

Activation of the Anticancer Prodrugs Cyclophosphamide and Ifosfamide: Identification of Cytochrome P450 2B Enzymes and Site-Specific Mutants with Improved Enzyme Kinetics

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ABSTRACT

Cyclophosphamide (CPA) and ifosfamide (IFA) are oxazaphosphorine anticancer prodrugs metabolized by two alternative cytochrome P450 (P450) pathways, drug activation by 4-hydroxylation and drug inactivation by *N*-dechloroethylation, which generates the neurotoxic and nephrotoxic byproduct chloroacetaldehyde. CPA and IFA metabolism catalyzed by P450s 2B1, 2B4, 2B5, and seven site-specific 2B1 mutants was studied in a reconstituted *Escherichia coli* expression system to identify residues that contribute to the unique activities and substrate specificities of these enzymes. The catalytic efficiency of CPA 4-hydroxylation by rat P450 2B1 was 10- to 35-fold higher than that of rabbit P450 2B4 or 2B5. With IFA, ~50% of metabolism proceeded via *N*-dechloroethylation for 2B1 and 2B4, whereas CPA *N*-dechloroethylation corresponded to only ~3% of total metabolism (2B1) or was absent (2B4, 2B5). Improved catalytic efficiency of CPA and IFA 4-hy-

droxylation was obtained upon substitution of 2B1 Ile-114 by Val, and replacement of Val-363 by Leu or Ile selectively suppressed CPA *N*-dechloroethylation $\geq 90\%$. P450 2B1-V367A, containing the Ala replacement found in 2B5, exhibited only ~10% of wild-type 2B1 activity for both substrates. Canine P450 2B11, which has Val-114, Leu-363, and Val-367, was therefore predicted to be a regioselective CPA 4-hydroxylase with high catalytic efficiency. Indeed, P450 2B11 was 7- to 8-fold more active as a CPA and IFA 4-hydroxylase than 2B1, exhibited a highly desirable low K_m (80–160 μM), and catalyzed no CPA *N*-dechloroethylation. These findings provide insight into the role of specific P450 2B residues in oxazaphosphorine metabolism and pave the way for gene therapeutic applications using P450 enzymes with improved catalytic activity toward these anticancer prodrug substrates.

The oxazaphosphorine cyclophosphamide (CPA) and its structural isomer ifosfamide (IFA) are DNA-alkylating agents commonly used in cancer chemotherapy (Sladek, 1994). These anticancer agents are administered as prodrugs that are activated by a liver cytochrome P450-catalyzed 4-hydroxylation reaction that yields active, cytotoxic metabolites. Several liver-expressed P450 enzymes catalyze this activation reaction; the phenobarbital-inducible rat P450 enzyme 2B1 (Clarke and Waxman, 1989) and its human counterpart 2B6 (Chang et al., 1993; Huang et al., 2000) show particularly high CPA 4-hydroxylase activity compared with other P450 forms. The primary metabolite, 4-OH-CPA or 4-OH-

IFA, equilibrates with the ring-open aldophosphamide and undergoes β -elimination to yield the therapeutically active, DNA cross-linking phosphoramidate mustard and the byproduct acrolein (4-hydroxylation pathway). CPA and IFA are also subject to an alternative, P450-catalyzed side chain oxidation that generates therapeutically inactive *N*-dechloroethylated metabolites and the neurotoxic and nephrotoxic byproduct chloroacetaldehyde (CAA; *N*-dechloroethylation pathway) (Furlanut and Franceschi, 2003).

In patients with cancer, CPA and IFA are primarily activated in the liver, a tissue rich in P450 activity, followed by transport of the activated metabolites to the tumor via blood flow. However, these activated metabolites also gain entry to normal body tissues, where they may induce host toxicity. These and related issues may be circumvented using a P450-

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ABBREVIATIONS: CPA, cyclophosphamide; IFA, ifosfamide; *N*-deCl, *N*-dechloroethylation; DTT, dithiothreitol; P450, cytochrome P450; P450 reductase, NADPH cytochrome P450 reductase; CAA, chloroacetaldehyde; SRS, substrate-recognition site; MOPS, 3-morpholinepropanesulfonic acid; CHAPS, 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction.

based gene therapy strategy whereby tumor cells are sensitized to CPA and IFA by introduction of cDNA coding for P450 2B1 or 2B6 using a suitable viral or nonviral vector (Chen and Waxman, 2002). This gene therapy has the potential to improve therapeutic efficacy by increasing tumor cell kill at lower systemic drug doses and with reduced host tissue toxicity. To take full advantage of this potential, it will be important to optimize the system through the discovery or design of P450 enzymes with increased activity at pharmacologically relevant drug levels. Desirable enzymes would display increased catalytic efficiency (V_{\max}/K_m) for 4-hydroxylation of CPA and IFA. P450 2B variants with a lower K_m would be especially desirable, in view of the high K_m values (~ 0.5 – 2 mM) of P450s 2B1 and 2B6 with CPA and IFA as substrates (Weber and Waxman, 1993; Huang et al., 2000) and the comparatively low peak plasma drug concentrations (≤ 100 – 200 μM) found in treated patients (Sladek, 1994; Furlanut and Franceschi, 2003). Decreasing metabolic flux through the *N*-dechloroethylation pathway would also be desirable, because the metabolic products of this pathway are neurotoxic and nephrotoxic and are generally believed to be devoid of antitumor activity (however, see Borner et al., 2000).

In recent years, there have been numerous examples of rational redesign of the substrate specificity of bacterial P450s through substitution of individual, or combinations of, active site residues identified by X-ray crystallography (Walsh et al., 2000; Li et al., 2001). In parallel with these advances, structure-function studies of mammalian P450 enzymes have revealed how substitutions of residues that map to the active sites of bacterial P450s and the mammalian enzyme P450 2C5 can enhance existing activities or confer new specificities (Domanski and Halpert, 2001). Many of these residues belong to five of the six substrate-recognition sites (SRSs) for P450 family 2 enzymes that were proposed on the basis of multiple sequence alignments and analogy with P450 101 (Gotoh, 1992). In the case of the P450 2B enzymes, residues 114, 363, and 367 account for many of the functional differences between rat 2B1 and 2B2 (Strobel and Halpert, 1997), rabbit 2B4 and 2B5 (He et al., 1996; Szklarz et al., 1996), rat 2B1 and human 2B6 (Domanski et al., 1999), and rat 2B1 and canine 2B11 (Hasler et al., 1994). These functional alterations can frequently be explained using three-dimensional homology models based on the structures of bacterial P450s (Szklarz and Halpert, 1997) or P450 2C5 (Wester et al., 2003). Most recently, the progesterone hydroxylation specificity of P450 2B1 was converted to that of P450 2C5 by simultaneous substitution of seven active site residues with the corresponding residues of 2C5, as predicted by a 2B1 homology model based on the structure of 2C5 (Kumar et al., 2003).

In the present study, rat 2B1, rabbit 2B4 and 2B5, and seven site-specific mutants of P450 2B1 at positions 114 (SRS-1), 363, and 367 (SRS-5) were characterized with respect to their steady-state kinetic properties using the anticancer prodrug substrates CPA and IFA. The data obtained identify these three SRS residues as important determinants of the catalytic activity and regioselectivity of oxazaphosphorine metabolism and led to the discovery of P450 2B11 as a highly efficient and regioselective catalyst of CPA 4-hydroxylation.

Materials and Methods

Materials. MOPS, δ -aminolevulinic acid, CHAPS, dilauroylphosphatidylcholine, EDTA, DTT, CPA, NADPH, adenosine, and 1- N^6 -ethenoadenosine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). CAA [50% (w/v) solution in water], 3-aminophenol, and hydroxylamine HCl were purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC-grade methanol was purchased from J. T. Baker (Phillipsburg, NJ). 4-Hydroperoxy-IFA and 4-hydroperoxy-CPA were provided by ASTA Pharma (Bielefeld, Germany). IFA was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD).

Construction of P450 2B1 Mutants and Expression in *Escherichia coli*. Construction of P450 2B1 mutants I114V, V363L, V363A, and V367A was described previously (He et al., 1995). P450 2B1-V363I was produced using a one-step PCR method. A mutagenic primer (GAAGATCTTATCCCTATTGGAG) containing a BglII site (underlined) was used with a pKK233-2 vector-specific 3'-primer in a PCR amplification reaction. The P450 2B1 mutant was then constructed in pKK233-2 (Amersham Biosciences Inc., Piscataway, NJ) by replacing a 380-base pair BglII fragment from wild-type P450 2B1 with the corresponding mutated PCR fragment. To construct the P450 2B1 double mutants, I114V/V363L and I114V/V363I, a BglII fragment of pKK-2B1-I114V was replaced with the corresponding fragment from pKK-2B1-V363L or pKK-2B1-V363I. All mutants generated in this study were verified by DNA sequencing to ensure the presence of the desired changes and the absence of extraneous mutations (Protein Chemistry Laboratory, University of Texas Medical Branch, Galveston, TX). P450 2B1 mutants and wild-type P450 enzymes 2B1, 2B4, 2B5, and 2B11 cloned into pKK233-2 (John et al., 1994; Szklarz et al., 1996) were expressed in *E. coli* strain Topp3 as described previously (He et al., 1995). Briefly, Topp3 cells were grown at 37°C with 250-rpm shaking in 250 ml of liquid Terrific Broth media to midlog phase before adding isopropyl 1-thio- β -D-galactopyranoside (final concentration of 1 mM) and δ -aminolevulinic acid (final concentration of 80 mg/l). Cells were harvested after an additional 72-h incubation at 30°C with 190-rpm shaking. CHAPS-solubilized membranes were prepared as described previously (John et al., 1994).

Rat NADPH-Cytochrome P450 Reductase Expression and Purification. cDNA encoding rat NADPH-P450 reductase was kindly provided by Dr. Todd Porter (College of Pharmacy, University of Kentucky, Lexington, KY). P450 reductase was expressed from a T7 expression plasmid [pET29a(+); Novagen, Madison, WI] and purified as described previously (Harlow and Halpert, 1997).

Reconstitution of *E. coli*-Expressed P450s. *E. coli*-derived P450 membrane fractions (10 pmol of P450 at 1–5 nmol of P450/ml) were mixed with 3 μl of freshly sonicated dilauroylphosphatidylcholine solution (1 $\mu\text{g}/\mu\text{l}$) and purified rat liver P450 reductase (~ 20 pmol) in 100 mM MOPS buffer, pH 7.3, containing 0.2 mM DTT, 1 mM EDTA, 0.5% CHAPS detergent, and 20 μl of 100 mM KPi buffer, pH 7.4, to give a final volume of 45 μl (20–40 mM MOPS, 44 mM KPi, pH 7.4, 0.25–0.5 mM EDTA, 40–80 μM DTT, and 0.1–0.2% CHAPS). The reconstituted mixture was then incubated at room temperature for 10 min and kept on ice before enzymatic analysis (Shimada and Yamazaki, 1998).

Metabolism of CPA and IFA. Reconstituted P450 samples (45 μl) prepared as described above were added to 30 μl of 100 mM KPi, pH 7.4, containing various concentrations of CPA or IFA (final assay concentrations of CPA and IFA were 0.3, 0.5, 0.8, 1.5, 3, 5, and 7 mM). Samples were preincubated for 4 min at 37°C. Reactions were then initiated with 25 μl of 4 mM NADPH to give a final volume of 100 μl . Samples were incubated for a further 15 min at 37°C, at which time the reactions were stopped by the sequential addition of 40 μl of 5.5% ZnSO_4 , 40 μl of saturated barium hydroxide, and 20 μl of 0.01 M HCl on ice. Samples were vortexed and then centrifuged at 14,000 rpm for 15 min. Two 80- μl aliquots were removed from the supernatant of each sample and placed in separate screw-cap Ep-

TABLE 1

Steady-state kinetics analysis: CPA metabolism catalyzed by P450s 2B1, 2B4, and 2B5

P450	4-Hydroxylation			N-Dechloroethylation			% N-deCl ^a
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	
	<i>mM</i>	<i>mol/min/mol</i> <i>P450</i>	<i>mol/min/mol</i> <i>P450/mM</i>	<i>mM</i>	<i>mol/min/mol</i> <i>P450</i>	<i>mol/min/mol</i> <i>P450/mM</i>	
2B1 ^b	1.45 ± 0.24	35.9 ± 6.2	24.9 ± 3.8	1.65 ± 0.09	1.1 ± 0.3	0.7 ± 0.2	3.2 ± 1.6
2B4 ^c	5.28 ± 0.37	13.5 ± 2.9	2.6 ± 0.4	N.D.	0	0	0
2B5 ^c	4.17 ± 1.82	2.8 ± 0.7	0.7 ± 0.1	N.D.	0	0	0

N.D., not determined; catalytic activity at or below limit of detection.

^a N-dechloroethylation as a percentage of total metabolism at V_{max} .^b Data are based on $n = 4$ independent experiments (4-hydroxylation) or $n = 3$ (N-dechloroethylation).^c Data are based on $n = 2$ independent experiments.

pendorf tubes. One aliquot was used for derivatization of CAA to 1- N^6 -ethenoadenosine by addition of 10 μ l of 100 mM adenosine (dissolved in 0.25 N HCl) and 10 μ l of 2 M sodium acetate, pH 4.5 (final pH, 4.2) followed by heating for 2.5 h at 80°C (Huang and Waxman, 1999). The second aliquot was used to derivatize acrolein (breakdown product of 4-hydroxy-CPA and 4-hydroxy-IFA) to 7-hydroxyquinoline by addition of 40 μ l of 3-aminophenol (60 mg of 3-aminophenol and 60 mg of hydroxylamine HCl dissolved in 10 ml of 1 N HCl) followed by heating for 25 min at 90°C (Bohnenstengel et al., 1997). Derivatized samples were stored at -20°C in the dark before analysis and were stable for at least 1 month. The conversion of metabolites to fluorescent products was linear from 0.25 to 10 nmol for acrolein and from 25 to 1000 pmol for CAA.

HPLC Analysis. The HPLC system used in this study was equipped with two Rainin Dynamax SD-200 pumps (Rainin Instruments, Woburn, MA), a Waters model WISP710B autosampler, and a Waters model 474 scanning fluorescence scanning detector (Waters, Milford, MA). HPLC pumps were controlled using Rainin Dynamax DA Controller software. Chromatography acquisition was controlled with Waters Millennium³² software. Samples were separated on a Luna C18(2) column (5 μ m, 150 × 4.6 mm; Phenomenex Inc., Torrance, CA). Dedicated HPLC columns were used to analyze each metabolite. For determination of CAA, 20 μ l of each derivatized sample was injected and eluted with 13% aqueous methanol in water at a flow rate of 1 ml/min. The fluorescent 1- N^6 -ethenoadenosine product eluted at ~8.6 min and was detected by fluorescence (excitation, 270 nm; emission, 411 nm; gain, 10; slit width, 18). The sensitivity of detection (defined as twice background) was 10 pmol of CAA per injection, corresponding to 0.55 μ M CAA in the enzyme assay. Samples containing <10 pmol of CAA gave variable results and were considered unreliable. For determination of acrolein, 20 μ l of each derivatized sample was injected onto the HPLC column and eluted under isocratic conditions with 18% methanol in aqueous 0.33% phosphoric acid solution at a flow rate of 1 ml/min. The fluorescent 7-hydroxyquinoline product eluted at 3.2 min and was detected by fluorescence (excitation at 350 nm and emission at 515 nm; gain, 10 and slit width, 18). The sensitivity of detection was 40 pmol of 4-OH-CPA per injection, corresponding to 2.4 μ M in the

enzyme assay. Quantitation was based on integrated peak areas determined using Millennium³² software.

Data Analysis. Data were managed using Excel (Microsoft Corp., Redmond, WA). Data were obtained from samples assayed at each substrate concentration, and background activity control samples (i.e., no NADPH, no drug, or no P450 enzyme) were converted to rate of metabolite produced (moles of 7-hydroxyquinoline per mole of P450 per minute or moles of 1- N^6 -ethenoadenosine per mole of P450 per minute). The highest of the three measured background activities was subtracted from each sample, and the results were used to calculate steady-state kinetic parameters using Kinetics v0.44 software (Jacek Stanislawski, Novato, CA). K_m and V_{max} values for each individual experiment, typically based on data for seven substrate concentrations, were determined by graphical analysis using the Eadie-Hofstee method. V_{max} values are expressed as moles of product formed per minute, normalized to moles of P450 included in each reaction, and thus correspond to k_{cat} activities. Mean values and standard deviations were determined based on three independent sets of determinations for each enzyme-substrate pair, except as noted. Statistical comparisons using a nonparametric t test were performed using Prism software (GraphPad Software, San Diego, CA).

Results

Profiles of CPA and IFA Metabolism by P450 2B1, 2B4, and 2B5. Previous studies of panels of rat and human P450 enzymes have established that rat P450 2B1 (Clarke and Waxman, 1989) and human P450 2B6 (Chang et al., 1993; Huang et al., 2000) are the most active catalysts of CPA 4-hydroxylation in each species. We therefore investigated whether P450 2B4 and 2B5, which are well studied rabbit P450 2B enzymes, also display high activity with CPA as substrate. Steady-state kinetic analysis showed that 4-hydroxylation was the predominant pathway of CPA metabolism catalyzed by all three mammalian P450 2B enzymes (Table 1). The alternative, N-dechloroethylation pathway corresponded to only ~3% of total CPA metabolism in the case of

TABLE 2

Steady-state kinetics analysis: IFA metabolism catalyzed by P450s 2B1, 2B4, and 2B5

P450	4-Hydroxylation			N-Dechloroethylation			% N-deCl ^a
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	
	<i>mM</i>	<i>mol/min/mol</i> <i>P450</i>	<i>mol/min/mol</i> <i>P450/mM</i>	<i>mM</i>	<i>mol/min/mol</i> <i>P450</i>	<i>mol/min/mol</i> <i>P450/mM</i>	
2B1 ^b	1.73 ± 0.44	13.2 ± 1.1	7.9 ± 1.8	1.87 ± 0.72	14.7 ± 1.7	8.8 ± 3.4	52.7 ± 2.4
2B4 ^c	4.78 ± 0.63	3.1 ± 0.1	0.65 ± 0.06	7.64 ± 0.54	3.1 ± 0.6	0.41 ± 0.05	50.1 ± 4.0
2B5 ^c	2.52 ± 0.31	0.95 ± 0.07	0.38 ± 0.08	N.D.	0	0	0

N.D., not determined; catalytic activity at or below limit of detection.

^a N-dechloroethylation as a percentage of total metabolism at V_{max} .^b Data are based on $n = 4$ independent experiments.^c Data are based on $n = 2$ independent experiments.

2B1 and was undetectable with 2B4 and 2B5 (Table 1). 2B1 displayed a V_{\max} value (36 mol/min/mol of P450) that was 2.7-fold higher than 2B4 and 13-fold higher than 2B5. In contrast to CPA, IFA was metabolized by 2B1 and 2B4 by both pathways, with 4-hydroxylation/*N*-dechloroethylation product ratios of $\sim 1:1$ at V_{\max} (Table 2). P450s 2B4 and 2B5 exhibited 10- to 20-fold lower catalytic efficiencies (V_{\max}/K_m) than 2B1 for IFA 4-hydroxylation, and 2B5 was unable to catalyze IFA *N*-dechloroethylation (Table 2).

Impact of Site-Specific P450 2B1 Mutations on CPA and IFA Metabolic Activities. We next investigated whether site-directed mutagenesis could be applied to improve the catalytic properties of P450 2B1 toward CPA and IFA, either by increasing 4-hydroxylation or by decreasing metabolism by *N*-dechloroethylation. We focused our efforts on three 2B1 active site residues, Ile-114 in SRS-1 and Val-363 and Val-367 in SRS-5, based on a large body of earlier studies showing the critical importance of the residues at these positions in dictating substrate specificity differences among P450 2B subfamily members (Hasler et al., 1994; He et al., 1996; Szklarz et al., 1996; Strobel and Halpert, 1997;

Domanski et al., 1999). Seven site-specific mutants were prepared and expressed in *E. coli*. Kinetic analysis of P450 2B1-I114V, chosen on the basis of a 4-fold increase in progesterone hydroxylation activity and an altered androstenedione metabolite profile (He et al., 1995), revealed a ~ 3 -fold lower K_m than wild-type P450 2B1 using CPA as substrate (Fig. 1A). Decreases in K_m were also seen with the double mutants I114V/V363L and I114V/V363I, where Val-363 was additionally replaced by either Leu (found in P450 2B6) or Ile (found in P450 2B4) (also see below). Decreases in K_m of up to 4-fold were also seen for IFA with all three I114V-containing mutants in both metabolic pathways (Figs. 1D and 2D). However, these changes in K_m were accompanied by decreases in V_{\max} for CPA and IFA 4-hydroxylation in all three proteins that contained the I114V substitution (up to a 60–75% decrease seen with I114V/V363L). Consequently, the overall improvement in catalytic efficiency was modest (a ~ 2 -fold increase for CPA with I114V and 2.5-fold increase for IFA with I114V/V363I) (Fig. 1, C and F).

We next investigated the impact of mutating the P450 2B1 SRS-5 residue Val-363 to Leu, Ile, or Ala. The V363L and

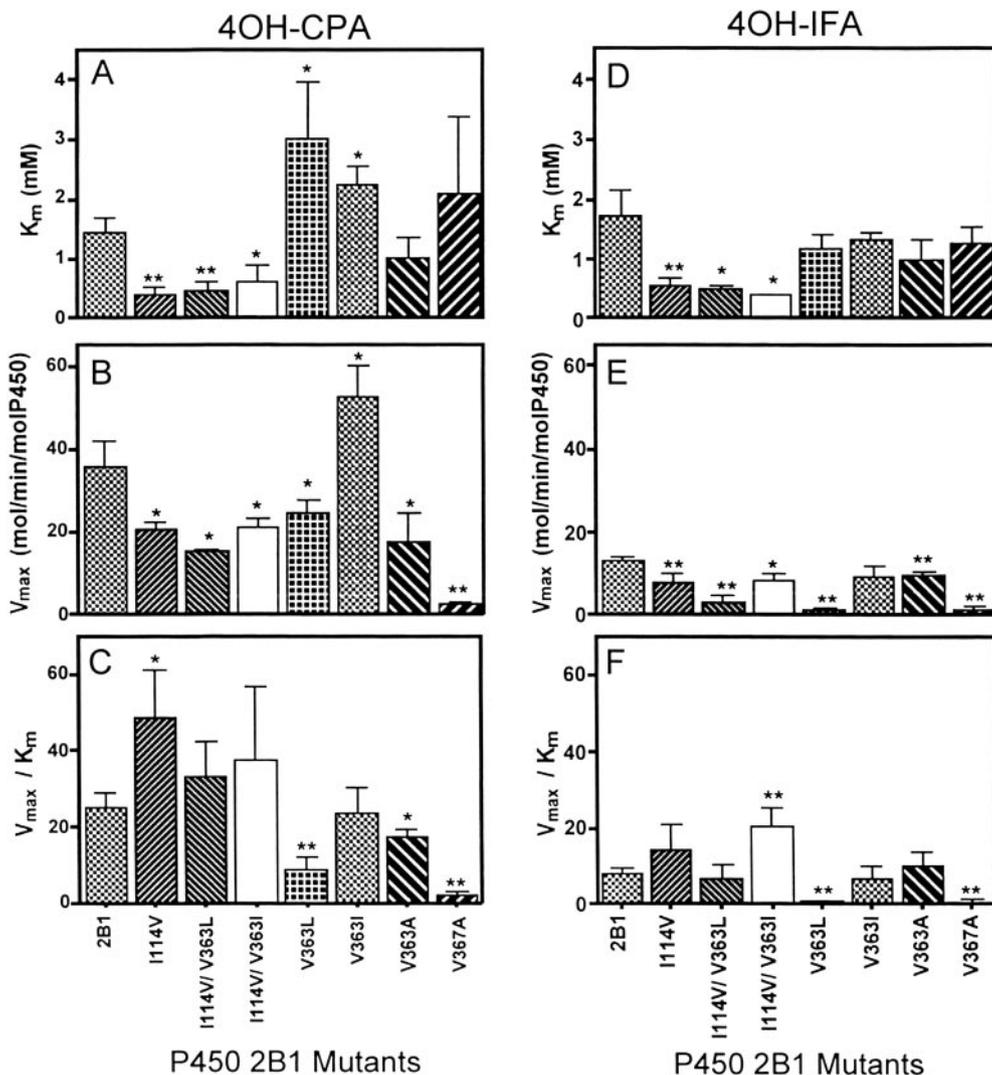


Fig. 1. Enzyme kinetic analysis: metabolism of CPA (A-C) and IFA (D-F) by 4-hydroxylation. Data are shown for P450 2B1 and seven site-specific mutants, as indicated at the bottom. Data shown are mean \pm S.D. values derived from Eadie-Hofstee analysis based on $n = 3$ independent sets of determinations, except in the case of wild-type P450 2B1 ($n = 4$) and P450 2B1 mutants I114V/V363L, I114V/V363I, and V363I ($n = 2$). *, $p < 0.05$; **, $p < 0.01$ for comparisons with P450 2B1. V_{\max}/K_m values are expressed as moles of product per minute per mole of P450 per millimolar.

V363I mutants showed modest differences in V_{\max} for CPA 4-hydroxylation compared with wild-type enzyme [≤ 2 -fold increase (V363I) or decrease (V363L); Fig. 1B]. Interestingly, however, both mutant 2B1 proteins totally lacked CPA *N*-dechloroethylation activity (Fig. 2B; Table 3). This loss of CPA *N*-dechloroethylation was also seen with the I114V/V363L double mutant. In contrast, with IFA as substrate, the V363L and V363I mutants showed no decrease in IFA *N*-dechloroethylation at V_{\max} (Fig. 2E). Moreover, because of the decrease in K_m (Fig. 2D), the overall catalytic efficiency of IFA *N*-dechloroethylation increased significantly in the case of I114V/V363L, I114V/V363I, and V363I (Fig. 2F). V363L showed a markedly reduced V_{\max} for IFA 4-hydroxylation, corresponding to $\sim 10\%$ of wild-type 2B1 (Fig. 1E) with no change in IFA *N*-dechloroethylation, such that overall metabolic flux via *N*-dechloroethylation increased from 52 to 90% of total metabolism (Table 3). A similar trend in IFA 4-hydroxylation was seen with the I114V/V363L double mutant.

Interestingly, although the replacement of V363 with Leu or Ile abolished CPA *N*-dechloroethylation, in the case of the

V363A mutant, the V_{\max} for this reaction increased 4-fold and the K_m for CPA decreased ~ 2 -fold, resulting in an 8-fold overall increase in catalytic efficiency (Fig. 2, A–C). Correspondingly, metabolism of CPA via the *N*-dechloroethylation pathway increased from 3 to $\sim 20\%$ of total metabolism (Table 3).

Finally, substitution of a second P450 2B1 SRS-5 residue, Val-367, with the Ala found in 2B5 markedly decreased metabolism of CPA and IFA by both metabolic pathways. The V_{\max} for CPA 4-hydroxylation was decreased to 8% of wild-type P450 2B1, and CPA *N*-dechloroethylation was undetectable (Figs. 1B and 2B). Similarly, the V_{\max} values for IFA 4-hydroxylation and *N*-dechloroethylation were decreased to 11 and 2.2% of wild-type 2B1, respectively (Figs. 1E and 2E).

P450 2B11 Is a High-Efficiency, Low K_m Catalyst of CPA and IFA Activation. The mutagenesis studies described above indicate that the Ile-114-to-Val substitution is beneficial, insofar as it decreases the K_m of P450 2B1 for CPA and IFA. The Val-363-to-Leu and Val-363-to-Ile substitutions are also beneficial, insofar as they suppress *N*-dechloro-

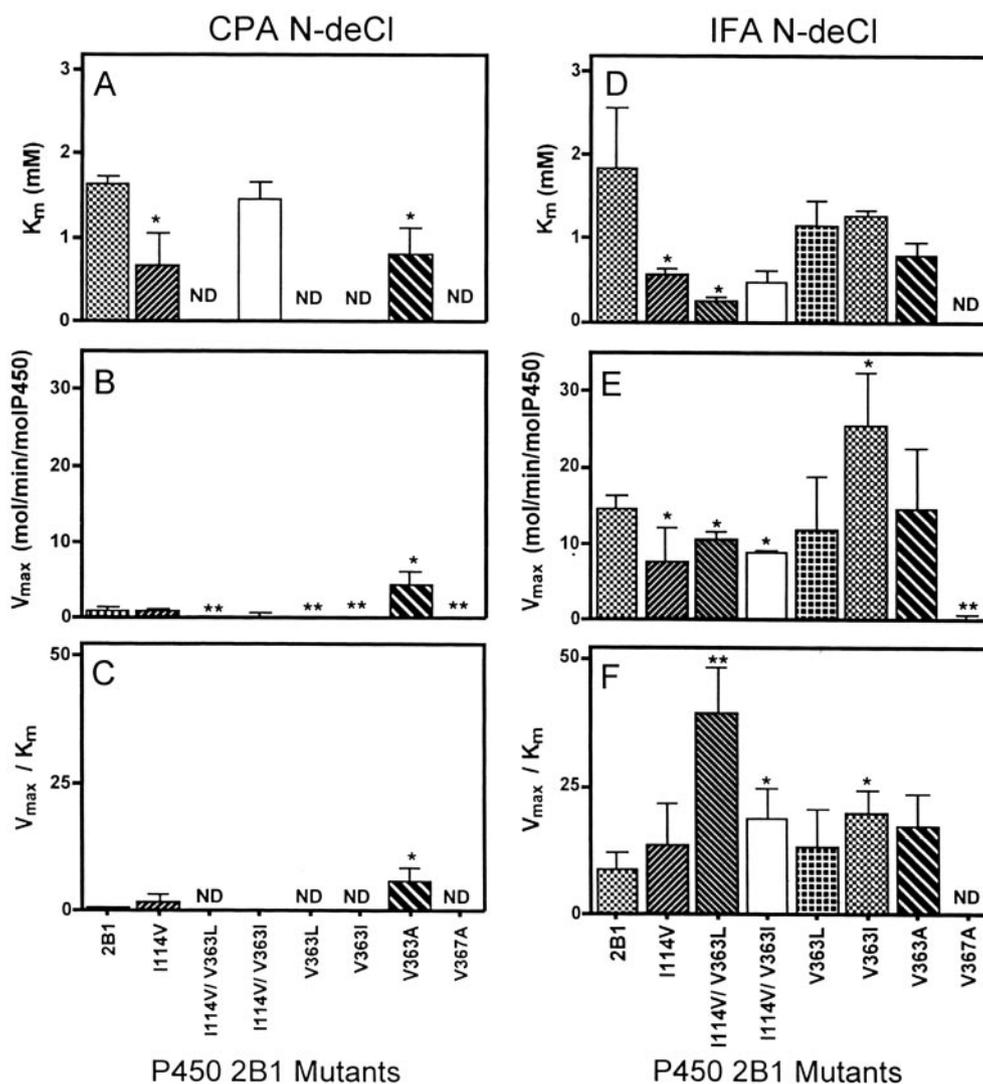


Fig. 2. Enzyme kinetic analysis: metabolism of CPA (A–C) and IFA (D–F) by *N*-dechloroethylation. Data are shown for P450 2B1 and seven site-specific mutants, as indicated at the bottom. Data shown are mean \pm S.D. values derived from Eadie-Hofstee analysis based on $n = 3$ independent sets of determinations, except as noted in Fig. 1. *, $p < 0.05$; **, $p < 0.01$ for comparisons with P450 2B1. ND, K_m and V_{\max}/K_m values were not determined for samples where *N*-dechloroethylation activity was at or below the limit of detection. V_{\max}/K_m values are expressed as moles of product per minute per mole of P450 per millimolar.

TABLE 3

N-dechloroethylation of CPA and IFA catalyzed by P450 2B1 and its site-specific mutants

Data for P450 2B1 are based on Tables 1 and 2 ($n = 3$ –4 independent experiments). Other data are based on Figs. 1 and 2 ($n = 3$ independent experiments, except as noted in Fig. 1).

Enzyme ^b	% <i>N</i> -Dechloroethylation ^a	
	CPA	IFA
P450 2B1	3.2 ± 1.6	52.7 ± 2.4
I114V	4.8 ± 0.4	46.0 ± 14.1
I114V/V363L	0	76.8 ± 4.9
I114V/V363I	1.6 ± 1.0	52.1 ± 5.4
V363L	0	90.3 ± 3.5
V363I	0	74.0 ± 1.1
V363A	20.6 ± 7.8	57.0 ± 15.7
V367A	0	27.1 ± 9.7

^a *N*-dechloroethylation as a percentage of total metabolism at V_{\max} .

^b Wild-type and site-specific mutants of P450 2B1, as indicated.

roethylation in the case of CPA. P450 2B11, which corresponds to a major phenobarbital-inducible enzyme in canine liver (Duignan et al., 1987), contains corresponding amino acid residues at both of these positions; i.e., it has the sequence Val-114 and Leu-363. We therefore investigated whether this P450 displays favorable kinetics of CPA and IFA metabolism as would be predicted by the P450 2B1 mutagenesis studies. Table 4 shows that P450 2B11 displays K_m values for CPA (160 μ M) and IFA (80–90 μ M) that are substantially lower than those of P450 2B1 (K_m , 1450–1870 μ M; Tables 1 and 2), in accordance with the effect of the P450 2B1 Ile-114-to-Val substitution. Moreover, CPA *N*-dechloroethylation was totally absent from 2B11, in accord with the effects of the P450 2B1 Val-363-to-Leu substitution. The V_{\max} values for 4-hydroxylation of CPA and IFA by 2B11 were lower than those of 2B1 by ~20 and 60%, respectively, in agreement with the 30 and 90% suppression of CPA and IFA 4-hydroxylation, respectively, in the case of P450 2B1-V363L. The catalytic efficiency of P450 2B11 for CPA and IFA 4-hydroxylation (V_{\max}/K_m) was 7- to 8-fold higher than that of P450 2B1 and ≥ 65 -fold higher than P450 2B4 and 2B5. Overall, P450 2B11 exhibits a highly favorable catalytic efficiency and regioselectivity for CPA 4-hydroxylation, in accord with predictions based on site-directed mutagenesis of P450 2B1.

Discussion

This study was undertaken in an effort to identify or design P450 2B enzymes that exhibit improved catalytic activity or substrate specificity with respect to activation of the anticancer prodrugs CPA and IFA. Initially, three naturally occurring P450 2B proteins were characterized, and site-

directed mutagenesis targeting three active site residues was carried out with P450 2B1. Striking differences among the enzymes were found in terms of V_{\max} and/or K_m for the 4-hydroxylation pathway, which generates active, cancer chemotherapeutic metabolites. Substantial differences were also seen in the *N*-dechloroethylation pathway, which inactivates these drugs and generates metabolites with neurotoxic and nephrotoxic activity. Incorporation of a Val residue at position 114 in 2B1 was found to lower the K_m for both pathways of CPA and IFA oxidation, and introduction of a Leu or Ile at position 363, or Ala at position 367, resulted in 100% regioselectivity for CPA 4-hydroxylation. The metabolite profiles of P450 2B1-V363L with CPA and IFA were very similar to those of human P450 2B6 (Huang et al., 2000). This human enzyme shares Ile-114 and Val-367 with 2B1 but has Leu at position 363. Based on these observations, it was predicted that canine P450 2B11, which incorporates two of the desired "mutations", Val-114 and Leu-363, would be a highly efficient and regioselective catalyst of the therapeutically beneficial 4-hydroxylation pathway.

Indeed, P450 2B11 exhibited a 9-fold lower K_m for CPA 4-hydroxylation than 2B1 with similar V_{\max} , such that the catalytic efficiency (V_{\max}/K_m) was 7-fold higher than 2B1. In addition, as predicted, 2B11 did not metabolize CPA by the undesirable *N*-dechloroethylation pathway. In the case of IFA, the decrease in K_m was ~20-fold compared with 2B1 and the increase in V_{\max}/K_m for 4-hydroxylation 8.5-fold. To our knowledge, this represents the first example where a species difference in P450 metabolic profile was correctly predicted based on the identification of active site residues. Interestingly, although improved, the catalytic efficiency for CPA 4-hydroxylation exhibited by P450 2B1-I114V/V363L was still 5-fold lower than that of P450 2B11, suggesting the importance of yet additional residues. One attractive possibility is amino acid 290, which is Ile in 2B1 and Asp in 2B11. This residue contributes to the unique ability of 2B11 to hydroxylate certain polychlorinated biphenyls (Hasler et al., 1994) and to the distinct steroid metabolite profiles that are characteristic of P450s 2B1 and 2B11 (Harlow et al., 1997). In terms of *in vitro*/*in vivo* correlations, the high catalytic efficiency of P450 2B11 for CPA 4-hydroxylation coupled with the high constitutive expression of this enzyme in canine liver (Duignan et al., 1987) may also explain why the dose of CPA used in treating lymphomas and carcinomas in dogs is generally lower than the standard treatment dose in humans [c.f., 200–250 mg CPA/m² for treatment of dogs (Chun et al., 2000) versus 500–1500 mg CPA/m² per treatment course in humans (Dorr and Von Hoff, 1994)].

Undesirable changes seen with some of the 2B1 mutants

TABLE 4

P450 2B11: steady-state kinetics analysis of CPA and IFA metabolism. Data are based on $n = 2$ independent experiments.

Substrate	4-Hydroxylation			<i>N</i> -Dechloroethylation			
	K_m <i>mM</i>	V_{\max} <i>mol/min/mol</i> P450	V_{\max}/K_m <i>mol/min/mol</i> P450/ <i>mM</i>	K_m <i>mM</i>	V_{\max} <i>mol/min/mol</i> P450	V_{\max}/K_m <i>mol/min/mol</i> P450/ <i>mM</i>	% <i>N</i> -deCl ^a
CPA	0.16 ± 0.01	28.2 ± 0.2	174.7 ± 8.4	N.D.	0	N.D.	0
IFA	0.08 ± 0.02	5.3 ± 0.5	66.8 ± 19.1	0.09 ± 0.01	5.0 ± 1.0	56.2 ± 5.8	48.2 ± 2.8

N.D., not determined; catalytic activity at or below limit of detection.

^a *N*-dechloroethylation as a percentage of total metabolism at V_{\max} .

investigated here included an increase in CPA *N*-dechloroethylation (V363A) and a substantial decrease in overall CPA and IFA metabolic activity (V367A). Thus, introduction into P450 2B1 of the Val-367-to-Ala substitution found in rabbit 2B5 recapitulated the low CPA and IFA 4-hydroxylase activity of that enzyme. P450 2B1-V367A is not inactive, however, as shown by the high steroid hydroxylase activity that it displays, albeit with altered stereo- and regiospecificity compared with wild-type 2B1 (Szklarz et al., 1996). Moreover, with IFA, none of the P450 2B1 mutants displayed increased partitioning in favor of 4-hydroxylation. Indeed, in the case of the V363L, V363I, and I114V/V363L mutants, IFA *N*-dechloroethylation was further increased, from ~50% (wild-type 2B1) to 74 to 90% of total metabolism. In the case of V363L, the alteration in overall metabolic profile largely reflects a 90% decrease in V_{max} for IFA 4-hydroxylation. P450 2B1-V367A exhibited the lowest percent of IFA *N*-dechloroethylation (27%), consistent with the absence of this pathway in the case of P450 2B5. Thus, in addition to providing new insights into the structural basis for species differences in CPA and IFA metabolism, the site-specific 2B1 mutants characterized here yielded intriguing findings as to how the two isomeric prodrug substrates orient themselves in P450 2B active sites.

With IFA as substrate, the large chloroethyl side chain at position *N*-3 of the oxazaphosphorine may inhibit substrate binding in an orientation that leads to hydroxylation at the adjacent C-4 carbon (4-hydroxylation). With CPA as substrate, however, both *N*-chloroethyl side chains are linked to the same exocyclic nitrogen atom. This may, in part, explain why CPA is preferentially metabolized by 4-hydroxylation by many liver P450s, whereas IFA is actively metabolized by both pathways. Previous efforts to examine CPA binding to P450 2B1 using a homology model based on a P450 102 template suggested that 2B1 residues 114 and 363 play a key role in orienting the substrate (Lewis and Lake, 1997), in agreement with our experimental results. However, it was not feasible to apply molecular modeling based on established mammalian P450 structures (Wester et al., 2003; Williams et al., 2003) to obtain a more detailed understanding of the changes in CPA and IFA metabolite profiles seen with the various P450 2B1 mutants (data not shown). CPA and IFA are both chiral compounds, such that when the racemates are used, as in the present study and in the clinic, each enantiomer may undergo both 4-hydroxylation and *N*-dechloroethylation. Moreover, in the case of the racemic IFA substrate, the CAA measured as a monitor of *N*-dechloroethylation can be derived by four different enzymatic pathways, (*R*)- and (*S*)-IFA 2-dechloroethylation, and (*R*)- and (*S*)-IFA 3-dechloroethylation (Granvil et al., 1999; Roy et al., 1999). The recent elucidation of a high-resolution X-ray crystal structure of ligand-free P450 2B4 (Scott et al., 2003) together with ongoing work on a ligand-bound structure bodes well for the generation of accurate P450 2B active site models. Such models, together with additional information derived from studies using single enantiomers of CPA and/or IFA, hold great promise for a structure-based approach to the design of even more efficient and selective catalysts of oxazaphosphorine activation than P450 2B11.

The present identification of P450 enzymes with enhanced efficiency for activation of CPA and IFA, such as P450 2B11 and some of the P450 2B1 mutants studied here, may lead to

improvements in P450-based gene therapies for cancer treatment. P450 prodrug activation-based gene therapies use viral or nonviral vectors to deliver a prodrug-activating P450 cDNA to tumor cells in vivo, which thereby acquire the capability to activate prodrugs, such as CPA and IFA, locally within the tumor. This circumvents the need for liver P450-catalyzed prodrug activation and enhances the killing of tumor cells in a selective manner (Chen and Waxman, 2002). This concept has been validated using P450s 2B1 and 2B6 in a variety of in vivo tumor models (Ichikawa et al., 2001; Kan et al., 2001; Jounaidi and Waxman, 2004) and has shown promise in early stage clinical trials (Lohr et al., 2001). P450 2C enzymes characterized by low K_m values for CPA and IFA (Chang et al., 1997) have also been evaluated, but their use is limited by low V_{max} values and hence low overall activity compared with P450 2B enzymes at expression levels that can be achieved in tumor cells (Jounaidi et al., 1998; Jounaidi and Waxman, 2004). Low K_m enzymes with high V_{max} values, such as P450 2B11 and the I114V-containing site-specific variants of P450 2B1 described here, may facilitate the introduction of P450 2B-based gene therapy applications using low prodrug doses, such as low-dose, metronomic CPA treatment (Man et al., 2002). In this way, liver P450-catalyzed prodrug activation, and its associated systemic toxicity, may be reduced while preserving intratumoral prodrug activation and its anticancer effect. Further studies will be required to determine the ultimate clinical effectiveness of this approach.

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