CYP2C9 Amino Acid Residues Influencing Phenytoin Turnover and Metabolite Regio- and Stereochemistry

Carrie M. Mosher, Guoying Tai, and Allan E. Rettie

Department of Medicinal Chemistry, University of Washington, Seattle, Washington

Received January 19, 2009; accepted March 2, 2009

ABSTRACT

Phenytoin has been an effective anticonvulsant agent for over 60 years, although its clinical use is complicated by nonlinear pharmacokinetics, a narrow therapeutic index, and metabolically based drug-drug interactions. Although it is well established that CYP2C9 is the major cytochrome P450 enzyme controlling metabolic elimination of phenytoin through its oxidative conversion to (S)-5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), nothing is known about the amino acid binding determinants within the CYP2C9 active site that promote metabolism and maintain the tight stereocontrol of hydroxy metabolite formation. This knowledge gap was addressed here through the construction of nine active site mutants at amino acid positions Phe100, Arg108, Phe114, Leu208, and Phe476 and in vitro analysis of the steady-state kinetics and stereo-

chemistry of *p*-HPPH formation. The F100L and F114W mutants exhibited 4- to 5-fold increases in catalytic efficiency, whereas the F100W, F114L, F476L, and F476W mutants lost >90% of their phenytoin hydroxylation capacity. This pattern of effects differs substantially from that found previously for (S)-warfarin and (S)-flurbiprofen metabolism, suggesting that these three ligands bind within discrete locations in the CYP2C9 active site. Only the F114L, F476L, and L208V mutants altered phenytoin's orientation during catalytic turnover. The L208V mutant also uniquely demonstrated enhanced 6-hydroxylation of (S)-warfarin. These latter data provide the first experimental evidence for a role of the F-G loop region in dictating the catalytic orientation of substrates within the CYP2C9 active site.

Phenytoin sodium (5,5-diphenylhydantoin, Dilantin) is a hydantoin anticonvulsant that initially gained favor because it was less sedating compared with the bromide and barbiturate drugs (Merritt and Putnam, 1938). With its high efficacy and low cost, phenytoin is still widely used for the treatment of complex partial seizures, secondarily generalized tonic-clonic seizures, and acute seizures (Rudis et al., 2004). However, clinical use is complicated by the drug's nonlinear pharmacokinetics, narrow therapeutic window, and metabolic drug-drug interactions. The latter mainly result from phenytoin's pronounced induction of CYP3A4 but

can also arise as a result of inhibition of P450 enzyme(s) responsible for the drug's clearance (Mather and Levy, 2000).

Phenytoin's primary metabolic pathway is hydroxylation by CYP2C9 to 5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH) (Veronese et al., 1993). Approximately 95% of the *p*-HPPH produced by CYP2C9 has the (S) configuration (Scheme 1). CYP2C19 is responsible for most of the (R)-*p*-HPPH formed (Giancarlo et al., 2001), but this isoform usually plays a minor role in phenytoin hydroxylation, unless the CYP2C9 pathway becomes saturated at higher doses of the drug (Bajpai et al., 1996). The dominance of CYP2C9 catalysis is evident from the urinary (S)/(R) ratio *p*-HPPH, which is ~24 under normal circumstances (Argikar et al., 2006).

Because CYP2C9 and CYP2C19 share >90% sequence similarity, it can be inferred that the cause of this difference in prochiral stereoselectivity lies within the substrate recognition site (SRS) regions, which are hypervariable areas responsible for substrate selectivity (Gotoh, 1992). These regions include the phenylalanine cluster in the active site of CYP2C9 composed of Phe100 and Phe114 (both B-C loop, SRS1), and Phe476 (β 4-fold, SRS6). In the recently solved crystal structures of CYP2C9 complexed with (S)-warfarin or

This work was supported by the National Institutes of Health National Institute of General Medical Sciences [Grants GM32165, GM07750].

Part of this work was previously presented as follows: Mosher CM and Rettie AE (2006) CYP2C9 active site determinants of phenytoin metabolism, orientation, and lansoprazole-mediated activation. *14th North American Meeting of the International Society for the Study of Xenobiotics*; 2006 Oct 22-26; San Juan, Puerto Rico. International Society for the Study of Xenobiotics, Washington, DC; and Tai G (2006) *Structural Determinants of CYP2C9's Genetic Variability, Substrate Specificity and Dioxygen Cleavage*. Ph.D. Thesis, University of Washington, Seattle, WA.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

doi:10.1124/jpet.109.150706.

ABBREVIATIONS: P450, cytochrome P450; *p*-HPPH, 5-(4-hydroxyphenyl)-5-phenylhydantoin; SRS, substrate recognition site; DLPC, dilaurylphosphatidylcholine; LC, liquid chromatography; MS, mass spectrometry; MRM, multiple reaction monitoring; HPPH, 5-hydroxyphenyl-5-phenylhydantoin; pdb, Protein Data Bank.

Scheme 1. In vitro metabolic pathways of phenytoin metabolism by CYP2C9. CYP2C9 is also known to catalyze formation of both (S)-*p*-HPPH and (R)-*p*-HPPH.

flurbiprofen, interactions were shown to occur between these ligands and one or more of the Phe residues (Williams et al., 2003; Wester et al., 2004). Mutagenesis studies have demonstrated the importance of these aromatic residues for binding and orientation of (S)-warfarin and several nonsteroidal anti-inflammatory drugs (Haining et al., 1999; Tracy et al., 2002; Melet et al., 2003; Mosher et al., 2008). However, beyond a single report that the I359L (CYP2C9.3) variant increases the percentage of (R)-*p*-HPPH produced (Takanashi et al., 2000), nothing is known about phenytoin's binding determinants within the active site of CYP2C9. This is especially surprising because one of the first studies employing site-directed mutagenesis to investigate the activity of CYP2C9 genetic variants involved phenytoin as the probe drug (Veronese et al., 1993).

Given the importance of the Phe cluster residues (Phe100, Phe114, Phe476) in the binding and orientation of (S)-warfarin (Mosher et al., 2008), we hypothesized that these residues would be at least as important to phenytoin turnover and might also be the primary determinants of phenytoin's high prochiral stereoselectivity for *p*-HPPH formation. Therefore, site-directed mutants were engineered at positions 100, 114, and 476, expressed and purified from *Escherichia coli*, and the kinetics, regioselectivity, and prochiral selectivity of phenytoin metabolism were examined. We also evaluated the influence of the non-Phe residues Arg108 and Leu208, which are known to be important for (S)-warfarin metabolism (Dickmann et al., 2004). The results are discussed in terms of multiple binding modes for ligand interactions with CYP2C9 during catalysis.

Materials and Methods

Materials. The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): phenytoin, dilaurylphosphatidylcholine

(DLPC), dithiothreitol, β -NADPH, isopropyl β -D-thiogalactoside, δ -aminolevulinic acid, ampicillin, catalase from bovine liver, and protease inhibitor cocktail. Platinum *Pfx* DNA polymerase, T4 DNA ligase, and MAX efficiency DH5 α F'IQ competent cells were purchased from Invitrogen (Carlsbad, CA). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA). Glacial acetic acid, ammonium hydroxide, and zinc sulfate, all American Chemical Society grade, and acetonitrile and methanol, optima grade, were purchased from Thermo Fisher Scientific (Waltham, MA). (S)-Warfarin was obtained by fractional crystallization, and the deuterated hydroxylated internal standards were synthesized as described previously (Bush et al., 1983). The *m*- and *o*-HPPH were gifts from Dr. Wendel L. Nelson (University of Washington, Seattle, WA), and the (S)- and (R)-*p*-HPPH were gifts from Dr. J. H. Maguire (University of North Carolina, Chapel Hill, NC).

Mutagenesis and Protein Expression. The engineering of the wild-type CYP2C9 was achieved through a single PCR, whereas the remaining mutants were produced by overlap extension polymerase chain reaction as previously described (Mosher et al., 2008). The mutants were engineered using wild-type CYP2C9 as a template. The forward mutagenic oligonucleotides consisted of the following sequences [the altered nucleotide(s) are in boldface italics]: L208V, 5'-C ATC AAG ATT **GTG** AGC AGC CCC TGG AT-3'; R108L, 5'-G GCT GAA AGA GCT AAC **TTA** GGA TTT GGA ATT GT-3'; and R108F, 5'-G GCT GAA AGA GCT AAC **TTT** GGA TTT GGA ATT GT-3'. The constructs were then cloned into the NdeI and SalI restriction sites of histidine-tagged pCWori+ and transformed into DH5 α cells for large-scale overexpression in *E. coli*. All plasmids had their integrity verified by DNA sequencing. Proteins were purified via nickel-nitrilotriacetic acid Superflow (QIAGEN, Valencia, CA) and hydroxyapatite affinity columns essentially as described previously, without the inclusion of stabilizing ligands (Cheesman et al., 2003).

Liquid Chromatography-Mass Spectrometry Analysis of Hydroxywarfarin Metabolites. Two different sets of warfarin incubations were performed: one set utilized a single saturating concentration of (S)-warfarin (500 μ M) and 50 pmol P450, whereas the other contained 20 or 50 pmol P450 and encompassed a range of

(S)-warfarin concentrations (2–200 μM) to determine kinetic constants. (S)-Warfarin turnover by CYP2C9 was assessed in incubation mixtures (0.5 ml) containing 25 mM potassium phosphate, pH 7.4, 0.02 pmol DLPC/pmol P450 extruded through a 0.2- μm filter, 200 μM dithiothreitol, reconstituted P450 enzyme, P450 reductase, and cytochrome b_5 , in a ratio of 1:2:1. Reactions were preincubated for 3 min at 37°C and then initiated by the addition of NADPH at a final concentration of 0.5 mM. After 20 min, reactions were quenched with 10 μl of perchloric acid, 100 ng of each internal standard (d_4 -4', d_5 -6-, d_5 -7-, and d_5 -8-hydroxywarfarin) was added, and the samples were centrifuged at 13,500 rpm for 4 min. Hydroxywarfarin concentrations were measured by liquid chromatography (LC)-mass spectrometry (MS) using a Hewlett Packard series 1100 MSD (Palo Alto, CA) system operating in the positive ion electrospray mode with selected ion monitoring and equipped with HP Chemstation data analysis software. The mobile phase consisted of 1.5% ammonium acetate, pH 4.7 (A) and acetonitrile (B) with a gradient elution at 0.25 ml/min. Gradient elution steps were as follows: 0 to 4 min, 35% B; 4- to 7-min linear gradient to 55% B; 7- to 8-min linear gradient to 80% B; and 8- to 9-min linear gradient to 35% B. Analyte separation was achieved through an Inertsil ODS-3 5- μm column (150 $\times$ 2.1 mm) (Alltech Associates, Deerfield, IL). Hydroxywarfarin metabolites were monitored at m/z 325, and the deuterated internal standards were monitored at m/z 329 and m/z 330. Quantitation was achieved by comparing the ratio of ion currents obtained for the d_0 -hydroxy metabolites and $d_{4/5}$ internal standards. Enzyme kinetics were determined by the Michaelis-Menten model:

$$v = \frac{V_{\max} \times S}{K_m + S} \quad (1)$$

using SigmaPlot 8.0 (Systat Software, Inc., San Jose, CA).

LC-MS/MS Analysis of Hydroxyphenytoin Metabolites. Two different sets of phenytoin incubations were performed. One set utilized a single saturating concentration of phenytoin (200 μM) and 200 pmol P450, whereas the other contained 20, 40, 100, or 200 pmol P450, depending on the rate of turnover, and encompassed a range of phenytoin concentrations (2–200 μM) to determine kinetic constants. Phenytoin turnover by CYP2C9 was assessed in incubation mixtures (0.2 ml) containing 25 mM potassium phosphate, pH 7.4, 0.02 pmol DLPC/pmol P450 extruded through a 0.2- μm filter, 200 μM dithiothreitol, 100 units of catalase, reconstituted P450 enzyme, P450 reductase, and cytochrome b_5 , in a ratio of 1:2:1. Reactions were preincubated for 3 min at 37°C and then initiated by the addition of NADPH at a final concentration of 0.5 mM. After 20 min, reactions were quenched with 100 μl of methanol; 10 μl of 25% acetic acid and 0.02 μg of the internal standard *o*-HPPH were added; and the samples were centrifuged at 13,500 rpm for 4 min. Analyte separation was achieved with an Inertsil ODS-3 5- μm column (50 $\times$ 2.1 mm) (Alltech Associates) and a mobile phase consisting of 20 mM ammonium acetate (A) and methanol (B). The flow rate was 0.3 ml/min. Gradient elution steps were as follows: 0 to 2 min, 30% B; 2- to 6-min linear gradient to 60% B; 6- to 7-min linear gradient to 80% B; and 7- to 8-min linear gradient to 30% B. Analytes were measured using a Shimadzu LC-10AD system (Shimadzu, Kyoto, Japan) coupled to a triple quadrupole Quattro II mass spectrometer (Waters, Milford, MA) and operated in the positive ion electrospray mode with Mass-Lynx 4.0 running in multiple reaction monitoring (MRM) mode. Two MRM transitions were monitored simultaneously (dwell time, 0.8 s): m/z 269 $\rightarrow$ 198, cone voltage = 25 V, collision energy = 20 eV (*p*- and *m*-HPPH); and m/z 269 $\rightarrow$ 209, cone voltage = 25 V, collision energy = 15 eV (*o*-HPPH, internal standard). Quantitation was achieved by comparison of the ion ratios for these MRM transitions and comparison with a standard curve generated with authentic metabolites. All conditions were the same for the chiral separation of HPPH for the determination of (S)- and (R)-*p*-HPPH, except that separation was achieved using a Nucleodex β -OH column (250 $\times$ 4.6

mm) (Phenomenex, Torrance, CA), with isocratic mobile phase (30% B) at 0.4 ml/min and 100- μl injection volume. Enzyme kinetics were determined by eq. 1 using SigmaPlot 8.0.

Docking Phenytoin into CYP2C9. The Protein Data Bank (pdb) file 1R9O (Wester et al., 2004) of CYP2C9 complexed with flurbiprofen was used as the starting point for docking studies. The flurbiprofen molecule was first removed, along with water and glycerol molecules, before polar hydrogen bonds were added. The phenytoin molecule [coordinates derived by drawing phenytoin in DS ViewerPro 6.0 (Accelrys, San Diego, CA) and saving as a pdb file] was then docked into the CYP2C9 active site defined by AutoGrid in AutoDock 3.0.5 via AutoDockTools (The Scripps Research Institute, La Jolla, CA). Standard AutoDock Lamarckian genetic algorithm conditions were used over 50 generations. The coordinates of the lowest energy result with the pro-(R) phenyl ring above the heme (as opposed to the nonmetabolized hydantoin ring above the heme) were then saved as a separate pdb file. Despite extensive permutations of parameters within AutoDock, no conformations were output that illustrated the pro-(S) phenyl ring above the heme (the catalytically productive orientation). It is possible that this conformation could only be achieved through the added use of molecular dynamics studies, which were not performed here. Therefore, both the 1R9O structure minus flurbiprofen and the lowest energy output were illustrated in UCSF Chimera 1.2540 (Regents of the University of California, Oakland, CA). The 1R9O molecule was then made inactive while the phenytoin ligand was rotated to the catalytically productive orientation with the pro-(S) ring positioned above the heme iron.

Results

Construct Selection and Protein Characterization.

The Phe cluster within the CYP2C9 active site has proven to be very important to the turnover and regioselectivity of (S)-warfarin metabolism (Mosher et al., 2008). Therefore, to probe the importance of aromaticity and steric factors in CYP2C9-dependent phenytoin metabolism, the phenylalanine $\rightarrow$ leucine or phenylalanine $\rightarrow$ tryptophan mutants were constructed at residues 100, 114, or 476. Additional mutants at positions 108 and 208 were constructed and tested in a similar manner based on previous findings that Arg108 and Leu208 influenced ligand binding in CYP2C9. In earlier studies, the CYP2C9 L208V mutant was found to alter the catalytically productive orientation of (S)-warfarin using baculovirus-infected insect cell-expressed protein (G. Tai, unpublished observations). In CYP2C19, Val is the naturally occurring residue at position 208, so the stereochemistry of *p*-HPPH produced from the CYP2C9 L208V mutant was of considerable interest. Arg108 has been identified as a critical binding determinant of flurbiprofen, providing a salt bridge to the carboxylate of this nonsteroidal anti-inflammatory drug in the crystal structure (Wester et al., 2004). Consistent with this observation, the R108F mutant (expressed in insect cells) was previously found to nearly eliminate (S)-warfarin and diclofenac turnover, while maintaining pyrene turnover (Dickmann et al., 2004). As a consequence, the R108F and R108L mutants were also expressed in *E. coli* for analysis of phenytoin metabolism. All mutants expressed well in *E. coli*, with protein levels of 200 to 400 nmol expressed protein/l. Protein specific content was between 8 and 15 nmol/mg, and mass data for the apoenzymes were determined by LC-MS agreed within $\leq 0.01\%$ of the calculated molecular weights (see Mosher et al., 2008).

Comparison of Phenytoin and (S)-Warfarin Turnover by CYP2C9 Mutants. The ability of wild-type CYP2C9 and several active site mutants to catalyze phenytoin hydroxylation was determined initially at a high concentration (100 μ M), and the results are shown in Fig. 1A. The turnover of (S)-warfarin by the corresponding proteins is adapted from data presented in Mosher et al. (2008) and shown in Fig. 1B for comparison. The bars represent the sum of all metabolites produced by each substrate. The F100W enzyme exhibited decreased turnover of both (S)-warfarin and phenytoin [to a greater extent with (S)-warfarin], whereas the F100L mutant demonstrated increased turnover of both substrates, although to a greater extent with phenytoin. The Phe114 $\rightarrow$ Leu and Phe476 $\rightarrow$ Leu mutations both had largely the same effect on each ligand, decreasing turnover to $\sim$ 30% or less. The greatest individual differences were seen with the Trp mutants. F114W decreased (S)-warfarin turnover slightly, yet increased phenytoin turnover 4-fold. Conversely, the

F476W mutant increased (S)-warfarin turnover 4-fold, whereas it almost eliminated turnover of phenytoin. L208V reduced turnover of both ligands by approximately 70%, whereas the activity of both R108L and R108F was below quantifiable levels.

Kinetics of Phenytoin Metabolism. Table 1 details the kinetic parameters for phenytoin turnover by the CYP2C9 mutants. F100L and F114W both increased $V_{\max}$ substantially, with no change in K_m ; therefore, the V/K values were elevated approximately 4-fold. For all other mutants, there was a considerable drop in catalytic efficiency relative to wild-type enzyme. F114L, F476L, F100W, and L208V each increased the K_m approximately 3-fold while decreasing the $V_{\max}$ 3–5-fold, resulting in V/K values that were $\sim$ 10% that of wild type. As noted above, turnover by the F476W, R108L, and R108F mutants was less than 10% of the wild-type value and below the limit of quantitation. For (S)-warfarin, V/K values were reported by Mosher et al. (2008), except for L208V, which produced 6-hydroxywarfarin as the major metabolite [$K_m = 29 \pm 3 \mu$ M, $V_{\max} = 27 \pm 1$ pmol min $^{-1}$ nmol P450 $^{-1}$, $V/K = 0.9 \mu$ l min $^{-1}$ nmol P450 $^{-1}$ (2% of wild-type value)], and the R108L and R108F mutants, whose activities were below quantifiable levels.

Regio- and Stereoselectivity of Phenytoin Metabolism. Wild-type CYP2C9 formed *p*-, *m*-, and *o*-HPPH in a ratio of 96:4:0. Of the mutant enzymes studied here, only F114L, F476L, and L208V increased the proportion of *m*-HPPH, but only by $\sim$ 2-fold (Fig. 2A). For the major *p*-HPPH metabolite, the same three mutants were the only proteins to increase the percentage of (*R*)-*p*-HPPH formed above 3% for wild-type (Fig. 2B). The F114L and F476L mutants increased the percentage to 8% of total HPPH, whereas the L208V mutant produced 24% (*R*)-*p*-HPPH. Figure 3 shows the near baseline separation of (*R*)- and (*S*)-*p*-HPPH for wild-type enzyme and the L208V mutant. It is unfortunate that analyte levels for the R108L and R108F mutants were again too low to permit accurate determination of their prochiral selectivity for *p*-HPPH formation.

Regioselectivity of (S)-Warfarin Metabolism. The (S)-warfarin data for the Phe mutants shown in Fig. 2, C and D, were adapted from Mosher et al. (2008), with new data for the L208V mutant included. Again, the F114L, F476L, and

Fig. 1. Metabolic turnover of wild-type CYP2C9 and Phe mutant enzymes with 0.1 mM phenytoin (A) and 0.5 mM (S)-warfarin (B) (Mosher et al., 2008). Bars equal the sum of major and minor metabolites. Values for R108L and R108F are omitted because of low turnover. Error bars, S.D. of triplicate incubations.

TABLE 1
Kinetic parameters for phenytoin *p*-hydroxylation by CYP2C9^a

	Phenytoin		
	K_m	$V_{\max}$	$V_{\max}/K_m$
	μ M	pmol min $^{-1}$ nmol $^{-1}$	μ l min $^{-1}$ nmol P450 $^{-1}$
Wild type	25 $\pm$ 3	180 $\pm$ 6	7 (100) ^b
F100L	22 $\pm$ 2	740 $\pm$ 20	33 (460)
F114L	82 $\pm$ 9	50 $\pm$ 2	0.6 (9)
F476L	83 $\pm$ 6	50 $\pm$ 1	0.6 (9)
F100W	57 $\pm$ 1	28 $\pm$ 2	0.5 (7)
F114W	21 $\pm$ 2	630 $\pm$ 10	30 (410)
F476W ^c		<5	
L208V	80 $\pm$ 2	70 $\pm$ 2	0.9 (13)
R108L		<15	
R108F		<5	

^a The error values represent the S.D. calculated from the best fit values of the curves derived from fitting to the Michaelis-Menten equation.

^b Boldface number in parentheses refers to percentage of wild-type V/K .

^c Values for F476W, R108L, and R108F were not determined due to $V_{\max}$ values < 10% of wild type.

Fig. 2. Fold change from wild-type CYP2C9 formation of percentage *m*-HPPH of total HPPH (wild-type percentage was 4 ± 0.02) (A), percentage (*R*)-*p*-HPPH of total *p*-HPPH (wild-type percentage was 3 ± 0.1) (B), 6-/7-hydroxywarfarin (C) (wild-type ratio was 0.3:1), or percentage 4'-hydroxywarfarin of total (*S*)-warfarin (D) (wild-type percentage was 3 ± 0.2). (Mosher et al., 2008). F476W values in A and B and R108L and R108F values in A to D are not reported because of low turnover. Error bars, propagated error from triplicate incubations.

L208V mutants demonstrated the most significant changes in product ratios relative to wild-type enzyme. However, although the F114L and F476L effects were manifest by an increased flux to the (*S*)-warfarin 4-hydroxy metabolite (Fig. 2D), the L208V mutant was unique in increasing the ratio of 6-hydroxy:7-hydroxy warfarin from $\sim 0.3:1$ for wild-type CYP2C9 (Rettie et al., 1992) to $\sim 3:1$ in the mutant, a notable 10-fold change (Fig. 2C).

Discussion

Before the present study, the only information available regarding specific CYP2C9 amino acids that may influence the interaction between the enzyme and phenytoin came from in vitro studies that evaluated the effect of CYP2C9 genetic variants on phenytoin turnover (Veronese et al., 1993; Rettie et al., 1999; Takanashi et al., 2000). These early studies demonstrated that expression of the *CYP2C9**2 (R144C) and *CYP2C9**3 (I359L) alleles resulted in diminished metabolism of phenytoin because of a reduction in K_m , K_{cat} , or a combination of these two kinetic effects. In recent years, X-ray crystallography and mutagenesis studies have demonstrated the importance of several active-site phenylalanine residues; Phe100, Phe114, and Phe476 in the binding, metabolism, and orientation of (*S*)-warfarin and several nonsteroidal anti-inflammatory drugs (Melet et al., 2003; Williams et al., 2003; Wester et al., 2004; Mosher et al., 2008). Because phenytoin contains two prochiral aromatic rings, one of which is the major site of metabolism, it seemed probable that

mutation within this Phe cluster would have a significant impact on substrate binding and orientation within the CYP2C9 active site. Therefore, we focused initially on Leu and Trp mutations at these loci, specifically to manipulate aromaticity and steric bulk.

The loss of aromaticity and steric bulk in the CYP2C9 Phe $\rightarrow$ Leu mutants had the same general effects on phenytoin turnover, as noted previously for (*S*)-warfarin and the NSAIDs, i.e., a substantial decrease in metabolic efficiency for the F114L and F476L mutants and a trend toward activation of metabolism with the F100L mutant, although this latter effect was much more pronounced for phenytoin. However, it was the Trp mutants that provided the most interesting behavior, particularly the F114W and F476W mutants. F114W more than restored the loss of catalytic efficiency observed with the F114L mutant, whereas F476W abolished phenytoin hydroxylation. These effects on phenytoin turnover were the reverse of those observed for (*S*)-warfarin (Fig. 1, Mosher et al., 2008). Therefore, the spectrum of metabolic changes for this battery of Phe mutants is unique for phenytoin relative to that presented previously for (*S*)-warfarin and flurbiprofen (Mosher et al., 2008). The ligand-specific nature of the metabolic profiles across the range of mutants studied suggests that phenytoin, (*S*)-warfarin, and the NSAIDs each interact in a unique fashion with CYP2C9 active-site amino acids during catalytic turnover. This conclusion complements that made by Liu et al. (2005), who found that the addition of (*R*)-lansoprazole had differential effects on the metabolism of these same three ligands.

Fig. 3. LC-MS/MS chromatograms showing separation of (*R*)- and (*S*)-*p*-HPPH from turnover of phenytoin by wild-type CYP2C9 (A) or L208V (B). Only one channel of the MRM analysis is shown (m/z 269 > 198). The peaks are as follows: 8.3 min, *m*-HPPH; 9.0 min, (*R*)-*p*-HPPH; and 9.7 min, (*S*)-*p*-HPPH.

Phenytoin is a very useful drug with which to probe substrate positioning during catalysis by CYP2C9 because both the regio- and stereochemistry of product formation can be monitored (Scheme 1). The present study confirmed that (*S*)-*p*-HPPH was by far the dominant metabolic product formed by wild-type CYP2C9. It is notable that only the F114L, F476L, and L208V mutants altered phenytoin's metabolite profile, increasing the flux to both *m*-HPPH and (*R*)-*p*-HPPH relative to wild-type enzyme. This is indicative of a relaxation of binding constraints in the CYP2C9 active site when hydrophobic/steric interactions between phenytoin and Phe114, Phe476, and Leu208 are disrupted. The same sites are clearly important to preserving (*S*)-warfarin's catalytic orientation in CYP2C9. However, with (*S*)-warfarin, the L208V mutation causes a change in hydroxylation regioselectivity from the 7-position to the 6-position of the coumarin ring (Fig. 2C), whereas the F114L and F476L mutations appear to cause a larger re-orientation, such that the C-9 phenyl ring becomes the main target for hydroxylation (Fig. 2D; Mosher et al., 2008). With phenytoin as substrate, the relative magnitude of the L208V and F114L/Phe476 effects on orientation are reversed, with L208V demonstrating the most significant changes in metabolite profile.

Leu208 is located in the F helix, just before the F-G loop which, in other CYP2 enzymes, has been speculated to play an important role in ligand specificity and orientation, possibly through effects on side-chain packing (Negishi et al., 1996; Uno et al., 1997). The data here provide the first experimental evidence for a role of the F-G loop region in

dictating the catalytic orientation of substrates within the CYP2C9 active site. L208V was thought initially to represent a genetic polymorphism in exon 4 that compromised (*S*)-warfarin metabolism and, therefore, decreased the warfarin dose requirement (Leung et al., 2001). However, it was subsequently determined to be a sequencing artifact (Zarza et al., 2002; Rettie et al., 2003). Val208 is found naturally in wild-type CYP2C19, an isoform that exhibits a greatly reduced prochiral selectivity for *p*-HPPH formation (Bajpai et al., 1996), so it would not be surprising if this residue is also important to phenytoin's binding to CYP2C19.

In summary, these data show that mutations within the CYP2C9 active site Phe cluster differentially affect phenytoin and (*S*)-warfarin metabolism, suggesting that these ligands sample different, albeit likely overlapping sets of amino acid binding determinants during turnover. In addition, it is clear that the Phe114, Phe476, and Leu208 residues, but not Phe100, are critical to the high regioselectivity and stereoselectivity of phenytoin hydroxylation. Based on these functional observations, Fig. 4 depicts a possible set of interactions between phenytoin and the CYP2C9 active site. The figure shows phenytoin oriented for *p*-hydroxylation on the pro-(*S*) phenyl ring "anchored" by a hydrophobic/aromatic interaction with Phe114, the pro-(*R*) ring interacting with both Leu208 and Phe476, and the hydantoin ring directed toward Arg108. This speculative model awaits further refinement, which may be obtained most clearly from a crystal structure of phenytoin bound to the enzyme in its catalytic orientation.

Fig. 4. UCSF Chimera rendering of phenytoin in the CYP2C9 active site. Phenytoin is pictured in green surrounded by residues that appear to influence its orientation in the active site, including Phe114 (blue), Phe476 (purple), Arg108 (yellow), and Leu208 (orange). The heme is red.

Acknowledgments

We thank Dr. Rory Rimmel for the use of the Nucleodex β -OH chiral column and Dr. Ross F. Lawrence and Tom F. Kalhorn for technical assistance with the chiral LC-MS/MS assay.

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Address correspondence to: Allan E. Rettie, Department of Medicinal Chemistry, University of Washington, Box 357610, Seattle, WA 98195. E-mail: rettie@u.washington.edu