasic* (eq. 2) equations and presented as a mean ± S.E. of the best fit.

<table>
<thead>
<tr>
<th>P450</th>
<th>Substrate</th>
<th>CYP2C9 Chemical Equivalent</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; pmol/min/mg protein</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; µM</th>
<th>V&lt;sub&gt;m1*&lt;/sub&gt; pmol/min/mg protein</th>
<th>K&lt;sub&gt;m1*&lt;/sub&gt; µM</th>
<th>CL&lt;sub&gt;int*&lt;/sub&gt; µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Ethoxyresorufin</td>
<td>*1/*1</td>
<td>997 ± 57</td>
<td>0.2</td>
<td>0.2</td>
<td>32 ± 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*1/*3</td>
<td>992 ± 68</td>
<td>0.3 ± 0.1</td>
<td>25 ± 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*3/*3</td>
<td>1074 ± 79</td>
<td>0.3 ± 0.1</td>
<td>24 ± 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2B6</td>
<td>Bupropion</td>
<td>*1/*1</td>
<td>340 ± 3</td>
<td>153 ± 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*1/*3</td>
<td>331 ± 3</td>
<td>150 ± 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*3/*3</td>
<td>321 ± 2</td>
<td>138 ± 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C8</td>
<td>Amodiaquine</td>
<td>*1/*1</td>
<td>1180 ± 22</td>
<td>3 ± 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*1/*3</td>
<td>1164 ± 22</td>
<td>3 ± 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*3/*3</td>
<td>1184 ± 18</td>
<td>3 ± 0.2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2C19</td>
<td>Omeprazole</td>
<td>*1/*1</td>
<td>76 ± 2</td>
<td>9 ± 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*1/*3</td>
<td>71 ± 1</td>
<td>9 ± 0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*3/*3</td>
<td>71 ± 2</td>
<td>9 ± 1</td>
<td></td>
<td></td>
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<tr>
<td>2D6</td>
<td>Bufuralol</td>
<td>*1/*1</td>
<td>146 ± 7</td>
<td>13 ± 2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>*1/*3</td>
<td>147 ± 6</td>
<td>14 ± 1</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>*3/*3</td>
<td>148 ± 6</td>
<td>15 ± 1</td>
<td></td>
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<tr>
<td>2D6</td>
<td>Dextromethorphan</td>
<td>*1/*1</td>
<td>480 ± 31</td>
<td>10 ± 2</td>
<td>1 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*1/*3</td>
<td>453 ± 5</td>
<td>10 ± 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*3/*3</td>
<td>429 ± 5</td>
<td>9 ± 0.5</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2E1</td>
<td>Chlorzoxazone</td>
<td>*1/*1</td>
<td>2789 ± 261</td>
<td>48 ± 18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*1/*3</td>
<td>3192 ± 190</td>
<td>58 ± 13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*3/*3</td>
<td>2986 ± 289</td>
<td>61 ± 22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3A4</td>
<td>Testosterone</td>
<td>*1/*1</td>
<td>640 ± 14</td>
<td>106 ± 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*1/*3</td>
<td>597 ± 21</td>
<td>97 ± 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*3/*3</td>
<td>629 ± 17</td>
<td>115 ± 8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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FIGURE 2