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Methadone N-demethylation by the common *CYP2B6* allelic variant CYP2B6.6

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Abbreviations: EDDP, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine

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Abstract

The long-acting opioid methadone displays considerable unexplained interindividual pharmacokinetic variability. Methadone metabolism clinically occurs primarily by N-demethylation to 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), catalyzed predominantly by cytochrome P450 2B6 (CYP2B6). Retrospective studies suggest an influence of the common allele variant *CYP2B6*6* on methadone plasma concentrations. The catalytic activity of CYP2B6.6, encoded by *CYP2B6*6,* is highly substrate-dependent. This investigation evaluated methadone N-demethylation by CYP2B6.6, and in comparison to that by wild-type CYP2B6.1. Methadone enantiomer and racemate N-demethylation by recombinant expressed CYP2B6.6 and CYP2B6.1 was determined. At substrate concentrations (0.25-2 μM) approximating plasma concentrations occurring clinically, rates of methadone enantiomer N-demethylation by CYP2B6.6, incubated individually or as the racemate, were one-third to one-fourth those by CYP2B6.1. For methadone individual enantiomers metabolism by CYP2B6.6 compared with CYP2B6.1, V_{max} was diminished, K_s was greater, the *in vitro* intrinsic clearance was diminished 5- to 6-fold. The intrinsic clearance for R- and S-EDDP formation from racemic methadone was diminished approximately 6-fold and 3-fold for R- and S-methadone. Both CYP2B6.6 and CYP2B6.1 showed similar stereoselectivity (S>R-methadone). Human liver microsomes with diminished CYP2B6 content due to a *CYP2B6*6* allele had lower rates of methadone N-demethylation. Results show 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. Diminished methadone N-demethylation by CYP2B6.6 may provide a mechanistic explanation for clinical observations of altered methadone disposition in individuals carrying the *CYP2B6*6* polymorphism.

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Introduction

The long-acting opioid methadone is used to treat opiate addiction, as well as acute, chronic, and cancer pain. Clinical use of methadone is challenging, however, because of considerable and unpredictable inter- and intra-individual variability in pharmacokinetics, including metabolism, clearance, and susceptibility to drug interactions (Ferrari et al., 2004; McCance-Katz et al., 2010). This can result in opiate withdrawal, inadequate analgesia, or drug accumulation and toxicity. Indeed, with increasing methadone use over the past decade there has been an epidemic of toxicity, including a nearly 1800% increase in adverse events and a 390% increase in fatalities, which persist today (2012).

Methadone in humans is cleared primarily by hepatic cytochrome P450 (CYP)-catalyzed metabolism, to the pharmacologically inactive metabolite 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), with some urinary excretion of unchanged drug. Methadone clearance and N-demethylation are stereoselective. After considerable research, a consensus has emerged that methadone N-demethylation *in vitro*, both by human liver microsomes and expressed CYPs, is catalyzed most efficiently by CYP2B6 and CYP3A4 (while CYP3A5 is comparatively inactive), and, CYP2B6 but not CYP3A4 N-demethylates methadone stereoselectively (Gerber et al., 2004; Kharasch et al., 2004; Totah et al., 2007; Totah et al., 2008; Chang et al., 2010). Clinical drug interaction studies indicate that CYP2B6, rather than CYP3A4, is a major or predominant CYP isoform responsible for clinical methadone disposition. CYP3A induction (Vourvahis et al., 2012), and very strong CYP3A inhibition (Kharasch et al., 2004; Kharasch et al., 2008; van Heeswijk et al., 2011; Kharasch et al., 2012), had no influence on (or in some studies even increased) methadone N-demethylation or clearance. However CYP2B6 induction or inhibition did correspondingly modulate methadone plasma concentrations, metabolism and clearance (Kharasch et al., 2004; Kharasch et al., 2008; Kharasch and Stubbert, 2013).

CYP2B6 comprises a small fraction of hepatic CYP content, but is responsible for metabolizing a much larger percentage of drugs and xenobiotics (Wang and Tompkins, 2008; Mo et al., 2009) *CYP2B6* is a highly polymorphic gene (Zanger et al., 2007), with numerous single nucleotide polymorphisms encoding thirty CYP2B6 protein variants identified to date. The *CYP2B6*6* allele (516G>T, Q172H;

785A>G, K262R) is of considerable interest, owing to its common occurrence (particularly in populations or descendants of Africans, Asians, and Hispanics) and therapeutic significance (Zanger et al., 2007). For example, *CYP2B6*6* influences the metabolism, pharmacokinetics, and clinical effects of efavirenz, nevirapine, cyclophosphamide, and bupropion (Mo et al., 2009) *CYP2B6*6* has been associated with diminished CYP2B protein expression in human liver microsomes (Xie et al., 2003; Desta et al., 2007; Hofmann et al., 2008) Expressed CYP2B6.6, compared with CYP2B6.1, has diminished catalytic activity towards bupropion and efavirenz (Zhang et al., 2011; Xu et al., 2012), modest-to-moderately increased activity towards cyclophosphamide (Xie et al., 2003; Ariyoshi et al., 2011; Raccor et al., 2012), and artemether (Honda et al., 2011), and indifferent metabolism of selegiline (Watanabe et al., 2010). Human liver microsomes from *CYP2B6*6* carriers had diminished metabolism of mephenytoin (Lang et al., 2001), bupropion (Hesse et al., 2004; Xu et al., 2012), and efavirenz (Desta et al., 2007; Xu et al., 2012), but not cyclophosphamide (Xie et al., 2003; Raccor et al., 2012). Thus, the influence of *CYP2B6*6* genotype on the direction (increased or decreased metabolism), magnitude, pharmacokinetic consequence, clinical implications and mechanism of CYP2B6-dependent biotransformation are complex, and, substrate-dependent.

Available clinical evidence suggests that *CYP2B6* polymorphisms can influence methadone disposition. Specifically, associations between *CYP2B6*6* genotype and higher dose-adjusted steady-state plasma methadone concentrations (Crettol et al., 2005; Crettol et al., 2006; Eap et al., 2007; Wang et al., 2011), or need for lower methadone doses (Hung et al., 2011; Levran et al., 2012), have been reported.

Despite these pharmacogenetic association studies evaluating single steady-state plasma methadone concentrations, the influence of *CYP2B6*6* alleles or *CYP2B6* polymorphism in general, on clinical methadone metabolism and clearance is unknown, as are their influence on human liver microsomal methadone metabolism. In addition, the activity of CYP2B6.6 towards methadone metabolism is also unknown. Therefore, the purpose of this investigation was to evaluate methadone N-demethylation by CYP2B6.6, and compare it to that by wild-type CYP2B6.1.

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Materials and Methods

Chemicals and reagents. EDDP and EDDP-d3 were purchased from Cerilliant (Round Rock, TX). Insect cell microsomes (Supersomes®) containing CYP2B6.1 coexpressed with human cytochrome P450 reductase and human cytochrome b_5 were purchased from BD Gentest Corporation (Woburn, MA). Microsomes containing CYP2B6.6 coexpressed with P450 reductase and cytochrome b_5 were a generous gift from BD Gentest Corporation. All other reagents were from Sigma-Aldrich (St. Louis, MO).

Methadone metabolism. Incubations (200 µl, 10 pmol/ml CYP2B6) with RS-, R-, or S-methadone were performed as previously described (Totah et al., 2007; Totah et al., 2008), with minor modifications. Reactions (10 min) were quenched with 20 µl 20% trichloroacetic acid containing internal standard (d3-EDDP, final concentration 4.55 ng/ml), centrifuged, and supernatant (200µl) processed immediately by solid-phase extraction as described previously (Kharasch et al., 2004), except that Strata-X-C 33 µm plates (Phenomenex, Torrance, CA) were used. EDDP analysis was performed on an API 3200 triple-quadrupole mass spectrometer with Turbo Ion Spray source (Applied Biosystems/MDS Sciex, Foster City, CA), Shimadzu HPLC (Columbia, MD), autosampler (Gerstel, Germany), and chiral AGP column (3 x 50mm, 5µm) with chiral AGP guard cartridge (3 x 10mm) (Chrom Tech, Apple Valley, MN), as described (Sharma et al., 2011), except that the mobile phase was 20mM ammonium formate (pH 5.0) and methanol. Retention times were 10.4 and 11.2 min, respectively, for R- and S-EDDP. EDDP was quantified using peak area ratios and standard curves prepared using calibration standards in buffer. Control incubations lacking NADPH and protein were included for all reactions to determine the background EDDP, which was subtracted from all results.

Data and Statistical Analysis. Results are the mean ± SD (3-6 replicates) unless otherwise indicated. Differences between groups were determined by analysis of variance followed by the Student-Newman-Keuls test (SigmaPlot 12.3 Systat, San Jose, CA). EDDP formation vs substrate concentration data were analyzed by nonlinear regression analysis (SigmaPlot 12.3) using the Adair-Pauling model, based on the recognition that CYP2B6 contains at least two binding sites, and as described previously (Totah et al., 2007; 2008). Results are the parameter estimate ± standard error of the estimate.

Results

N-demethylation of racemic methadone and individual enantiomers was evaluated at concentrations (0.25-2 μM) approximating those in patients receiving low and high doses of methadone, respectively, for treatment of pain (typically 10-20 mg) or substance abuse (60-100 mg). Rates of methadone enantiomer N-demethylation by CYP2B6.6 were typically one-third those by CYP2B6.1, when enantiomers were incubated individually (Figure 1A). When racemic methadone N-demethylation was evaluated (Figure 1B), rates of R- and S-EDDP formation by CYP2B6.6 were even lower, approximately one-fourth those by CYP2B6.1. N-demethylation by CYP2B6.1 was stereoselective, with S-methadone metabolism exceeding that of R-methadone, both with individual enantiomers and the racemate. Although EDDP formation by CYP2B6.6 was much lower than that by CYP2B6.1, stereoselectivity (S>R-methadone) was preserved, with individual enantiomers and the racemate.

Concentration-dependence of methadone N-demethylation was determined for racemic methadone and the individual enantiomers (Figure 2). As observed previously (Totah et al., 2008), Eadie-Hofstee plots for racemic methadone N-demethylation by CYP2B6.1 were not strictly linear, nor were those for R- and S-methadone, indicating that the nonlinearity of the racemate did not represent the two enantiomers interacting differently with a single enzyme site, but rather, each enantiomer interacting with two apparent enzyme sites. Nonlinear Eadie-Hofstee plots were also observed for CYP2B6.6-catalyzed racemic methadone and methadone enantiomers N-demethylation. The Adair-Pauling equation, which allows for two binding sites, was used to model EDDP formation from individual methadone enantiomers, and kinetic parameters are provided in Table 1. For CYP2B6.6, V_{max} was diminished to approximately one-third to one-fifth that for CYP2B6.1, K_s greater than for CYP2B6.1, and the *in vitro* intrinsic clearance (Cl_{int}, V_{max}/K_s), was diminished 5- to 6-fold. The Adair-Pauling equation was also used to model EDDP enantiomer formation from racemic methadone. For CYP2B6.6, the apparent K_s for both enantiomers was approximately 50% greater than for CYP2B6.1, V_{max} was diminished more for R-methadone (to one fourth) than S-methadone (to approximately half), and the *in vitro* intrinsic clearance was diminished approximately 6-fold and 3-fold for R- and S-methadone, respectively.

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Discussion

The major finding of this investigation is that the N-demethylation of methadone catalyzed by CYP2B6.6, the CYP2B6 variant encoded by the *CYP2B6*6* polymorphism, is catalytically deficient compared with wild-type CYP2B6.1 With CYP2B6.6 compared with CYP2B6.1, EDDP formation from both individual methadone enantiomers was diminished, K_s was increased, V_{max} was reduced, and the *in vitro* intrinsic clearance was diminished to approximately one-fifth that for the wild-type enzyme. With racemic methadone, EDDP formation from both methadone enantiomers was also lower with CYP2B6.6, V_{max} was reduced, and the *in vitro* intrinsic clearance was diminished to approximately one-fifth to one-half that for wild-type CYP2B6.1. At substrate concentrations approximating total plasma methadone concentrations (0.25-0.5 μM each enantiomer) occurring clinically, for both individual enantiomers and racemic methadone, rates of N-demethylation by CYP2B6.6 were generally only one-third those for CYP2B6.1. Although rates of methadone metabolism by CYP2B6.6 were diminished compared with CYP2B6.1, the stereoselectivity of metabolism (S-methadone > R-methadone) seen previously with CYP2B6.1 (Gerber et al., 2004; Totah et al., 2007; Totah et al., 2008; Chang et al., 2010), was preserved with CYP2B6.6.

Modeling of methadone N-demethylation by CYP2B6 is complex (Totah et al., 2007). Methadone enantiomers N-demethylation by CYP2B6.1 showed apparent multiple-site or multiple-affinity binding with complex allosteric kinetics or homotropic cooperativity, which was best described using the Adair-Pauling equation (Totah et al., 2007). This approach was used to model methadone enantiomers N-demethylation by CYP2B6.1 and CYP2B6.6 in the present investigation. With CYP2B6.6, at the highest substrate concentrations, the possibility of substrate or product inhibition cannot be eliminated, however there are insufficient data with which to evaluate such models in an identifiable fashion, and hence it most appropriate to use the simplest model for which there is precedent. With racemic methadone, a previous investigation with CYP2B6.1 found a competitive inhibitory interaction, with each enantiomer in the racemate (R- or S-) inhibiting the metabolism of its antipode (S- or R-methadone). The Adair-Pauling model was found to be mis-specified for racemic methadone, and a

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more complex model was required to describe methadone metabolism, however that required evaluating metabolism of a complex matrix of individual antipode concentrations at each enantiomer concentration. In the present investigation, only racemic methadone metabolism by CYP2B6.6 was evaluated, precluding application of the complex CYP2B6 model, and the simpler Adair-Pauling equation used to model the data, accepting some misspecification in the parameter estimates. Thus for racemic methadone the reported K_s and V_{max} are best considered as apparent parameters. Use of a Michaelis-Menten model did not result in improved fits to the data (not shown).

The catalytic behavior of CYP2B6.6 is substrate-dependent, and the mechanism of altered CYP2B6.6-catalyzed biotransformation, when it has been observed, is not well understood. Some information is available from studies using expressed CYP2B6.6, those using liver microsomes of individuals carrying the *CYP2B6*6* allele, and from clinical pharmacogenetic studies. In COS-1 cells expressing CYP2B6.6, Cl_{int} for 7-ethoxy-4-trifluoromethylcoumarin O-deethylation was double that for CYP2B6.1 (Jinno et al., 2003). In COS-7 cells expressing CYP2B6.6, Cl_{int} for 7-ethoxy-4-trifluoromethylcoumarin O-deethylation, selegiline N-demethylation, and selegiline N-depropagylation was not different from that for CYP2B6.1 (Watanabe et al., 2010), and the activities towards bupropion and artemether were significantly less (Hofmann et al., 2008), and greater (Honda et al., 2011), respectively. In an insect cell CYP2B6 expression system co-expressing P450 reductase but not cytochrome b_5, Cl_{int} for efavirenz 8-hydroxylation by CYP2B6.6 was half that compared with CYP2B6.1, while the Cl_{int} for cyclophosphamide 4-hydroxylation was 60% greater (Ariyoshi et al., 2011). In a CYP2B6 reconstitution system with P450 reductase but not cytochrome b_5, the catalytic efficiency (k_{cat}/K_m) of CYP2B6.6 for 7-ethoxy-4-trifluoromethylcoumarin, bupropion 4-hydroxylation, and efavirenz 8-hydroxylation was decreased to two-thirds, one-half, and one-fifth that compared with CYP2B6.1 (Zhang et al., 2011). In a CYP2B6 expression system co-expressing P450 reductase and cytochrome b_5, CYP2B6.6-catalyzed efavirenz 8-hydroxylation was not significantly different and the Cl_{int} for bupropion 4-hydroxylation was reduced by approximately one-third compared with CYP2B6.1 (Xu et al., 2012). In the absence of b_5, efavirenz 8-hydroxylation Cl_{int} was approximately half for

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CYP2B6.6 compared with CYP2B6.1, and bupropion 4-hydroxylation Cl_{int} approximately 50% greater (Xu et al., 2012). In the present insect cell CYP2B6 expression system, containing both co-expressed P450 reductase and cytochrome b_5, the catalytic difference between CYP2B6.6 and CYP2B6.1 for methadone enantiomer metabolism was generally greater than that observed previously for other substrates, with a 5-fold lower Cl_{int} for N-demethylation. Thus methadone appears to be one of the most susceptible substrates to the diminished catalytic efficiency of CYP2B6.6.

In human liver, the *CYP2B6*6* allele causes aberrant splicing (Hofmann et al., 2008), resulting in reduced functional mRNA and low hepatic CYP2B expression (Lang et al., 2001; Desta et al., 2007; Hofmann et al., 2008). Thus both diminished CYP content and deficient catalytic efficiency may combine to cause the phenotype of decreased CYP2B6-catalyzed biotransformation in CYP2B6*6 carriers. Human liver microsomes may therefore show greater catalytic differences between CYP2B6.1 and CYP2B6.6 and the effect of the *CYP2B6*6* polymorphism, compared with expressed enzyme systems. Indeed, in human liver microsomes from individuals with *6 genotypes, there was markedly diminished CYP2B6 protein expression, and cyclophosphamide 4-hydroxylation (Xie et al., 2003), bupropion hydroxylation (Hesse et al., 2004; Xu et al., 2012), and efavirenz 8-hydroxylation (Desta et al., 2007; Xu et al., 2012). Clinically, numerous investigations have shown an association between *CYP2B6*6* genotypes and increased efavirenz plasma concentrations, diminished metabolism and clearance, and greater neurotoxicity and hepatotoxicity (Haas et al., 2004; Holzinger et al., 2012; Turpeinen and Zanger, 2012). Bupropion metabolism was similarly diminished in *CYP2B6*6* carriers, based on lower plasma hydroxybupropion/bupropion AUC ratios (Chung et al., 2011).

We previously evaluated the influence of CYP2B6 content and genetic polymorphisms on human liver microsomal methadone metabolism (Totah et al., 2008). There was no apparent consistent relationship between methadone N-demethylation and CYP2B6 content (or genotype), and no definitive conclusions could be drawn regarding *CYP2B6* genotype and methadone metabolism. Since then, microsomal CYP3A content was re-quantified (Raccor et al., 2012), and, when pairs of livers were re-matched for CYP3A content but different CYP2B6 content (or genotype) and methadone enantiomers

metabolism again compared, a clear influence of *CYP2B6* genotype became apparent (Supplemental Table 1). For example, microsomes from human livers 141 and 144 both had high CYP3A content, but high (*CYP2B6*1/*1*) and moderate CYP2B6 (*CYP2B6*1/*6*) content, respectively, and lower N-demethylation of both methadone enantiomers was observed in HLM 144. Livers 124 and 148 both had high CYP3A content, but high (*CYP2B6*1/*6*) and low CYP2B6 (*CYP2B6*6/*6*) content, respectively, and lower methadone metabolism was observed in HLM 148. HLM 142 and 164 both had low CYP3A, but moderate (*CYP2B6*1/*4*) and low CYP2B6 (*CYP2B6*1/*6*) content, and lower methadone N-demethylation was observed with HLM 164. These data suggest that *CYP2B6* genotype, specifically the **6* allele, can influence human liver microsomal methadone N-demethylation.

Diminished methadone metabolism by expressed CYP2B6.6 and livers from individuals with the *CYP2B6**6 allele is consistent with previous reports of a genetic influence of CYP2B6 on methadone plasma concentrations. Dose-adjusted steady-state trough and peak plasma S- methadone concentrations were greater in homozygous carriers of *CYP2B6*6*, compared with heterozygotes and non-carriers (Crettol et al., 2005; Crettol et al., 2006; Eap et al., 2007). Dose-adjusted steady-state trough S-methadone concentrations were 2-fold higher in **6/*6* genotypes than non-carriers (Crettol et al., 2005). Another investigation found that *CYP2B6*6* homozygotes similarly needed lower methadone doses (Hung et al., 2011). *CYP2B6*6* carriers had higher plasma S-methadone concentrations and a higher concentration-to-dose ratio for both enantiomers (Wang et al., 2011). Mean methadone doses required by methadone maintenance patients were significantly lower in *CYP2B6*6/*6* genotypes than in heterozygotes or non-carriers (Levran et al., 2012). In a series of methadone-related deaths, whole blood RS-methadone concentrations were significantly (approximately 2-fold) higher in *CYP2B6*6* carriers than non-carriers (Bunten et al., 2010). Together these reports suggest that the *CYP2B6*6* allele influences methadone disposition, although there have been no published studies investigating the influence of *CYP2B6*6* or other polymorphisms on clinical methadone metabolism or clearance. Diminished methadone N-demethylation by CYP2B6.6 further supports these clinical observations, and may provide a mechanistic explanation.

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Authorship Contributions:

Participated in research design: Gadel, Kharasch

Conducted experiments: Gadel, Crafford, Regina

Performed data analysis: Gadel, Crafford, Regina, Kharasch

Wrote or contributed to the writing of the manuscript: Gadel, Crafford, Regina, Kharasch

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Footnote

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Legends for Figures

Figure 1

Recombinant CYP2B6-catalyzed methadone N-demethylation at therapeutic concentrations. Results are shown for (A) metabolism of individual methadone enantiomers (0.25-1 μM each) and (B) racemic methadone (0.5-2 μM, corresponding to 0.25-1 μM of each enantiomer) by CYP2B6.1 and CYP2B6.6. Results are the mean ± SD of 3-6 determinations. *Significantly different vs CYP2B6.1 ($p<0.05$).

Figure 2

Concentration-dependence and kinetics of recombinant CYP2B6-catalyzed N-demethylation of methadone to EDDP. Results are shown for metabolism of (A and B) individual methadone enantiomers (0.25-500 μM each) and (C and D) racemic methadone (0.5-1000 μM, corresponding to 0.25-500 μM of each enantiomer) by CYP2B6.1 and CYP2B6.6. Corresponding Eadie-Hofstee plots are shown in B and D. For ease of comparison to enantiomers metabolism (A and B), racemic methadone is shown as the concentration of the individual enantiomers (C and D). Symbols represent CYP2B6.1-catalyzed EDDP formation from R-methadone (△) and S-methadone (▽) (single enantiomers or the racemate) and CYP2B6.6-catalyzed EDDP formation from R-methadone (▲) and S-methadone (▼) (single enantiomers or the racemate). Each data point is the mean ± SD of 3-6 determinations. Lines represent rates predicted from nonlinear regression analysis of data using the Adair-Pauling equation.

Table 1 Kinetic Parameters for methadone *N*-demethylation

Parameter	Single Enantiomer Metabolism				Racemate Metabolism			
	CYP2B6.1		CYP2B6.6		CYP2B6.1		CYP2B6.6	
	R-EDDP formation	*S*-EDDP formation	*R*-EDDP formation	*S*-EDDP formation	*R*-EDDP formation	*S*-EDDP formation	*R*-EDDP formation	*S*-EDDP formation
K_s (μM)	75 ± 18	25 ± 5	97 ± 7	40 ± 7	40 ± 20	13 ± 3	57± 6	20 ± 3
V_{max} (nmol/nmol P450/min)	20.8 ± 0.6	14.6 ± 1.0	5.7 ± 0.3	3.5 ± 0.3	18.9± 7.4	8.7 ± 0.2	4.7 ± 0.6	5.1 ± 0.2
Cl_{int} (ml/min/nmol)	0.3 ± 0.1	0.6 ± 0.1	0.06 ± 0.01	0.09± 0.02	0.5 ± 0.3	0.7 ± 0.2	0.08 ± 0.01	0.3 ± 0.1

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Figure 1

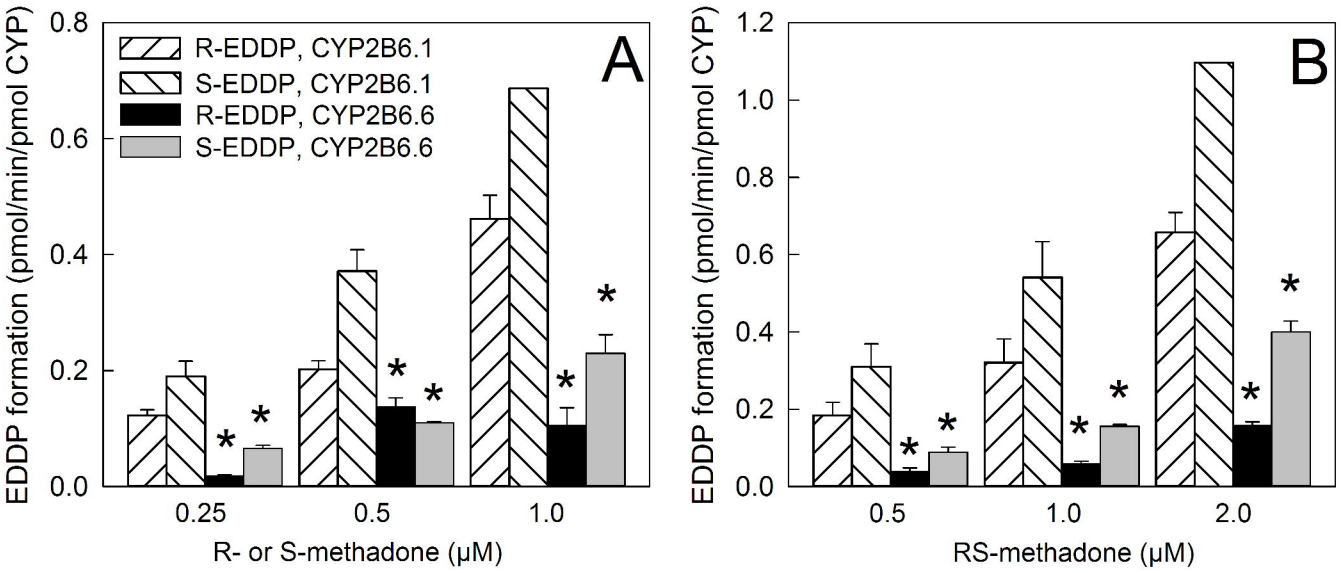

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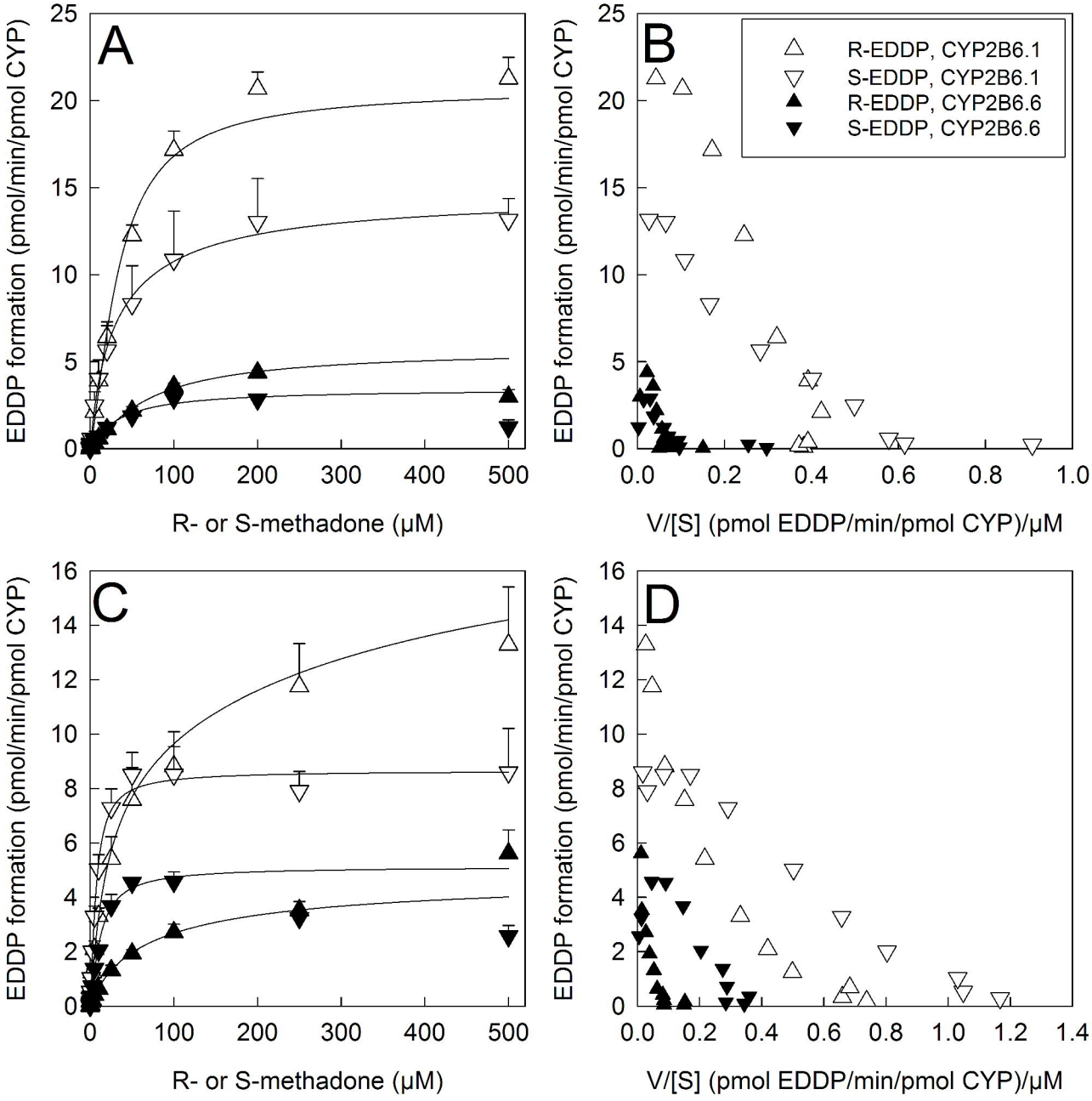
Figure 2



Methadone N-demethylation by the common *CYP2B6* allelic variant CYP2B6.6

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Drug Metabolism and Disposition 50625

Supplemental Table 1. *CYP2B6* genotype and human liver microsomal methadone metabolism

Liver pair	Liver	*CYP2B6* genotype	Description	CYP content (pmol/mg protein)		EDDP formation (pmol/mg/min)	
				CYP3A	CYP2B6	R-methadone	S-methadone
A	141	*1/*1	High 3A, high 2B6	381	69	26.2	55.5
	144	*1/*6	High 3A, moderate 2B6	351	27	20.4	27.0
B	124	*1/*6	High 3A, high 2B6	176	50	7.9	9.7
	148	*6/*6	High 3A, low 2B6	171	12	3.6	3.7
C	149	*1/*6	Moderate 3A, moderate 2B6	154	32	13.4	20.5
	166	*1/*1	Moderate 3A, low 2B6	146	14	2.3	3.7
D	139	*1/*4	Low 3A, high 2B6	70	81	10.7	22.8
	120	*1/*1	Low 3A, low 2B6	71	16	2.8	7.8
E	142	*1/*4	Low 3A, moderate 2B6	35	29	5.0	6.5
	164	*1/*6	Low 3A, low 2B6	36	7	0	1.4

Microsome pairs from different livers were matched to contain similar CYP3A protein content and high and low CYP2B6 contents. Liver microsomal CYP2B6 protein contents and formation of EDDP (2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine) from individual methadone enantiomers (1 μM) were originally reported by Totah *et al. (*Totah RA, Allen KE, Sheffels P, Whittington D and Kharasch ED (2007) Enantiomeric metabolic interactions and stereoselective human methadone metabolism. *J Pharmacol Exp Ther* **321:**389-399). Microsomal CYP3A protein content in liver pairs was reanalyzed and reported by Raccor *et al* (Raccor BS, Claessens AJ, Dinh JC, Park JR, Hawkins DS, Thomas SS, Makar KW, McCune JS and Totah RA (2012) Potential contribution of cytochrome P450 2B6 to hepatic 4-hydroxycyclophosphamide formation in vitro and in vivo. *Drug Metab Dispos* **40:**54-63). Results are the mean of two determinations.