

Role of CYP2B6 in Stereoselective Human Methadone Metabolism

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Background: Metabolism and clearance of racemic methadone are stereoselective and highly variable, yet the mechanism remains largely unknown. Initial *in vitro* studies attributed methadone metabolism to cytochrome P4503A4 (CYP3A4). CYP3A4 was also assumed responsible for methadone clearance *in vivo*. Nevertheless, recent clinical data do not support a primary role for CYP3A4 and suggest that CYP2B6 may mediate methadone clearance. Expressed CYP2B6 and also CYP2C19 *N*-demethylate methadone *in vitro*. This investigation tested the hypothesis that CYPs 2B6, 3A4, and/or 2C19 are responsible for stereoselective methadone metabolism in human liver microsomes and *in vivo*.

Methods: *N*-demethylation of racemic methadone and individual enantiomers by expressed CYPs 2B6, 2C19, and 3A4 was evaluated. Stereoselective microsomal methadone metabolism was quantified, compared with CYP 2B6 and 3A4 content, and probed using CYP isoform-selective inhibitors. A crossover clinical investigation (control, CYP2B6 and CYP3A4 induction by rifampin, CYP3A inhibition by troleandomycin and grapefruit juice) evaluated stereoselective methadone disposition.

Results: At clinical concentrations, methadone enantiomer *N*-demethylation by recombinant CYPs 2B6, 3A4, and 2C19 was $S > R$, $S = R$, and $S \ll R$. Greater stereoselective metabolism ($S > R$) occurred in livers expressing high levels of CYP2B6 compared with CYP3A4. Clopidogrel, troleandomycin, and (+)-*N*-3-benzyl-nirvanol, selective inhibitors of CYPs 2B6, 3A4, and 2C19, respectively, inhibited microsomal methadone metabolism by 50–60%, 20–30%, and less than 10%. Only inhibition by clopidogrel was stereoselective. Clinically, rifampin diminished both *R*- and *S*-methadone plasma concentrations, but troleandomycin and grapefruit juice altered neither *R*- nor *S*-metha-

done concentrations. Plasma *R/S*-methadone ratios were increased by rifampin but unchanged by CYP3A inhibition.

Conclusions: These results suggest a significant role for CYP2B6, but not CYP3A, in stereoselective human methadone metabolism and disposition.

METHADONE is an efficacious, inexpensive, cost effective, utilitarian μ -opioid agonist with clinical application in adults, children and even neonates. It is effective in the treatment of acute, chronic, neuropathic, and cancer pain and can be administered *via* intravenous, oral, nasal, rectal, epidural, and other routes.^{1,2} Methadone is used in opioid rotation strategies, is increasingly being used as a first-line analgesic, and is a mainstay in the treatment of opioid addiction. Methadone is usually administered as a racemic mixture of *R*- and *S*-enantiomers, although nearly all μ -opioid agonist activity resides in the *R*-enantiomer.³

Predictable and reproducible attainment and maintenance of methadone concentrations within therapeutic ranges is confounded by the considerable and unpredictable interindividual variability in methadone pharmacokinetics (particularly clearance), susceptibility to drug interactions, and a long elimination half-life.⁴ The potential consequences are inadequate treatment or unwanted side effects, such as withdrawal or respiratory depression. Methadone is mainly cleared by hepatic metabolism, with minor urinary excretion of unchanged drug.⁵ The primary metabolic route is *N*-demethylation by cytochrome P450 (CYP) enzymes to 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), which is pharmacologically inactive.⁵ There is large interindividual variability (20- to 100-fold) in methadone clearance.^{4,6} Moreover, methadone drug interactions occur with CYP inducers such as rifampin^{7,8} and phenytoin,⁹ and CYP inhibitors such as antidepressants, antifungal agents, and antiretroviral drugs.⁴

For more than a decade, methadone clearance has been attributed to CYP3A4. Early *in vitro* studies demonstrated the involvement of CYP3A4 in methadone *N*-demethylation by human liver or intestinal microsomes and complementary DNA (cDNA)-expressed P450s.^{10–15} Based on these *in vitro* studies and well-intended extrapolation, methadone metabolism and clearance *in vivo* have been attributed to CYP3A4.^{4,14,16} Some evidence supports this supposition. For example, methadone clearance is affected by CYP3A4 inducers such as rifampin and inhibitors such as antifungals.¹⁴ Nevertheless, rifampin is not a specific inducer of CYP3A4 (inducing other CYP isozymes, most notably

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CYP2B6^{17,18}), and antifungal agents including fluconazole inhibit several other P450s in addition to CYP3A4.¹⁹ *In vivo* comparisons of methadone clearance with CYP3A4 activity produced unconvincing correlations.^{13,14,20} Therefore, **clinical evidence to support an *in vivo* role for CYP3A4 is weak.** Furthermore, more direct evidence against a primary role for CYP3A4 in clinical methadone metabolism and clearance was recently published.¹³ Another consideration is that **methadone metabolism *in vivo* is stereoselective,**⁴ yet **methadone *N*-demethylation by CYP3A4 *in vitro* was not stereoselective.**^{11,21} All of these data engender marked doubt for a major role for CYP3A4 in clinical methadone metabolism and clearance.

Recent evidence suggests an important role for CYP2B6 in methadone metabolism. Expressed CYP2B6 was recently shown to metabolize methadone *in vitro*, with activity at least as high as CYP3A4,^{11,13,21} and was suggested to be a major enzyme responsible for clinical methadone metabolism and clearance.¹³ **CYP2B6 is expressed in human liver, accounts for up to 10% of total hepatic P450, is variably and polymorphically expressed, and is affected by many of the same inhibitors and inducers (*i.e.*, phenobarbital and rifampin) as CYP3A4.**²²

Nevertheless, **the identity of the CYPs responsible for methadone metabolism and clearance remains highly controversial.** In addition, the mechanism of stereoselective methadone metabolism and disposition *in vivo* remains incompletely understood. Insufficient understanding of methadone metabolism and clearance is a barrier to predictable dosing and successful therapeutic outcomes.

This investigation tested the hypothesis that CYP2B6 and/or CYP3A4 are responsible for stereoselective human methadone metabolism in liver microsomes and *in vivo*. Because **CYP2C19 has also been reported to metabolize methadone, this isoform was also evaluated.**^{11,21} Experiments evaluated the metabolism of methadone racemate and individual enantiomers by expressed CYPs 2B6, 2C19, and 3A4, and the role of these isoforms in human liver microsomal metabolism of methadone racemate and enantiomers. The influence of CYP3A4 and/or CYP2B6 on the stereoselective disposition of methadone *in vivo* was evaluated by reanalyzing samples from a previously published clinical investigation of methadone pharmacokinetics and CYP induction or inhibition.

Materials and Methods

Materials

(*RS*)-(6-Dimethylamino-4,4-diphenyl-heptan-3-one) hydrochloride (*RS*-methadone), (*S*)-(6-dimethylamino-4,4-diphenyl-heptan-3-one) hydrochloride (*S*-methadone, 99% enantiomeric excess), troleandomycin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and β -NADP were purchased from Sigma (St. Louis, MO). (*R*)-(6-Dimethylamino-4,4-diphenyl-heptan-3-one) hydrochloride (*R*-methadone, 99% enantiomeric excess) was purchased

from Research Biochemicals Inc. (Natick, MA). (*RS*)-6-Dimethylamino-4,4-diphenyl-1,1-trideuterioheptan-3-one (d3-methadone) was purchased from Cerilliant (Austin, TX). (\pm)-2-Ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine perchlorate (EDDP) and [ethyl-2',2',2'-²H₃]-3,3-diphenyl-2-ethyl-5-methyl-1-pyrroline hydrochloride (d3-EDDP) were obtained from the National Institute on Drug Abuse through Research Triangle Institute (Research Triangle Park, NC). Clopidogrel hydrogen sulfate was obtained from ChemPacific (Baltimore, MD), and (+)-*N*-3-benzyl-nirvanol was a generous gift from Allan Rettie, Ph.D. (Professor and Chairman, Department of Medicinal Chemistry, University of Washington, Seattle, WA). Chromatography-grade methanol, zinc sulfate, glacial acetic acid, and ammonium formate were purchased from Fisher Scientific (Pittsburgh, PA). Outdated human plasma was obtained from Puget Sound Blood Center (Seattle, WA) and was pooled from several donors. Baculovirus-insect cell microsomes (Supersomes[®]) containing expressed CYP3A4, CYP2B6, or CYP2C19 enzymes with coexpressed human cytochrome P-450 reductase and coexpressed human cytochrome *b*₅ were purchased from BD Gentest Corporation (Woburn, MA). Human liver microsomes (HLMs) were prepared as described previously.²³ Specific anti-CYP2B6 immunoquantitation (catalog No. 458226) antibody was also from BD Gentest Corporation. All stock drug solutions, buffers, and mobile phase were prepared using Milli-Q-grade water (Millipore, Bedford, MA).

Determination of CYP2B6 Protein Content in Microsomes

Human liver samples (n = 60) were obtained from the University of Washington School of Pharmacy Human Tissue Bank. Relevant information about the bank tissues has been published previously.²³ Microsomes were prepared as described previously.²⁴ Protein concentrations were determined by the method of Lowry using bovine serum albumin as a reference. Immunoquantitation of CYP2B6 was performed by Western blot analysis using a specific CYP2B6 antibody (BD Gentest Corporation) as described.²⁴ Control experiments independently confirmed that the CYP2B6 antibody did not cross react with CYP1A2, CYP2C9/19, CYP3A4, or CYP3A5. The secondary antibody was an alkaline phosphatase-conjugated antibody, and quantitation was performed by integrating the optical density for each alkaline phosphatase substrate 5-bromo-4-cholor-3-indolyl phosphate/nitro blue tetrazolium developed band using a ChemiDoc Imager (Bio-Rad, Hercules, CA) and Quantity One Software (Bio-Rad). Unknown CYP2B6 protein levels were determined by comparison to a standard curve using CYP2B6 Supersomes[®] (BD Gentest; specific content 140 pmol/mg protein). Reported CYP2B6 protein contents are the average of three individual determinations measured on different occasions which did not vary by more than 20%.

CYP3A4 and CYP3A5 protein content for the same HLMs were measured and reported elsewhere.²³

DNA Analysis

To assess the effect of CYP2B6 genotype on methadone metabolism, an array of liver samples was analyzed as follows: DNA was isolated from liver samples using commercially available kits (QIAGEN GmbH, Hilden, Germany). All single nucleotide polymorphism detection assays were performed in the DNA Sequencing and Gene Analysis Center at the University of Washington using an Applied Biosystems HT SDS Real-Time PCR System (Foster City, CA). Samples were genotyped using 5'-nuclease assays using mgb-probes 5' labeled with either VIC or FAM and a black hole quencher (BHC) on the 3' terminus. Samples were analyzed for the 785 A>G (CYP2B6*4, *6, or *7), 516 G>T (CYP2B6*9, *6, or *7), and 1459C>T (CYP2B6*5 or *7) variants. The primer and probe sets for 516 G>T and 1459C>T were purchased from Applied Biosystems under their Drug Metabolizing Enzymes genotyping product line. Genotyping for CYP2B6 785 A>G variant was performed using polymerase chain reaction followed by DNA sequencing (Big Dye version 3.1) on a capillary sequencing instrument (Applied Biosystems 3100) as described by Lang *et al.*²⁵

General Incubation Conditions

Typical incubation conditions were as follows: All incubations were performed in 96-well plates using HLMs, CYP2B6, CYP3A4, or CYP2C19 Supersomes[®]. Incubations were performed in 100 mM potassium phosphate buffer (pH 7.4) and contained enzyme (0.1 mg/ml HLM, 10 pmol/ml CYP3A4 or CYP2B6, or 20 pmol/ml CYP2C19) and *RS*-, *R*-, or *S*-methadone. Incubations were equilibrated in a shaking water bath (37°C) for 5 min before the reaction was initiated with a nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system (final concentrations: 10 mM glucose-6-phosphate, 1 mM β -NADP, 1 U/ml glucose-6-phosphate dehydrogenase, and 5 mM magnesium chloride, preincubated at 37°C for 10 min), unless otherwise indicated. Typically, incubations were quenched after 10 min (CYP2B6, CYP3A4) or 20 min (CYP2C19) with 15% zinc sulfate (for chiral analysis) or 20% trichloroacetic acid (for achiral analysis), and internal standard (d3-EDDP, 250 ng/ml) was added and then placed on ice before centrifugation for 10 min at 1,800g, unless other conditions are specified. Both the quench and the internal standard were added at 10% of the final incubation volume. The supernatant was transferred to a clean 450 μ l conical bottom 96-well plate for EDDP analysis. Results were background subtracted (due to 0.2% and 0.4% EDDP in *R*- and *S*-methadone, respectively) using controls lacking NADPH.

Specific Chemical Inhibition of CYP3A4, CYP2B6, and CYP2C19

To determine the contribution of individual isozymes to methadone metabolism in microsomal preparations, experiments using specific inhibitors for CYP2B6, CYP3A4, and CYP2C19 were performed. Incubations (final volume 0.25 ml), containing HLM (0.1 mg/ml), *RS*-methadone (2 μ M) as substrate, and (+)-*N*-3-benzyl-nirvanol (final concentration 1 μ M), were preincubated at 37°C for 5 min before initiation of the reaction by adding the NADPH regenerating system. Similar mixtures containing troleandomycin (final concentration 100 μ M) and/or clopidogrel (final concentration 1 μ M) were preincubated with enzyme, inhibitor, and NADPH regeneration system for 15 min before initiation of the reaction by adding methadone. Incubations containing all three inhibitors were performed similar to incubations containing troleandomycin alone. Control incubations (containing 0.17% methanol) were treated identically to those with inhibitor and were performed for all experiments. After 10 min reaction with substrate, incubations were quenched and prepared for chiral EDDP analysis as described above in General Incubation Conditions.

Determination of K_m and V_{max} in HLMs

Incubations with HLMs (final volume 0.2 ml) were performed as described under General Incubation Conditions using a range of *RS*-methadone concentrations (0.5–1,500 μ M). Experiments were performed under conditions where the rate of EDDP formation was linear with protein concentration (0.1 mg/ml) and time of incubation (10 min). Reactions were initiated by adding NADPH regenerating system and terminated after 10 min. Control incubations contained 100 mM potassium phosphate buffer instead of the NADPH regenerating system and were performed for all the concentrations used to generate the K_m and V_{max} . Determination of *R*- and *S*-EDDP by chiral analysis was performed as described below in Chiral Analysis of EDDP.

Achiral Analysis of EDDP

EDDP formation from *R*- or *S*-methadone metabolism was measured by liquid chromatography-mass spectrometry using selected ion monitoring after solid phase extraction, as described previously.²⁶ Incubations were quenched with 20% trichloroacetic acid and internal standard added as above. The quenched mixture was then applied to an MCX extraction cartridge for clean-up.²⁶ Samples (10 μ l) were applied on to a Zorbax Eclipse XBD column (2.1 \times 50 mm, 5 μ m) with a Zorbax Eclipse C8 guard column (2.1 \times 12.5 mm, 5 μ m) (Agilent, Palo Alto, CA) and analyzed on an Agilent 1100 high-pressure liquid chromatograph-mass spectrometer. The mobile phase gradient (0.25 ml/min) started at 32% acetonitrile in 0.05% trifluoroacetic acid for 4 min, increased sharply to 50% at 5 min and to 90% at 5.5 min,

and was held at 90% for 1.5 min before reequilibrating at 32% acetonitrile for 3.5 min. Under these conditions, EDDP and d3-EDDP eluted at 3.6 min and were monitored at m/z 278 and 281, respectively. The nitrogen drying gas was at 325°C and 6 l/min, fragmentor at 100 V, nebulizer pressure 20 psi, and the capillary at 2000 V. Standard curves were linear ($r^2 > 0.99$) over the range of 1–1,000 ng/ml EDDP. The intraday and interday coefficients of variation were 15% or less for all quality control standards.

Chiral Analysis of EDDP

Chiral analysis of *R*- and *S*-EDDP formed from *RS*-methadone metabolism was achieved using a liquid chromatography–mass spectrometry method, developed for measuring EDDP in plasma,²⁷ and modified as described under Clinical Investigation, with the exception that the supernatant from the deproteinized incubation mixture (20–85 μ l injection) was analyzed. Analytes were quantified using standard curves of peak area ratios. Standard curves were linear ($r^2 > 0.99$) over the range of 2.5–500 ng/ml for each EDDP enantiomer. The intraday and interday variations were 15% or less for all quality control standards.

Clinical Investigation

Plasma samples from a previous clinical investigation of methadone disposition (stored at -20°C), originally analyzed using an achiral assay, were reanalyzed using a chiral assay. Healthy volunteers received simultaneous oral deuterium-labeled and intravenous unlabeled methadone, in a randomized, balanced, four-way crossover after pretreatment with rifampin (hepatic/intestinal CYP induction), troleandomycin (hepatic/intestinal CYP3A inhibition), grapefruit juice (selective intestinal CYP3A inhibition), or nothing.¹³ Plasma methadone and EDDP enantiomer concentrations were determined by deproteination, on-line solid phase extraction, chiral chromatography, and mass spectrometry as previously described²⁷ with the following changes: Samples were reconstituted with 225 μ l aqueous ammonium formate (20 mM, adjusted to a pH of 5.7 with formic acid), the injection volume was 75 μ l, and the final concentration of methanol in the gradient was 35%. Retention times were 12.5 and 14 min for *R*- and *S*-EDDP and 13.0 and 13.9 min for *R*- and *S*-methadone, respectively. Chiral concentrations were obtained by application of enantiomeric ratios to the original achiral quantitation.

Data and Statistical Analysis

Analytes were quantified using standard curves of peak area ratios. Minimum validation criteria were as follows: calibration curves $r^2 > 0.95$, quality control standards $\leq 15\%$ deviation and $< 20\%$ approaching the limit of quantitation. Incubations were performed in triplicate and results reported as the mean \pm SD unless otherwise indicated. Differences between groups were determined

by analysis of variance followed by the Student-Newman-Keuls test (SigmaStat 3.5.1; Systat, Point Richmond, CA). Microsomal velocity-*versus*-substrate concentration data were analyzed by nonweighted nonlinear regression analysis (SigmaPlot 9.01; Systat) using several models (two-site, two-substrate model, Hill cooperative model, *etc.*) based on the recognition that CYPs 3A4 and 2B6 contain at least two binding sites, as described previously.^{21,26,28–31} Choice of model and goodness of fit were guided by the Eadie-Hofstee curves and r^2 , distribution of residuals, SE of the parameter estimates, and corrected Akaike information criterion. When Eadie-Hofstee plots were linear, velocity-*versus*-substrate data were analyzed using a simple Michaelis-Menten model:

$$V = (V_{\max} \cdot S)/(K_m + S).$$

Results are expressed as the parameter estimate \pm SE of the estimate. Correlations were analyzed using the Pearson product-moment test, or the Spearman rank order test for nonnormally distributed data. Terminal slopes of plasma concentration-*versus*-time data were determined using WinNonlin (Pharsight Corp., Mountain View, CA).

Results

Stereoselective Methadone Metabolism by Recombinant P450s and HLMs

N-Demethylation of (*RS*) and *R*- and *S*-methadone to EDDP at therapeutic concentrations (2 or 1 μM) was determined. Stereoselectivity of EDDP enantiomer formation from (*RS*)-methadone and methadone enantiomers catalyzed by recombinant CYPs 2B6, 3A4, and 2C19 was $S > R$, $S = R$, and $S \ll R$ EDDP (fig. 1A). There was variability between various lots of recombinant CYP2B6 in the absolute rates of EDDP formation but consistency in the stereoselectivity of methadone *N*-demethylation ($S > R$). There was relative consistency between various lots of CYP3A4 in the absolute rates of EDDP formation and in the lack of stereoselectivity. There was no evidence for metabolic interconversion (*i.e.*, no *S*-EDDP was formed from *R*-methadone, and no *R*-EDDP was formed from *S*-methadone).

Human liver microsomal methadone metabolism generally showed greater EDDP formation from *S*-methadone, whether as the single enantiomer or in the racemate (fig. 1B). There was, however, variability between livers in the degree of enantioselectivity. Microsomes with CYP2B6 content which was comparable to or higher than that of CYP3A4 (for protein content, see table 1) generally metabolized *S*-methadone at higher rates than *R*-methadone, whether from the racemate or from individual enantiomers. Conversely, microsomes with lower CYP2B6 content compared with CYP3A4 generally metabolized both *R*- and *S*-methadone enanti-

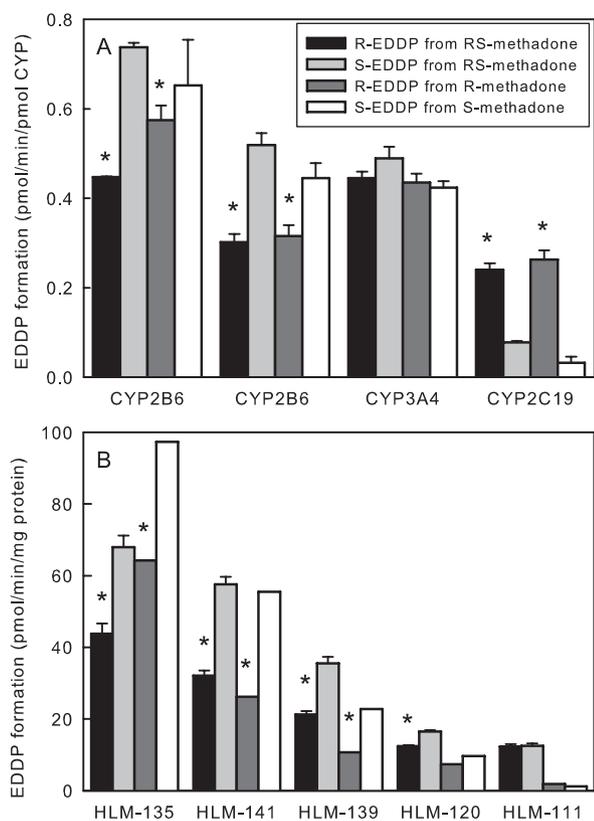


Fig. 1. Selective formation of R-EDDP from RS and R- and S-methadone by recombinant P450 (CYP; A) or human liver microsomes (HLMs; B). Methadone (1 μM single enantiomer or 2 μM racemate) was incubated with 5 pmol recombinant CYP or 0.1 mg/ml microsomal protein. Data are the mean \pm SD ($n = 3$ or 4) or mean of duplicates where SD is not shown. Results are shown for two different lots of CYP2B6. * Significant difference between R- and S-EDDP formation ($P < 0.05$). EDDP = 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine.

omers at similar rates and without stereoselectivity (*i.e.*, HLM-111 and -120).

To assess further the role of CYP2B6 in methadone metabolism, the entire University of Washington School of Pharmacy human liver bank ($n = 60$) was screened for CYP2B6 content. CYP2B6 protein was detected in all liver samples (fig. 2). Median CYP2B6 protein content was 14.2 pmol/mg protein; however, expression was highly variable (6.1–95.6 pmol/mg protein). CYP2B6 content was positively and significantly correlated with CYP3A4 protein content ($r = 0.58$, $P < 0.001$; fig. 2, inset).

From the entire set of HLMs shown in figure 2, a subset of HLM pairs was identified in which each pair contained similar CYP3A4 protein content but disparate (high and low) levels of CYP2B6. Rates of EDDP formation from R- and S-methadone (1 μM) were then determined to assess the significance of CYP2B6 in microsomal methadone metabolism (table 1). In general, in the livers containing greater amounts of CYP2B6, N-demethylation of both methadone enantiomers was greater, and greater stereoselectivity ($S > R$) in methadone metabolism was observed. Furthermore, in this limited data set, a better correlation was observed between methadone enantio-

Table 1. EDDP Formation from R- or S-Methadone by Human Liver Microsomes

HLM (Genotype)	Cytochrome P450 Content, pmol/mg protein		EDDP Formation, pmol \cdot min ⁻¹ \cdot mg ⁻¹	
	CYP3A4	CYP2B6	R-Methadone	S-Methadone
HLM-139 (*1/*4)	67	81	10.7	22.8
HLM-120 (*1/*1)	69	16	7.41	9.67
HLM-141 (*1/*1)	90	71	26.2	55.5
HLM-111 (*1/*5)	95	15	1.87	1.2
HLM-124 (*1/*6)	171	50	7.9	9.7
HLM-148 (*6/*6)	170	12	3.6	3.7
HLM-118 (*1/*6)	20	35	1.5	2.1
HLM-128 (*1/*1)	20	7	BLQ	2.7
HLM-142 (*1/*1)	32	29	5	6.5
HLM-164 (*1/*6)	34	7	BLQ	1.4
HLM-149 (*1/*6)	151	32	13.4	20.5
HLM-166 (*1/*1)	144	14	2.3	3.7
HLM-170 (*1/*6)	197	53	7.3	6.9
HLM-144 (*1/*6)	187	27	20.4	27.0

Microsome pairs from different livers were matched to contain similar CYP3A4 protein content and high and low CYP2B6 content. Microsomes (0.1 mg protein/ml) were incubated with individual methadone enantiomers (1 μM , 0.5 ml final volume, 10-min incubation). Results are the mean of two incubations. BLQ = below the limit of quantification (1 ng/ml EDDP); EDDP = 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine; HLM = human liver microsome sample.

mer N-demethylation and CYP2B6 content (Pearson $r = 0.63$ and 0.66 for R- and S-methadone, respectively; both $P < 0.02$) than with CYP3A4 ($r = 0.38$ and 0.16 for R- and S-methadone, respectively; not significant) (not shown). Stepwise forward or reverse regression analysis of EDDP formation versus CYP2B6 protein content was not improved by including CYP3A4 in the analysis. CYP3A5 protein content was not included in the analysis because CYP3A5 did not catalyze methadone N-demethylation (not shown), as observed previously.¹³

Effect of CYP2B6 Genotype on Methadone N-Demethylation

The entire liver bank was analyzed for CYP2B6 *4, *5, *9, *6, and *7 alleles, which represent the most common polymorphisms.³² There was no consistent relation between CYP2B6 genotype and methadone N-demethylation (table 1). Only one HLM sample (HLM-148) carried the *6/*6 genotype, which has been associated with slow metabolizer status for some CYP2B6 substrates.³³ Therefore, no definitive conclusions could be drawn from this limited data set on CYP2B6 genotype and methadone metabolism *in vitro*.

Effect of Selective Chemical Inhibitors on Methadone Enantiomer Metabolism by HLMs

The role of CYP2B6, CYP3A4, and CYP2C19 in methadone enantiomer metabolism by HLMs was investigated using isoform-selective chemical inhibitors and 2 μM RS-methadone (fig. 3). Each pair of HLMs contained

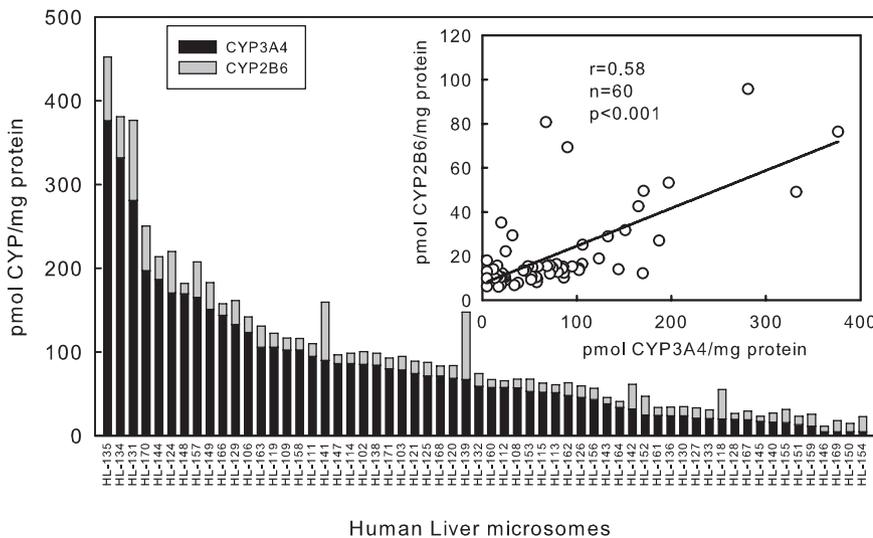


Fig. 2. Expression of CYP2B6 (gray bars) and CYP3A4 (black bars) protein in human liver microsomes. The samples were previously characterized for CYP3A4 and CYP3A5 protein content.²³ Correlation between CYP2B6 and CYP3A4 protein contents is shown in the inset. The Spearman rank order coefficient was significant. There was no improvement in the correlation when CYP2B6 was compared with total (CYP3A4 + CYP3A5) CYP3A content (not shown).

comparable CYP3A4 protein content and CYP2C19 activity (characterized using *S*-mephenytoin as a CYP2C19 probe; data not shown) but disparate CYP2B6 protein contents. The CYP2B6-, CYP3A4-, and CYP2C19-selective chemical inhibitors (used at concentrations and conditions described previously) were clopidogrel,³⁴ troleandomycin,³⁵ and (+)-*N*-3-benzyl-nirvanol,³⁶ respectively. *N*-Benzyl-nirvanol nonsignificantly inhibited less than 15% of *R*- and *S*-EDDP formation, although control reactions showed that greater than 90% of cDNA-expressed CYP2C19 activity was inhibited under similar experimental conditions (not shown). Troleandomycin inhibited 27–66% of EDDP formation and had comparable effects on the two methadone enantiomers. Clopidogrel caused the greatest decrease in methadone metabolism, inhibiting 14–54% of *R*-EDDP and 34–80% of *S*-EDDP formation. Inhibition was greatest in HLMs containing more CYP2B6 compared with CYP3A4, with greater inhibition of *S*- versus *R*-EDDP formation. Together, clopidogrel and troleandomycin inhibited 60–90% of *R*- and *S*-EDDP formation, strongly suggesting that CYP3A4 and CYP2B6 are the major isozymes responsible for methadone metabolism in HLMs.

Influence of CYP2B6 on HLM Methadone N-Demethylation Kinetics

RS-Methadone metabolism was determined in two HLM samples (139 and 148) containing contrasting amounts of CYP2B6 (fig. 4). Both HLMs exhibited saturation kinetics and apparently linear Eadie-Hofstee curves; therefore, kinetic data were analyzed using a single enzyme Michaelis-Menten model (table 2).# Me-

tabolism of both methadone enantiomers by HLM-139 was twofold to threefold greater than by HLM-148. The CYP2B6 content of HLM-139 was substantially greater than that of HLM-148, and CYP3A content was less than half. In HLM-139, with a high CYP2B6 content compared with CYP3A4, *S*-methadone intrinsic clearance was greater than that of *R*-methadone. Conversely, HLM-148, with relatively little CYP2B6 relative to CYP3A4, metabolized both methadone enantiomers similarly.

Effect of CYP2B6 and CYP3A4 Modulation on Clinical Methadone Enantiomer Disposition

In a previous clinical investigation in healthy human volunteers, simultaneous intravenous and oral (deuterium-labeled) racemic methadone was dosed after pretreatment with rifampin (CYP induction), troleandomycin or grapefruit juice (CYP3A inhibition), or nothing, and plasma was analyzed using an achiral assay.¹³ There was **no effect of CYP3A inhibition, whereas CYP3A/2B6 induction increased methadone clearance.** Nevertheless, this study did not evaluate potential enantioselective effects of CYP2B6 and CYP3A4 modulation. Therefore, the samples were re-analyzed using a chiral assay to quantify *R*- and *S*-methadone enantiomers. **There were no significant differences between controls and troleandomycin- or grapefruit juice-treated subjects in the plasma concentration–time profile of either *R*- or *S*-methadone after either intravenous or oral administration** (fig. 5). However, rifampin pretreatment caused a significant decrease in both *R*- and *S*-methadone concentrations after both oral and intravenous administration. Careful inspection of figure 5 shows differences in the slopes of the elimination curves for *R*- and *S*-methadone, and it is known that the plasma *R/S*-methadone ratio increases over time.⁶ Replotting results from figure 5 as the *R/S*-methadone ratio showed the expected time-dependent increase in the ratio (fig. 6). This increase was augmented by rifampin pretreatment compared with controls, whereas troleandomycin and grapefruit juice had no significant effect.

Human liver microsomes contain at least two P450s that metabolize methadone (2B6 and 3A4), and previous investigations reported biphasic kinetics and nonlinear Eadie-Hofstee curves.^{13,26} Nonetheless, for HLM-139 and HLM-148, Eadie-Hofstee plots were linear. Data analysis using a dual-substrate–dual-site model or the Hill cooperative model yielded extremely poor fits (based on r^2 values and residuals analysis), and a single enzyme Michaelis-Menten kinetic model provided improved fits over the more complex models.

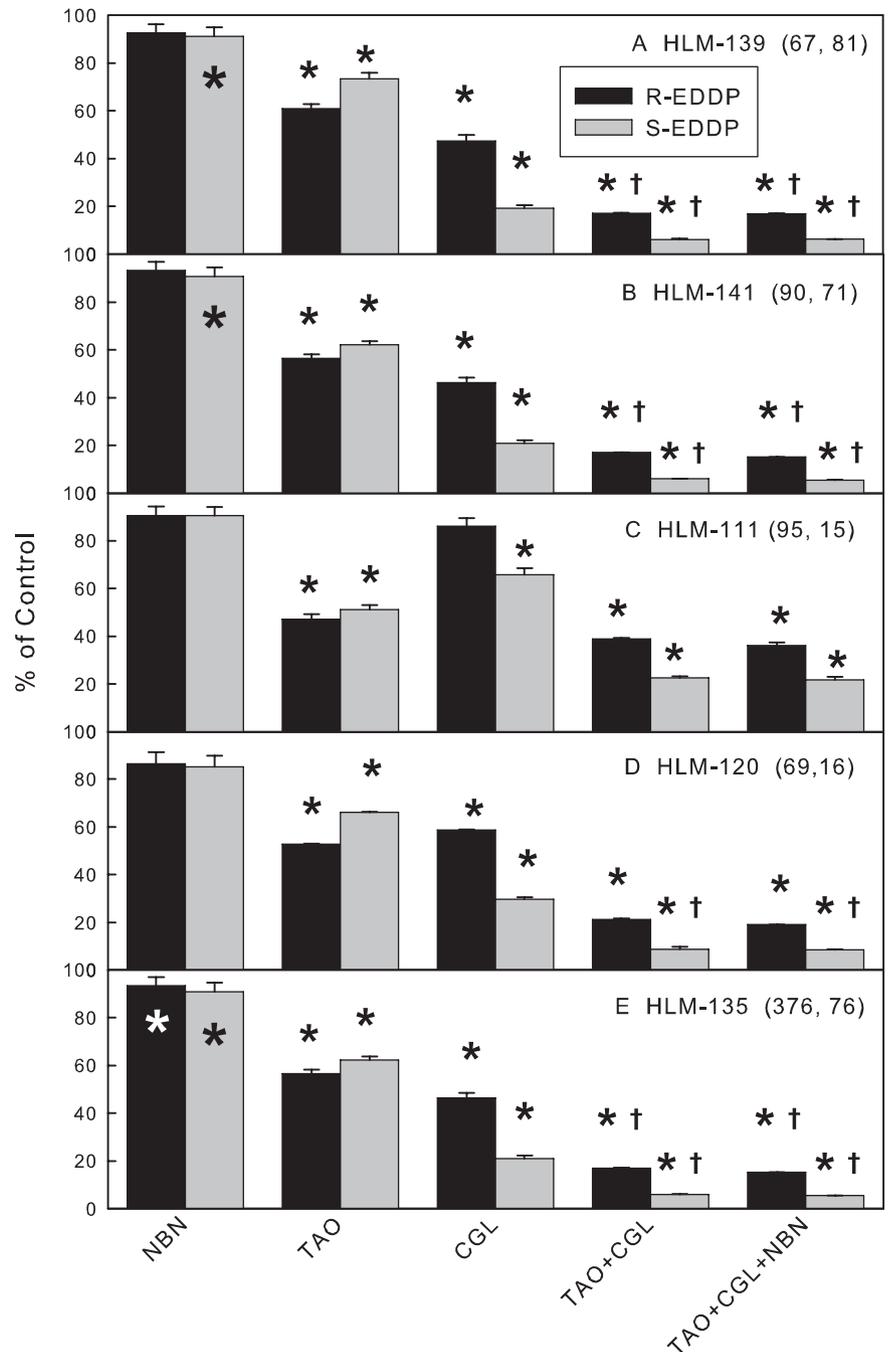


Fig. 3. Influence of P450 isoform-selective chemical inhibitors on methadone *N*-demethylation by human liver microsomes (HLMs). Results are shown for five livers (A–E). CYP3A4 and CYP2B6 contents (respectively, pmol/mg protein) are shown in parentheses. Inhibitors were troleanomycin (TAO, 100 μ M), clopidogrel (CGL, 1 μ M), and (+)-*N*-3-benzyl-nirvanol (NBN, 1 μ M). *R*- and *S*-EDDP formation was determined from 2 μ M *RS*-methadone. Results are the mean \pm SD ($n = 3$). Control rates of *R*- and *S*-EDDP formation ($\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) were as follows: HLM-139, 21 \pm 1 and 35 \pm 2; HLM-141, 32 \pm 1 and 58 \pm 2; HLM-111, 12 \pm 1 and 13 \pm 1; HLM-120, 12 \pm 1.0 and 16 \pm 1; HLM-135, 44 \pm 3 and 68 \pm 3, respectively. * Significantly different from control ($P < 0.05$). † Significantly different from troleanomycin ($P < 0.05$). EDDP = 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine.

Discussion

One major finding of this investigation is that CYP2B6 is a major contributor to human liver microsomal *N*-demethylation of methadone at steady state therapeutic concentrations (1–2 μ M).⁶ CYP2B6 protein was quantifiable in all liver samples examined from a panel of 60 HLMs. EDDP formation rates from *R*- and *S*-methadone were significantly correlated with CYP2B6 but not CYP3A4 protein contents. The CYP2B6-selective inhibitor clopidogrel exhibited the largest inhibitory effect on methadone *N*-dealkylation in HLMs. Recombinant CYP2C19 catalyzed significantly less metabolism than CYP2B6 or CYP3A4, and CYP2C19 inhibition had little effect on HLM methadone metabolism,

suggesting that CYP2C19 is relatively nonparticipatory in HLM methadone metabolism.

An equally major finding is that CYP2B6, but not CYP3A4, is responsible for the stereoselective *N*-demethylation of methadone in HLMs and by recombinant enzymes. At clinically relevant substrate concentrations, CYP2B6 *N*-dealkylated *S*-methadone at higher rates than *R*-methadone, consistent with the higher *in vivo* clearance of *S*-methadone.³⁷ CYP2C19 had the opposite stereoselectivity, and CYP3A4 showed no enantiomeric preference. Microsomal samples with a high ratio of CYP2B6 to CYP3A4 protein content metabolized *S*-methadone at a higher rate than *R*-methadone, whereas mi-

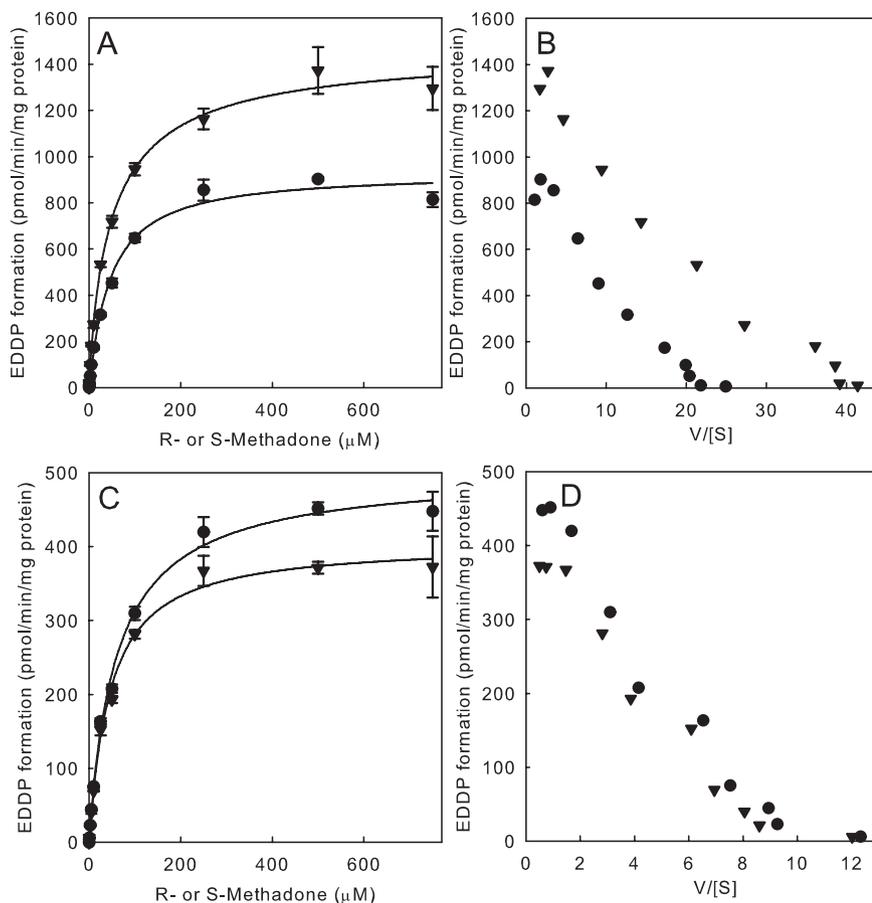


Fig. 4. Human liver microsomal *N*-demethylation of *RS*-methadone. Microsomes were incubated with 0.5–1,500 μM racemic methadone and EDDP enantiomer concentrations determined by stereoselective liquid chromatography–mass spectrometry. Results are shown for *R*-EDDP (\bullet) and *S*-EDDP (\blacktriangledown) formation as a function of individual enantiomer concentrations (*A* and *C*) and the corresponding Eadie-Hofstee plots (*B* and *D*). *A* and *B* show HLM-139 (CYP3A4 and CYP2B6 content 67 and 81 pmol/mg, respectively). *C* and *D* show HLM-148 (CYP3A4 and CYP2B6 content 170 and 12 pmol/mg, respectively). Data points are the mean \pm SD ($n = 3$). Lines are rates predicted by nonlinear regression analysis using a single enzyme Michaelis-Menten model. Kinetic parameters are reported in table 2. EDDP = 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine.

rosomes high in CYP3A4 compared with CYP2B6 metabolized both enantiomers similarly. Furthermore, inhibition of HLM methadone *N*-demethylation by CYP2C19- and CYP3A4-selective chemical inhibitors was not stereoselective, whereas CYP2B6 inhibition by clopidogrel was clearly stereoselective.

The current results agree with previous studies on methadone metabolism by expressed CYP isoforms and HLM.^{11,13} Both previous investigations found that expressed CYP2B6 catalyzed methadone *N*-demethylation at rates approximating or greater than those for CYP3A4. P450s (without coexpressed cytochrome *b*₅) showed methadone stereoselectivities, as assessed by substrate depletion ($S > R$, $S = R$, and $S \ll R$ for CYPs 2B6, 3A4, and 2C19) or EDDP formation ($S > R$ and $S \ll R$ for

CYPs 2B6 and 2C19),¹¹ similar to those herein. In microsomes from two human livers with high and low CYP2B6 content, the former generated more EDDP, despite equivalent CYP3A4 contents.¹¹ Anti-CYP2B6 antibody inhibited EDDP formation in microsomes from a liver with high, but not low, CYP2B6 content.¹³ Nevertheless, most previous investigations evaluated racemic methadone metabolism, using either achiral or chiral assays, whereas the current investigation is the first comprehensive evaluation of both racemic and methadone enantiomer metabolism, using a large panel of HLMs, P450s with coexpressed cytochrome *b*₅, and CYP-specific chemical inhibitors. All results demonstrate a significant role for CYP2B6 in methadone *N*-demethylation and in its stereoselectivity.

Table 2. Kinetic Parameters for *RS*-Methadone *N*-Demethylation to EDDP by Human Liver Microsomes

HLM Sample	CYP3A4, pmol/mg	CYP2B6, pmol/mg	<i>R</i> -EDDP			<i>S</i> -EDDP		
			K_m , μM	V_{max} , $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$	V_{max}/K_m	K_m , μM	V_{max} , $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$	V_{max}/K_m
HLM-139	67	81	48 \pm 3	948 \pm 17	20	45 \pm 3	1,407 \pm 23	31
HLM-148	170	12	62 \pm 4	501 \pm 10	8	50 \pm 4	412 \pm 9	8

Kinetic parameters were obtained by nonlinear regression analysis using a single enzyme Michaelis-Menten model. Values are the parameter estimates and SE of the estimate.

EDDP = 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine; HLM = human liver microsome sample; K_m = Michaelis-Menten coefficient; V_{max} = maximum velocity.

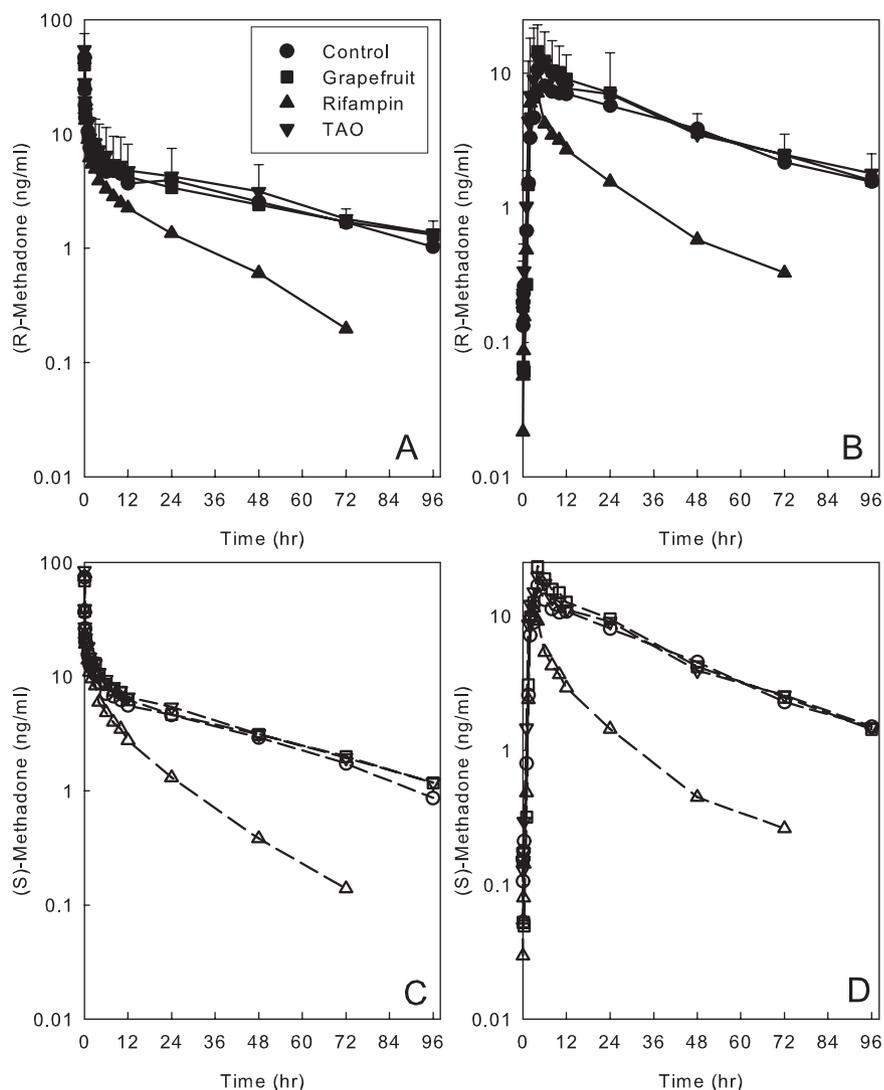


Fig. 5. Influence of rifampin, grapefruit juice, and troleandomycin (TAO) on the disposition of methadone enantiomers in humans *in vivo* from intravenous methadone (A and C) and oral methadone (B and D). Solid symbols and lines depict (R)-methadone (A and B), and open symbols and dotted lines depict (S)-methadone (C and D). Results are the mean \pm SD (n = 10 subjects). Some SDs are omitted for clarity.

The third major finding of this investigation is that CYP3A does not seem to be a major CYP isoform responsible for clinical methadone enantiomer metabolism and elimination in humans. The CYP3A-selective inhibitor troleandomycin (which has no effect on CYP2B6 activity³⁸) was previously shown to have no effect on plasma *RS*-methadone or EDDP concentrations or *RS*-methadone clearance.¹³ That investigation, however, used an achiral assay that measured total methadone, rather than individual enantiomers. The current investigation showed that troleandomycin had no effect on either *R*- or *S*-methadone enantiomer plasma concentrations or clearance. Additional evidence refuting CYP3A as a major determinant of stereoselective methadone elimination is that neither of the CYP3A inhibitors (troleandomycin and grapefruit juice) altered plasma *R/S*-methadone concentration ratios.

The fourth major finding is an apparent role for CYP2B6 as a major determinant of stereoselective methadone elimination in humans, as articulated previously,^{11,13} evidenced by rifampin induction of methadone elimina-

tion together with the lack of CYP3A inhibitor effects. Rifampin (which induces hepatic CYP2B6 threefold¹⁸ in addition to inducing CYP3A4 and other P450s) decreased plasma concentrations of both methadone enantiomers and altered the plasma *R/S*-methadone concentration ratio. This clinical result is consistent with previous model predictions, based on methadone metabolism by CYPs 2B6 and 3A4 and scaling factors, that the rate of increase with time of the plasma methadone *R/S* ratio would be proportional to the hepatic CYP2B6 content, that CYP2B6 induction would further increase the *R/S*-methadone ratio, and that CYP3A inhibition would have minimal effect on the *R/S*-methadone ratio.²¹ Although HLM CYP2B6 protein content (1-10% of total CYP) is generally lower than CYP3A (as much as 50% of total CYP), these data nonetheless strongly implicate CYP2B6 in methadone disposition. Rifampin can induce CYP2C19, but CYP2C19 is unlikely to be responsible for rifampin alterations in the *R/S* ratio because the stereoselectivity of CYP2C19 (*R* > *S* *in vitro*) is opposite that observed clinically. Stereoselective methadone transport

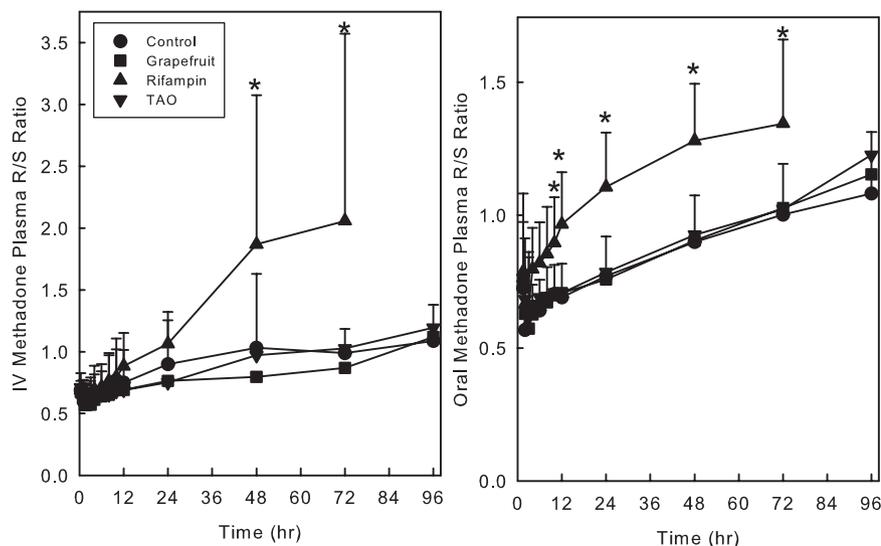


Fig. 6. Effect of rifampin, grapefruit juice, and troleandomycin (TAO) on stereoselective intravenous (IV) and oral methadone disposition in humans *in vivo*. Results are the (*R*)-methadone/(*S*)-methadone plasma concentration ratio. Results are the mean \pm SD ($n = 10$). * $P < 0.05$ versus control. Initial *R*- and *S*-methadone concentrations differ because of enantiomeric differences in volumes of distribution (not shown). Methadone enantiomer concentrations were below the limit of quantification at 96 h in rifampin-treated subjects.

by P-glycoprotein and/or other influx or efflux transporters and induction by rifampin cannot, however, be excluded by the current results.

Previous evidence supporting a clinical role for CYP3A4 in methadone disposition has been variable. Correlations between CYP3A4 activity and either methadone clearance or doses required for maintaining opioid abstinence yielded inconsistent results. Some studies suggested that patients with higher CYP3A4 activity required a higher methadone dose to prevent withdrawal symptoms.^{4,14} Attempts to correlate methadone clearance or maintenance dose with CYP3A4 activity (using xenobiotic probes such as midazolam or endogenous probes such as the 6-OH/17-OH corticosteroid ratio in urine) were unsuccessful.^{13,20} The CYP3A4 inhibitors atazanavir, saquinavir, and ritonavir did not inhibit human methadone clearance, also suggesting that CYP3A4 is not the main enzyme responsible for methadone disposition.^{13,20,39,40} Although fluconazole increased methadone plasma concentrations, this cannot be attributed solely to CYP3A4 inhibition because fluconazole is not a specific CYP3A4 inhibitor.⁴¹ A major role for CYP2B6 in methadone disposition may help to explain these inconsistent and perplexing observations.

Why were extrapolations of *in vitro* and liver microsomal data misleading and suggestive of a clinical role for CYP3A4? Why do some clinical reports suggest a role for CYP3A4?^{4,16} In the current investigation, CYP2B6 protein content was significantly correlated with that of CYP3A4. In addition, CYP2B6 is affected by many of the same inhibitors (*i.e.*, protease inhibitors) and inducers (*i.e.*, phenobarbital and rifampin)^{17,18} as CYP3A4.^{9,42-44} Therefore, CYP3A4 may simply serve as a reporter for CYP2B6 activity *in vivo* under certain conditions. Apparent correlations between methadone disposition and CYP3A4 may instead represent a dependence on CYP2B6 activity. The possibility of coordinate regulation of CYPs 2B6 and 3A4 has been suggested previously.³⁸

This may explain lower methadone plasma concentrations in patients with greater CYP3A activity (midazolam metabolic ratio).¹⁶ Alteration in the plasma methadone *R/S* ratio by rifampin but not by CYP3A inhibitors, the failure of CYP3A inhibitors such as troleandomycin and antiretroviral drugs to increase *R*- or *S*-methadone plasma concentrations, and the lack of meaningful correlations between methadone disposition and CYP3A are consistent with this hypothesis. Recent evidence also supports a predominant role for CYP2B6 in clinical methadone disposition.^{16,45}

Potential limitations of this investigation merit address. Most, but not all, microsomes pairs showed greater methadone *N*-demethylation and stereoselectivity with higher CYP2B6 content. This may be attributable to potential differences in apoprotein *versus* holoprotein in the Western blot analysis, which may also influence CYP correlations. Plasma samples were reanalyzed from a previous study.¹³ Quality control samples from that study were not available to formally validate this approach. Nonetheless, much evidence supports the results and conclusions from these data. Subsequent to the original study using achiral analysis, we conducted several studies of methadone disposition using chiral analysis. Results (for controls) from the chiral reanalysis are similar to those from the subsequent chiral studies¹³ and values published by others.³⁷ In addition, the time course of plasma *R/S* ratios (for controls) from the chiral reanalysis is nearly identical to that published previously.⁶ Last, differences between control and rifampin-treated subjects (and lack of difference in troleandomycin- or grapefruit juice-treated subjects) in plasma methadone enantiomer concentrations and *R/S* ratios cannot be explained by analytical confounding.

The potential clinical significance of these finding is manifold, and results may have implications for clinical methadone dosing guidelines. Despite decades of research, the mechanism of interindividual variability in

methadone pharmacokinetics has remained largely unknown. There is reticence in the clinical use and dosing of methadone because of uncertainty in the pharmacokinetics and fear of toxicity. This is due in part to concerns about drug interactions which have been classically attributed to CYP3A4.⁴ The current results suggest that statements identifying methadone as a clinical CYP3A4 substrate and warnings in the product label about clinical effects of CYP3A4 induction and/or inhibition, which are based on extrapolations of *in vitro* data, require reevaluation. Such apparently incorrect extrapolations reinforce the need to confirm *in vitro* observations with clinical investigations.

A role for CYP2B6 in methadone disposition may help to explain the large interindividual variation observed in methadone plasma concentrations and methadone dosing. CYP2B6 protein is variably expressed in the liver, said to account for 1–10% of total hepatic P450s, and mirrored by the variable content (6–95 pmol/mg protein) among livers in this investigation. Furthermore, genetic polymorphisms in CYP2B6²² could also contribute to variable methadone clearance. To date, 53 haplotypes and 28 alleles have been identified for CYP2B6, with 7 variant alleles occurring at a frequency greater than 1%. There is considerable ethnic and racial variability in the occurrence of variant alleles. For example, CYP2B6*6, whose carriers have up to fourfold lower CYP2B6 expression and activity, occurs in approximately 25% of whites, one third of Africans and African-Americans, and up to 40% of Chinese.^{22,46} CYP2B6 polymorphisms have been shown to affect the pharmacokinetics of other CYP2B6 substrates, such as efavirenz.^{32,46} There is also a potential for CYP2B6-mediated drug–drug interactions, causing either induction or inhibition of methadone metabolism and clearance, exemplified by methadone interactions with antiretrovirals, antifungals, and antiepileptics.^{4,47,48}

In summary, these results show that CYP3A4 and CYP2B6 but not CYP2C19 contribute to the overall metabolism of methadone in HLMs, that CYP2B6 is responsible for stereoselective methadone metabolism in HLMs, and that CYP2B6 seems responsible for clinical methadone metabolism and clearance. Future studies with specific CYP2B6 probes are needed to validate the clinical role of CYP2B6 in methadone disposition.

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References

- Peng PW, Tumber PS, Gourlay D: Review article: Perioperative pain management of patients on methadone therapy. *Can J Anaesth* 2005; 52:513–23
- Ripamonti C, Bianchi M: The use of methadone for cancer pain. *Hematol Oncol Clin North Am* 2002; 16:543–55
- Kristensen KCC, Christrup L: The mu1, mu2, delta, kappa opioid receptor binding profiles of methadone stereoisomers and morphine. *Life Sci* 1995; 56:PLA5–PL50
- Eap CB, Buclin T, Baumann P: Interindividual variability of the clinical

pharmacokinetics of methadone: Implications for the treatment of opioid dependence. *Clin Pharmacokinet* 2002; 41:1153–93

5. Begre S, von Bardeleben U, Ladewig D, Jaquet-Rochat S, Cosendai-Savary L, Golay KP, Kosel M, Baumann P, Eap CB: Paroxetine increases steady-state concentrations of (R)-methadone in CYP2D6 extensive but not poor metabolizers. *J Clin Psychopharmacol* 2002; 22:211–5

6. Foster DJ, Somogyi AA, Dyer KR, White JM, Bochner F: Steady-state pharmacokinetics of (R)- and (S)-methadone in methadone maintenance patients. *Br J Clin Pharmacol* 2000; 50:427–40

7. Iribarne C, Dreano Y, Bardou LG, Menez JF, Berthou F: Interaction of methadone with substrates of human hepatic cytochrome P450 3A4. *Toxicology* 1997; 117:13–23

8. Niemi M, Backman JT, Fromm MF, Neuvonen PJ, Kivisto KT: Pharmacokinetic interactions with rifampicin: Clinical relevance. *Clin Pharmacokinet* 2003; 42:819–50

9. Faucette SR, Wang H, Hamilton GA, Jolley SL, Gilbert D, Lindley C, Yan B, Negishi M, LeCluyse EL: Regulation of CYP2B6 in primary human hepatocytes by prototypical inducers. *Drug Metab Dispos* 2004; 32:348–58

10. Foster DJ, Somogyi AA, Bochner F: Methadone N-demethylation in human liver microsomes: Lack of stereoselectivity and involvement of CYP3A4. *Br J Clin Pharmacol* 1999; 47:403–12

11. Gerber JG, Rhodes RJ, Gal J: Stereoselective metabolism of methadone N-demethylation by cytochrome P4502B6 and 2C19. *Chirality* 2004; 16:36–44

12. Iribarne C, Berthou F, Carlhant D, Dreano Y, Picart D, Lohezic F, Riche C: Inhibition of methadone and buprenorphine N-dealkylations by three HIV-1 protease inhibitors. *Drug Metab Dispos* 1998; 26:257–60

13. Kharasch ED, Hoffer C, Whittington D, Sheffels P: Role of hepatic and intestinal cytochrome P450 3A and 2B6 in the metabolism, disposition, and mitotic effects of methadone. *Clin Pharmacol Ther* 2004; 76:250–69

14. Shinderman M, Maxwell S, Brawand-Amey M, Golay KP, Baumann P, Eap CB: Cytochrome P4503A4 metabolic activity, methadone blood concentrations, and methadone doses. *Drug Alcohol Depend* 2003; 69:205–11

15. Wang JS, DeVane CL: Involvement of CYP3A4, CYP2C8, and CYP2D6 in the metabolism of (R)- and (S)-methadone *in vitro*. *Drug Metab Dispos* 2003; 31:742–7

16. Crettol S, Deglon JJ, Besson J, Croquette-Krokar M, Hammig R, Gothuey I, Monnat M, Eap CB: ABCB1 and cytochrome P450 genotypes and phenotypes: Influence on methadone plasma levels and response to treatment. *Clin Pharmacol Ther* 2006; 80:668–81

17. Faucette SR, Sueyoshi T, Smith CM, Negishi M, Lecluyse EL, Wang H: Differential regulation of hepatic CYP2B6 and CYP3A4 genes by constitutive androstane receptor but not pregnane X receptor. *J Pharmacol Exp Ther* 2006; 317:1200–9

18. Lobo KK, Gross AS, Williams KM, Liauw WS, Day RO, Bliervnicht JK, Zanger UM, McLachlan AJ: Cytochrome P450 2B6 activity as measured by bupropion hydroxylation: Effect of induction by rifampin and ethnicity. *Clin Pharmacol Ther* 2006; 80:75–84

19. Niwa T, Shiraga T, Takagi A: Effect of antifungal drugs on cytochrome P450 (CYP) 2C9, CYP2C19, and CYP3A4 activities in human liver microsomes. *Biol Pharm Bull* 2005; 28:1805–8

20. Charlier C, Dessalles MC, Plomteux G: Methadone maintenance treatment: Is it possible to adapt the daily doses to the metabolic activity of the patient? *Ther Drug Monit* 2001; 23:1–3

21. Totah RA, Allen KE, Sheffels P, Whittington D, Kharasch ED: Enantiomeric metabolic interactions and stereoselective human methadone metabolism. *J Pharmacol Exp Ther* 2007; 321:389–99

22. Zanger UM, Klein K, Saussele T, Bliervnicht JMHH, Schwab M: Polymorphic CYP2B6: Molecular mechanisms and emerging clinical significance. *Pharmacogenomics* 2007; 8:743–59

23. Lin YS, Dowling AL, Quigley SD, Farin FM, Zhang J, Lamba J, Schuetz EG, Thummel KE: Co-regulation of CYP3A4 and CYP3A5 and contribution to hepatic and intestinal midazolam metabolism. *Mol Pharmacol* 2002; 62:162–72

24. Paine M, Khalighi M, Fisher J, Shen D, Kunze K, Marsh C, Perkins J, Thummel KE: Characterization of inter- and intra-intestinal differences in human CYP3A-dependent metabolism. *J Pharmacol Exp Ther* 1997; 283:1552–62

25. Lang T, Klein K, Fischer J, Nussler AK, Neuhaus P, Hofmann U, Eichelbaum M, Schwab M, Zanger UM: Extensive genetic polymorphism in the human CYP2B6 gene with impact on expression and function in human liver. *Pharmacogenetics* 2001; 11:399–415

26. Oda Y, Kharasch ED: Metabolism of methadone and levo-alpha-acetylmethadol (LAAM) by human intestinal cytochrome P450 3A4 (CYP3A4): Potential contribution of intestinal metabolism to presystemic clearance and bioactivation. *J Pharmacol Exp Ther* 2001; 298:1021–32

27. Whittington D, Sheffels P, Kharasch ED: Stereoselective determination of methadone and the primary metabolite EDDP in human plasma by automated on-line extraction and liquid chromatography mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004; 809:313–21

28. Hosea NA, Miller GP, Guengerich FP: Elucidation of distinct ligand binding sites for cytochrome P450 3A4. *Biochemistry* 2000; 39:5929–39

29. Korzekwa KR, Krishnamachary N, Shou M, Ogai A, Parise RA, Rettie AE, Gonzalez FJ, Tracy TS: Evaluation of atypical cytochrome P450 kinetics with two-substrate models: Evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry* 1998; 37:4137–47

30. Shou M, Dai R, Cui D, Korzekwa KR, Baillie TA, Rushmore TH: A kinetic model for the metabolic interaction of two substrates at the active site of cytochrome P450 3A4. *J Biol Chem* 2001; 276:2256-62
31. Ekins S, Wrighton SA: The role of CYP2B6 in human xenobiotic metabolism. *Drug Metab Rev* 1999; 31:719-54
32. Rotger M, Tegude H, Colombo S, Cavassini M, Furrer H, Decosterd L, Bliedernicht J, Saussele T, Gunthard HF, Schwab M, Eichelbaum M, Telenti A, Zanger UM: Predictive value of known and novel alleles of CYP2B6 for efavirenz plasma concentrations in HIV-infected individuals. *Clin Pharmacol Ther* 2007; 81:557-66
33. Desta Z, Saussele T, Ward B, Bliedernicht J, Li L, Klein K, Flockhart DA, Zanger UM: Impact of CYP2B6 polymorphism on hepatic efavirenz metabolism *in vitro*. *Pharmacogenomics* 2007; 8:547-58
34. Turpeinen M, Nieminen R, Juntunen T, Taavitsainen P, Raunio H, Pelkonen O: Selective inhibition of CYP2B6-catalyzed bupropion hydroxylation in human liver microsomes *in vitro*. *Drug Metab Dispos* 2004; 32:626-31
35. Newton DJ, Wang RW, Lu AY: Cytochrome P450 inhibitors: Evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metab Dispos* 1995; 23:154-8
36. Suzuki H, Kneller MB, Haining RL, Trager WF, Rettie AE: (+)-N-3-benzyl-nirvanol and (-)-N-3-benzyl-phenobarbital: New potent and selective *in vitro* inhibitors of CYP2C19. *Drug Metab Dispos* 2002; 30:235-9
37. Boulton DW, Arnaud P, DeVane CL: Pharmacokinetics and pharmacodynamics of methadone enantiomers after a single oral dose of racemate. *Clin Pharmacol Ther* 2001; 70:48-57
38. Faucette SR, Hawke RL, Shord SS, Lecluyse EL, Lindley CM: Evaluation of the contribution of cytochrome P450 3A4 to human liver microsomal bupropion hydroxylation. *Drug Metab Dispos* 2001; 29:1123-9
39. Friedland G, Andrews L, Schreiberman T, Agarwala S, Daley L, Child M, Shi J, Wang Y, O'Mara E: Lack of an effect of atazanavir on steady-state pharmacokinetics of methadone in patients chronically treated for opiate addiction. *AIDS* 2005; 19:1635-41
40. Shelton MJ, Cloen D, DiFrancesco R, Berenson CS, Esch A, de Caprariis PJ, Palic B, Schur JL, Bugge CJ, Ljungqvist A, Espinosa O, Hewitt RG: The effects of once-daily saquinavir/minidose ritonavir on the pharmacokinetics of methadone. *J Clin Pharmacol* 2004; 44:293-304
41. Cobb MN, Desai J, Brown LS Jr, Zannikos PN, Rainey PM: The effect of fluconazole on the clinical pharmacokinetics of methadone. *Clin Pharmacol Ther* 1998; 63:655-62
42. Martin H, Sarsat JP, de Waziers I, Housset C, Ballardur P, Beaune P, Albaladejo V, Lerche-Langrand C: Induction of cytochrome P450 2B6 and 3A4 expression by phenobarbital and cyclophosphamide in cultured human liver slices. *Pharm Res* 2003; 20:557-68
43. Parkinson A, Mudra DR, Johnson C, Dwyer A, Carroll KM: The effects of gender, age, ethnicity, and liver cirrhosis on cytochrome P450 enzyme activity in human liver microsomes and inducibility in cultured human hepatocytes. *Toxicol Appl Pharmacol* 2004; 199:193-209
44. Pascussi JM, Gerbal-Chaloin S, Drocourt L, Maurel P, Vilarem MJ: The expression of CYP2B6, CYP2C9 and CYP3A4 genes: A tangle of networks of nuclear and steroid receptors. *Biochim Biophys Acta* 2003; 1619:243-53
45. Crettol S, Deglon JJ, Besson J, Croquette-Krokkar M, Gothuey I, Hammig R, Monnat M, Huttemann H, Baumann P, Eap CB: Methadone enantiomer plasma levels, CYP2B6, CYP2C19, and CYP2C9 genotypes, and response to treatment. *Clin Pharmacol Ther* 2005; 78:593-604
46. Rotger M, Colombo S, Furrer H, Bleiber G, Buclin T, Lee BL, Keiser O, Biollaz J, Decosterd L, Telenti A: Influence of CYP2B6 polymorphism on plasma and intracellular concentrations and toxicity of efavirenz and nevirapine in HIV-infected patients. *Pharmacogenet Genomics* 2005; 15:1-5
47. Bruce RD, Altice FL, Gourevitch MN, Friedland GH: Pharmacokinetic drug interactions between opioid agonist therapy and antiretroviral medications: Implications and management for clinical practice. *J Acquir Immune Defic Syndr* 2006; 41:563-72
48. Liu P, Foster G, Labadie R, Somoza E, Sharma A: Pharmacokinetic interaction between voriconazole and methadone at steady state in patients on methadone therapy. *Antimicrob Agents Chemother* 2007; 51:110-8