POLYMORPHIC VARIANTS (CYP2C9*3 AND CYP2C9*5) AND THE F114L ACTIVE SITE MUTATION OF CYP2C9: EFFECT ON ATYPICAL KINETIC METABOLISM PROFILES

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ABSTRACT:

CYP2C9 wild-type protein has been shown to exhibit atypical kinetic profiles of metabolism that may affect in vitro-in vivo predictions made during the drug development process. Previous work suggests a substrate-dependent effect of polymorphic variants of CYP2C9 on the rate of metabolism; however, it is hypothesized that these active site amino acid changes will affect the kinetic profile of a drug's metabolism as well. To this end, the kinetic profiles of three model CYP2C9 substrates (flurbiprofen, naproxen, and piroxicam) were studied using purified CYP2C9*1 (wild-type) and variants involving active site amino acid changes, including the naturally occurring variants CYP2C9*3 (Leu359) and CYP2C9*5 (Glu360) and the man-made mutant CYP2C9 F114L. CYP2C9*1 (wild-type) metabolized each of the three compounds with a distinctive profile reflective of typical hyperbolic (flurbiprofen), biphasic (naproxen), and substrate inhibition (piroxicam) kinetics.

CYP2C9*3 metabolism was again hyperbolic for flurbiprofen, of a linear form for naproxen (no saturation noted), and exhibited substrate inhibition with piroxicam. CYP2C9*5-mediated metabolism was hyperbolic for flurbiprofen and piroxicam but linear with respect to naproxen turnover. The F114L mutant exhibited a hyperbolic kinetic profile for flurbiprofen metabolism, a linear profile for naproxen metabolism, and a substrate inhibition kinetic profile for piroxicam metabolism. In all cases except F114L-mediated piroxicam metabolism, turnover decreased and the K_m generally increased for each allelic variant compared with wild-type enzyme. It seems that the kinetic profile of CYP2C9-mediated metabolism is dependent on both substrate and the CYP2C9 allelic variant, thus having potential ramifications on drug disposition predictions made during the development process.

CYP2C9 is an important cytochrome P450 isoform found in human liver and is responsible for the metabolism of several commonly used drugs, including *S*-warfarin (Rettie et al., 1992), phenytoin (Veronese et al., 1991), and the nonsteroidal anti-inflammatory drugs (Zhao et al., 1992; Leemann et al., 1993; Tracy et al., 1995; Hamman et al., 1997). Recent work has demonstrated that CYP2C9, like many of the other P450[2] enzymes, can exhibit atypical kinetic profiles for drug metabolism (Korzekwa et al., 1998; Hutzler et al., 2001). Examples of atypical kinetic profiles include autoactivation, activation, partial inhibition, and substrate inhibition. In vitro-in vivo correlations depend on the accurate determination of the K_m and V_{max} of a compound's in vitro metabolism for the extrapolation to the in vivo situation. Misassignment of the kinetic model may provide incorrect parameter estimates that can profoundly affect the prediction of a drug's dispo-

sition characteristics in humans (Houston and Kenworthy, 2000). Thus, it is important to the drug development process that the correct kinetic profile is determined for each new chemical entity since the profile observed is frequently substrate-dependent. As the frequency of polymorphic variants of P450 enzymes becomes more apparent, it would be helpful to understand whether these variants exhibit kinetic profiles of metabolism different than the wild-type enzyme because this may also affect drug development.

The most studied allelic variants of CYP2C9, apart from the wild-type protein CYP2C9*1 [Arg144Ile359], that are present with significant frequencies in Caucasians and contribute to human CYP2C9-mediated metabolism are CYP2C9*2 (Cys144) and CYP2C9*3 (Leu359) (Furuya et al., 1995; Wang et al., 1995; Stubbins et al., 1996; Sullivan-Klose et al., 1996; Miners and Birkett, 1998). Recently, another allelic variant of CYP2C9 has been reported, CYP2C9*5 (Glu360), that seems to be preferentially expressed at low levels in African Americans (Dickmann et al., 2001).

The Leu359 and Glu360 variants seem to reside within the CYP2C9 active site (Von Wachenfeldt and Johnson, 1995) and thus would be expected to produce changes in substrate metabolism. In vitro, the Leu359 substitution has been reported to decrease the V_{max} of tolbutamide, phenytoin, and *S*-warfarin metabolism while increasing the K_m (Haining et al., 1996; Bhasker et al., 1997). Similar reductions in metabolizing capacity were observed in patients heterozygous for the Leu359 allele and receiving phenytoin (Hashimoto

et al., 1996). The homozygous Leu359 allele has been associated with the poor metabolizer phenotype for tolbutamide (Sullivan-Klose et al., 1996; Bhasker et al., 1997), warfarin (Steward et al., 1997), phenytoin and glipizide (Kidd et al., 1999), and losartan (Spielberg et al., 1996). With respect to the Glu360 variant (CYP2C9*5), Dickmann et al. (2001) reported that this amino acid substitution increases the K_m for the in vitro metabolism of S-warfarin, diclofenac, and lauric acid, with more modest effects on V_{max}. To date, in vivo characterization of the effects of the Glu360 variant has not been reported.

Haining et al. (1999) expressed a mutant CYP2C9 enzyme with a leucine substituted for phenylalanine at the 114 position (Leu114). This location is presumed to be involved in π-stacking interactions of substrates and has been used to better define active site interactions. When studied with respect to warfarin metabolism, this mutant dramatically altered the metabolite profile of warfarin metabolism and resulted in a lower V_{max} and higher K_m with respect to this substrate. In contrast, the metabolism of lauric acid (which should not depend on π-stacking interactions) was not affected. Finally, the K_m for diclofenac metabolism was substantially increased, but the V_{max} was unaffected. These studies provide additional evidence that effects resulting from active site changes are substrate-dependent.

Since active site amino acid changes can affect rates of metabolism, it was hypothesized that these amino acid changes could also affect the kinetic profile of drug metabolism. Previous work by our group has demonstrated that naproxen demethylation exhibits atypical kinetics (biphasic) (Tracy et al., 1997; Korzekwa et al., 1998), whereas flurbiprofen behaved in typical Michaelis-Menten fashion (Tracy et al., 1995, 1996; Korzekwa et al., 1998). Using these two NSAIDs and the structurally dissimilar NSAID piroxicam, for which preliminary experiments in our lab demonstrated substrate inhibition kinetics, we studied the effects of active site amino acid changes (Leu359, Glu360, and Leu114) on rates of metabolism and kinetic profile of these NSAIDs. The results of these studies with model NSAID substrates are reported here.

Materials and Methods

Chemicals and Reagents. (S)-Naproxen and desmethylnaproxen were a gift from Syntex Co. (Palo Alto, CA). (S)-Flurbiprofen, 4′-hydroxyflurbiprofen, and 2-fluoro-4-biphenyl acetic acid were gifts of the Pharmacia, Corp. (Kalamazoo, MI). Piroxicam was purchased from the Sigma Chemical Co. (St. Louis, MO) and 5′-hydroxypiroxicam was a gift from Pfizer, Inc. (Groton, CT). All other chemicals were obtained from commercial sources and were of the highest purity available.

Baculovirus Expression and Purification. The expression and purification of CYP2C9 wild-type [WT], the I359L allelic variant, and the D360E variant (CYP2C9*1, CYP2C9*3, and CYP2C9*5, respectively) from a *Trichoplusia ni*/baculovirus system have been described previously (Haining et al., 1996; Dickmann et al., 2001). Expression of the F114L mutation was carried out in an analogous manner, according to the methods of Haining et al. (1999). Viral amplification, expression in suspension cultures of *T. ni* cells, and purification of CYP2C9*3, *5, and CYP2C9 F114L were conducted as described previously for CYP2C9WT (Haining et al., 1996). Each CYP2C enzyme was purified to apparent homogeneity and a specific content in excess of 10 nmol/mg of protein.

Enzyme Reconstitution and Incubation Conditions. The P450s were incubated with either (S)-flurbiprofen, (S)-naproxen, or piroxicam at various concentration ranges in the presence of 50 mM potassium phosphate buffer, pH 7.4, and NADPH for 20 min (or 45 min for piroxicam) at 37°C. Purified enzymes were reconstituted in dilauroylphosphatidylcholine vesicles (extruded through a 200-nm pore size membrane), P450 reductase (Panvera, Madison, WI), and cytochrome b_5 (Panvera, Madison, WI) in a ratio of 1:2:1. Enzyme amounts of 5 to 40 pmol of P450 were used per incubation depending on the isoform and substrate. Previous experiments had shown these conditions to be linear with respect to time and P450 concentration. All experiments were performed in duplicate, and variances of the velocity values were less than 15%.

HPLC Assay of 4′-Hydroxyflurbiprofen. Only (S)-flurbiprofen was used for these experiments since there seems to be very little enantioselectivity in microsomal oxidation of the two enantiomers of flurbiprofen (Tracy et al., 1995). The measurement of 4′-hydroxyflurbiprofen after incubation of (S)-flurbiprofen was carried out according to the method of Tracy et al. (1995) with minor modifications. The samples were quenched with 200 μl of acetonitrile containing 36 ng of 2-fluoro-4-biphenyl acetic acid (internal standard) and acidified with 20 μl of H_3PO_4. The samples were then centrifuged at 10,000 rpm for 4 min, and 10 to 50 μl was injected onto the HPLC system (Waters Alliance 2690XE HPLC system and Waters 474 fluorescence detector; Milford, MA). The mobile phase consisted of acetonitrile/10 mM K_2HPO_4, pH 3.0 (40:60), pumped at 1 ml/min through a Brownlee Spheri-5 C_{18}, 4.6 × 100-mm column (PerkinElmer Instruments, Norwalk, CT), with detection of analytes accomplished using a fluorescence detector set at an excitation wavelength of 260 nm and an emission wavelength of 320 nm.

HPLC Assay of Desmethylnaproxen. The measurement of desmethylnaproxen after incubation of (S)-naproxen was carried out as previously described (Tracy et al., 1997) with minor modifications. The incubation reaction was terminated by the addition of 200 μl of acetonitrile. To the samples, 480 ng of 2-fluoro-4-biphenyl acetic acid (internal standard) and 20 μl of H_3PO_4 were added. The samples were centrifuged at 11,000g for 4 min, and 5 to 22 μl was directly injected onto the HPLC system. Samples were quantitated by fluorescent detection at an excitation wavelength of 230 nm and an emission wavelength of 340 nm. The mobile phase consisted of (37:63) acetonitrile/20 mM K_2HPO_4, pH 3, pumped at 1 ml/min through a Brownlee Spheri-5 C_{18}, 4.6 × 100-mm column.

HPLC Assay of 5′-Hydroxypiroxicam. Formation of 5′-hydroxypiroxicam was measured by HPLC by the following methods. Incubations (45 min) were terminated by addition of 20 μl of perchloric acid, vortexed, and immediately placed on ice. The samples were then centrifuged for 4 min, and the supernatant was injected onto the HPLC system. The mobile phase was (50:50) acetonitrile/K_2HPO_4, pH 3, pumped at 1 ml/min through a Phenomenex Luna C_{18}, 5-μm column (4.6 × 150 mm; Torrance, CA). Samples were quantitated using ultraviolet detection at 365 nm. Retention times of 5′-hydroxypiroxicam and piroxicam were 4.3 and 4.9 min, respectively.

Kinetic Data Analysis. The formation of 4′-hydroxyflurbiprofen, desmethylnaproxen and 5′-hydroxypiroxicam from the respective parent compounds were fit according to either a typical Michaelis-Menten equation $v = (V_m \cdot S)/(K_m + S)$ (model 1), a linear equation $v = (V_m/K_m)S$ (model 2), or the following equation, which describes a substrate binding to two sites within a single enzyme molecule: $v = [(V_{m1} \cdot S) + (CL_{int} \cdot S^2)]/(K_{m1} + S)$ (model 3) (Korzekwa et al., 1998), where CL_{int} is referred to as intrinsic clearance and represents the linear portion of the biphasic kinetic curve (V_{max2}/K_{m2}). Substrate inhibition was modeled using a simple substrate inhibition equation $v = V_m/(1 + K_m/S + S/K_i)$ (model 4), where K_i represents the binding constant for the drug molecule at the inhibitory site of the enzyme. All data fitting was performed using SigmaPlot 7.0 (SPSS, Inc., Chicago IL), and appropriateness of the fits was determined by examination and comparison of the residuals, residual sum of squares, coefficients of determination, and F values.

Results

The primary oxidative metabolites for flurbiprofen, naproxen, and piroxicam are depicted in Fig. 1. All three compounds tested were substrates for all CYP2C9 forms studied, albeit at different rates. The formation of 4′-hydroxyflurbiprofen by CYP2C9*1, CYP2C9*3, CYP2C9*5, and CYP2C9 F114L is depicted in Fig. 2. Standard Michaelis-Menten kinetics (i.e., hyperbolic) (model 1) were noted for flurbiprofen metabolism by CYP2C9*1 and all studied CYP2C9 forms having amino acid changes in the active site (CYP2C9*3, CYP2C9*5, and CYP2C9 F114L). The kinetic parameter estimates and R^2 values of the fits of these data are listed in Table 1.

Figure 3 depicts the kinetic profiles for the CYP2C9-mediated formation of desmethylnaproxen from (S)-naproxen. Metabolism by CYP2C9*1 was best fit using the equation for two binding sites, with
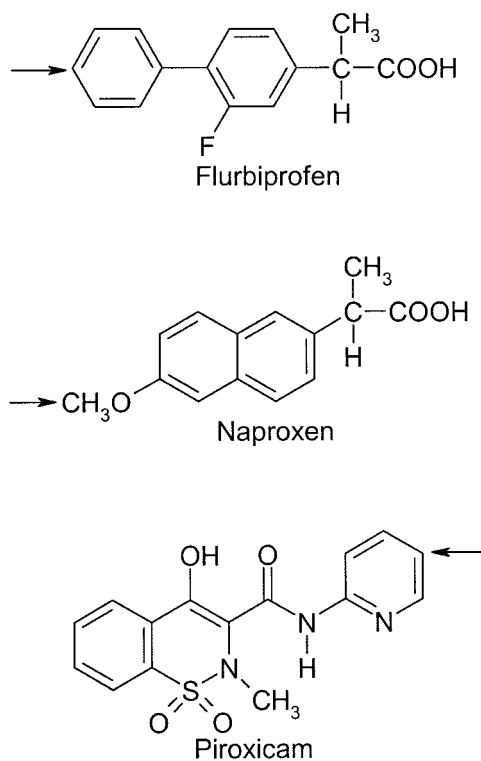
FIG. 1. *Structures of flurbiprofen, naproxen, and piroxicam.*
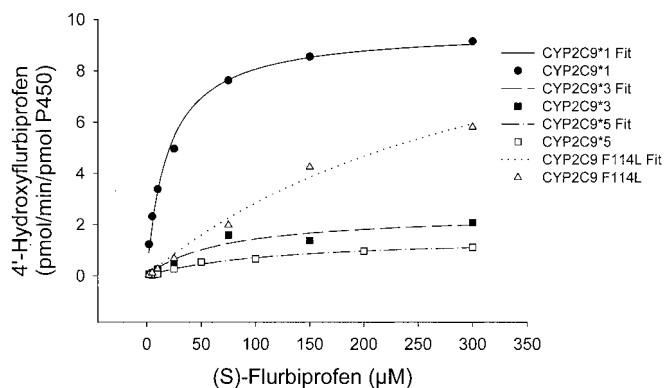Arrows indicate site of metabolism.

FIG. 2. *Formation of 4′-hydroxyflurbiprofen from (S)-flurbiprofen by CYP2C9*1, CYP2C9*3, CYP2C9*5, and the site-specific mutant CYP2C9 F114L purified from a baculovirus expression system.*

Symbols indicate actual data points, and lines indicate the fit of the data, as described under *Materials and Methods* and *Results*.

TABLE 1

Kinetic parameter estimates for 4′-hydroxylation of (S)-flurbiprofen by the CYP2C9 variants

Values are the parameter estimates ± S.E. of the estimate.

	K_m	V_{max}	CL_{int}[b]	R^2
	μM	pmol/min/pmol P450	μl/min/pmol P450	
CYP2C9*1[a]	19.4 ± 2.1	9.6 ± 0.3	0.49	0.992
CYP2C9*3[a]	71.7 ± 31.9	2.5 ± 0.4	0.03	0.944
CYP2C9*5[a]	119.7 ± 21.7	1.6 ± 0.1	0.01	0.989
CYP2C9 F114L[a]	360 ± 111	13.1 ± 2.5	0.04	0.991

[a] Fitted to the standard Michaelis-Menten equation (model 1).
[b] CL_{int} calculated as the ratio of V_{max}/K_m, a measure of the efficiency of the enzyme.
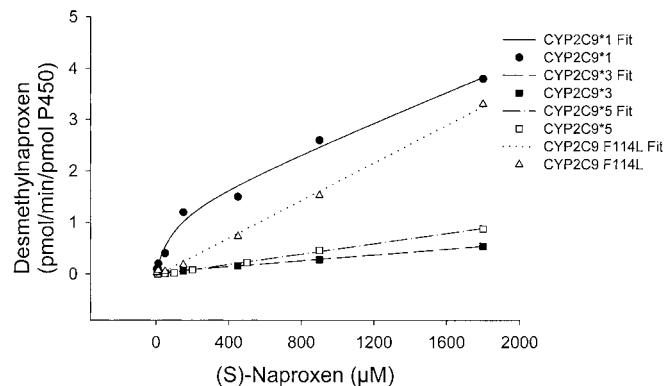

FIG. 3. *Formation of desmethylnaproxen from (S)-naproxen by CYP2C9*1, CYP2C9*3, CYP2C9*5, and the site-specific mutant CYP2C9 F114L purified from a baculovirus expression system.*

Symbols indicate actual data points, and lines indicate the fit of the data, as described under *Materials and Methods* and *Results*.

TABLE 2

Kinetic parameter estimates for demethylation of (S)-naproxen by the CYP2C9 variants

Values are the parameter estimates ± S.E. of the estimate.

	K_m	V_{max}	CL_{int}	R^2
	μM	pmol/min/pmol P450	μl/min/pmol P450	
CYP2C9*1[a]	89.1 ± 66.0	1.39 ± 0.44	0.002 ± 0.0002	0.990
CYP2C9*3[b]	—[c]	—[c]	0.0003 ± 0.00001	0.990
CYP2C9*5[b]	—[c]	—[c]	0.0005 ± 0.00001	0.998
CYP2C9 F114L[b]	—[c]	—[c]	0.002 ± 0.0001	0.997

[a] Fitted to the two-binding site model (model 3) so that K_m and V_{max} represent kinetics at binding site 1 and CL_{int} represents kinetics at binding site 2.
[b] Fitted to linear function (model 2), where CL_{int} is the turnover rate for that particular isoform.
[c] Not applicable since linear kinetics observed. CL_{int} calculated as slope of the line.

one type of substrate molecule suggestive of biphasic kinetics, i.e., a K_m and V_{max} describing a low K_m, low V_{max} component of the metabolism with some hyperbolic characteristics and a linear (V_{max}/K_m, intrinsic clearance) component (model 3). It should be noted that even at substrate concentrations of 1800 μM, saturation was not achieved. In contrast, the formation of desmethylnaproxen by the CYP2C9*3 and CYP2C9*5 variants and the F114L mutant were best fit by a linear equation only (no evidence of any hyperbolic tendency), again, up to 1800 μM substrate concentrations. Table 2 lists the kinetic parameter estimates and R^2 values of the fits for these data.

Finally, piroxicam hydroxylation by CYP2C9 is depicted in Fig. 4. Substrate inhibition was noted for the hydroxylation of piroxicam by CYP2C9*1, CYP2C9*3, and the F114L mutant. The data were fit to model 4 (substrate inhibition), and the parameter estimates and R^2 values for the fits of these reactions are listed in Table 3. However, piroxicam hydroxylation by CYP2C9*5 was best described by a hyperbolic equation (model 1), with no evidence of substrate inhibition noted. These findings suggest that single amino acid changes have substantial effects on both the relative activity of the allelic variants and the piroxicam kinetic profile observed. A summary of the kinetic profiles observed for each model substrate with respect to CYP2C9*1 and the various polymorphic variants and F114L mutant are presented in Table 4.

Discussion

It has been well established that oxidative metabolism of several of the NSAIDs occurs via CYP2C9 (Zhao et al., 1992; Leemann et al.,
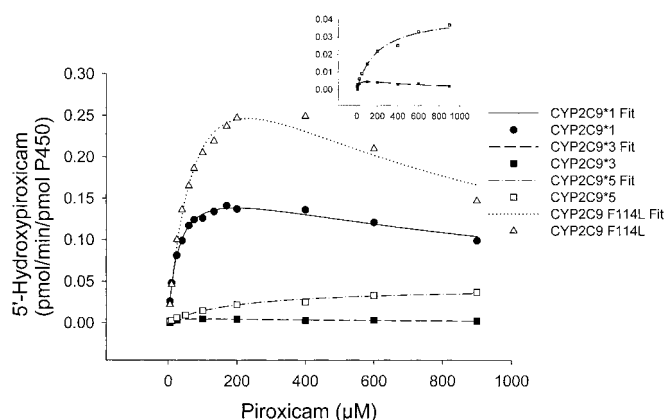
FIG. 4. *Formation of 5′-hydroxypiroxicam from piroxicam by CYP2C9*1, CYP2C9*3, CYP2C9*5, and the site-specific mutant CYP2C9 F114L purified from a baculovirus expression system.*

Inset is the data for the *3 and *5 variants in an expanded form to better allow visual inspection of the shape of the curves. Symbols indicate actual data points, and lines indicate the fit of the data, as described under *Materials and Methods* and *Results*.

1993; Tracy et al., 1995; Hamman et al., 1997). Although many NSAIDs, such as flurbiprofen, exhibit typical (i.e., Michaelis-Menten-type) kinetic profiles, in the cases of naproxen and tenoxicam (an analog of piroxicam), biphasic and substrate inhibition kinetics, respectively, have been observed (Zhao et al., 1992; Korzekwa et al., 1998). However, it is still unknown whether these kinetic profiles (both typical and atypical) are affected by amino acid changes within the active site, such as occurs with some allelic variants and following site-directed mutagenesis. In the present work, it was demonstrated that each of the studied NSAIDs exhibited a different kinetic profile (flurbiprofen, standard Michaelis-Menten; naproxen, biphasic; and piroxicam, substrate inhibition) when metabolized by the wild-type enzyme. Furthermore, active site amino acid changes, either through natural mutations (e.g., CYP2C9*3 or CYP2C9*5) or following site-directed mutagenesis (e.g., Leu114), in some cases altered the kinetic profile observed depending on the substrate in addition to their effects on the rate of metabolism.

It seems that the I359L and D360E alterations observed in the CYP2C9*3 and CYP2C9*5 variants occur in SRS5 (Von Wachenfeldt and Johnson, 1995), located within the K-helix, and thus serve to explain changes in K_m and V_{max} when these substitutions are observed. Likewise, modeling studies have predicted that the phenylalanine at position 114 is located in the active site B′-C loop region, which corresponds to SRS1, and is responsible for π-stacking interactions with substrates (Jones et al., 1996; Haining et al., 1999; Rao et al., 2000; Williams et al., 2000) Consequently, a change at the 114 position to a nonaromatic amino acid (e.g., F114L as used here in the site-directed mutagenesis studies) would be predicted to disrupt π-stacking interactions, thus altering substrate binding and consequently kinetic parameter estimates.

Flurbiprofen has been reported to be metabolized primarily by CYP2C9 and obeys standard Michaelis-Menten kinetics in both human liver microsomes and in HepG2 cells expressing the wild-type (CYP2C9*1) enzyme (Tracy et al., 1995, 1996). In the current study, flurbiprofen 4′-hydroxylation displayed standard Michaelis-Menten kinetics in the CYP2C9*1 enzyme, with similar results observed for both the CYP2C9*3 and CYP2C9*5 variants and the F114L mutant, suggesting that these changes in active site amino acid composition did not affect the kinetic profile observed, although different affinities and turnover rates were noted. Like results observed previously with

S-warfarin (Haining et al., 1996), the Leu359 variant had both a decreased catalytic efficiency toward flurbiprofen 4′-hydroxylation and a substantially higher K_m, similar to results reported with other CYP2C9 substrates (Takanashi et al., 2000). Similarly, the Glu360 variant (CYP2C9*5) also had dramatically reduced flurbiprofen hydroxylase activity with an even higher K_m and lower V_{max} compared with the Leu359 allele. These results with the Glu360 allele contrast slightly to those recently reported with the NSAID diclofenac in which this allele increased the K_m value but had minimal effect on V_{max}, although they are similar to results reported for lauric acid (Dickmann et al., 2001). It is unclear at this time why the results concerning effect on V_{max}, naproxen, and piroxicam differ from those reported with diclofenac, but ongoing work in our lab (T.S. Tracy, J. Hutzler, and M. Hummel, unpublished observations) suggests that diclofenac may exhibit different binding characteristics (e.g., orientation, binding region, etc.) within the CYP2C9 active site. Regarding the site-directed mutant F114L (Leu114), the K_m for flurbiprofen 4′-hydroxylation was increased approximately 18-fold compared with wild-type, but minimal changes in V_{max} were noted (Table 1). This finding of increased K_m and little change in V_{max} for the F114L mutant is similar to previous results seen with diclofenac (another NSAID) hydroxylation in which the K_m increased by 10-fold, but the V_{max} for the reaction remained unchanged (Haining et al., 1999). These results suggest that at least for some compounds, a change in amino acid at this purported π-stacking region can substantially alter the affinity of the enzyme for the compound but not change the maximum turnover.

With respect to naproxen demethylation, the kinetic results were in marked contrast to those observed with flurbiprofen. Previous results in our laboratory have demonstrated that in human liver microsomes, naproxen is primarily oxidatively metabolized by CYP2C9 but that P450s 1A2 and 2C8 can also contribute to demethylation (Tracy et al., 1997). Thus, in liver microsomes, the nonhyperbolic nature of the reaction could possibly be attributed to multiple enzyme involvement. However, in the experiments reported here, data were obtained using purified recombinant CYP2C9 as the enzyme source. The data from these experiments demonstrate nonhyperbolic (biphasic) kinetics for the wild-type enzyme (Fig. 3). We propose that these atypical kinetics are due to two substrate molecules binding within the CYP2C9 active site simultaneously (positive homotropic cooperativity) (Korzekwa et al., 1998), with each binding region displaying different K_m and V_{max} values. This biphasic kinetic profile and lack of saturation, even at high substrate concentrations, is in agreement with our previous findings using wild-type CYP2C9 expressed in HepG2 cells (Tracy et al., 1997). In contrast, demethylation of naproxen by the CYP2C9*3 and CYP2C9*5 variants and the F114L mutant was best described by a linear equation because no curvature in the data was observed and the formation velocity did not reach saturation, even at 1800 μM (Fig. 3). This observation may be due either to a large increase in K_m or because a complete picture of Michaelis-Menten kinetics cannot be obtained due to an inability to solubilize naproxen at sufficiently high concentrations to allow achievement of maximal velocity. The velocity of formation noted with the CYP2C9*3 and CYP2C9*5 variants was markedly lower than observed for the wild-type isoform. However, the formation velocity for naproxen demethylation noted with the F114L mutant seemed to reach levels comparable to the wild-type enzyme (Fig. 3), although obviously with lower affinity, similar to results reported with diclofenac (Haining et al., 1999). That K_m was increased substantially and V_{max} decreased substantially for naproxen demethylation by the CYP2C9*3 variant is in agreement with results noted for a number of CYP2C9 substrates (e.g., Bhasker et al., 1997; Takanashi et al., 2000).

TABLE 3

Kinetic parameter estimates for 5′-hydroxylation of piroxicam by the CYP2C9 variants

Values are the parameter estimates ± S.E. of the estimate.

	K_m	V_{max}	CL_{int}[c]	K_i	R^2
	μM	*pmol/min/pmol P450*	*μl/min/pmol P450*	*μM*	
CYP2C9*1[a]	30.5 ± 2.6	0.181 ± 0.005	0.0059	1277 ± 146	0.996
CYP2C9*3[a]	29.5 ± 17.1	0.007 ± 0.002	0.0002	396 ± 230	0.866
CYP2C9*5[b]	202 ± 34.4	0.043 ± 0.003	0.0002		0.986
CYP2C9 F114L[a]	94.9 ± 18.0	0.447 ± 0.005	0.0047	570 ± 125	0.984

[a] Data sets were best fit to a substrate inhibition model (model 4).
[b] Fitted to standard Michaelis-Menten equation (model 1).
[c] CL_{int} calculated as the ratio of V_{max}/K_m, a measure of the efficiency of the enzyme.

TABLE 4

*Summary of the kinetic profiles observed for each of the model CYP2C9 substrates with respect to metabolism by CYP2C9*1 (wild-type enzyme), the polymorphic variants, and a man-made mutant CYP2C9 F114L involving active site amino acid changes*

	Flurbiprofen	Naproxen	Piroxicam
CYP2C9*1	Hyperbolic	Biphasic	Substrate inhibition
CYP2C9*3	Hyperbolic	Linear	Substrate inhibition
CYP2C9*5	Hyperbolic	Linear	Hyperbolic
CYP2C9 F114L	Hyperbolic	Linear	Substrate inhibition

Metabolism of the third NSAID studied, piroxicam, demonstrated substrate inhibition profiles for piroxicam hydroxylation by three variants of CYP2C9 (*1, *3, and F114L), whereas the CYP2C9*5 variant displayed hyperbolic kinetics (Fig. 4). Even with the F114L variant and maximum formation rates, only 0.29% of the substrate had been converted to product, further suggesting that the reduction in metabolite formation at higher substrate concentrations is due to substrate inhibition and not product inhibition. To our knowledge, this is the first report of substrate inhibition kinetics for piroxicam hydroxylation. This type of kinetic profile has been reported for metabolism of the analog tenoxicam by human liver microsomes (Zhao et al., 1992), although no substrate inhibition was observed for lornoxicam hydroxylation (Bonnabry et al., 1996) by human liver microsomes. Observation of substrate inhibition with all but one of the variants suggests that piroxicam may exhibit dual substrate binding regions within the active site, resulting in these atypical kinetic profiles (Korzekwa et al., 1998). Thus, changes in amino acids at positions 359 (*3) and 114 (Leu mutant) do not seem to affect the kinetic profile, as was observed with flurbiprofen and naproxen, yet the Glu360 variant does seem to have a substantial effect on the profile, as seen by a change from substrate inhibition kinetics to a hyperbolic kinetic profile. However, changes in each of these amino acid positions did have profound effects on the turnover rates of piroxicam. The turnover of piroxicam was greatly enhanced in the F114L mutant compared with the wild-type enzyme (Fig. 4), in contrast to flurbiprofen and naproxen metabolism in which the turnover was either decreased or just slightly lower. We speculate that removal of the aromatic ring from position 114 (i.e., substitution of leucine for phenylalanine) reduces some steric hindrance to piroxicam binding and thus allows the piroxicam molecule to bind in a more productive orientation, resulting in greater substrate turnover. However, as observed with (*S*)-warfarin and diclofenac (Haining et al., 1999), as well as flurbiprofen and naproxen, changes at this amino acid position result in a decrease in binding affinity (K_m). Thus, π-stacking interactions with the 114-position phenylalanine that either decrease [warfarin (Haining et al., 1999) and flurbiprofen] or have no effect on substrate turnover [diclofenac (Haining et al., 1999)] seem to produce the opposite effect with piroxicam. As observed with the

other two compounds studied, turnover of piroxicam was markedly decreased in the CYP2C9*3 and CYP2C9*5 variants, but surprisingly with the CYP2C9*3 variant, the K_m remained unchanged (Table 3). Interestingly, K_i values for the inhibition of piroxicam metabolism by itself were highest for the wild-type enzyme and about one-half that value for the two variants involving changes in the active site. This suggests that substrate inhibition may be more likely in these variants presumably because these changes allow a tighter binding of a second substrate molecule that can result in more pronounced substrate inhibition characteristics. The estimation of K_i values being higher than the corresponding K_m values is in agreement with the observations of substrate inhibition kinetics reported recently by Lin and colleagues (2001). This suggests that the affinity for the site of inhibition is less than that for metabolism, and thus, inhibition was observed with an increase in substrate concentration.

In summary, the metabolism kinetic profiles produced by CYP2C9 and its allelic variants seem to be both substrate- and allelic variant-dependent. It is possible that this same allelic variant dependence occurs with other P450 isoforms as well. Drug development is increasingly dependent on in vitro-in vivo correlations for decision making concerning a compound's potential for exhibiting a favorable in vivo disposition profile. The presence of atypical kinetic profiles complicates these predictions since incorrect characterizations can greatly affect the accuracy of the disposition predictions. As identification of an individual's drug metabolism enzyme genotype becomes more viable, knowledge of a drug's metabolism kinetic profile by enzyme variants may allow even better individualization of therapy to improve therapeutic outcomes, minimize toxicities, and reduce drug interactions.

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