Effects of the CYP2B6*6 Allele on Catalytic Properties and Inhibition of CYP2B6 In Vitro: Implication for the Mechanism of Reduced Efavirenz Metabolism and Other CYP2B6 Substrates In Vivo

Cong Xu, Evan T. Ogburn, Yingying Guo, and Zeruesenay Desta

Indiana University School of Medicine, Indianapolis, Indiana (C.X., E.T.O., Z.D.); and Drug Disposition Laboratories and Business Operations, Lilly Research Laboratories, Indianapolis, Indiana (Y.G.)

Received August 17, 2011; accepted January 9, 2012

ABSTRACT:
The mechanism by which CYP2B6*6 allele alters drug metabolism in vitro and in vivo is not fully understood. To test the hypothesis that altered substrate binding and/or catalytic properties contribute to its functional consequences, efavirenz 8-hydroxylation and bupropion 4-hydroxylation were determined in CYP2B6.1 and CYP2B6.6 proteins expressed without and with cytochrome b5 (Cyt b5) and in human liver microsomes (HLMs) obtained from liver tissues genotyped for the CYP2B6*6 allele. The susceptibility of the variant protein to inhibition was also tested in HLMs. Significantly higher \( V_{\text{max}} \) and \( K_m \) values for 8-hydroxyefavirenz formation and -2-fold lower intrinsic clearance (\( C_{\text{int}} \)) were noted in expressed CYP2B6.6 protein (−b5) compared with that of CYP2B6.1 protein (−b5); this effect was abolished by Cyt b5. The \( V_{\text{max}} \) and \( C_{\text{int}} \) values for 4-hydroxybupropion formation were significantly higher in CYP2B6.6 than in CYP2B6.1 protein, with no difference in \( K_m \), whereas coexpression with Cyt b5 reversed the genetic effect on these kinetic parameters. In HLMs, CYP2B6*6/*6 genotype was associated with markedly lower \( V_{\text{max}} \) (and moderate increase in \( K_m \)) and thus lower \( C_{\text{int}} \) values for efavirenz and bupropion metabolism, but no difference in catalytic properties was noted between CYP2B6*1/*1 and CYP2B6*1/*6 genotypes. Inhibition of efavirenz 8-hydroxylation by voriconazole was significantly greater in HLMs with the CYP2B6*6 allele (\( K_i = 1.6 \pm 0.8 \mu M \)) than with HLMs with CYP2B6*1/*1 genotype (\( K_i = 3.0 \pm 1.1 \mu M \)). In conclusion, our data suggest the CYP2B6*6 allele influences metabolic activity by altering substrate binding and catalytic activity in a substrate- and Cyt b5-dependent manner. It may also confer susceptibility to inhibition.

Introduction
The CYP2B6 enzyme catalyzes several clinically important drugs, other xenobiotics, and endogenous compounds (Mo et al., 2009). Studies in human liver tissues indicate that the expression and activity of CYP2B6 are highly variable in part due to genetic polymorphisms. Subsequent to the demonstration that CYP2B6 is the principal clearance mechanism of efavirenz in vitro (Ward et al., 2003), several studies have documented that the CYP2B6*6 allele or its tagging SNP is at increased risk for higher efavirenz exposure and/or adverse effects (Haas et al., 2004; Tsuchiya et al., 2004; Zanger et al., 2003; Hesse et al., 2004; Desta et al., 2007). In addition, this variant has also been associated with functional consequences in expressed CYP2B6.6 than in CYP2B6.1 protein, with no difference in \( K_m \) whereas coexpression with Cyt b5 reversed the genetic effect on these kinetic parameters. In HLMs, CYP2B6*6/*6 genotype was associated with markedly lower \( V_{\text{max}} \) (and moderate increase in \( K_m \)) and thus lower \( C_{\text{int}} \) values for efavirenz and bupropion metabolism, but no difference in catalytic properties was noted between CYP2B6*1/*1 and CYP2B6*1/*6 genotypes. Inhibition of efavirenz 8-hydroxylation by voriconazole was significantly greater in HLMs with the CYP2B6*6 allele (\( K_i = 1.6 \pm 0.8 \mu M \)) than with HLMs with CYP2B6*1/*1 genotype (\( K_i = 3.0 \pm 1.1 \mu M \)). In conclusion, our data suggest the CYP2B6*6 allele influences metabolic activity by altering substrate binding and catalytic activity in a substrate- and Cyt b5-dependent manner. It may also confer susceptibility to inhibition.

This work was supported by the National Institutes of Health, National Institute of General Medical Sciences [Grants GM078501, GM078501-04S1, 2R56-GM067308-09A1].

1 Current affiliation: SSCI, West Lafayette, Indiana.

Abbreviations: SNP, single nucleotide polymorphism; HLMs, human liver microsomes; P450, cytochrome P450; Cyt b5, cytochrome b5; HPLC, high-performance liquid chromatography; POR, P450 oxidoreductase; LC/MS/MS, liquid chromatography/tandem mass spectrometry; \( C_{\text{int}} \), in vitro intrinsic clearance; CV, coefficient of variation.
may not explain the functional consequences of this allele. For substrates that include cyclophosphamide, this allele is associated with enhanced metabolism despite reduced protein expression (Xie et al., 2003), which appears due to substantially lower $K_m$ in the variant versus wild-type protein (Ariyoshi et al., 2011).

Other in vitro studies, mostly in expression systems, have also reported that the CYP2B6*6 allele or the amino acids harbored in it influence catalytic properties, although the extent and direction of effect appears to depend on the substrate and the enzyme sources used (Ariyoshi et al., 2001; Jinno et al., 2003; Bumpus and Hollenberg, 2008; Watanabe et al., 2010; Zhang et al., 2011). Therefore, in addition to reduced protein expression, altered protein structure due to amino acid changes may contribute to altered substrate metabolism.

The first purpose of this study was to examine the influence of the CYP2B6*6 allele on catalytic properties measured by efavirenz 8-hydroxylation (Ward et al., 2003) and bupropion 4-hydroxylation (Fauchet et al., 2000) as probes of activity using expressed enzymes and HLMs. As has been shown for other cytochromes P450 (P450s) such as CYP2C8 (Kaspera et al., 2011) and CYP2C9 (Kumar et al., 2006), several factors inherent to specific enzyme sources that include differences in cytochrome b5 (Cyt b5) contents may influence in vitro kinetic parameters and inhibition constants in a substrate-dependent manner. Cyt b5 has been reported to activate several P450s including CYP2B6 (Reed and Hollenberg, 2003; Jushchyshyn et al., 2005), but its influence on the catalytic properties of CYP2B6.6 protein has not been studied. Therefore, the second purpose was to test the influence of Cyt b5 on metabolic activities of expressed CYP2B6.1 and CYP2B6.6 proteins. In addition, it has been shown that amino acid substitutions, such as those found in the variant of CYP2B6*6 allele, may also alter the degree of susceptibility to competitive metabolic inhibitors for certain CYP2B6 variants (Bumpus et al., 2006; Bumpus and Hollenberg, 2008; Talakad et al., 2009). Therefore, the third aim was to test whether the variant protein is more or less susceptible to metabolic inhibition.

Materials and Methods

Chemicals. Efavirenz, 8-hydroxyefavirenz, bupropion, 4-hydroxybupropion, nevirapine, ritonavir, voriconazole, and clopidogrel were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and NADP were purchased from Sigma-Aldrich (St. Louis, MO). All of the other chemicals were of high-performance liquid chromatography (HPLC) grade.

Microsomal Preparations. Expressed CYP2B6.1 and CYP2B6.6 proteins. CYP2B6.1 and CYP2B6.6 proteins with coexpression of human P450 oxidoreductase (POR) (without and with coexpression of Cyt b5) and plasmid-transfected negative controls were produced by BD Biosciences (Woburn, MA) and kindly provided by Dr. Guo (Eli Lilly and Company, Indianapolis, IN). In those proteins expressed without Cyt b5, the protein content, P450 content, and Cytochrome c reductase activity were 26.6 mg/ml, 1451 pmol/ml, and 1739 nmol/(min⋅mg protein) for CYP2B6.1, and 33.5 mg/ml, 1582 pmol/ml, and 1489 nmol/(min⋅mg protein) for CYP2B6.6. Assuming that a specific activity of 3.0 micromoles of Cytochrome c reduced per minute per nanomole of reductase (Parikh et al., 1997), the molar ratios of P450/reductase for CYP2B6.1 and CYP2B6.6 were 1:10.6 and 1:10.5, respectively. In those proteins coexpressed with Cyt b5, the protein content, P450 content, Cytochrome c reductase activity, and Cyt b5 content were 9.0 mg/ml, 1000 pmol/ml, 1900 nmol/(min⋅mg protein), and 220 pmol/mg protein for CYP2B6.1, whereas they were 2.7 mg/ml, 1000 pmol/ml, 851 nmol/(min⋅mg protein), and 370 pmol/mg protein for CYP2B6.6. The molar ratio of P450: reductase/Cyt b5 of CYP2B6.1 was 1.5:4:2, and that of CYP2B6.6 was 1.0:73:1.

Human liver microsomes. HLMs obtained from liver tissues with CYP2B6*6/*1, CYP2*1/*6, and CYP2*6/*6 genotypes were used for the metabolism and inhibition studies. Two HLM sources were used. HLMs that were obtained from the Medical College of Wisconsin (Milwaukee, WI), Medical College of Virginia (Richmond, VA), Indiana University School of Medicine (Indianapolis, IN), and University of Pittsburgh (Pittsburgh, PA) under protocols approved by the appropriate committees for the conduct of human research were prepared by Eli Lilly and Company (Indianapolis, IN) and kindly provided by Dr. Guo. Liver microsomes were prepared by differential centrifugation (van der Hoeven and Coon, 1974). Additional HLMs for inhibition study were obtained from in-house human liver tissues, which were medically unsuitable for transplantation and were prospectively collected in the Division of Clinical Pharmacology by Dr. Hall through the liver transplantation units of Indiana University hospitals. HLMs were prepared from these liver tissues by ultracentrifugation, and protein concentrations were determined using a bicinchoninic acid protein assay (Ding et al., 1998). Genotyping for the CYP2B6*6 allele was performed in those human liver tissues from which HLMs were prepared. Liver samples were homogenized, and genomic DNA was isolated using a QiAamp DNA Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer’s protocol. The concentration of DNA was determined using the PicoGreen assay, and the quality of DNA was checked by agarose gel and polyacrylamide chain reaction. The DNA samples were stored at −80°C until analysis. The two SNPs tagging CYP2B6*6 allele, 516G>T and 785A>G, were genotyped using either the Affymetrix DMET Premier Pack (Affymetrix, Santa Clara, CA) or TaqMan SNP-genotyping assays (Applied Biosystems, Foster City, CA) according to the respective manufacturer’s protocols. CYP2B6*6 was designated as the allele without these two tagging SNPs. Other microsomal preparations (HLMs, expressed enzymes, and plasmid-transfected negative controls) were obtained from BD Biosciences (San Jose, CA). All microsomal preparations were stored at −80°C until analysis.

General Incubation Conditions in Expressed Enzymes and HLMs. Efavirenz 8-hydroxylation and bupropion 4-hydroxylation (Fig. 1) have been shown to be mainly catalyzed by CYP2B6 (Fauchet et al., 2000; Ward et al., 2003). Therefore, we used these two probe reactions to determine CYP2B6 activity in expressed CYP2B6 proteins (CYP2B6.1 and CYP2B6.6) and HLMs obtained from human liver tissues genotyped for the CYP2B6*6 allele. Efavirenz and bupropion were dissolved and diluted in methanol to the required concentrations (1–200 μM efavirenz and 10–1000 μM bupropion), and methanol was removed by drying in a speed vacuum before the addition of the incubation components. The reaction components contain 200 mM potassium phosphate buffer (pH 7.4), expressed CYP2B6 (10–15 pmol) or 25 μl of HLMs (2.5 mg/ml), and a substrate (efavirenz or bupropion) (total incubation volume of 250 μl). The incubation mixture was prewarmed for 5 min at 37°C. The reaction was initiated by adding a NADPH-regenerating system (1.3 mM NADP, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂, and 1 μl/mg glucose-6-phosphate dehydrogenase). Reaction was allowed to proceed for 15 min and then was terminated by placing tubes on ice and immediate addition of 500 μl of acetonitrile. After an internal standard was added, the sample was vortexed and centrifuged at 14,000 rpm for 5 min. Ritonavir (50 μl of 0.01 mg/ml) and nevirapine (50 μl of 500 ng/ml) were used as an internal standard for 8-hydroxyefavirenz assay by HPLC and liquid chromatography/tandem mass spectrometry (LC/MS/MS) methods, respectively. For 4-hydroxybupropion assay, nevirapine (25 μl of 50 μM) was used as an internal standard for the HPLC assay and 25 μl of 5 μM nevirapine was used as an internal standard for the LC/MS/MS assay. The supernatant layer was extracted with 500 μl of 0.5 ml glycine/NaOH buffer (pH 11.3) and 6 ml of ethyl acetate and then centrifuged at 36,000 rpm for 15 min. The organic layer was dried and reconstituted with mobile phase, and an aliquot was injected into an HPLC or LC/MS/MS (see Quantification of Efavirenz Metabolites). Quantification of Efavirenz Metabolites. 8-Hydroxyefavirenz formed from efavirenz incubations in expressed CYP2B6.1 and CYP2B6.6 was quantified by an HPLC/UV system as described previously (Ward et al., 2003). Due to the slow formation rates of 8-hydroxyefavirenz in HLMs samples, particularly in those with CYP2B6*6/*6 genotype, a sensitive and selective LC/MS/MS method was developed to assay 8-hydroxyefavirenz from HLMs incubation and was implemented as described in our previous publication (Ogburn et al., 2010). The MS/MS system was an API 2000 MS/MS triple quadrupole system (Applied Biosystems, Foster City, CA) equipped with a turbo ion spray and was coupled with a Shimadzu (Columbia, MD) HPLC system consisting of an LC-20AB pump and SIL-20A HT autosampler, all controlled by Analyst 1.4.2 software (Applied Biosystems/MDS Sciex, Foster City, CA) in conjunction with Windows 2000 (Microsoft, Redmond, WA). Hydroxyefavirenz and nevirapine were detected using multiple reactions monitoring at a m/z of 352.2/248.3 and 267.1/226.4 in positive ion mode, respectively.
Quantitative of 4-Hydroxybupropion. An HPLC assay method with UV detection was developed for the quantification of 4-hydroxybupropion from bupropion incubation in expressed enzymes. The HPLC system consisted of a Shimadzu LC-10AT pump, SIL-10AD autosampler, SCL-10A system controller, and SPD-10A UV-VIS detector. The separation system consisted of a Zorbax SB-C18 column (150 × 4.6 mm, 3.5-μm particle size; Phenomenex, Torrance, CA), a Luna C18 guard column (30 × 4.6 mm, 5 μm; Phenomenex), and a mobile phase composed of 85% 10 mM KH₂PO₄ (adjusted to pH 3 with 85% phosphoric acid) and 15% (v/v) acetonitrile (flow rate, 1 ml/min). The column eluate was monitored by UV detection at 214 nm for 4-hydroxybupropion and 282 nm for internal standard (nevirapine). A LC/MS/MS assay was developed for the quantification of 4-hydroxybupropion metabolite in HLM incubation samples. The MS/MS system was the same as that for efavirenz metabolites quantification described above. In brief, bupropion, 4-hydroxybupropion, and the internal standard (nevirapine) were separated using Zorbax SB-C18 column (100 × 2.00 mm, 3-μm particle size), a Luna C18 guard column (30 × 4.6 mm, 5 μm), and an isotropic mobile phase that consisted of 75% formic acid (0.1% in H₂O) and 25% acetonitrile (flow rate, 0.3 ml/min). 4-Hydroxybupropion and nevirapine were detected using multiple reactions monitoring at m/z 256.1/238.0 and 267.2/224.4 in positive ion mode, respectively.

Inhibition of CYP2B6 by Voriconazole and Clopidogrel in HLMs. To test the impact of the CYP2B6*6 allele on the inhibition of CYP2B6, inhibition experiments were performed in HLMs obtained from human liver tissues genotyped for the CYP2B6*6 allele. IC₅₀ values for the inhibition of CYP2B6 by voriconazole and clopidogrel were determined by incubating efavirenz (10 μM) with a NADPH-generating system and 25 μl of HLMs (2.5 mg/ml) at 37°C for 15 min in the absence or presence of voriconazole (0.01–4 μM) and clopidogrel (0.003–2.5 μM) (total incubation volume of 250 μl). Dixon plots for the inhibition of CYP2B6 by bupropion were determined by incubating efavirenz (10–100 μM) with a NADPH-generating system and 25 μl of HLMs (2.5 mg/ml) at 37°C for 15 min in the absence or presence of voriconazole (0.1–10 μM) (total incubation volume of 250 μl). The samples were processed, and formation of 8-hydroxyefavirenz was quantified by LC/MS/MS as described above.

Data Analysis. Apparent kinetic constants (Kₘ and Vₘₐₓ) were estimated by fitting formation rates of metabolites versus substrate concentrations to simple single-site Michaelis-Menten equation by nonlinear regression analysis using Prism version 5.0 software (GraphPad Software Inc., San Diego, CA). In vitro intrinsic clearance (Clᵢ) was given as Vₘₐₓ/Kₘ. Inhibition constants (Kᵢ) values were calculated by fitting the inhibition data to different models of enzyme inhibition (competitive, noncompetitive, and uncompetitive) using nonlinear least-squares regression analysis. The appropriate type of inhibition or metabolism model for each data set was selected on the basis of visual inspection of the plots, the size of the sum of squares of residuals, the Akaike information criterion, and the 95% confidence interval of the parameter estimates.

Statistical Analysis. Statistical comparisons of metabolism and inhibition kinetic parameters among genotypes were performed using one-way analysis of variance with Dunn’s post hoc test for multiple comparison correction. Independent t test was used to compare parametric data from two groups. Mann-Whitney U test or Wilcoxon test was performed for nonparametric data. Correlation analysis was performed by a nonparametric test (Spearman’s rank correlation test). All statistical tests were performed using GraphPad. P < 0.05 was considered statistically significant.

Results

Efavirenz 8-Hydroxylation and Bupropion 4-Hydroxylation by CYP2B6.1 and CYP2B6.6 Proteins without Coexpression of Cyt b5. The catalytic properties of expressed CYP2B6.1 and CYP2B6.6 proteins were determined using efavirenz and bupropion as probes. The kinetic profiles for formation of 8-hydroxyefavirenz from efavirenz and 4-hydroxybupropion from bupropion in these proteins are depicted in Fig. 2. The kinetic parameters estimated are summarized in Table 1. The Kₘ and Vₘₐₓ values for the formation of 8-hydroxye
were significantly higher in the CYP2B6.1 than in the CYP2B6.1 protein (Table 1). In the in vitro intrinsic clearance ($V_{max}/K_m$ or $Cl_{int}$) in the CYP2B6.6 protein was significantly lower than in the CYP2B6.1 protein (Table 1). As shown in Fig. 2B and Table 1, $V_{max}$ value for the formation of 4-hydroxybupropion in the CYP2B6.6 protein was significantly higher than that estimated from CYP2B6.1 protein, whereas there was no statistically significant difference in the $K_m$ values between the variant and the wild-type proteins. Accordingly, the $Cl_{int}$ for the formation of 4-hydroxybupropion was significantly increased in the CYP2B6.6 protein compared with that of the CYP2B6.1 protein (Table 1).

Efavirenz 8-Hydroxylation and Bupropion 4-Hydroxylation by CYP2B6.1 and CYP2B6.6 Proteins Coexpressed with Cyt b5. To evaluate the potential effect of Cyt b5 on catalytic properties of the variant versus wild-type protein, kinetic parameters for the formation of 8-hydroxyefavirenz and 4-hydroxybupropion were determined in CYP2B6.1 and CYP2B6.6 proteins that were coexpressed with Cyt b5, and the results were compared with those data obtained from CYP2B6.1 and CYP2B6.6 proteins without coexpression of Cyt b5. Kinetic profiles for the formation of 8-hydroxyefavirenz and 4-hydroxybupropion in CYP2B6.1 and CYP2B6.6 proteins are shown in Fig. 3. The corresponding kinetic parameters are summarized in Table 1. In contrast to the significant changes by the CYP2B6.6 allele observed in expressed system without coexpression of Cyt b5, the $V_{max}$ value for the formation of 8-hydroxyefavirenz was not significantly different ($P = 0.20$) between CYP2B6.1 and CYP2B6.6 proteins coexpressed with Cyt b5 (Fig. 3A; Table 1). Although the $K_m$ value in CYP2B6.6 protein was higher by 81% than that in the CYP2B6.1 protein and the $Cl_{int}$ value was decreased by 43% consistent with the data obtained from system without Cyt b5 (Table 1), the differences did not reach a statistically significant level (Fig. 3A; Table 1) ($P = 0.059$). When bupropion 4-hydroxylation was used as a reaction probe, a significantly lower $V_{max}$ value was observed in CYP2B6.6 protein compared with the value obtained from the CYP2B6.1 protein (Fig. 3B; Table 1). The $K_m$ value was increased from 9.06 $\mu M$ in the CYP2B6.1 protein to 110.0 $\mu M$ in the variant protein, but this did not reach a statistically significant difference ($P = 0.27$). A significant decrease in $Cl_{int}$ was observed in the CYP2B6.6 protein compared with the CYP2B6.1 protein.

As described in the preceding paragraph, the catalytic properties of CYP2B6 seemed to be genotype- and Cyt b5-dependent. To gain further insight regarding the differential effect of Cyt b5 on the formation of 8-hydroxyefavirenz and bupropion 4-hydroxylation obtained in the presence of Cyt b5 were compared with those values obtained in P450 systems without Cyt b5 (Table 1). In the CYP2B6.1 protein, none of the kinetic parameters of efavirenz 8-hydroxylation were significantly different compared with values obtained from CYP2B6.1 protein without coexpression of Cyt b5. Coexpression of Cyt b5 with CYP2B6.6 protein significantly decreased the $V_{max}$ value for the formation of 8-hydroxyefavirenz compared with the value obtained from CYP2B6.6 protein without coexpression of Cyt b5. However, the $K_m$ value also tended toward decrease in the CYP2B6.6 protein, the $Cl_{int}$ value in the CYP2B6.6 protein coexpressed with Cyt b5 was not significantly different than that obtained from the CYP2B6.6 protein without Cyt b5 (Table 1). Similar to the findings with efavirenz 8-hydroxylation, differential effects of Cyt b5 on the catalytic properties of CYP2B6.1 and CYP2B6.6 were observed with bupropion 4-hydroxylation (Table 1). Compared with the CYP2B6.1 protein without Cyt b5, the CYP2B6.1 protein coexpressed with Cyt b5 exhibited modest increases in the $V_{max}$ and $K_m$ values for the formation of 4-hydroxybupropion, the $Cl_{int}$ tended toward decrease (Table 1). The presence of Cyt b5 with CYP2B6.6 protein decreased the $V_{max}$ value to 11.77 pmol $\cdot$ min$^{-1} \cdot$ pmol$^{-1}$ of P450 from 18.55 pmol $\cdot$ min$^{-1} \cdot$ pmol$^{-1}$ in the CYP2B6.6 protein without Cyt b5 ($P = 0.0002$). A significant increase was observed in $K_m$ value of

![Fig. 3. Efavirenz concentrations versus formation rate of 8-hydroxyefavirenz (A) and bupropion concentrations versus formation rate of 4-hydroxybupropion (B) in microsomes containing cDNA-expressed CYP2B6.1 and CYP2B6.6 with coexpression of Cyt b5. Efavirenz (1–200 $\mu M$) or bupropion (10–1000 $\mu M$) was incubated with reconstituted systems containing either CYP2B6.1 or CYP2B6.6 with coexpression of Cyt b5 (15 pmol) and a NADPH-generating system for 15 min at 37°C. The formation rate of 8-hydroxyefavirenz and 4-hydroxybupropion (pmol/min/pmol P450) versus substrate concentrations were fit to the simple single-site Michaelis-Menten equation. Each point represents mean ± S.D. of three replicates. 8-OHEFV, 8-hydroxyefavirenz; 4-OHBUP, 4-hydroxybupropion.](image-url)
CYP2B6.6 protein coexpressed with Cyt b5 compared with that of CYP2B6.6 protein coexpressed without Cyt b5. As a result, \( C_l \) for the formation of 4-hydroxybupropion was significantly decreased in CYP2B6.6 coexpressed with Cyt b5 (Table 1).

We recognized that recombinant protein systems have limitations, which include differences in cofactor expression between variants and wild type or even between batches of the same protein. In the expressed enzymes used in this experiment, the amounts of Cyt b5 expressed in CYP2B6.1 and CYP2B6.6 proteins were slightly different (220 and 370 pmol/mg protein, respectively). However, we have observed substrate-dependent effects for the CYP2B6*6 allele in the presence of Cyt b5, i.e., no significant differences were found in \( V_{\text{max}} \) and \( K_m \) comparing CYP2B6.1 to CYP2B6.6 using efavirenz as substrate, whereas \( V_{\text{max}} \) was significantly lower in CYP2B6.6 using bupropion. These data suggest that expression differences in Cyt b5 do not seem to significantly contribute to the differences in kinetic parameters observed.

**Catalytic Properties of CYP2B6 in HLMs Genotyped for the CYP2B6*6 Allele.**
To further evaluate the effect of CYP2B6*6 allele on catalytic properties, the kinetics of efavirenz 8-hydroxylation and bupropion 4-hydroxylation were characterized in 15 HLM samples with CYP2B6*1/*1, CYP2B6*1/*6, and CYP2B6*6/*6 genotypes (\( n = 5 \) for each genotype).

In Fig. 4A, efavirenz concentrations versus formation rate of 8-hydroxyefavirenz in the different genotypes are shown. Formation rates of 8-hydroxyefavirenz versus efavirenz concentrations were fit into a Michaelis-Menten equation to estimate kinetic parameters. The mean \( \pm \) S.D. of these parameters for each genotype are listed in Table 2. The kinetic parameters for individual HLMs are summarized in Supplemental Table 2. Similar to that observed for the kinetics of 8-hydroxyefavirenz, the values of \( V_{\text{max}} \) and \( C_l \) for the formation of 4-hydroxybupropion in HLMs also exhibited a large variability. HLMs with CYP2B6*6/*6 and CYP2B6*1/*6/*6 genotypes showed higher variability (CV of more than 100%) than that in HLMs with CYP2B6*6/*6 (CV of approximately 60 and 65%, respectively). The average \( V_{\text{max}} \) values for the formation of 4-hydroxybupropion were also much lower in HLMs with CYP2B6*6/*6 genotype than that in wild type and heterozygotes. The HLMs with CYP2B6*6/*6/*6 exhibited lower average \( K_m \) values compared with HLMs with CYP2B6*1/*6/*6, although this difference did not reach statistical significance (Table 3). The \( C_l \) in CYP2B6*6/*6 genotype was decreased by over 95% compared with CYP2B6*1/*6/*6 and CYP2B6*1/*6 (Table 3).

**Inhibition of 8-Hydroxyefavirenz Formation in HLMs Genotyped for the CYP2B6*6 Allele.** To test whether susceptibility to metabolic inhibitors differs between HLMs that carry the CYP2B6*6 allele and the wild type, inhibition potency of voriconazole and

---

**TABLE 2**

<table>
<thead>
<tr>
<th>HLMs</th>
<th>( V_{\text{max}} ) (pmol · min(^{-1}) · mg protein(^{-1}))</th>
<th>( K_m ) (( \mu )M)</th>
<th>( C_l ) (pmol · min(^{-1}) · mg protein(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B6*1/*1</td>
<td>87.1 ± 87.4</td>
<td>11.2 ± 6.7</td>
<td>14.5 ± 22.6</td>
</tr>
<tr>
<td>CYP2B6*1/*6</td>
<td>100.6 ± 143.7</td>
<td>15.2 ± 8.0</td>
<td>6.4 ± 7.8</td>
</tr>
<tr>
<td>CYP2B6*6/*6</td>
<td>25.0 ± 6.7</td>
<td>24.0 ± 31.3</td>
<td>2.4 ± 1.6</td>
</tr>
</tbody>
</table>

---

Kinetic analyses for the formation of 4-hydroxybupropion were also performed in the same 15 HLM samples that were used for the characterization of efavirenz metabolism. In Fig. 4B, bupropion concentrations versus formation rate of 4-hydroxybupropion in the different genotypes are shown. Formation rates of 4-hydroxybupropion versus bupropion concentrations were fit into a Michaelis-Menten equation to estimate kinetic parameters. The mean \( \pm \) S.D. of these parameters for each genotype are listed in Table 3. The kinetic parameters for individual HLMs are summarized in Supplemental Table 2. Similar to that observed for the kinetics of 8-hydroxyefavirenz, the values of \( V_{\text{max}} \) and \( C_l \) for the formation of 4-hydroxybupropion in HLMs also exhibited a large variability. HLMs with CYP2B6*6/*6/*6 and CYP2B6*1/*6/*6/*6 genotypes showed higher variability (CV of more than 100%) than that in HLMs with CYP2B6*6/*6/*6 (CV of approximately 60 and 65%, respectively). The average \( V_{\text{max}} \) values for the formation of 4-hydroxybupropion were also much lower in HLMs with CYP2B6*6/*6/*6 genotype than that in wild type and heterozygotes. The HLMs with CYP2B6*6/*6/*6/*6 exhibited lower average \( K_m \) values compared with HLMs with CYP2B6*1/*6/*6/*6, although this difference did not reach statistical significance (Table 3). The \( C_l \) in CYP2B6*6/*6/*6 genotype was decreased by over 95% compared with CYP2B6*1/*6/*6/*6 and CYP2B6*1/*6/*6 (Table 3).
clopidogrel towards efavirenz 8-hydroxylation was determined in HLMs obtained from tissues genotyped for the CYP2B6*6 allele. Inhibition of CYP2B6 by voriconazole and clopidogrel in HLMs with CYP2B6*1/*1 and CYP2B6*6/*6 is shown in Fig. 5. IC₅₀ values for voriconazole inhibition in HLMs with CYP2B6*1/*1 and CYP2B6*6/*6 were 0.40 and 0.16 μM, respectively. IC₅₀ value for clopidogrel inhibition of efavirenz 8-hydroxylation in HLMs with CYP2B6*1/*1 (IC₅₀ = 0.14 μM) was also higher than that in HLMs with CYP2B6*6/*6 (IC₅₀ = 0.05 μM). Representative Dixon plots for the inhibition of efavirenz 8-hydroxylation in the HLMs with CYP2B6*1/*1, CYP2B6*6/*6, and CYP2B6*6/*6 genotypes are shown in Fig. 6. As shown in Fig. 7, there was a statistically significant difference among the Kᵢ values estimated from the three genotypes (P = 0.04). There was no significant difference between the Kᵢ values estimated from CYP2B6*1/*1 (average Kᵢ value = 1.55 μM) and CYP2B6*6/*6 (average Kᵢ value = 1.64 μM) (P = 0.85). However, the Kᵢ values estimated from HLMs with CYP2B6*6/*6 (P = 0.04) and CYP2B6*1/*6 (P = 0.04) genotypes were both significantly lower than that estimated from CYP2B6*1/*1 genotype (average Kᵢ value = 3.03 μM) (Fig. 7). When the data from HLMs with CYP2B6*6/*6 and CYP2B6*1/*6 genotypes were combined and compared against HLMs with the CYP2B6*1/*1 genotype, the Kᵢ values for the inhibition of 8-hydroxylation by voriconazole in the HLMs with CYP2B6*1/*1 and CYP2B6*6/*6 was significantly lower (P = 0.009) than those observed in HLMs with CYP2B6*1/*1 genotype (data not shown).

Discussion

In this study, we have shown that the CYP2B6*6 allele is associated with altered binding affinity and/or catalytic activity. Cyt b5 affects the kinetic profiles of CYP2B6 in genotype- and substrate-dependent manner. Our data also suggest that the variant protein is more susceptible to metabolic inhibition. These findings suggest that the mechanism by which the CYP2B6*6 allele is associated with altered substrate metabolism and drug interaction may be in part due to amino acid changes that modify catalytic properties of the variant versus wild-type protein.

Our data suggest that the amino acid changes harbored in CYP2B6*6 allele may influence substrate binding with pronounced effect on efavirenz than bupropion. The average Kᵢ value for the formation of 8-hydroxyefavirenz in CYP2B6*6 protein expressed without b5 was increased by 175% and in HLMs with CYP2B6*6/*6 genotype was also increased by 58 and 114% than in HLMs with CYP2B6*1/*1 and CYP2B6*6/*6 genotypes, respectively. These data concur with a recent report by Zhang et al. (2011). Kᵢ value for bupropion hydroxylation did not differ between expressed variant and wild-type proteins. A slight increase in Kᵢ for bupropion 4-hydroxylation was noted in HLMs with CYP2B6*1/*6 and CYP2B6*6/*6 genotypes than HLMs with CYP2B6*1/*1 genotype. However, because some Kᵢ values derived from HLMs with CYP2B6*1/*1 and CYP2B6*6/*6 were outliers and may have skewed the average data, these data should be interpreted with caution.

Consistent with previous reports in various expressed systems (Ariyoshi et al., 2001; Jinno et al., 2003; Bumpus et al., 2006), we noted that the Vₘₐₓ values for the formation of 8-hydroxyefavirenz and 4-hydroxybupropion were significantly higher in CYP2B6*6 than in CYP2B6*1 proteins expressed without Cyt b5. However, Vₘₐₓ values for the formation of 8-hydroxyefavirenz and 4-hydroxybupropion were substantially decreased (by ~70%) in HLMs with CYP2B6*6/*6 genotype versus HLMs with CYP2B6*1/*1 and CYP2B6*1/*6 genotypes. Our interpretation is that the expressed variant protein inherently increases catalytic activity for most substrates, whereas the decreased Vₘₐₓ value in HLMs is probably mainly due to reduced protein expression by the CYP2B6*6/*6 genotype (Hesse et al., 2004; Desta et al., 2007; Hofmann et al., 2008).

In vivo, the CYP2B6*6/*6 genotype is associated with >3-fold increase in efavirenz exposure compared with CYP2B6*1/*1 genotype (Rotger et al., 2007), but its effect on plasma exposure of bupropion or 4-hydroxybupropion was marginal (Kirchheiner et al., 2003). Our in vitro data mirror these clinical observations. It is well recognized that variants in other P450s that change amino acids affect metabolic activity in a substrate-dependent manner. However, the CYP2B6*6 allele seems unique in that its effect on catalytic activity is not only substrate-dependent but also results in opposite effects. This variant has been associated with enhanced cyclophosphamide metabolism in vitro (Xie et al., 2003; Ariyoshi et al., 2011) and in vivo (Nakajima et al., 2007), which seems to be primarily driven by the

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vₘₐₓ (μM)</th>
<th>Kᵢ (μM)</th>
<th>Clint (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B6*6/*1</td>
<td>492.8</td>
<td>86.0</td>
<td>18.8</td>
</tr>
<tr>
<td>CYP2B6*6/*6</td>
<td>441.6</td>
<td>212.1</td>
<td>21.8</td>
</tr>
<tr>
<td>CYP2B6*1/*1</td>
<td>112.9</td>
<td>204.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**Table 3**

Kinetic parameters (mean ± S.D.) for the formation of 4-hydroxybupropion from bupropion in 15 human liver microsomal samples with CYP2B6*1/*1, CYP2B6*1/*6, and CYP2B6*6/*6 genotypes (n = 5 HLMs for each genotype)

Bupropion (10–1000 μM) were incubated with HLM samples (0.25 mg/ml) with CYP2B6*1/*1, CYP2B6*1/*6, and CYP2B6*6/*6 genotypes (n = 5 HLMs for each genotype) and a NADPH-generating system at 37°C for 15 min in duplicate. Kinetic parameters (Vₘₐₓ, Kᵢ, and Clint) for the formation of 4-hydroxybupropion were estimated by fitting the velocity versus bupropion concentrations to the simple single-site Michaelis-Menten. In vitro Clint was calculated as Vₘₐₓ/Kᵢ. The kinetic parameters (Vₘₐₓ, Kᵢ, and Clint) for each genotype group are listed in Supplemental Table 2. The data presented here are mean ± S.D. calculated from five individual HLM values for each genotype.

**Fig. 5.** Inhibition of CYP2B6 by voriconazole (A) and clopidogrel (B) in HLMs with CYP2B6*1/*1 and CYP2B6*6/*6. Efavirenz (10 μM) was incubated with HLMs (0.25 mg/ml) and the NADPH-generating system for 15 min without or with voriconazole (0–4 μM) and clopidogrel (0–2.5 μM). Each point represents the mean of duplicate.
significantly lower \( K_m \) for cyclophosphamide 4-hydroxylation in CYP2B6.6 than in CYP2B6.1 proteins (Ariyoshi et al., 2011). The CYP2B6*6 allele appears to alter substrate metabolism in two ways: 1) by decreasing (e.g., cyclophosphamide) or increasing (e.g., efavirenz) substrate binding (Ariyoshi et al., 2011; Zhang et al., 2011; present data) probably due to changes in the three-dimensional structures of the protein; and 2) by reducing catalytic efficiency secondary to reduced protein expression (Hofmann et al., 2008). Overall, altered substrate binding and/or catalytic activity as a result of amino acid changes seem to play a critical role in determining the substrate-dependent functional consequences of the CYP2B6*6 allele.

Our data show that Cyt b5 affects catalytic properties in a genotype- and substrate-dependent manner and highlight the fact that interpretation of in vitro studies performed with expressed proteins may vary depending on the presence or absence of Cyt b5, substrate used, and underlying genotype. The ability of Cyt b5 to influence P450-mediated drug oxidation (increase, inhibit, or no effect) has been described for multiple P450s (Schenkman and Jansson, 2003). The mechanisms by which Cyt b5 might alter substrate metabolism include the following: providing the second electron during the catalytic cycle of P450s; interacting physically with P450s and thus modifying conformation of the protein, which, in turn, influences interaction with the substrate or reductase; or by competing for same binding site with P450 reductase, thereby preventing reduction of ferric P450 and initiation of the catalytic cycle (Zhang et al., 2008). In this study, Cyt b5 had no impact on CYP2B6.1-catalyzed efavirenz 8-hydroxylation. However, in sharp contrast to the results obtained from CYP2B6.6 without Cyt b5, \( V_{\text{max}} \) for efavirenz 8-hydroxylation was significantly reduced by Cyt b5 (with no effect on \( K_m \)) in CYP2B6.6 protein, effectively abolishing the genotype-dependent effect observed in CYP2B6.6 protein expressed without Cyt b5. The kinetic properties were different for bupropion 4-hydroxylation. Cyt b5 significantly increased \( V_{\text{max}} \) and \( K_m \) values for bupropion 4-hydroxylation in CYP2B6.1 protein compared with CYP2B6.1 without Cyt b5. In CYP2B6.6, the \( K_m \) for bupropion 4-hydroxylation was significantly increased and \( V_{\text{max}} \) was significantly reduced by Cyt b5, leading to marked reduction in Clint in the CYP2B6.6 protein (Table 1). For both substrates, CYP2B6.1 exhibited similar or increased catalytic activities with coexpression of Cyt b5 compared with that without Cyt b5, whereas Cyt b5 significantly decreased \( V_{\text{max}} \) values in CYP2B6.6. These data suggest an overlapping binding site between P450 reductase and Cyt b5 in CYP2B6.6 but probably not in CYP2B6.1. The possibility that the observed effect of Cyt b5 could be due to differences in the expression of Cyt b5 or POR among the genotypes cannot be excluded. In our study, the POR level was relatively lower in CYP2B6.6 with coexpression of Cyt b5 than that without coexpression of Cyt b5. Thus, the possibility that the lower expression of POR in the CYP2B6.6 protein may influence the magnitude of effect of Cyt b5 among the genotypes and substrates cannot be fully excluded. However, variation in kinetic parameters were observed even when the POR level was balanced between the variant and wild-type protein (Ariyoshi et al., 2011; Zhang et al., 2011). However, POR is much less functionally variable in general population than hepatic drug-
variant proteins may influence substrate metabolism was suggested by b5. That altered electron transfer from P450 reductase to CYP2B6 electron is generally thought to be transferred by P450 reductase, enzyme and its redox partner, P450 reductase (Bumpus and Hollenberg, 2008; Gay et al., 2010). The oxidation reaction catalyzed by P450s requires transferring of two electrons from NADPH. The first electron is generally thought to be transferred by P450 reductase, whereas the second can be transferred by either P450 reductase or Cyt b5. That altered electron transfer from P450 reductase to CYP2B6 variant proteins may influence substrate metabolism was suggested by a recent study (Zhang et al., 2011). It is reasonable to suggest that amino acid changes may influence the interaction between the P450s and electron transfer proteins and thus alter the catalysis of substrates in a Cyt b5- and substrate-dependent manner.

The same property of the variant that influences substrate metabolism may also influence inhibition drug interactions. The fact that the Ks values for CYP2B6 inhibition by voriconazole was significantly lower in HLMs with CYP2B6*6 allele than in those with the CYP2B6*1/*1 genotype suggests that the variant protein is more susceptible to metabolic inhibition than the wild type. This suggestion is further supported by our data using clopidogrel as an inhibitor (2.8-fold lower IC₅₀). Our data are in contrast to a previous study that reported decreased susceptibility of CYP2B6 protein to metabolic inhibition (Talakad et al., 2009). However, the different type and composition of the proteins, substrates, and inhibitors used in our study versus the other study preclude direct comparison of the data.

In summary, we have provided in vitro evidence that amino acid changes harbored in the CYP2B6*6 allele alter substrate binding and/or catalytic activity. In addition to reduced total enzyme pool, this variant allele may alter drug clearance and drug interaction via changes in three-dimensional protein structures. This in vitro suggestion is further supported by in vivo observation that showed association of CYP2B6*6 allele with reduced clearance [e.g., efavirenz (Zanger et al., 2007)], increased metabolism [e.g., cyclophosphamide (Nakajima et al., 2007)], or no effect [e.g., bupropion (Kirchheiner et al., 2003)]. Our data also showed that CYP2B6*6 allele may influence susceptibility to metabolic inhibition. In conclusion, predicting functional consequences of the CYP2B6*6 allele seems complex and depends on the substrate (or inhibitor) and enzyme sources used. These factors should be taken in to account when predicting the influence of the CYP2B6*6 allele on substrate metabolism and drug interactions.

References


Bumpus NN, Kent UM, and Ogburn PF (2006) Metabolism of efavirenz and 8-hydroxyefa-
virenz by P450 2B6 leads to inactivation by two distinct mechanisms. J Pharmacol Exp Ther 318:345–351.


Authorship Contributions

Participated in research design: Xu and Desta.
Conducted experiments: Xu and Ogburn.
Contributed new reagents or analytic tools: Guo.
Performed data analysis: Xu and Desta.
Wrote or contributed to the writing of the manuscript: Xu, Guo, and Desta.


Address correspondence to: Dr. Zeruesenay Desta, Associate Professor of Medicine, Pharmacology and Toxicology, Department of Medicine/Division of Clinical Pharmacology, Indiana University School of Medicine, 1001 West 10th Street, WD Myers Bldg., W7123, Indianapolis, IN 46202. E-mail: zdesta@iupui.edu