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Re-engineering of CYP2C9 to Probe Acid-Base Substrate Selectivity

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Abstract

A common feature of many CYP2C9 ligands is their weak acidity. As revealed by crystallography, the structural basis for this behavior involves a charge-pairing interaction between an anionic moiety on the substrate and an active site R108 residue. In the present study we attempted to re-engineer CYP2C9 to better accept basic ligands by charge reversal at this key residue. We expressed and purified the R108E and R108E/D293N mutants and compared their ability with that of native CYP2C9 to interact with (*S*)-warfarin, diclofenac, pyrene, propranolol, and ibuprofen amine. As expected, the R108E mutant maintained all the native enzyme's pyrene 1-hydroxylation activity, but catalytic activity toward diclofenac and (*S*)-warfarin was abrogated. In contrast, the double mutant displayed much less selectivity in its behavior toward these control ligands. Neither of the mutants displayed significant enhancement of propranolol metabolism, and all three preparations exhibited a type II (inhibitor) rather than type I (substrate) spectrum with ibuprofen amine, although binding became progressively weaker with the single and double mutants. Collectively, these data underscore the importance of the amino acid at position 108 in the acid substrate selectivity of CYP2C9, highlight the accommodating nature of the CYP2C9 active site, and provide a cautionary note regarding facile re-engineering of these complex cytochrome P450 active sites.

CYP2C9 is a major form of human liver cytochrome P450 (P450) involved in the metabolism of ~15% of all the therapeutic agents that are cleared by oxidative metabolic processes (Williams et al., 2004). Many of these drugs, including (*S*)-warfarin, phenytoin, and a wide variety of nonsteroidal anti-inflammatory drugs (NSAIDs), are hydrophobic and weakly acidic (Miners and Birkett, 1998; Rettie and Jones, 2005). Early site-directed mutagenesis studies suggested that CYP2C9 residues F114, V113, and F476 were involved in hydrophobic interactions with the substrates (*S*)-warfarin, diclofenac, and the competitive inhibitor sulfaphenazole (Haining et al., 1999; Melet et al., 2003). In addition, the I helix residues S286 and N289 have been implicated as important determinants of CYP2C9-dependent NSAID metabolism and high affinity sulfaphenazole binding (Jung et al., 1998; Klose et al., 1998). Mutagenesis of the charged residues at R97, R108, D293, and D360 was also found to alter CYP2C9 substrate binding and metabolism (Ridderström et al., 2000; Flanagan et al., 2003; Dickmann et al., 2004). In particular, the R108A mutant displayed a 100-fold lower activity toward diclofenac compared with wild-type (Ridderström et al., 2000), and previous studies from this laboratory showed that the R108F mutant exhibited diminished catalytic activity

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toward diclofenac and (*S*)-warfarin while maintaining most of the wild-type activity toward the uncharged molecule pyrene (Dickmann et al., 2004). This latter finding of ligand-dependent activity changes showed that activity alterations were not a global phenomenon and indicated that R108 plays a critical role in the acid substrate selectivity of CYP2C9. Moreover, the crystal structure of CYP2C9 with flurbiprofen bound reveals that a side-chain nitrogen on R108 forms a salt bridge with the carboxylate of flurbiprofen (Wester et al., 2004), thereby cementing the critical role of R108 in CYP2C9 selectivity for organic anions.

The above findings prompt the question, can charge reversal at this site alter ligand specificity for CYP2C9? Literature precedent exists for this scenario in studies conducted previously with CYP2D6. In contrast to CYP2C9, CYP2D6 exhibits a well recognized preference for the binding and metabolism of basic compounds. Site-directed mutagenesis studies have implicated the charged acidic residues E216 and D301 in providing critical electrostatic interactions with basic ligands (Ellis et al., 1995; Guengerich et al., 2002). This interpretation appears compatible with the ligand-free crystal structure of CYP2D6 (Rowland et al., 2006). Although wild-type CYP2D6 is incapable of metabolizing the NSAID diclofenac, the CYP2D6 E216Q/D301Q double mutant gained up to 16% of CYP2C9's activity toward this substrate (Paine et al., 2003). These data underscore the importance of these acidic residues in CYP2D6 for the binding and metabolism of basic compounds and encouraged us to attempt a similar approach to the rational re-engineering of CYP2C9's substrate specificity by targeting the critical R108 residue. We also examined the influence of D293 because this I-helix residue maps to D301 in CYP2D6 and forms a hydrogen bond with R108 when flurbiprofen is bound in the active site (Wester et al., 2004).

The study design involved charge reversal and neutralization of these apparently critical charged amino acids in the active site of CYP2C9, and so the R108E and R108E/D293N double mutants were expressed in insect cells and purified to near homogeneity. These proteins were used in comparison to wild-type CYP2D6 and CYP2C9 to explore the metabolism (and in some cases spectral binding) of the control ligands: diclofenac, pyrene, and *S*-warfarin; ibuprofen amine: a “designer” drug wherein the carboxylate of the CYP2C9 substrate ibuprofen has been replaced with a basic amine; and propranolol: a prototypic CYP2D6 substrate (see Fig. 1).

Materials and Methods

Chemicals and Reagents

4-Hydroxy propranolol, 5-hydroxy propranolol, and desisopropyl propranolol metabolites were gifts from Dr. Wendel L. Nelson (University of Washington). Ibuprofen amine was synthesized as previously described (Davies et al., 2004). Oligonucleotides, dNTPs, and Cellfectin reagent were purchased from Invitrogen (Carlsbad, CA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). EX-CELL 420 insect serum-free medium was purchased from JRH Bioscience, Inc. (Lenexa, KS). Octyl-Sepharose, δ -aminolevulinic acid, ferric citrate, dithiothreitol, sodium dithionite, NADPH, and diclofenac were obtained from Sigma-Aldrich (St. Louis, MO). 4'-Hydroxydiclofenac was provided by Novartis (Basel, Switzerland). Hydroxyapatite was purchased from Bio-Rad (Hercules, CA). Pyrene and 1-hydroxypyrene were purchased from Supelco (Bellefonte, PA) and Acros Organics (Santa Clara, CA), respectively. (*S*)-Warfarin was obtained by fractional crystallization, and deuterium-labeled 3'-, 4'-, 6-, 7-, and 8-hydroxy warfarins were synthesized as described previously (Bush and Trager, 1983). Dilaurylphosphatidylcholine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). High-performance liquid chromatography (HPLC)-grade solvents were from Fisher Scientific Co. (Fair Lawn, NJ).

Protein Expression

CYP2C9 was subcloned into the pFastBac vector. Mutagenesis was performed using overlap extension polymerase chain reaction for the R108E mutation and QuikChange II XL (Stratagene, La Jolla, CA) protocols for the D293N mutation, respectively. Mutagenic oligonucleotides consisted of the following sequences: R108E (forward), 5'-GAG CTA ACG AAG GAT TTG GAA TTG-3'; R108E (reverse), 5'-AAA CAA TTC CAA ATC CTT CGT TAG CTC-3'; D293N (forward), 5'-GCT TGG AAA ACA CTG CAG TTA ATT TGT TTG GAG CTG GGA CA-3'; and D293N (reverse), 5'-TGT CCC AGC TCC AAA CAA ATT AAC TGC AGT GTT TTC CAA GC-3'. The target mutation and integrity of the gene were confirmed by DNA sequencing of the entire gene in both the pFastBac vector and in DNA isolated from infected insect cells. Recombinant baculovirus construction and protein expression in *Trichoplusia ni* and *Sf9* cells were performed according to the Bac-to-Bac baculovirus expression system protocol from Invitrogen using EX-CELL 420 medium supplemented with 0.5% fetal bovine serum. δ -Aminolevulinic acid and ferric citrate were added to the cell medium at 0.3 and 0.2 mM final concentrations, respectively, after the cells had been infected for 16 to 24 h.

Enzyme Preparations

Purification of wild-type CYP2C9 and the R108E, R108E/D293N mutants were carried out as described previously (Haining et al., 1999) on octyl-Sepharose and hydroxyapatite with omission of the DEAE-Sepharose step. P450 spectral measurements (Estabrook and Werringloer, 1978) were taken with an Agilent 8453E UV-visible spectrometer (Agilent Technologies, Palo Alto, CA). Protein concentrations were measured by the Lowry method (Lowry et al., 1951).

Protein Mass Spectrometry

Liquid chromatography (LC)/electrospray ionization mass spectrometry (MS) analysis was performed to obtain the mass of the mutant proteins as described previously (Henne et al., 2001). In brief, instruments included a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass, Ltd., Manchester, UK) coupled to a Shimadzu (Kyoto, Japan) LC consisting of two LC-10ADvp pumps, an SPD-10Avp UV-visible detector, an SCL-10Avp controller, and an SIL-10ADvp auto injector. The mass spectrometer was run in electrospray ionization mode at a cone voltage ranging from 45 to 55 V, source block temperature of 100°C, and a desolvation temperature of 350°C. Two hundred to 500 pmol of proteins were injected on a self-packed 2.1 $\times$ 150-mm narrow-bore POROS R2 column (Applied Bio-systems, Foster City, CA). The mobile phase used consisted of 0.05% trifluoroacetic acid (TFA) (A) and acetonitrile containing 0.05% TFA (B). The flow rate was set at 0.2 ml/min, and a linear gradient was established that increased from 20% B to 100% B over 12 min. Under these conditions, proteins eluted between 7 and 8 min. Data analysis was carried out using Micromass Mass-Lynx version 3.4 software.

UV-Visible Spectroscopy

Ibuprofen amine binding spectra were obtained on an Aminco DW-2 conversion UV-visible spectrophotometer (OLis On-line Instruments, Bogart, GA), with the slit width set to 2.0 nm, reads per data set to 20, and number of increments of 150. With temperature controlled at 37°C, scans were performed from 500 to 350 nm at a scan rate of 1.0 nm/s. Purified P450s were diluted to 0.5 μ M in 100 mM potassium phosphate buffer with 1 mM EDTA and 20% glycerol and 50 μ M dilaurylphosphatidylcholine at pH 7.4, and equally divided between sample and reference cuvettes (900 μ l). Concentrations of ibuprofen amine in acetonitrile were increased from 0.62 μ M to 232 μ M with successive aliquot additions to sample cuvette. The same volumes of acetonitrile were added correspondingly to the reference cuvette. The difference in

absorbance between the peak (~430 nm) and the trough (~410 nm) of the type II spectra obtained was then plotted versus ibuprofen amine concentration and analyzed by nonlinear regression (GraphPad Prism 4, GraphPad Software Inc., San Diego, CA) to estimate the K_s (spectral binding constant) from the titration binding curve using the following equation: $\Delta A = B_{\max}S/K_s + S$.

Thermal Stability

P450 preparations (1 μ M) were preincubated at 48°C in 100 mM potassium phosphate buffer, pH 7.4, for 0 to 30 min. At various time points aliquots were removed, and the reduced CO difference spectra were recorded relative to the zero time point value. Half-lives for the thermally induced conversion of P450 to P420 were determined according to first-order decay kinetics as previously described (Dickmann et al., 2004).

General Metabolic Incubation Conditions

Reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.4, 5 to 100 pmol of purified P450s, 10 to 200 pmol of P450 reductase, and 5 to 100 pmol of cytochrome b_5 (i.e., such that P450/reductase/cytochrome b_5 ratios were 1:2:1), 200 μ M dithiothreitol, 0.5 mM NADPH, and substrate in 0.2 to 0.5 ml final volume. Incubations were run from 5 to 30 min. All the incubation samples were centrifuged at 1300 rpm for 5 min using an Eppendorf centrifuge 5414 D (Brinkmann Instruments, Westbury, NY), and the supernatant was subjected to LC/MS or HPLC analysis.

Metabolic Assays

Diclofenac 4'-hydroxylation and pyrene 1-hydroxylation—These were performed according to the HPLC-UV (diclofenac) and HPLC-fluorescence (pyrene) assays described previously (Dickmann et al., 2004).

(S)-Warfarin hydroxylation—(S)-Warfarin metabolite profiling and subsequent steady-state kinetic studies were carried out by LC/MS assay using an Agilent Technologies LC/MSD series 1100 quadrupole mass spectrometer equipped with an Inertsil ODS-3 analytical column (2.1 $\times$ 150 mm i.d., 5- μ m particle size, GL Sciences Inc., Tokyo, Japan). Concentrations were 100 μ M for the metabolite profiling studies. Deuterium-labeled 4', 6-, 7-, and 8-hydroxy warfarins (10–100 ng) were used as internal standards. The mobile phases were (A) 1.5% acetic acid in water, pH 4.7, and (B) 100% acetonitrile. Gradient runs were programmed as follows: 35% B for 10 min, increasing to 50% B over 2 min and then to 80% B over a further 4 min before re-equilibration with 35% B for 2 min, all at a flow rate of 0.2 ml/min. Under these conditions, 4'-hydroxy warfarin eluted at ~8 min, 3'-hydroxy warfarin at ~9 min, 6-hydroxy warfarin at ~10 min, 8-hydroxy warfarin at ~13 min, and 7-hydroxy warfarin at ~14 min. GraphPad Prism 4 was used for kinetic analysis.

Propranolol hydroxylation and N-dealkylation—Propranolol concentrations ranged from 1 to 100 μ M and 50 to 2000 μ M for determination of K_M and $V_{\max}$ for CYP2D6 and CYP2C9 (including its mutants), respectively. Enzymatic reactions were initiated by the addition of NADPH and allowed to proceed at 37°C for 5 to 30 min. Ten microliters of 70% perchloric acid was added to terminate reactions followed by 10 μ l of 5 mg/ml solution of ascorbic acid to stabilize the hydroxylated propranolol metabolites. Racemic propranolol and its hydroxylated metabolites, 4-hydroxypropranolol and 5-hydroxypropranolol, and the N-dealkylated metabolite desisopropyl propranolol were separated on an XTerra RP18 analytical column (5 μ m, 4.6 $\times$ 150 mm) using a protocol modified from a published procedure (Uphagrove and Nelson, 2001). Analysis was carried out using a Shimadzu LC instrument equipped with an RF-10A XL fluorescence detector. Mobile phases were 0.2% TEA and 0.5%

phosphoric acid, pH 2.2 (A) and acetonitrile (B). Gradient runs were programmed as follows: 0 to 10 min, 10 to 20% B; 11 to 20 min, 20 to 70% B. The effluent was monitored fluorometrically with an excitation wavelength of 295 nm and an emission wavelength of 380 nm at a flow rate 1 ml/min. Under these conditions, 5-hydroxypropranolol eluted at 13 min, 4-hydroxypropranolol at 14 min, and desisopropyl propranolol at 16 min. GraphPad Prism 4 was used for kinetic analysis.

LC/MS assay for ibuprofen amine metabolism—Exploratory metabolic incubations were conducted with up to 100 μ M substrate and 200 pmol of P450. After 30 min at 37°C, reactions were extracted at either pH 7 or pH 10 using 3 $\times$ 2 ml of ethyl ether. Extracts were dried under nitrogen and reconstituted with 50 μ l of acetonitrile for MS analysis on a Micromass Quattro II tandem quadrupole mass spectrometer equipped with a Zorbax RX-C8 analytical column (2.1 $\times$ 150 mm i.d., 5- μ m particle size, Agilent). The mobile phases were (A) 0.05% TFA in water and (B) 100% acetonitrile. Gradient runs were programmed as follows: from 0 to 15 min, 30% B to 40% B; then 100% B for 2 min. Flow rate was 1 ml/min but split such that 0.2 ml/min flowed into the mass spectrometer. Under these conditions, ibuprofen amine eluted at $\sim$ 13 min. Ibuprofen amine and potential hydroxy metabolites were detected by LC/MS in the selected ion recording mode. The ions monitored were m/z 208 [(M + 16) + H]⁺, 192 [M+H]⁺ 191 and 190. Data were analyzed with Micromass Masslynx software.

Results

Heme Content, Accurate Mass Analysis, and Thermal Stability of Wild-Type and Mutant CYP2C9 Proteins

Native CYP2C9 and the R108E and R108E/D293N mutants expressed well in insect cells (up to 300 nmol holoenzyme/l) and purified readily through standard protocols, with average final yields ranging from 50 to 80%. Enzymes were characterized with regard to their specific heme content, accurate mass, and thermal stability (Table 1). Mass data obtained by LC/electrospray ionization/MS analysis of the apoenzymes were in agreement with calculated molecular weights with an error of <0.015%. Specific heme contents ranged from 3.4 to 11 nmol/mg protein, with the lowest specific contents evident for the single and double mutants. The half-lives for thermally induced conversion of P450 to P420 also decreased with the mutant proteins. Collectively, these data show that charge reversal/neutralization at these active site residues is tolerated by CYP2C9, but the mutant proteins show decreased stability and heme incorporation relative to the wild-type enzyme.

Functional Activities of CYP2C9 Mutants

The functional capabilities of the three CYP2C9 preparations were investigated with several types of ligands.

Control ligands—Metabolism of the prototypic acidic CYP2C9 substrates (*S*)-warfarin and diclofenac were tested together with the uncharged planar aromatic compound pyrene. Reactions were conducted at single substrate concentrations (50–100 μ M) that were well in excess of the wild-type K_M for each ligand. Compared with native CYP2C9, the R108E mutant displayed very little activity toward (*S*)-warfarin and diclofenac, whereas the metabolism of pyrene was unchanged (Fig. 2). These data show that R108E is a catalytically competent enzyme and that charge reversal provides the metabolic profile toward acidic and uncharged substrates expected from earlier studies with the R108F mutant (Dickmann et al., 2004). In contrast, the R108E/D293N retained 10 to 30% activity toward *S*-warfarin, diclofenac, and pyrene, indicating that the double mutant possesses little of the ligand dependence evident for the R108E mutant.

Propranolol—Propranolol metabolism was analyzed because it is a well recognized substrate for CYP2D6 forming multiple products (Nelson and Burke, 1978; Talaat and Nelson, 1988). In the current study, CYP2D6 displayed a high turnover number for propranolol (~70/min) and favored aromatic hydroxylation over *N*-dealkylation by a factor of >15:1 (Fig. 3). These data confirm that, under catalytic conditions, the propranolol molecule is principally oriented in CYP2D6 with the basic nitrogen directed away from the heme iron. In contrast, wild-type CYP2C9 displayed low activity and practically no regioselective metabolism of propranolol (Fig. 3). Disappointingly, the R108E mutant showed no tendency toward a CYP2D6 profile, either in terms of regioselectivity of metabolism or catalytic efficiency (Fig. 3; Table 2). The R108E/D293N double mutant did exhibit a slight increase in propranolol turnover coupled with a shift in the regioselectivity that appeared to better favor a CYP2D6 profile (Fig. 3, insert). However, a more detailed kinetic study revealed no gain in efficiency of propranolol metabolism by this CYP2C9 mutant (Table 2).

Ibuprofen amine analog—Because this analog has the same skeleton as ibuprofen, a prototypical CYP2C9 substrate, it was thought that CYP2C9 mutants might be better able to adapt to binding of the amine derivative of this molecule than to binding of the propranolol. However, although CYP2D6 metabolized this compound to a hydroxylated metabolite (as assessed by a gain of 16 atomic mass units for a more polar, uncharacterized metabolite), neither CYP2C9 nor either of the mutants formed any of this metabolite (data not shown). Subsequent spectral binding studies showed that ibuprofen amine forms a strong type II spectral complex with all three of these CYP2C9 preparations (Fig. 4). Interestingly, binding titration studies showed a gradation in K_s for ibuprofen amine from 4.5 to 7.4 to 13.5 μ M for CYP2C9 wild-type, the single and double mutants, respectively, potentially indicative of weaker binding in the unproductive orientation for the engineered forms of CYP2C9.

Discussion

The objective of this study was to alter the substrate selectivity of CYP2C9 toward basic ligands by site-directed mutagenesis of key active site amino acids known to bind acidic substrates like flurbiprofen (Wester et al., 2004). Many examples of successful re-engineering of other enzyme activities are known. For example, reciprocally switching amino acid F208 in rat monoamine oxidase (MAO)-A and its corresponding residue in rat MAO-B, I199, was sufficient to switch the substrate and inhibitor preferences of rat MAO-A and MAO-B (Tsugeno and Ito, 1997). Moreover, as noted earlier, in the P450 field, Paine et al. (2003) showed that a CYP2D6 double mutant E216Q/D301Q was able to confer on the enzyme modest CYP2C9-like diclofenac 4'-hydroxylase activity, although the overall catalytic efficiency achieved was only ~1% that of wild-type CYP2C9. The present study design drew on the (partial) success of the CYP2D6 mutagenesis experiments as we attempted to increase the selectivity of CYP2C9 for basic ligands by charge reversal and neutralization of R108 and D293, respectively.

Data from both site-directed mutagenesis and the flurbiprofen-bound CYP2C9 crystal structure indicate that the positively charged R108 interacts electrostatically with the carboxylic acid group of the NSAID (Dickmann et al., 2004; Wester et al., 2004). To investigate whether this electrostatic bonding with an acidic substrate is obligatory, we asked whether CYP2C9 could adapt to binding basic ligands if R108 was replaced by a negatively charged amino acid, i.e., the R108E mutant. It seemed possible that incorporation of a new, acidic amino acid in the CYP2C9 active site might engender some unfavorable contacts with other residues, such as the negatively charged D293. Therefore, we also expressed and purified the E108/N293 double mutant to neutralize any potential charge repellency. Fairly conservative changes at D293 are necessary because of the apparent role of this amino acid in protein stability (Flanagan et al., 2003; Dickmann et al., 2004), which were further highlighted in the present study (Table 1).

Both the single and double mutants expressed reasonably well in insect cells; however, the low specific heme contents and increased thermal lability, relative to wild-type CYP2C9, suggest that these mutations place a strain on the active site environment and hinder holoenzyme formation. Consequently, additional mutagenesis involving other potentially interacting active-site residues, such as N289, was not pursued at this time.

Functional data for “control” ligands showed that, as expected, the R108E mutant lost activity toward (*S*)-warfarin and diclofenac but maintained activity toward the uncharged molecule pyrene (Fig. 2). However, when metabolism of the prototypic CYP2D6 substrate propranolol was examined, the R108E single mutant exhibited the same low degree of turnover and lack of regioselectivity displayed by wild-type CYP2C9. Some slight improvement was evident with the double mutant in that regioselectivity shifted toward a CYP2D6 profile where aromatic hydroxylation predominates over *N*-dealkylation. Closer examination of the kinetics for these reactions, however, provided no indication of enhanced CYP2D6-type catalysis (Table 2).

One of the “control” ligands whose metabolism was examined is (*S*)-warfarin. It has been suggested that warfarin interacts with CYP2C9 as a ring-closed hemiketal, which is less acidic than the ring-opened form (He et al., 1999). Therefore, one may anticipate a reduced effect of mutations at R108 on (*S*)-warfarin metabolism. However, as had been seen previously for the R108F mutant (Dickmann et al., 2004), (*S*)-warfarin metabolism was practically abolished by the R108E mutation. Possibly, R108 impacts (*S*)-warfarin metabolism through a different type of interaction, either with substrate or the other active site residues, or perhaps the different tautomeric forms interconvert in the active site. The detailed mechanism underlying these observations requires further investigation.

Before the availability of CYP2C9 crystal structures and the emergence of R108 as a key active site residue, Davies et al. (2004) evaluated the role of two other basic amino acids, R97 and K72, in the ligand selectivity of CYP2C9. An interesting approach used in this study was the attempted spectral characterization of the amine analogs of the classical CYP2C9 substrates phenytoin, naproxen, and ibuprofen with the mutant enzymes generated. The rationale here was that a re-engineered CYP2C9 active site might more readily accommodate a basic ligand that had the same skeleton as an established CYP2C9 substrate and exhibit a type I (substrate-like) spectrum. However, no classical spectral perturbations were obtained in this earlier study, perhaps because of the relatively low levels of enzyme expression obtained for many of the mutant proteins.

In the present study, relatively large amounts of purified enzymes and mutants were available, and so we also examined spectral binding of the ibuprofen amine analog to each of the CYP2C9 preparations. Ibuprofen amine bound tightly to wild-type CYP2C9 and exhibited the expected type II binding spectrum indicative of nitrogen ligation to the heme. However, both CYP2C9 mutants also displayed this type II binding interaction, which indicates that a dominant orientation of this basic ligand in the CYP2C9 active site is not altered by providing a complementary acidic group in the distal cavity. Interestingly, we did note a trend toward increasing K_s for the binding of this amine to the single and double mutants. Although small, these changes could be reflective of weaker binding of the amine in the unproductive orientation to charge-modified CYP2C9, as might be expected for CYP2D6. However, when considered together with reduced specific heme contents, enhanced thermal lability, and a loss of ligand-specific metabolic effects, especially for R108E/D293N, this might simply be reflective of a more general disordering of the CYP2C9 active site in the mutant enzymes.

In summary, the functional studies conducted here further highlight the importance of the amino acid at position 108 in CYP2C9 in the binding and metabolism of acidic ligands. However, despite the increasingly well documented conformational flexibility of mammalian

P450 active sites (Ekroos and Sjögren, 2006; Muralidhara et al., 2006) and the experimentally shown tolerance of significant change in electrostatic charge distribution within the CYP2C9 active site, the attempted re-engineering of CYP2C9's ligand selectivity by charge reversal at this key residue provided little gain in terms of a CYP2D6 profile that favors basic ligands. Interestingly, a recent report showed that *very* extensive re-engineering (16 amino acid changes) was required to confer even modest (*S*)-mephenytoin 4'-hydroxylase activity—a CYP2C19 marker activity—on CYP2C9 (Wada et al., 2008). This result speaks to the complex, and likely interactive, nature of mammalian P450 active sites and suggests that successful re-engineering of P450 substrate specificity across subfamilies, as attempted here, will not be a trivial exercise.

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Abbreviations

P450	cytochrome P450
NSAID	nonsteroidal anti-inflammatory drug
HPLC	high-performance liquid chromatography
LC	liquid chromatography
MS	mass spectrometry

TFA

trifluoroacetic acid

MAO

monoamine oxidase

Diclofenac

Pyrene

Warfarin

Propranolol

Ibuprofen amine

Fig. 1.
Structures of CYP2C9 and CYP2D6 substrate probes.

Fig. 2. Catalytic activities of CYP2C9 and the R108E and R108E/D293N mutants toward (S)-warfarin, diclofenac, and pyrene. Metabolic incubations were conducted with 5 to 100 pmol of P450 and 50 to 100 μ M substrates, as described under *Materials and Methods*.

Fig. 3. Catalytic activity toward propranolol for CYP2D6, CYP2C9, and CYP2C9 mutants. Propranolol metabolism was assayed as described under *Materials and Methods* at 100 μ M (CYP2D6) or 2 mM (CYP2C9) preparations. 4-OH P, 4-hydroxy-propranolol; DIP, desisopropyl-propranolol.

Fig. 4.

Type II binding spectra generated from the ibuprofen amine analog with CYP2C9 (A), R108E (B), and R108E/D293N (C). Difference spectra were obtained with 0, 0.62, 1.2, 2.5, 5.0, 11.2, 23.5, 48.1, 110, 171, and 232 μ M ibuprofen amine and 0.5 μ M P450 as detailed under *Materials and Methods*. K_s values were 4.5, 7.4, and 13.5 μ M for CYP2C9, R108E, and R108E/D293N, respectively.

TABLE 1

Characterization of CYP2C9 mutants

Enzyme	Molecular Mass		Sp. Content	Thermal Half-Life [*]
	Expected	Observed (Δ)		
	<i>Da</i>	<i>Da</i>	<i>nmol/mg</i>	<i>min</i>
CYP2C9	55628	55627 (+1)	11	10
R108E	55601	55601 (+0)	3.1	3
R108E/D293N	55600	55592 (+8)	4.1	<1

* Half-life for the conversion of spectral P450 to P420 at 48°C.

TABLE 2**Kinetic parameters for propranolol metabolite formation**

Kinetic constants were determined over the concentration ranges 1 to 100 μM (CYP2D6) or 50 to 2000 μM (CYP2C9 preparations).

Enzyme	4-Hydroxy-propranolol		Desisopropyl-propranolol	
	K_M	V_m	K_M	V_m
	μM	min^{-1}	μM	min^{-1}
CYP2D6	22 ± 7	66 ± 11	14 ± 6	5 ± 0.07
CYP2C9	>900	<0.5	>150	<0.5
R108E	>900	<0.5	>150	<0.5
R108E/D293N	>900	<0.5	>150	<0.5