Methadone *N*-Demethylation by the Common *CYP2B6* Allelic Variant *CYP2B6.6*^S

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Received December 8, 2012; accepted January 8, 2013

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 *CYP2B6*. Retrospective studies suggest that the common allele variant *CYP2B6*6* may influence methadone plasma concentrations. The catalytic activity of *CYP2B6.6*, encoded by *CYP2B6*6*, is highly substrate-dependent. This investigation compared methadone *N*-demethylation by *CYP2B6.6* with 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 μ M) 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 and metabolism by *CYP2B6.6* compared with *CYP2B6.1*, $V_{\max}$ was diminished, K_s was greater and 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, respectively. 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.

Introduction

The long-acting opioid methadone is used to treat opiate addiction, as well as acute and chronic pain related to cancer and other conditions. Clinical use of methadone is challenging, however, because of considerable and unpredictable inter- and intraindividual 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 (CDC, 2012).

Methadone in humans is cleared primarily by hepatic cytochrome P450 (P450)-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 CYP isoforms, is catalyzed most efficiently by *CYP2B6* and *CYP3A4* (whereas

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, 2008; Chang et al., 2011). 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 Stubbett, 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 30 *CYP2B6* protein variants identified to date. The *CYP2B6*6* allele (S16G>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

This work was supported by the National Institutes of Health [Grants R01-DA14211 and K24-DA0041].

dx.doi.org/10.1124/dmd.112.050625.

^SThis article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: Cl_{int} , intrinsic clearance; EDDP, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine; HLM, human liver microsome; K_s , equilibrium dissociation constant for the enzyme-substrate complex; P450, cytochrome P450.

(Xie et al., 2003; Desta et al., 2007; Hofmann et al., 2008). Expressed CYP2B6.6, compared with CYP2B6.1, has diminished catalytic activity toward bupropion and efavirenz (Zhang et al., 2011; Xu et al., 2012), modest-to-moderately increased activity toward 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), and a need for lower methadone doses (Hung et al., 2011; Levran et al., 2012) have also 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 is their influence on human liver microsomal methadone metabolism. In addition, the activity of CYP2B6.6 toward methadone metabolism is also unknown. Therefore, the purpose of this investigation was to evaluate methadone *N*-demethylation by CYP2B6.6 and to compare it with that by wild-type CYP2B6.1.

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 P450 reductase and human cytochrome *b₅* were purchased from BD Gentest Corporation (Woburn, MA). Microsomes containing CYP2B6.6 coexpressed with P450 reductase and cytochrome *b₅* were a generous gift from BD Gentest Corporation. All other reagents were from Sigma-Aldrich (St. Louis, MO).

Methadone Metabolism. Incubations (200 μ l, 10 pmol/ml CYP2B6) with RS-, R-, or S-methadone were performed as previously described (Totah et al., 2007, 2008), with minor modifications. Reactions (10-minute) were quenched with 20 μ l 20% trichloroacetic acid containing internal standard (d3-EDDP, final concentration 4.55 ng/ml) and centrifuged; the supernatant (200 μ l) was processed immediately by solid-phase extraction as described previously (Kharasch et al., 2004), except that Strata-X-C 33 μ m 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 high-pressure liquid chromatographer (Columbia, MD), Gerstel autosampler (Baltimore, MD), and Chrom Tech chiral AGP column (3 $\times$ 50 mm, 5 μ m) with chiral AGP guard cartridge (3 $\times$ 10 mm) (Apple Valley, MN), as described (Sharma et al., 2011), except that the mobile phase gradient was 20 mM ammonium formate (pH 5.0) and methanol. Retention times were 10.4 and 11.2 minutes, 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 $\pm$ S.D. (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 versus substrate concentration data were analyzed by nonlinear regression analysis (SigmaPlot 12.3) as

described previously (Totah et al., 2007, 2008) using the Adair-Pauling model, based on the recognition that CYP2B6 contains at least two binding sites. Results are the parameter estimate $\pm$ standard error of the estimate.

Results

N-demethylation of racemic methadone and individual enantiomers was evaluated at concentrations (0.25–2 μ M) 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 (Fig. 1A). When racemic methadone *N*-demethylation was evaluated (Fig. 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 (Fig. 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 was 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 Cl_{int} was diminished approximately 6- and 3-fold for R- and S-methadone, respectively.

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. In comparing CYP2B6.6 with CYP2B6.1, EDDP formation from both individual methadone enantiomers was diminished, K_s was increased, $V_{\max}$ was reduced, and the in vitro Cl_{int} 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 Cl_{int} 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 μ M 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.

Fig. 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 ± S.D. of 3–6 determinations. *Significantly different versus CYP2B6.1 ($P < 0.05$).

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, 2008; Chang et al., 2011), was preserved with CYP2B6.6.

Modeling of methadone *N*-demethylation by CYP2B6 is complex (Totah et al., 2007). Methadone enantiomer *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 enantiomer *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 which to evaluate such models in an identifiable fashion. Consequently, this investigation used the simplest model for which there is precedent. In a previous investigation of racemic methadone metabolism by CYP2B6.1, a competitive inhibitory interaction was found; each enantiomer in the racemate (R- or S-) inhibited the metabolism of its antipode (S- or R-methadone) (Totah et al., 2007). The Adair-Pauling model was found to be misspecified for racemic methadone, and a more complex model was required to describe methadone metabolism; however, this model required evaluating metabolism of a complex matrix of individual

Fig. 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 with 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 ± S.D. 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	
	<i>R</i> -EDDP Formation	<i>S</i> -EDDP Formation	<i>R</i> -EDDP Formation	<i>S</i> -EDDP Formation	<i>R</i> -EDDP Formation	<i>S</i> -EDDP Formation	<i>R</i> -EDDP Formation	<i>S</i> -EDDP Formation
K_s (μ M)	75 $\pm$ 18	25 $\pm$ 5	97 $\pm$ 7	40 $\pm$ 7	40 $\pm$ 20	13 $\pm$ 3	57 $\pm$ 6	20 $\pm$ 3
$V_{\max}$ (nmol/nmol P450/min)	20.8 $\pm$ 0.6	14.6 $\pm$ 1.0	5.7 $\pm$ 0.3	3.5 $\pm$ 0.3	18.9 $\pm$ 7.4	8.7 $\pm$ 0.2	4.7 $\pm$ 0.6	5.1 $\pm$ 0.2
Cl_{int} (ml/min/nmol)	0.3 $\pm$ 0.1	0.6 $\pm$ 0.1	0.06 $\pm$ 0.01	0.09 $\pm$ 0.02	0.5 $\pm$ 0.3	0.7 $\pm$ 0.2	0.08 $\pm$ 0.01	0.3 $\pm$ 0.1

Cl_{int} , intrinsic clearance; EDDP, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine; K_s , equilibrium dissociation constant for the enzyme-substrate complex; P450, cytochrome P450.

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. The simpler Adair-Pauling equation was used to model the data, accepting some mis-specification 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, from others 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*-de-ethylation was double that for CYP2B6.1 (Jinno et al., 2003). In COS-7 cells expressing CYP2B6.6, the Cl_{int} for 7-ethoxy-4-trifluoromethylcoumarin *O*-de-ethylation, selegiline *N*-demethylation, and selegiline *N*-depropagation was not different from that for CYP2B6.1 (Watanabe et al., 2010); the activity toward bupropion was significantly less (Hofmann et al., 2008), whereas the activity toward artemether was significantly greater (Honda et al., 2011). In an insect cell CYP2B6 expression system co-expressing P450 reductase but not cytochrome *b*₅, the Cl_{int} for efavirenz 8-hydroxylation by CYP2B6.6 was half that of CYP2B6.1, whereas 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, respectively, two-thirds, one-half, and one-fifth of that seen 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. 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} by CYP2B6.6 was approximately one-half that seen with CYP2B6.1, and bupropion 4-hydroxylation Cl_{int} was approximately 50% greater (Xu et al., 2012). In the present insect cell CYP2B6 expression system, which contained both coexpressed 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. The Cl_{int} for *N*-demethylation was approximately 5-fold lower. 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 P450 content and deficient catalytic efficiency may combine to cause the phenotype of decreased CYP2B6-catalyzed biotransformation in *CYP2B6**6 carriers. Compared with expressed enzyme systems, human liver microsomes may therefore show greater catalytic differences between CYP2B6.1 and CYP2B6.6 and the effect of the *CYP2B6**6 polymorphism. Indeed, in human liver microsomes from individuals with *6 genotypes, there was markedly diminished CYP2B6 protein expression, 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 requantified (Raccor et al., 2012); when pairs of livers were rematched for CYP3A content but different CYP2B6 content (or genotype) and methadone enantiomer metabolism was 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; lower *N*-demethylation of both methadone enantiomers was observed in human liver microsome (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 than in heterozygotes and noncarriers (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 in noncarriers (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 noncarriers (Levrano 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 in noncarriers (Bunten et al., 2011). 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.

Acknowledgments

The authors gratefully appreciate the generous gift of CYP2B6.6 Super-somes provided by Chris Patten, BD Gentest Corporation.

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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