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Differences in Methadone Metabolism by CYP2B6 Variants

Sarah Gadel, Christina Friedel, and Evan D. Kharasch

Department of Anesthesiology, Division of Clinical and Translational Research (S.G., C.F., E.D.K.) and the Department of Biochemistry and Molecular Biophysics (E.D.K.), Washington University in St. Louis, St. Louis, Missouri

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ABSTRACT

Methadone is a long-acting opioid with considerable unexplained interindividual variability in clearance. Cytochrome P450 2B6 (CYP2B6) mediates clinical methadone clearance and metabolic inactivation via *N*-demethylation to 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP). Retrospective studies suggest that individuals with the *CYP2B6*6* allelic variant have higher methadone plasma concentrations. Catalytic activities of CYP2B6 variants are highly substrate- and expression-system dependent. This investigation evaluated methadone *N*-demethylation by expressed human CYP2B6 allelic variants in an insect cell coexpression system containing P450 reductase. Additionally, the influence of coexpressing cytochrome b_5, whose role in metabolism can be inhibitory or stimulatory depending on the P450 isoform and substrate, on methadone metabolism, was evaluated. EDDP formation from therapeutic (0.25–1 μM) *R*- and *S*-methadone concentrations was CYP2B6.4 ≥ CYP2B6.1 ≥ CYP2B6.5 >> CYP2B6.9 ≈ CYP2B6.6, and undetectable from CYP2B6.18. Coexpression of b_5 had small and variant-specific effects at therapeutic methadone concentrations but at higher concentrations stimulated EDDP formation by CYP2B6.1, CYP2B6.4, CYP2B6.5, and CYP2B6.9 but not CYP2B6.6. In vitro intrinsic clearances were generally CYP2B6.4 ≥ CYP2B6.1 > CYP2B6.5 > CYP2B6.9 ≥ CYP2B6.6. Stereoselective methadone metabolism (*S*>*R*) was maintained with all CYP2B6 variants. These results show that methadone *N*-demethylation by CYP2B6.4 is greater compared with CYP2B6.1, whereas CYP2B6.9 and CYP2B6.6 (which both contain the 516G>T, Q172H polymorphism), are catalytically deficient. The presence or absence of b_5 in expression systems may explain previously reported disparate catalytic activities of CYP2B6 variants for specific substrates. Differences in methadone metabolism by *CYP2B6* allelic variants provide a mechanistic understanding of pharmacogenetic variability in clinical methadone metabolism and clearance.

Introduction

Methadone is a long-duration opioid used (primarily as a racemate) to treat multiple types of acute, chronic, and cancer pain, as well as opiate addiction. Methadone use, particularly for pain, has grown exponentially over the past decades. However, the incidence of unanticipated methadone toxicity, and related fatalities, has grown disproportionately, even more so than the increase in methadone use (Paulozzi et al., 2012). There is considerable inter- and intraindividual variability in constitutive methadone metabolism and clearance, and also susceptibility to drug interactions, with the greatest risk related to unanticipated accumulation (Ferrari et al., 2004; Bruce et al., 2013). Variable disposition complicates the clinical use of methadone, and, despite considerable research, mechanisms of variability remain insufficiently understood.

Hepatic methadone *N*-demethylation to the inactive metabolite 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) is the major route of systemic clearance. Both methadone clearance and *N*-demethylation are stereoselective. Methadone *N*-demethylation in vitro, by human liver microsomes and by expressed cytochrome P450s (P450s), is catalyzed most efficiently by CYP2B6 and CYP3A4, and only CYP2B6 *N*-demethylates methadone stereoselectively (*S*>*R*) (Gerber et al., 2004; Kharasch et al., 2004; Totah et al., 2007, 2008; Chang et al., 2011; Gadel et al., 2013).

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Although both P450s metabolize methadone in vitro, it has become clear that CYP2B6, rather than CYP3A4, is the predominant P450 responsible for clinical methadone disposition. Evidence derives from drug interaction and genetic studies (Greenblatt, 2014). CYP2B6 induction or inhibition correspondingly modulated methadone metabolism, clearance, and plasma concentrations (Kharasch et al., 2004, 2008a,b; Kharasch and Stubbert, 2013b). In contrast, strong CYP3A inhibitors (Kharasch et al., 2004, 2008a, 2012; van Heeswijk et al., 2013; Kharasch and Stubbert, 2013a) failed to diminish (and sometimes increased) methadone *N*-demethylation and clearance, and CYP3A induction also had no effect (Vourvahis et al., 2012). *CYP2B6* polymorphisms may influence clinical methadone disposition. Gene-association studies suggested that the *CYP2B6*6* polymorphism was associated with higher dose-adjusted steady-state plasma methadone concentrations (Crettol et al., 2005, 2006; Eap et al., 2007; Wang et al., 2011) or use of lower methadone doses (Hung et al., 2011; Levran et al., 2013). Formal determination of methadone *N*-demethylation and clearance showed that both were greater and lesser than wild-types, respectively, in *CYP2B6*4* and *CYP2B6*6* carriers (Kharasch et al., 2014).

Whereas the fraction of total hepatic P450 represented by CYP2B6 is small, it nonetheless metabolizes a disproportionately greater percentage of drugs (Wang and Tompkins, 2008; Mo et al., 2009). The *CYP2B6* gene is highly polymorphic (Zanger and Klein, 2013), with thirty-eight CYP2B6 protein variants identified to date (http://www.cypalleles.ki.se/cyp2b6.htm). The functional consequences of *CYP2B6* allelic variants on catalytic activity in vitro are allele-, substrate-, and expression system–dependent (Turpeinen and Zanger, 2012; Zanger and

ABBREVIATIONS: EDDP, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine; P450, cytochrome P450; POR, P450 reductase.

Klein, 2013). P450 function can also be influenced (or not) by coexpression of cytochrome b_5 (Xu et al., 2012), and allelic variant effects in vivo are additionally influenced by quantitative differences in CYP2B6 protein expression (Turpeinen and Zanger, 2012; Zanger and Klein, 2013). Among the more well studied variants, *CYP2B6*4* (785A>G, K262R) is described as causing increased expression and variably increased or decreased enzymatic activity, *CYP2B6*5* (1459C>T, R487C) causing decreased expression and increased specific activity, *CYP2B6*6* (516G>T, Q172H; 785A>G, K262R) causing markedly reduced expression and substrate-dependent changes in activity, and *CYP2B6*18* (983T>C, I328T) having reduced expression and activity (Turpeinen and Zanger, 2012; Zanger and Klein, 2013). The *CYP2B6*6* allele is of particular interest, owing to its frequent occurrence (particularly in African, Asian, and Hispanic populations) and therapeutic significance for the metabolism, pharmacokinetics, and clinical effects of efavirenz, cyclophosphamide, and bupropion (Turpeinen and Zanger, 2012; Zanger and Klein, 2013). We recently reported that methadone *N*-demethylation catalyzed by CYP2B6.6, the CYP2B6 variant encoded by the *CYP2B6*6* polymorphism, is catalytically deficient compared with wild-type CYP2B6.1, and that human liver microsomes with diminished CYP2B6 content owing to a *CYP2B6*6* allele had lower rates of methadone *N*-demethylation (Gadel et al., 2013).

The purpose of the present investigation was to further characterize methadone *N*-demethylation by *CYP2B6* allelic variants, including CYP2B6.1, CYP2B6.4, CYP2B6.5, CYP2B6.6, CYP2B6.9, and CYP2B6.18, coexpressed with NADPH cytochrome P450 reductase in an insect cell system. The second purpose was to evaluate the influence of coexpressed cytochrome b_5 on the methadone *N*-demethylase activities of wild-type and variant CYP2B6 proteins.

Materials and Methods

Chemicals and Reagents. EDDP and EDDP-d3 were purchased from Cerilliant (Round Rock, TX). *R*- and *S*-methadone were from the National Institute on Drug Abuse (Bethesda, MD). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Construction of Plasmids. Human CYP2B6, P450 reductase (POR), and cytochrome b_5 were polymerase chain reaction–amplified from the Human Liver QUICK-Clone cDNA library (Clontech, Mountain View, CA). CYP2B6 variants (2B6.4, 2B6.5, 2B6.6, 2B6.9, 2B6.18) were made from CYP2B6.1 DNA using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA). CYP2B6, POR, and b_5 DNA were individually inserted into the pVL1393 vector using the In-Fusion HD Cloning system (Clontech, Mountain View, CA). All sequences were verified by the Protein and Nucleic Acid Chemistry Laboratory (PNACL) at Washington University in St. Louis.

Generation of Virus. *Spodoptera frugiperda* (SF9) cells (ATCC, Manassas, VA) were maintained in 500 ml polycarbonate Erlenmeyer flasks with vented caps (Corning, Corning, NY) shaken at 115 rpm and 27°C in Sf-900 III SFM (Life Technologies, Carlsbad, CA). The pVL1393/CYP2B6, pVL1393/POR, and pVL1393/b_5 vectors were each individually cotransfected in SF9 cells using the BestBac 2.0 Baculovirus Cotransfection Kit (Expression Systems, Davis, CA). Briefly, 2 ml of SF9 cells were plated at $4{,}6 \times 10^5$ cells/ml in a six-well tissue culture plate and allowed to adhere for 30 minutes. pVL1393/CYP2B6, pVL1393/POR, or pVL1393/b_5 DNA was then combined with linearized viral DNA, Expression Systems transfection media, and Expression Systems transfection reagent. The media was removed from the cells, the transfection solution was added, and plates were incubated at 27°C for 4.5 hours. After 4.5 hours, 3 ml of media was added to each well and the plates were incubated for 5 days at 27°C. The resulting cells and supernatant were collected as the p0 viral generation. Subsequent viral generations, up to p3, were performed in SF9 cells grown in suspension. All viral titers were determined using the BacPAK Baculovirus Rapid Titer Kit (Clontech).

Recombinant Protein Expression. *Trichoplusia ni* (High Five) cells (Life Technologies) were maintained in Express Five serum-free medium (Life Technologies) supplemented with 16 mM L-glutamine, in 500 ml polycarbonate Erlenmeyer flasks with vented caps (Corning) at 27°C with shaking at 115 rpm. CYP2B6 and POR with or without b_5 were coexpressed in High Five cells. On day 0, 100 ml of High Five cells were seeded at a density of 1×10^6 cells/ml. On day 1, cells were counted and infected with the following multiplicities of infection: 4:2:1 (CYP2B6/P450 reductase/cytochrome b_5) or 4:2 (CYP2B6/P450 reductase) plaque-forming units per milliliter (pfu/ml) in the presence of 100 μM δ-aminolevulinic acid and 100 μM ferric citrate (Lee et al., 1995). After 72 hours, infected cells were harvested by centrifugation for 15 minutes at 3000*g* and washed two times with phosphate-buffered saline and pelleted between each wash. The cell pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.4) and homogenized for 2 minutes on ice using a TissueRuptor (Qiagen, Hilden, Germany). Aliquots (500 μl) were stored at –70°C. Cytochrome P450 content, P450 reductase activity, and cytochrome b_5 content of the various expression systems are provided in Table 1.

Characterization of Expressed Protein. Protein concentrations of all infections were determined using Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA), following manufacturer's instructions. For electrophoresis, 20 μg of protein from whole cell lysate was mixed with 4× Protein Loading Buffer (LI-COR Biosciences, Lincoln, NE) and subjected to electrophoresis on a precast NuPAGE 4–12% Bis-Tris Gel (Life Technologies). Precision Plus Protein Dual Color Prestained Standard (10 μl; Bio-Rad, Hercules, CA) was used as a molecular weight standard. Proteins were transferred to a nitrocellulose membrane using the iBlot Transfer System (Life Technologies), per manufacturer's instructions. Membranes were blocked (1 hour at room temperature) in Blocking Buffer (LI-COR). Blocked membranes were incubated overnight at 4°C with rabbit anti-CYP2B6 (H-110) antibody (1:1000 dilution), rabbit anti-CYPOR (H-300) antibody (1:2500 dilution), and

TABLE 1

Characteristics of the CYP2B6 variant constructs

	P450 content[a]	Cytochrome *c* reductase activity	Cytochrome b_5 content	P450/reductase/b_5 ratio
	pmol/mg	*μmol/min per milligram protein*	*nmol/mg protein*	
CYP2B6.1 + P450 reductase + cytochrome b_5	77	4.5	0.25	1:18:3
CYP2B6.4 + P450 reductase + cytochrome b_5	107	3.3	0.28	1:10:3
CYP2B6.5 + P450 reductase + cytochrome b_5	172	4.1	0.49	1:7:3
CYP2B6.6 + P450 reductase + cytochrome b_5	458	4.0	0.28	1:2:1
CYP2B6.9 + P450 reductase + cytochrome b_5	198	5.9	0.32	1:9:2
CYP2B6.18 + P450 reductase + cytochrome b_5	0	2.1	0.34	
CYP2B6.1 + P450 reductase	64	3.6		1:18
CYP2B6.4 + P450 reductase	28	2.6		1:30
CYP2B6.5 + P450 reductase	29	2.6		1:28
CYP2B6.6 + P450 reductase	37	2.9		1:24
CYP2B6.9 + P450 reductase	28	3.0		1:34
CYP2B6.18 + P450 reductase	0	1.9		

[a]Determined spectrophotometrically from CO difference spectra, as described in *Materials and Methods*.

mouse anti-cytochrome b_5 (36) antibody (1:1000 dilution) (Santa Cruz Biotechnology, Dallas, TX) in blocking buffer containing 0.1% Tween-20. After washing, membranes were incubated with goat anti-rabbit IRDye 680 (1:10000 dilution) and goat anti-mouse IRDye 800CW (1:10000 dilution) (LI-COR) for 30 minutes at room temperature in blocking buffer containing 0.1% Tween-20. CYP2B6, POR, and b_5 were all visualized using an Odyssey Infrared Imager (LI-COR). P450 concentration was determined as previously described with minor modifications (Matsubara et al., 1976). Briefly, cell lysate was diluted to 1 mg/ml, bubbled with CO, and the baseline absorbance reading was taken from 400–500 nm using a Synergy MX Microplate Reader (Biotek, Winooski, VT). Sodium dithionite (final concentration 25 mM) was added immediately after the reference baseline was obtained, and the final absorbance reading was taken after 2 minutes. Cytochrome *c* reductase activity was determined using 0.3 M potassium phosphate buffer (pH 7.7) at 37°C (Dignam and Strobel, 1977) and the Synergy MX Microplate Reader. Cytochrome b_5 content was determined as previously described (McLaughlin et al., 2010). The P450/reductase ratio was calculated on the basis of an assumed specific activity of 3200 nmol of cytochrome *c* reduced/min per nanomole reductase (Parikh et al., 1997).

Methadone Metabolism. Incubations (200 μl) with *R*- or *S*-methadone were performed as previously described (Gadel et al., 2013), with minor modifications. Preliminary experiments showed that *N*-demethylation was linear for up to 45 minutes; routine incubations were 10 minutes. Reactions (10 minutes) were quenched with 40 μl 20% trichloroacetic acid containing internal standard (d3-EDDP, final concentration 1.6 ng/ml), and centrifuged for 5 minutes at 2500*g*; the supernatant (150 μl) was processed immediately by solid-phase extraction as described previously (Kharasch et al., 2004), except that Strata-X-C 33-μm, 30 mg/well plates (Phenomenex, Torrance, CA) were used. EDDP and methadone achiral analysis was performed on an Agilent 6140 single quadrupole mass spectrometer with an electrospray ionization source, Agilent 1100 series high-pressure liquid chromatography system equipped with a 96-well plate autosampler (Agilent Technologies, Inc., Santa Clara, CA), and a Sunfire C18 column (2.1 × 50 mm, 3.5-μm) (Waters, Milford, MA) with a 2-μm column filter guard (Supleco Analytical, Bellefonte, PA). Sample injections were 25 μl and the column oven was held at 30°C. Mobile phase A was 4.5 mM ammonium acetate in Milli-Q water, pH 4.5, and mobile phase B was 4.5 mM ammonium acetate in acetonitrile. The mobile phase gradient (0.4 ml/min) was 25% B for 0.5 minutes, linear gradient to 75% B between 0.5 and 4.2 minutes, held at 75% B until 5.0 minutes, immediately decreased back to 25% B and re-equilibrated at initial conditions for 3.0 minutes. Under these conditions, EDDP retention time was 4.6 minutes. Mass spectrometer parameters were: positive ion mode, nitrogen drying gas at 12 l/min and 300°C, nebulizer pressure of 35 psig, capillary voltage 3000 V, and fragmentor voltage of 80 V for EDDP 70 V for d3-EDDP. All ions were monitored in the same ion group: *m/z* of 278.2 and 281.2 for EDDP and d3-EDDP, respectively. Analytes were quantified using peak area ratios and standard curves prepared using calibration standards in buffer. Control incubations lacking enzyme were included for all reactions to determine background EDDP content, which was subtracted from all results.

Data and Statistical Analysis. Results are the mean ± S.D. (3–6 replicates) unless otherwise indicated. EDDP formation by CYP2B6 variants was compared by analysis of variance. EDDP formation versus substrate concentration data were analyzed by nonlinear regression analysis (SigmaPlot 12.5; Systat, San Jose, CA) evaluating a single-enzyme Michaelis-Menten, Adair-Pauling, or substrate (or product inhibition) model as described previously (Totah et al., 2007, 2008; Gadel et al., 2013). The choice of model was based on whether the Eadie-Hofstee plots were linear or nonlinear and the goodness of fit regression diagnostics. Modeling results are the parameter estimate plus or minus standard error of the estimate. Parameter estimates for CYP2B6 variants and CYP2B1.1 were compared using an unpaired *t* test. Significance was assigned at $P < 0.05$.

Results

CYP2 2B6.1, 2B6.4, 2B6.6, 2B6.9, and 2B6.18 were successfully coexpressed with cytochrome P450 reductase, with or without coexpressed cytochrome b_5. Expression of all proteins was confirmed by Western blot (Fig. 1). CYP2B6.18 protein was expressed but did not generate a CO-difference spectrum. P450 content, P450 reductase activity, and b_5 content of the various constructs are provided in Table 1.

Methadone enantiomer *N*-demethylation was evaluated at plasma concentrations (0.25–1 μM) typically occurring in patients receiving low and high doses of methadone for treatment of pain or substance abuse, respectively (Fig. 2). EDDP formation from therapeutic (0.25–1 μM) *R*- and *S*-methadone concentrations was CYP2B6.4 = CYP2B6.1 = CYP2B6.5 > CYP2B6.9 ≈ CYP2B6.6, and undetectable from CYP2B6.18 in expression systems with P450, P450 reductase, and coexpressed cytochrome b_5, and CYP2B6.4 > CYP2B6.1 ≥ CYP2B6.5 > CYP2B6.9 ≈ CYP2B6.6, and undetectable from CYP2B6.18 in expression systems with P450 and P450 reductase but without cytochrome b_5. EDDP formation by CYP2B6.6 and CYP2B6.9 was one-half to one-third that by CYP2B6.1. Coexpression of b_5 had little effect at therapeutic methadone concentrations. EDDP formation ratios with/without b_5 were 1.3, 0.7, 1.7, 0.9, and 1.2 for CYP2Bs 6.1, 6.4, 6.5, 6.6, and 6.9, respectively. With all CYP2B6 variants and expression systems, methadone *N*-demethylation was stereoselective (*S*>*R*). With coexpressed cytochrome b_5, the *S/R* ratio averaged 2.1, whereas it was slightly less (1.8) without b_5.

The concentration-dependence of methadone enantiomers *N*-demethylation was determined both in the presence and absence of coexpressed cytochrome b_5 for CYP2Bs 6.1, 6.4, 6.5, 6.6, and 6.9 (Fig. 3). There was evidence for substrate or product inhibition at the highest concentration of *S*-methadone (but not *R*-methadone), as observed previously (Gadel et al., 2013). Eadie-Hosfstee plots were generally linear, with or without coexpressed b_5 (not shown). Linear plots were interpreted as methadone binding to a single site, and analyzed with the Michaelis-Menten model, with substrate inhibition where apparent. Kinetic parameters are provided in Table 2. In vitro intrinsic clearances (Cl_{int}) were generally greater for *S*- versus *R*-methadone *N*-demethylation, regardless of CYP2B6 variant or the absence or presence of b_5. Cl_{int} was generally of the order CYP2B6.4

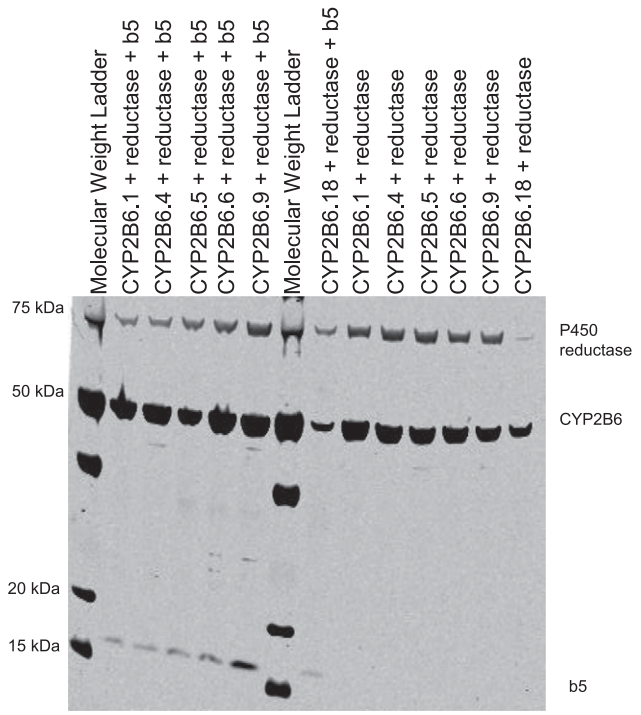

Fig. 1. Western blot showing expression of CYP2B6, P450 reductase, and cytochrome b_5.

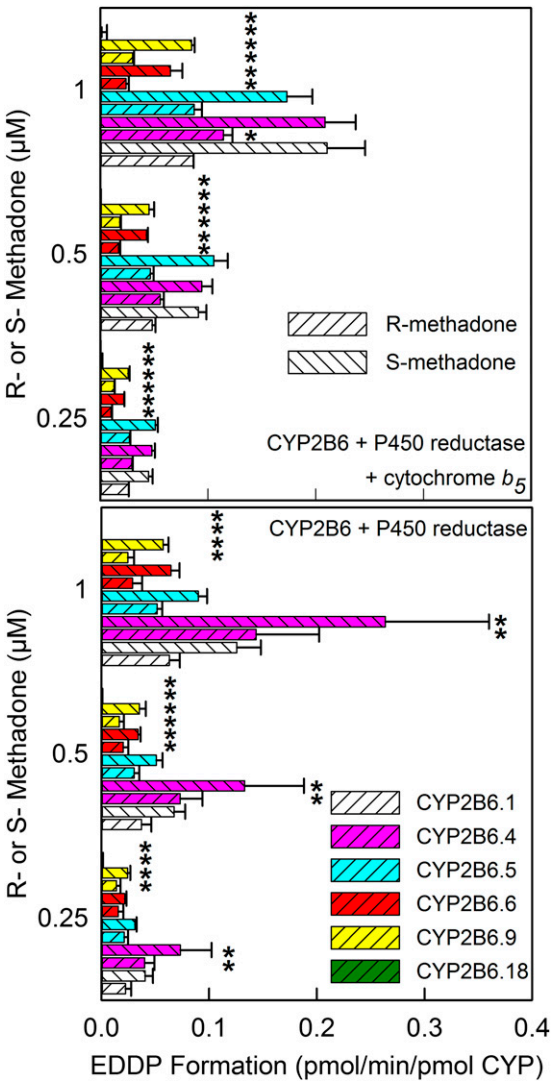

Fig. 2. Recombinant CYP2B6-catalyzed *N*-demethylation of methadone enantiomers at therapeutic concentrations. Results are shown for CYP2B6 and cytochrome P450 reductase, with (top) or without (bottom) coexpressed cytochrome b_5. Results are the mean ± S.D. of 5–6 determinations. *Significantly different from CYP2B6.1 ($P < 0.05$).

≥ CYP2B6.1 > CYP2B6.5 > CYP2B6.9 ≥ CYP2B6.6. Figure 3 also shows the influence of cytochrome b_5 coexpression on *N*-demethylase activity for each CYP2B6 variant. For CYP2B6.1, 6.4, and 6.5, cytochrome b_5 was stimulatory, and Cl_{int} was increased 20–80% compared with expression systems omitting b_5. Coexpression of b_5 had little effect on methadone *N*-demethylation by CYP2B6.6 and CYP2B6.9.

Discussion

The major finding was that at therapeutic concentrations, *N*-demethylation of *R*- and *S*-methadone by the variant CYP2B6 isoform CYP2B6.4 was greater than that by wild-type CYP2B6.1, whereas EDDP formation by CYP2B6.6 and CYP2B6.9 was less than CYP2B6.1, and CYP2B6.18 was catalytically incompetent. Assessed over broader methadone concentrations, in vitro intrinsic clearances for CYP2B6.6 and CYP2B6.9 were significantly lower than for CYP2B6.1. Stereoselectivity of methadone metabolism was maintained in all CYP2B6 variants, with *S*-methadone *N*-demethylation 2-fold greater than that of *R*-methadone, similar to that with CYP2B6.1 (Gerber et al., 2004; Totah et al., 2007, 2008; Chang et al., 2011; Gadel et al., 2013). Lower rates of CYP2B6.6-catalyzed methadone *N*-demethylation in the present investigation, in which insect cells were transfected with individual virus constructs for CYP2B6, P450 reductase, and cytochrome b_5, were also seen in an insect cell system transfected with a single virus containing all three proteins (Gadel et al., 2013).

The kinetics of methadone *N*-demethylation by CYP2B6 are complex (Totah et al., 2007; Gadel et al., 2013). Previous experiments with expressed CYP2B6.1 and CYP2B6.6 suggested apparent multisite or multiple-affinity methadone binding with complex allosteric kinetics or homotropic cooperativity, modeled best using the Adair-Pauling equation (Totah et al., 2007; Gadel et al., 2013). Although there appeared to be substrate or product inhibition with CYP2B6.6 at the highest *S*-methadone concentration, this could not be modeled (Gadel et al., 2013). In the present investigation, substrate (or product) inhibition with *S*-methadone was more apparent, and more amenable to modeling, and Michaelis-Menten kinetics with substrate inhibition provided better fits to the data. Nonetheless, differences between kinetic models did not materially affect the intrinsic clearances or conclusions of this investigation that metabolism by CYP2B6.4 was greater than by CYP2B6.1, CYP2B6.5 was lower, and CYP2B6.6 and CYP2B6.9 were substantially less.

CYP2B6 expression levels in the triple protein constructs differed between allelic variants, resulting in varying P450/P450 reductase molar ratios. Nonetheless, this did not explain the differences between variants in methadone metabolism. Such varying ratios were also seen previously, with CYP2B6 systems using a single virus for the three proteins (P450, reductase, and b_5) (Xu et al., 2012; Gadel et al., 2013). When reductase levels are sufficient for catalytic activity, overexpression of reductase may not be a factor (Nakajima et al., 2002).

The influence of *CYP2B6* allelic mutations on catalytic activity, in general, is CYP2B6 variant- and substrate-dependent (Mo et al., 2009; Zanger and Klein, 2013). In an insect cell system with coexpressed P450 reductase and b_5, CYP2B6.6 Cl_{int} for bupropion 4-hydroxylation was lower, whereas efavirenz 8-hydroxylation was less but not significantly different from CYP2B6.1 (Xu et al., 2012). CYP2B6.6 activity in other systems (*E. coli*, Cos-1, Cos-7) was lower toward bupropion and efavirenz (Zhang et al., 2011), and ketamine (Li et al., 2013), but greater for cyclophosphamide (Xie et al., 2003; Ariyoshi et al., 2011; Raccor et al., 2012) and artemether (Honda et al., 2011), and unchanged for selegiline (Watanabe et al., 2010). The present and previous (Gadel et al., 2013) experiments show that methadone is one of the most catalytically diminished substrates for CYP2B6.6. A novel finding herein is that CYP2B6.9 is also catalytically deficient toward methadone. Data regarding the functional activity of CYP2B6.9 are said to be rare (Zanger and Klein, 2013). In a COS-1 system, the activities of CYP2B6.6 and CYP2B6.9, in which 516G>T is the common polymorphism, were both extremely low, but this was attributed to minimal expression (Hofmann et al., 2008). CYP2B6.9 activity toward artemether was somewhat lower than CYP2B6.1 in COS-7 cells (Honda et al., 2011), and toward 7-ethoxy-4-trifluoromethylcoumarin, bupropion, and efavirenz in a reconstituted bacterial expression system (Zhang et al., 2011). The present results suggest that methadone is also one of the more catalytically diminished substrates with CYP2B6.9. Although CYP2B6.5 has been reported to have shown increased catalytic activity that compensates for lower expression (Zanger and Klein, 2013), this was not observed with methadone. CYP2B6.5 activities are variable with other substrates. CYP2B6.5 cyclophosphamide 4-hydroxylation in *E. coli*, Cos-1, and Cos-7 systems was half that of CYP2B6.1 (Raccor et al.,

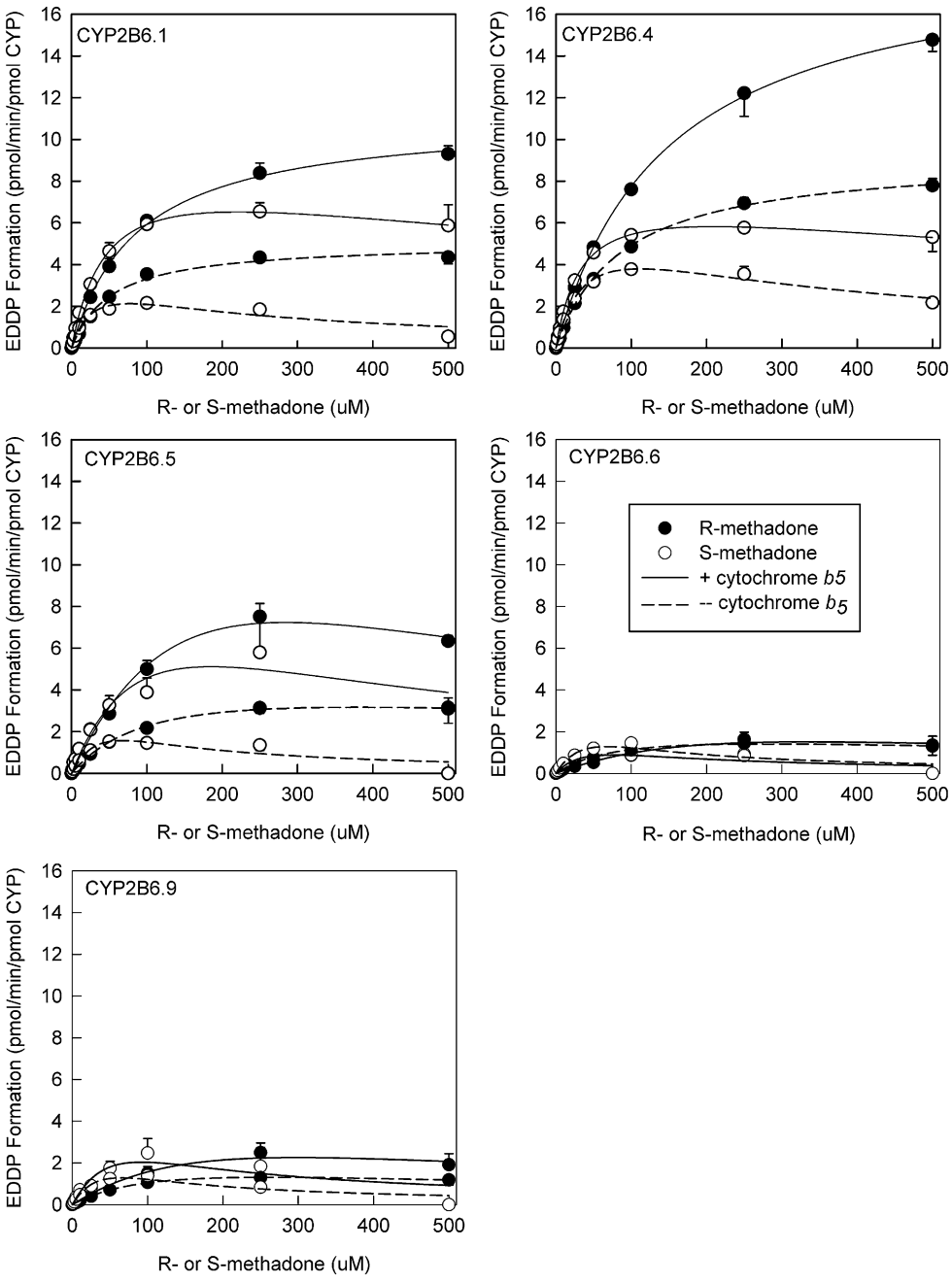

Fig. 3. Concentration-dependence of recombinant CYP2B6-catalyzed *N*-demethylation of methadone enantiomers to EDDP. Each data point is the mean ± S.D. of 3–6 determinations. Solid and open symbols show *R*-EDDP and *S*-EDDP, respectively. Solid and open lines show the presence and absence of cytochrome b_5. Lines represent rates predicted from nonlinear regression analysis of observed metabolite formation. Kinetic parameters are summarized in Table 2.

2012), and artemether and selegiline metabolism was diminished (Watanabe et al., 2010; Honda et al., 2011), whereas 7-ethoxy-4-trifluoromethylcoumarin, bupropion, and efavirenz metabolism was decreased by one-third, half, and increased, respectively, compared with CYP2B6.1 (Zhang et al., 2011; Radloff et al., 2013). CYP2B6.4 results are even more substrate-dependent. In *E. coli*, Cos-1, and Cos-7 systems, CYP2B6.4 cyclophosphamide 4-hydroxylation was 25% lower than CYP2B6.1 (Ariyoshi et al., 2011; Raccor et al., 2012), the catalytic efficiency for 7-ethoxy-4-trifluoromethylcoumarin, bupropion, and efavirenz was decreased to half, one-third, and unchanged, respectively (Zhang et al., 2011), and artemether and selegiline metabolism was almost doubled (Watanabe et al., 2010; Honda et al., 2011). Methadone metabolism by CYP2B6.4 in the present investigation was greater than by CYP2B6.1. Thus, whereas 785A>G (K262R) alone (CYP2B6.4) caused increased methadone *N*-demethylation, 785A>G together with 516G>T (Q172H) (CYP2B6.6), and 516G>T alone (CYP2B6.9) diminished methadone metabolism. Catalytic incompetence of CYP2B6.18 toward methadone is consistent with other CYP2B6 substrates (Zanger and Klein, 2013).

The influence of *CYP2B6* allelic mutations on catalytic activity appears dependent on the enzyme system used. In addition to differences noted above between various expression systems, most have also not included cytochrome b_5, and the absence or presence of b_5 may influence results. Efavirenz metabolism by both CYP2B6.1 and CYP2B6.6 was unchanged by b_5, whereas bupropion metabolism by CYP2B6.1 was unchanged by b_5 but metabolism by CYP2B6.6 was diminished (Xu et al., 2012). The present results showed that b_5 increased methadone Cl_{int} by CYP2B6.1 and CYP2B6.4 but not metabolism at therapeutic concentrations. Effects of b_5 on CYP2B6-catalyzed metabolism may underlie, at least in part, different results

TABLE 2

Kinetic parameters for methadone N-demethylation

Results are the parameter estimate ± standard error of the estimate.

Parameter	CYP2B6.1		CYP2B6.4		CYP2B6.5		CYP2B6.6		CYP2B6.9	
	R-EDDP formation	*S*-EDDP formation	*R*-EDDP formation	*S*-EDDP formation	*R*-EDDP formation	*S*-EDDP formation	*R*-EDDP formation	*S*-EDDP formation	*R*-EDDP formation	*S*-EDDP formation
CYP2B6 + P450 reductase + cytochrome b_5										
V_{max} (pmol/min per picomole P450)	11 ± 1	9 ± 1	19 ± 1*	8 ± 1*	22 ± 4*	13 ± 5	4 ± 3*	2 ± 1*	7 ± 4	6 ± 4
K_m (μM)	87 ± 4	48 ± 6	148 ± 6*	34 ± 3*	284 ± 73*	138 ± 78	337 ± 290	54 ± 46	298 ± 204	92 ± 91
Cl_{int} (ml/min per nanomole)	0.13 ± 0.01	0.19 ± 0.03	0.13 ± 0.01	0.23 ± 0.02	0.08 ± 0.03	0.09 ± 0.06	0.01 ± 0.01*	0.04 ± 0.04*	0.02 ± 0.02*	0.07 ± 0.08
CYP2B6 + P450 reductase										
V_{max} (pmol/min per picomole P450)	5 ± 1	4 ± 1	9 ± 1*	7 ± 1*	5 ± 1	5 ± 1	2 ± 1*	4 ± 2	3 ± 1*	4 ± 2
K_m (μM)	52 ± 3	33 ± 8	87 ± 3*	43 ± 5	119 ± 33*	67 ± 45	96 ± 32	67 ± 44	132 ± 32*	65 ± 36
Cl_{int} (ml/min per nanomole)	0.10 ± 0.01	0.12 ± 0.04	0.11 ± 0.01	0.16 ± 0.02	0.04 ± 0.01*	0.07 ± 0.06	0.02 ± 0.01*	0.06 ± 0.05	0.02 ± 0.01*	0.06 ± 0.04

*Significantly different versus CYP2B6.1 ($P < 0.05$)

with various expression systems, and the kinetic consequences of *CYP2B6* polymorphisms (Zanger and Klein, 2013). This investigation appears to be one of the first to use an insect cell system with a panel of CYP2B6 variants and coexpressed P450 reductase and b_5. Use of fully competent CYP2B6 systems, containing both P450 reductase and cytochrome b_5, may be advantageous.

Although the present investigation evaluated only catalytic activity, some clinical implications can be inferred regarding the influence of *CYP2B6* variants on methadone disposition. Clinical consequences of *CYP2B6* allelic variants will depend on both the intrinsic activity of the mutant protein and its level of expression. The *CYP2B6*6* allele causes low human hepatic CYP2B expression (Lang et al., 2001; Desta et al., 2007; Hofmann et al., 2008), thus both deficient catalytic efficiency and decreased P450 content combine to cause a diminished CYP2B6 metabolizer phenotype in *CYP2B6*6* carriers. Liver microsomes from *CYP2B6*6* carriers had diminished methadone *N*-demethylation (Gadel et al., 2013). The lower CYP2B6.9 and CYP2B6.18 activities toward methadone suggest that similar results might be expected with *CYP2B6*9* and *CYP2B6*18* carriers; however, microsomal methadone metabolism by livers from carriers of variants other than *CYP2B6*6* have not been reported. Clinical genetic association studies of methadone plasma concentrations are consistent with diminished methadone *N*-demethylation by CYP2B6.6 and liver microsomes from *CYP2B6**6 carriers. In patients, methadone doses were lower (Hung et al., 2011; Levran et al., 2013), and dose-adjusted steady-state *S*-methadone concentrations were greater (Crettol et al., 2005, 2006; Eap et al., 2007; Wang et al., 2011) in *CYP2B6*6* homozygotes compared with heterozygotes and non-carriers. While these observations suggested that *CYP2B6* allelic variants might influence clinical methadone pharmacokinetics, only recently has this been confirmed. Methadone enantiomer *N*-demethylation and clearance were increased and decreased, respectively, in *CYP2B6*4* and *CYP2B6*6* carriers (Kharasch et al., 2014). Thus in vitro methadone metabolism by CYP2B6 variants does predict clinical methadone metabolism and clearance.

An interesting finding is that b_5 coexpression influenced methadone metabolism, and this was CYP2B6 variant–specific. Coexpression of b_5 stimulated *R*- and *S*-methadone metabolism by CYP2B6.1, CYP2B6.4, and CYP2B6.5, with less or minimal effects on CYP2B6.6 and CYP2B6.9. In the P450 catalytic cycle, involving two sequential one-electron transfers from NADPH to P450 and substrate, P450 reductase transfers the first electron, and the second electron may be transferred by P450 reductase or by b_5 (Hildebrandt and Estabrook, 1971; Schenkman and Jansson, 2003). Effects of b_5 are however variable, stimulating, inhibiting, or not affecting metabolism, depending on the P450 isoform, substrate, and experimental conditions (Schenkman and Jansson, 2003; Finn et al., 2008; Im and Waskell, 2011). Recently this variability was shown for the first time to extend to P450 variants, with some *CYP1A2* mutants quite affected by b_5 (Palma et al., 2013). On the basis of several kinetic, mutagenesis, and NMR binding studies of wild-type rabbit CYP2B4, Waskell et al. proposed a model for an electron transfer complex between the acidic convex surface of b_5 and the concave basic proximal surface of CYP2B4 (Im and Waskell, 2011; Ahuja et al., 2013). More specifically, the b_5 binding site is on the CYP2B4 C-helix and β-bulge, with Asp65 and Val66 of b_5 in contact with Arg122, Arg126, and Lys433 of CYP2B4, with Arg133 also critical for interaction of the two proteins. Other sites, specifically Met137 and Lys139, which were important for CYP2B4 binding to b_5, are thought to perturb the C-helix, and R422 also affected CYP2B4 binding (Ahuja et al., 2013). On the basis of sequence alignments, the CYP2B4 residues Arg122, Arg126, Met137, Lys139, and Arg422, which influence binding to b_5,

correspond to Lys122, Arg126, Met137, Lys139, and Lys422 of CYP2B6. The mutated residues in CYP2B6.6 (Gln172His, Lys262Arg), however, do not correspond to the b_5 binding site identified on CYP2B4. With CYP1A2, the variant Gly299Ser (CYP1A2.13) had the most altered b_5 response compared with wild-type, with a lesser influence of the variants Thr83Met (CYP1A2.9), Ile386Phe (CYP1A2.4), and Cys406Tyr (CYP1A2.5) (Palma et al., 2013). Gly299Ser was reported to be on the surface of the heme domain near the interaction site with b_5, and close to the CYP1A2 C-helix, which is also thought to be important in interaction with b_5 (Palma et al., 2013). On the basis of sequence alignment of CYP2B6 with CYP1A2, Thr83, Gly299, Ile386, and Cys406 of CYP1A2 correspond to CYP2B6 Arg73, His280, Val367, and Ile387. These residues are not mutated in the CYP2B6 variants with altered response to b_5. Thus there appears no consistent pattern to explain P450 mutant–specific effects of b_5. Another suggested explanation is that b_5 stimulates metabolism of poorer substrates but not those more avidly metabolized (Im and Waskell, 2011). The present results show that b_5 enhanced metabolism by the more active CYP2B6 variants. As stated previously, the role of cytochrome b_5 remains enigmatic (Schenkman and Jansson, 2003).

In summary, methadone *N*-demethylation by CYP2B6 variants was in the order CYP2B6.4 $\geq$ CYP2B6.1 $>$ CYP2B6.5 $>$ CYP2B6.9 $\geq$ CYP2B6.6 $>>$ CYP2B6.18. Differences in methadone metabolism by *CYP2B6* allelic variants provide a mechanistic understanding for pharmacogenetic variability in clinical methadone metabolism and clearance.

Authorship Contributions

Participated in research design: Gadel, Kharasch.
Conducted experiments: Gadel, Friedel.
Contributed new reagents or analytic tools: Gadel.
Performed data analysis: Gadel, Friedel, Kharasch.
Wrote or contributed to the writing of the manuscript: Gadel, Kharasch.

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Address correspondence to: Dr. Evan D. Kharasch, Department of Anesthesiology, Washington University in St. Louis, 660 S. Euclid Ave., Campus Box 8054, St. Louis, MO 63110. E-mail: kharasch@wustl.edu