Mechanism-Based Inactivation of Cytochrome P450 2B6 by Methadone Through Destruction of Prosthetic Heme

Hemali T. Amunugama, Haoming Zhang, and Paul F. Hollenberg

Department of Pharmacology, University of Michigan, Ann Arbor, MI 48109 (HTA, HZ, and PFH).
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Send Correspondence to: Paul F. Hollenberg, PhD.

University of Michigan

2301 MSRB III

1150 W. Medical Center Drive

Ann Arbor, MI 48109-5632

E-mail: phollen@umich.edu

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Nonstandard abbreviations: 7-EFC, 7-ethoxy-4-trifluoromethylcoumarin; 7-HFC, 7-hydroxy-4-trifluoromethylcoumarin; CYP, cytochrome P450; CPR, NADPH-dependent cytochrome P450 reductase; ESI-LC/MS, electro-spray ionization liquid chromatography mass spectrometry;
ABSTRACT

Methadone is a µ-opioid receptor agonist widely used in the treatment of narcotic addiction and chronic pain conditions. Methadone is metabolized predominantly in the liver by cytochrome P450s (CYPs) to its pharmacologically inactive primary metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine. Initial in vitro data suggested that CYP3A4 is the major isoform responsible for the in vivo clearance of methadone in humans. However, recent clinical data have identified that CYP2B6 is actually the major isoform responsible for methadone metabolism and clearance in vivo. In this study, methadone was shown to act as a mechanism-based inactivator of CYP2B6. Methadone inactivates CYP2B6 in a time-, concentration- and NADPH- dependent manner with a $K_i = 10.0 \, \mu M$ and $k_{inact} = 0.027 \, \text{min}^{-1}$. The loss of CYP2B6 activity in the presence of methadone and NADPH occurred with a concomitant loss of the reduced CO spectrum of the P450. Moreover, there was a good correlation between the loss of CYP2B6 activity and the loss of the CO-binding spectrum. HPLC analysis of the native heme of the inactivated CYP2B6 demonstrated that approximately 75% loss of heme was accompanied by a comparable inactivation of CYP2B6. LC/MS analysis did not reveal the formation of a protein adduct during the inactivation. The evidence strongly suggests that destruction of prosthetic heme is the underlying mechanism leading to the inactivation of CYP2B6 by methadone.
INTRODUCTION

Methadone, a synthetic opioid with a long elimination half life, is widely used in the treatment of narcotic addiction and pain management. Methadone was first introduced to the United States in 1947. Since then, the clinical use of methadone has increased rapidly. The total number of methadone prescriptions has increased by 1300% from 1997 to 2006 (Kharasch et al., 2009; Lu et al., 2010). As a consequence of this increased usage of methadone, the number of methadone-associated fatalities has gone up by 390% and the amount of adverse events has gone up by 1800% from 1997 to 2004 (Kharasch et al., 2009). These statistics are presumably due to the usage of methadone in the management of various pain conditions rather than in the treatment of addiction (Kharasch et al., 2009). Despite all of the negative attention based on these statistics, methadone is a very useful drug with unique pharmacological properties including its high potency and efficacy, high oral bioavailability, no known formation of biologically active metabolites and faster onset than morphine (Clark, 2008; Li et al., 2008).

Methadone is administered as a racemic mixture of R- and S- isomers although the R-isomer is believed to be the isomer responsible for analgesic activity. R-Methadone binds to the µ-opioid receptor with high affinity and causes analgesia. Moreover both R- and S- isomers bind at the N-methyl-D-aspartate (NMDA) receptor and inhibit the reuptake of serotonin and noradrenaline (Leppert, 2009). Methadone is predominantly metabolized in the liver by several CYPs to its pharmacologically inactive primary metabolite 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and subsequently to its secondary metabolite 2-ethyl-5-methyl-3,3-diphenyl-pyrroline (EMDP) as illustrated in...
Figure 1 (Sporkert and Pragst, 2000). Cytochrome P450 (CYP) mediated p-hydroxylation of one of the aromatic rings has also been reported (Sullivan and Due, 1973; Anggard et al., 1975; Foster et al., 1999). Initial in vitro data suggested that CYP3A4 is the major isoform responsible for the in vivo clearance of methadone in humans (Iribarne et al., 1996; Iribarne et al., 1997). However, recent clinical data have challenged the major role of CYP3A4 in methadone metabolism (Kharasch et al., 2009). Furthermore, the in vitro metabolism of methadone by CYP3A4 has shown to be nonstereoselective which is contrary to the metabolism of methadone in vivo which is stereoselective. The metabolism of methadone by CYP2C19, CYP2D6 and CYP19 has also been reported (Gerber et al., 2004; Lu et al., 2010).

Recent clinical data suggest that CYP2B6 is actually the major isoform responsible for methadone metabolism and clearance in vivo (Totah et al., 2008; Kharasch et al., 2009). CYP2B6 is expressed predominantly in the liver and it is also expressed at lower levels in brain, lung, nasal mucosa and trachea (Walsky et al., 2006). Although CYP2B6 was initially believed to play a relatively minor role in human drug metabolism, recent studies have demonstrated that the contribution of CYP2B6 to hepatic drug metabolism is several fold higher than previously estimated (Wang and Tompkins, 2008; Mo et al., 2009). CYP2B6 has been shown to play an important role in the metabolism of several therapeutically relevant drugs including bupropion, efavirenz and clopidogrel. Moreover the discovery of numerous other substrates of this highly inducible CYP2B6, the relatively higher occurrence of genetic polymorphisms has given CYP2B6 a prominent place in studies of human drug metabolism.
The large interindividual variability which has been observed in response to methadone treatment has been attributed to genetic polymorphisms in the genes coding for CYPs involved in methadone metabolism as well as the polymorphisms in the transporter proteins and µ-opioid receptors (Li et al., 2008; Bunten et al., 2011).

Although methadone is a versatile drug with some very beneficial properties in certain situations, it has also been shown to exhibit harmful pharmacological properties. Our current understanding of the metabolism of methadone in humans is incomplete; our inability to accurately predict pharmacokinetics in individuals leads to adverse effects, significant drug-drug interactions, and to a number of fatalities. A better understanding of the identity of the CYPs involved in methadone metabolism, their relative contributions to methadone clearance, and their stereoselectivity for methadone metabolism will greatly enhance our ability to administer this drug safely.

In this report, the mechanism-based inactivation of CYP 2B6 in the reconstituted system by methadone is described. Attempts were made to determine whether the inactivation proceeded through covalent modification of protein or heme. The absence of apoprotein adducts or stable heme adducts and the loss of the CO-difference spectrum implies that the destruction of prosthetic heme is the mechanism for inactivation of CYP2B6 by methadone.
MATERIALS AND METHODS

Chemicals. Catalase, NADPH, and glutathione were purchased from Sigma-Aldrich (St. Louis, MO). 7-Ethoxy-4-(trifluoromethyl) coumarin (EFC) was purchased from Invitrogen (Carlsbad, CA). All other chemicals and solvents were of the highest purity available from commercial sources. Racemic methadone was a generous gift from Dr. James Woods (The University of Michigan, Ann Arbor).

Overexpression and Purification of CYP2B6 and CPR.

Plasmid pLW01 expressing human CYP2B6 with a C-terminal H4 tag and lacking 21 N-terminal residues was expressed in Escherichia coli C41 (DE3) cells and purified using a nickel-affinity column (Sigma-Aldrich, St. Louis MO) followed by a CM-Sepharose cation exchange column as described in (Scott et al., 2001; Zhang et al., 2011). The NADPH-dependent cytochrome P450 reductase (CPR) was overexpressed and purified as described previously (Zhang et al., 2009).

Kinetics for the Mechanism-Based Inactivation of P450 2B6 by Methadone.

CYP2B6 was reconstituted with CPR in a 1:2 ratio by incubating the two proteins at room temperature for 40 minutes (Kenaan et al., 2010). The kinetics for the inactivation of CYP2B6 by methadone were determined at 37°C in 50 mM potassium phosphate buffer, pH 7.4. The primary reaction mixtures contained CYP2B6 (1 µM) and CPR (2 µM), 130 units of catalase and varying concentrations of methadone (0-100 µM). The reactions were initiated by the addition of NADPH to a final concentration of 1 mM. At designated times, aliquots of 6 µl of the primary reaction mixture were transferred to a secondary reaction mixture (150 µL) that contained 0.1 mM 7-EFC and 0.3 mM NADPH.
in 50 mM potassium phosphate, pH 7.4. The secondary reactions were terminated after incubation for 10 min by the addition of 50 µl of ice-cold acetonitrile. The fluorescent intensity of the 7-hydroxy-4-trifluoromethylcoumarin (7-HFC) product was measured at 510 nm with excitation at 410 nm using a Victor II microtiter plate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA). The fluorescent intensity was used to calculate the activity remaining of the CYP2B6. The results were expressed as a percentage of the control sample from which methadone was omitted (Kenaan et al., 2010).

**Partition Ratio for the Mechanism-Based Inactivation of P450 2B6 by Methadone.**

To determine the partition ratio, the primary reaction mixtures containing CYP2B6, CPR, catalase, NADPH (1 mM) and various concentrations of methadone (0 µM to 300 µM) were incubated for 60 min at 37°C until the inactivations were complete. The activity remaining after the inactivation of CYP2B6 was analyzed using the secondary reaction mixture as described above. The partition ratio was then determined as described previously (Kent et al., 1997).

**Analyses of the Methadone-inactivated CYP2B6 Protein by ESI-LC/MS.**

CYP2B6 was reconstituted with CPR in a 1:2 ratio as described above for the inactivation reactions. The primary reaction mixture containing 0.5 nmol of CYP2B6 and 1 nmol of CPR was inactivated with 50 µM methadone and 1.3 mM NADPH in 100 mM potassium phosphate buffer at pH 7.4. A control sample was incubated with methadone in the absence of NADPH. After incubating the samples at 30 °C for 30 min, a 50 µl aliquot of the reaction mixture was injected onto a reverse-phase C3 column (2×150
mm, 5 \mu m) (Agilent Technologies Santa Clara, CA) and eluted in to a LCQ classic ion-trap mass spectrometer (Thermo Fisher Scientific). CYP2B6 was separated from the other components of the reaction mixture by eluting the column with a binary solvent containing 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) using the following gradient: 30% B for 5 min, the gradient was then increased to 90% B using a linear gradient over 20 min and was held at 90% B for 30 min. The flow rate was 0.25 ml/min. The molecular masses of the inactivated (+NADPH) and (-NADPH) CYP2B6 were determined by deconvolution of the apoprotein charge envelopes using the Bio-works software (Thermo Fisher Scientific).

**Spectral Analysis of the Inactivated CYP2B6 and Irreversibility of Inactivation.**

The primary reaction mixture was incubated with 100 \mu M methadone in the presence of NADPH (inactivated sample) or absence of NADPH (control sample) and the reduced CO difference spectra of the proteins were determined by scanning from 400 to 500 nm on a Shimadzu UV 2500PC spectrophotometer (Omura and Sato, 1964). The primary reaction mixture was incubated with 100 \mu M methadone in the presence (inactivated sample) or absence of NADPH (control sample) and the irreversibility of inactivation was determined by dialyzing overnight as described previously (Blobaum et al., 2002).

**Time-Dependent Loss of Enzyme Activity and CO Binding of CYP2B6**

The time dependent loss of CYP2B6 activity due to inactivation by methadone was compared with the loss of CO binding of CYP2B6 over a time period of 50 min. The primary reaction mixture was incubated with 50 \mu M methadone and 1 mM NADPH at 37 \degree C. At designated times, aliquots were withdrawn for CO binding and enzyme activity
assays. The CO binding assay was carried out as described above and the enzyme activity assay was carried out in a manner similar to that described in the section on the kinetics for the mechanism-based inactivation of P450 2B6 by methadone.

**HPLC Analysis of Heme.**

The loss of the native heme and the possible formation of heme adducts were investigated by using a Shimadzu high-performance liquid chromatography system (LC-20AB). Aliquots containing 100 pmol of the inactivated (+NADPH) and control (-NADPH) CYP2B6 that had been incubated with 50 µM of methadone for 15 min at 37°C as described above for the inactivation reactions, were analyzed using a C4 reverse-phase column (5 µm, 4.6×250 mm, 300 Å; Phenomenex, Torrance, CA). The solvent system used was Solvent A [0.1% trifluoroacetic acid (TFA) in water] and Solvent B (0.05% TFA in acetonitrile). The column was eluted with a linear gradient from 30% to 80% B over 30 min at a flow rate of 1 ml/min, and the column eluant was monitored for heme at 400 nm.
RESULTS

Mechanism-Based Inactivation of CYP2B6 by Methadone

The kinetics for the inactivation of CYP2B6 by methadone were determined by measuring the loss of 7-EFC \( \text{O-deethylation} \) activity in the reconstituted system. As shown in Figure 2, CYP2B6 was inactivated by methadone in a time- and concentration-dependent manner. The activity for CYP2B6 remained unchanged with time in the absence of methadone while the remaining activity of CYP2B6 changed significantly with time and with increasing concentrations of methadone. From the data in Figure 2, it was calculated that methadone inactivates CYP2B6 with a \( K_i = 10.0 \mu\text{M} \) and \( k_{\text{inact}} = 0.027 \text{ min}^{-1} \) and \( t_{1/2} \) of 25 min. The presence of cytochrome \( b_5 \) did not alter the kinetic parameters for the inactivation CYP2B6 by methadone (data not shown). Therefore, all the subsequent experiments were carried out without cytochrome \( b_5 \).

Partition Ratio for the Mechanism-Based Inactivation of CYP2B6 by Methadone

CYP2B6 was incubated with various concentrations of methadone for 60 min allowing the inactivation to reach completion. The percentage of activity remaining was plotted as a function of the molar ratio of methadone to CYP2B6. The partition ratio was estimated from the intercept of the linear regression line obtained for the lower ratios of methadone to CYP2B6 with the straight line derived for the higher ratios of methadone to CYP2B6. The estimated partition ratio was approximately 22 for the inactivation of CYP2B6 by methadone (Figure 3). The residual activity of inactivated CYP2B6 was approximately 20%. Complete inactivation of the enzyme could not be achieved by increasing the ratio of methadone/CYP2B6. The inability to achieve complete
inactivation has previously been observed with other mechanism-based inactivators (Lin et al., 2005; Gay et al., 2011).

**ESI-LC-MS Analysis of the Inactivated Protein.**

To investigate whether the mechanism-based inactivation of CYP2B6 by methadone was due to the formation of an adduct with the apoprotein, the reconstituted CYP2B6 system was incubated with methadone in the presence or absence of NADPH for 30 min and the proteins were analyzed by ESI-LC-MS. The mass spectra obtained after 30 min of incubation are shown in Figure 4 (Traces A and B). The deconvoluted mass spectrum of CYP2B6 in the absence of NADPH has a molecular mass of approximately 54423.0 Da, which corresponds to the molecular mass of the unmodified CYP2B6 as calculated from the amino acid sequence. The deconvoluted mass spectrum of CYP2B6 incubated with NADPH exhibits a molecular mass of approximately 54434.0 Da. Since the error in the ESI-LC-MS experiment is ± 16 Da, there is no significant modification in the mass of apoprotein has occurred during the inactivation of CYP2B6 by methadone. If there had been a protein adduct the molecular mass of the protein should increase by the molecular mass of the reactive intermediate which in this case is approximately 294Da.

**Changes in the Reduced-CO Difference Spectrum and the Absolute Spectrum of the Methadone-Inactivated CYP2B6.**

As shown in Figure 5, the reduced-CO difference spectrum of the methadone-inactivated CYP2B6 decreased by approximately 61% after incubation of CYP2B6 with methadone in the presence of NADPH in the reconstituted system while the catalytic
activity of the enzyme decreased approximately by 58%. Therefore, the decrease in the reduced-CO difference spectrum is comparable to the loss in the catalytic activity of the enzyme. The removal of methadone and its reaction products by overnight dialysis of the inactivated and control samples did not lead to a significant recovery of catalytic activity or the reduced-CO difference spectra (data not shown) demonstrating that the inactivation of CYP2B6 by methadone is irreversible.

**Correlation between Time-Dependent Enzyme Activity Loss and Loss of CO Binding of CYP2B6 in the Presence of Methadone.**

As shown in Figure 6, the percentage activity remaining and the CO binding of the reduced CYP2B6 inactivated by incubating with methadone and NADPH decreased over time. The loss of enzyme activity is comparable to the loss of CO binding. This type of correlation between CO loss and enzyme activity loss has been observed with other mechanism-based inactivators (Foti et al., 2011).

**HPLC Analysis of Heme.**

HPLC analysis was performed on CYP2B6 following incubation with 50 µM methadone in the absence or presence of NADPH at 37°C for 20 min as shown in Figure 7. The elution profiles were monitored at 400 nm for intact heme. The native heme of both the control (-NADPH) and inactivated (+NADPH) P450s eluted at approximately 18 min. A significant loss (~ 75 %) in native heme in the inactivated sample was observed compared to the control sample. Stable heme adducts or cross-linked heme eluting either before or after the native heme peak at 18 min could not be detected indicating
that this inactivation reaction may lead to heme fragmentation or to very unstable heme adducts that cannot be detected by our HPLC methods.
DISCUSSION.

Although methadone has gained increasing acceptance as a treatment for various types of pain and for narcotic addiction, effective use of methadone is hampered by its relatively complicated and incompletely understood pharmacokinetics. A knowledge of the identity of the CYPs involved in methadone metabolism, their relative contributions to methadone clearance, and their stereoselectivity for methadone metabolism could provide valuable information for predicting potential *in vivo* drug-drug interactions and thus this knowledge should prove to be very beneficial in the rational use of methadone (Totah et al., 2008). Studies by Totah and co-workers have demonstrated that CYP2B6 plays a significant role in the stereoselective metabolism of methadone and that CYP2B6 preferentially metabolizes the S-isomer over the R-isomer (Totah et al., 2008).

Our results demonstrate that methadone is a mechanism-based inactivator of CYP2B6. The loss of catalytic activity of CYP2B6 is time- and concentration-dependent and requires metabolism of methadone. The values for $K_i$ and $k_{inact}$ are 10 µM and 0.027 min$^{-1}$, respectively, indicating that methadone is a relatively potent mechanism-based inhibitor of CYP2B6. Extensive dialysis of the methadone-inactivated CYP2B6 did not lead to recovery of the catalytic activity. Furthermore, the partition ratio for this inactivation is approximately 22, which suggests that on average twenty-two catalytic turnovers leading to product formation occur before one inactivation event.

The mechanism-based inactivation of CYP19 by racemic methadone was reported by Lu and co-workers in the reconstituted system (Lu et al., 2010). They demonstrated that racemic methadone and EDDP, the primary metabolite of methadone both efficiently
inactivate CYP19 in a time- and concentration- dependent manner whereas EMDP, the secondary metabolite was found to be a much weaker inactivator of CYP19. The reported $K_i$ and $k_{inact}$ values for racemic methadone are 40.0 $\mu$M and 0.06 min$^{-1}$ respectively. No stereoselectivity was observed in methadone metabolism by CYP19. In addition, in their study the mechanism by which the inactivation proceeds was not addressed.

There are three different mechanisms by which a reactive intermediate may inactivate a P450; covalent modification of an amino acid residue in the apoprotein, modification of the prosthetic heme moiety leading to the destruction of the heme group, and thirdly cross-linking of the apoprotein with the heme moiety by the reactive intermediate leading to the inactivation of the enzyme (Blobaum, 2006; Hollenberg et al., 2008). In addition to these three major mechanisms, an alternate mechanism for inactivation of CYP450s by alkyl and aromatic amines has been well established. During the CYP mediated oxidation of variety of secondary and tertiary amines, N-dealkylated intermediates are generated. These intermediates are believed to form pseudo-irreversible complexes (MI complexes) with the ferrous iron of the prosthetic heme group of the CYP450s (Sharma et al., 1996; Jones et al., 1999; Chatterjee and Franklin, 2003; Hutzler et al., 2006). While methadone initially undergoes N-dealkylation of the tertiary amine group to a secondary amine during metabolism by CYP2B6, the absence of spectroscopic evidence for the formation of an MI complex i.e. increase of absorbance at 455 nm with time (data not shown) rules out the possibility of MI complex formation as being responsible for the inactivation of CYP2B6 by methadone.
Another possible route for mechanism-based inactivation of CYP450s is through covalent modification of the apoprotein. A tertiary amine, phencyclidine is a mechanism-based inactivator that inactivates CYP2B1, CYP2B4, and CYP2B6 through protein modification (Ward et al., 1982; Hoag et al., 1987; Crowley and Hollenberg, 1995; Jushchyshyn et al., 2003; Jushchyshyn et al., 2006). In these inactivations, the phencyclidine iminium species has been proposed as the reactive intermediate that binds to the apoprotein. The absence of apoprotein adducts in the deconvoluted mass spectrum of the methadone-inactivated CYP2B6 suggest that the inactivation of CYP2B6 by methadone does not proceed through protein modification.

The loss of CYP2B6 activity in the presence of methadone and NADPH occurred with a concomitant loss of the reduced CO difference spectrum of the P450. Moreover, a good correlation was observed between the loss of CYP2B6 activity and the loss of CO-binding. HPLC analysis of the intact heme prosthetic groups of methadone-inactivated and control CYP2B6 showed identical retention times and shapes. However, there was approximately 75% less intact heme present in the methadone-inactivated CYP2B6 when compared to the control under conditions where there was a loss of 75% of the activity. The reduction in the reduced CO binding spectrum and the loss of native heme collectively suggest that the inactivation proceeds via modification of the prosthetic heme. However, no modified heme peaks were observed in the HPLC profile of methadone-inactivated CYP2B6. Successful isolation of heme adducts using HPLC as monitored by ultra-violet or mass spectroscopic approaches has previously been reported (He et al., 1996a; He et al., 1996b). Following similar approaches TFA/butanone extracts of methadone inactivated CYP2B6 and controls were analyzed by UV
spectroscopy and mass spectroscopy. However, methadone-modified heme derived substances were not detected in the TFA/butanone extracts. It has also been reported that CYP450s can be inactivated by mechanisms which involve destruction of prosthetic heme without the formation of stable, isolable heme adducts. This kind of heme destruction has been reported with inactivators such as carbon tetrachloride, resveratrol, diclofenac, and mibebradil (Guzelian and Swisher, 1979; Macdonald et al., 1982; Davies et al., 1986; Chang et al., 2001; Foti et al., 2011). Loss of the heme chromophore due to heme fragmentation has also been observed with P450 enzymes in the presence of cumene hydroperoxide (He et al., 1998). Thus methadone appears to behave in a manner similar to these inactivators in that it does not lead to any stable heme adducts as observed by UV and mass spectroscopy. In summary, methadone is an effective mechanism-based inhibitor of CYP2B6. The absence of covalently labeled protein, stable heme adducts, or the formation of a MI complex when taken together with the loss of the CO binding by the reduced P450 strongly suggests that destruction of the prosthetic heme is the underlying mechanism leading to the inactivation of CYP2B6 by methadone.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Amunugama H, Zhang H, Hollenberg PF

Conducted experiments: Amunugama H, Zhang H

Performed data analysis: Amunugama H, Zhang H

Contributed to the writing of the manuscript: Amunugama H, Zhang H, Hollenberg PF
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Footnotes

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B. Please send reprint requests to: Paul F. Hollenberg, PhD, Department of Pharmacology, The University of Michigan, 1150 W. medical Center Dr. 2200 MSRB III, Ann Arbor, MI 48109-5632. Email: phollen@umich.edu
FIGURE LEGENDS

Figure 1. CYP-mediated pathways for the N-demethylation of methadone. The asterisk denotes the position of the asymmetric carbon.

Figure 2. Kinetics for the mechanism-based inactivation of CYP2B6 by methadone. The inactivation reactions were performed at 37°C in the reconstituted system as described in Materials and Methods. The concentrations of methadone in the primary reaction mixture were 0 (●), 5 (■), 10 (▲), 40 (♦), 60 (▼) and 100 (□) µM. In the inset, the rates for the time-dependent decreases in the fluorescent intensity of 7-HFC at the various concentrations of methadone were fit to the Michaelis-Mention equation to obtain the $K_i$ and $k_{inact}$ for the inactivation.

Figure 3. Partition ratio determination for the inactivation of CYP2B6 by methadone. The percentage of the catalytic activity remaining was determined as a function of the molar ratio of methadone to CYP2B6. Samples were incubated with various concentrations of methadone for 45 min with NADPH until the inactivation reaction was essentially complete. The extrapolated partition ratio was determined from the intercept of the linear regression line for the lower methadone concentration ratios and the straight line obtained for the higher methadone concentration ratios. The value of the partition ratio is approximately 22.

Figure 4. ESI-LC/MS analysis of the inactivated protein formed during the mechanism-based inactivation of CYP2B6 by methadone. Inactivation and ESI-LC/MS analysis were performed as described in Materials and Methods. Trace A is the deconvoluted spectrum in the absence of NADPH and trace B is the deconvoluted spectrum in the
presence of NADPH. No significant alteration in the mass of the inactivated apoprotein was observed suggesting that protein adduct formation is not involved in the inactivation of CYP2B6 by methadone.

Figure 5. Reduced-CO difference spectra of reconstituted CYP2B6 incubated with methadone in the absence (a) and presence of NADPH (b). CYP2B6 (0.5 nmol/ml) was incubated with 100 µM methadone in the reconstituted system at 37ºC for 15 min and the spectra of the proteins were determined as described in Materials and Methods.

Figure 6. Percentage of enzyme activity remaining compared with the loss of CO binding of CYP2B6 following incubation at various time points as described under Materials and Methods. There is a good correlation between the loss of CO-binding and the loss of enzyme activity of CYP2B6 during the inactivation.

Figure 7. HPLC elution profiles for the CYP2B6 reconstituted system after incubation with 50 µM methadone in the absence and presence of NADPH. The elution profiles were monitored at 400 nm for prosthetic heme.
Figure 1

Methadone

\[
\text{H}_3\text{C}^\text{N}^\text{*} \text{CH}_3 \quad \text{N-demethylation} \quad \text{N-demethylation}
\]

\[
\text{H}_3\text{C}^\text{NCH}_3 \quad \text{Cyclization} \quad \text{N-Demethylation}
\]

\[
\text{H}_3\text{C}^\text{NCH}_3 \quad \text{Deprotonation}
\]

\[
\text{H}_3\text{C}^\text{NCH}_3
\]

2-Ethyl-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP)
Figure 3

![Graph showing % Activity Remaining vs. [Methadone]/[CYP2B6]](image)

- % Activity Remaining on the y-axis.
- [Methadone]/[CYP2B6] on the x-axis.

Data points are plotted with a line connecting them, indicating a decrease in activity as the ratio increases, followed by a plateau.
Figure 4

A

Intensity x 10^5

54423.0

Mass (Da)

B

Intensity x 10^5

54434.0

Mass (Da)
Figure 6

![Graph showing the percentage activity remaining over time for EFC and CO.](link)