Methadone: A Substrate and Mechanism-Based Inhibitor of CYP19 (Aromatase)

Wenjie Jessie Lu, Robert Bies, Landry Kamden Kamden, Zeruesenay Desta, David A Flockhart

Division of Clinical Pharmacology
Departments of Medicine and Pharmacology
Indiana University School of Medicine
Indianapolis, IN 46202
Methadone Inhibition of CYP19

Corresponding author:

Wenjie Jessie Lu
Division of Clinical Pharmacology
Room 7123 Myers,
1001 West 10th Street,
Indianapolis, IN 46250
317-630-8795
317-630-8185 (Fax)
lu20@iupui.edu

Pages: 27
Tables: 0
Figures: 5
References: 36

Words:
Abstract: 243
Introduction: 646
Discussion: 1140

Abbreviations:
CYP: cytochrome P450. EDDP: 2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine. EMDP: 2-ethyl-5-methyl-3, 3-diphenylpyrroline.
Abstract

The peripheral conversion of testosterone to estradiol by aromatase is the primary source of endogenous estrogen in post-menopausal women. Studies indicating that placental aromatase is able to metabolize methadone to its primary metabolite, EDDP led us to test the hypothesis that methadone is able to act as an inhibitor of aromatase. Using recombinant human CYP19, we examined the ability of methadone to bring about either reversible or mechanism-based inhibition of the conversion of testosterone to estradiol. To test for reversible inhibition, racemic methadone, its metabolite EDDP or EMDP were incubated for 30 min with testosterone at the $K_m$ (4 µM). To test for mechanism-based inhibition, microsomal pre-incubations were carried out for up to 30 minutes using racemic methadone (1 µM – 1000 µM), R- or S-methadone (0.5 µM – 500 µM), EDDP or EMDP (10, 100 µM) followed by incubation with testosterone at a $V_{max}$ concentration (50 µM). Racemic methadone, EDDP and EMDP did not act as competitive inhibitors of CYP19. Preincubation of methadone, EDDP or EMDP with CYP19 resulted in time and concentration-dependant inhibition, indicating a mechanism based reaction that destroys CYP19 activity. The $K_i$ and $k_{inact}$ values for racemic methadone were calculated to be $40.6 \pm 2.8$ µM, and $0.061 \pm 0.001 \text{min}^{-1}$ respectively. No stereoselectivity was observed. Methadone is metabolized by CYP19, and may act as a potent inhibitor of CYP19 in vivo. This may contribute to variability in methadone clearance, to drug-drug interactions, and to side effects observed in individual patients.
Introduction

Methadone is a synthetic analgesic that is distinguished by its long duration of action, a property that makes it ideal for the treatment of chronic pain and for opioid withdrawal (Fredheim, et al., 2008). The use of methadone is on the increase. Prescriptions having grown in the United States by 1300% between 1997 and 2006 (Kharasch et al, 2009), primarily as the result of increasing use as a first-line analgesic. In addition, methadone maintenance therapy is the mainstay for the treatment of opioid addiction, but it is estimated that fewer than 10% of individuals who are addicted to heroin and prescription opioids are actually receiving methadone (Kleber, 2008).

Despite its advantages and widespread utility, the use of methadone is made difficult by complex pharmacokinetic characteristics that include a long elimination half-life and susceptibility to pharmacokinetic drug-drug interactions (Weschules, et al., 2008). The goal of predictable and reproducible, effective dosing is confounded by considerable inter-individual variability in methadone pharmacokinetics, particularly in its clearance (up to 100-fold) (Totah, et al., 2008). This variability is further complicated by stereoselectivity in methadone pharmacokinetics. While methadone is generally administered as a racemic mixture, consisting of equal amount of two enantiomers; the R-, levo-, 1-or (+) and the S-, dextro-, d- or (-) methadone, these enantiomers differ in pharmacokinetic and pharmacodynamic properties. The unpredictability of methadone’s effects due to these multiple characteristics results in a high incidence of inappropriate over- and under-dosing. Inappropriate dosing can clearly cause severe adverse events such as withdrawal symptoms, respiratory depression, and electrocardiographic QT interval prolongation that can result in sudden cardiac death (Sims, et al., 2007), (Kharasch, et al., 2009)Kharasch, et al., 2009,(Kalutkar, et al., 2007), (Krantz, et al., 2003).
The dominant metabolism of methadone to its primary demethylated metabolite, EDDP, and then to its secondary metabolite, EMDP, is well documented (Figure 1) (Pohland, et al., 1971), but the enzymatic route by which these reactions occur in the body is controversial (Shiran, et al., 2009). Since understanding of the metabolic pathways responsible for drug interactions or environmental effects is incomplete (Weschules, et al., 2008), it is difficult to predict methadone pharmacokinetics, and adverse events during methadone therapy are often considered “idiopathic”. Patients taking methadone are often coprescribed many other medications and are therefore vulnerable to drug-drug interactions (Weschules, et al., 2008). Poorly understood drug-drug interactions may thus contribute to the notable increase in methadone-related deaths (Sims, et al., 2007) that has attended the increase in the drug’s use over the past decade (Kharasch, et al., 2009). In addition, the effects of methadone itself on the metabolism or pharmacokinetics of other medications is understudied.

While potential interactions with methadone via CYP3A and CYP2B6 have been carefully studied in vitro (Iribarne, et al., 1997), (Foster, et al., 1999), (Wang and Devane, 2003), (Kharasch, et al., 2004) and in vivo (Eap, et al., 2002), (Totah, et al., 2008), it is clear that these enzymes cannot fully explain variability in methadone pharmacokinetics. Other potential routes of metabolism may be important. Methadone has been shown to be metabolized by aromatase (CYP19), notably in the placenta (Hieronymus, et al., 2006), (Nanovskaya, et al., 2004). However, drug interactions involving drugs that alter methadone clearance via CYP19 and interactions involving effects of methadone on CYP19 that might alter metabolism of androgens or other drugs have not been described or tested for. Side effects of methadone may be explained in part by these interactions with CYP19. In addition, patients with metastatic breast cancer who are in pain may often be co-prescribed methadone with a potent aromatase
inhibitor. Lastly, the variable pharmacokinetics of methadone via auto-inhibition or auto-
induction may be explained in part by its effects on aromatase.

For these reasons, and as a first step towards obtaining a more complete understanding of methadone’s interactions with CYP19, we tested the ability of methadone to inhibit CYP19 under conditions in which either reversible or mechanism-based inhibition could be quantified.
Methods

Chemicals and Reagents

\(R^-(−)-\text{methadone}\) and \(S^+(+)-\text{methadone}\) were generously provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine iodide (EDDP iodide), and 2-ethyl-5-methyl-3,3-diphenylpyrroline hydrochloride, (EMDP hydrochloride) were obtained from Alltech (Deerfield, IL). Norgestrol was obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Racemic methadone hydrochloride, 17-\(\beta\)-estradiol, testosterone, \(\beta\)-NADP, glucose-6-phosphate dehydrogenase, and glucose-6-phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Monobasic and dibasic sodium phosphate, monobasic potassium phosphate, magnesium chloride, HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh PA). Glycine (electrophoresis purity reagent) was obtained from Bio-Rad Laboratories (Hercules, CA, USA). All solutions of methadone, testosterone, 17-\(\beta\)-estradiol, norgestrol, and letrozole were prepared by dissolving each compound in methanol, and were stored at −20 °C.

Recombinant human CYP19 microsomal preparations: Baculovirus-insect cell-expressed human CYP19 (with oxidoreductase) were purchased from BD Biosciences (San Jose, CA). All microsomal preparations were stored at −80°C until used.

Testosterone and Methadone Metabolism \textit{in vitro} by Recombinant CYP19

General incubation conditions: All incubations were carried out using incubation times and protein concentrations that were within the linear range for reaction velocity. Testosterone and methadone were dissolved in methanol and diluted with the same solvent to the required concentrations. Any methanol in the incubation tubes was removed by drying in speed vacuum
before the incubation. All incubations contained recombinant human CYP19 in 100mM sodium phosphate buffer (pH = 7.4), with a NADPH-generating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate, 3.3 mM MgCl2, and 0.4 U/ml glucose 6-phosphate dehydrogenase) and various concentrations of CYP19 substrate in a final volume of 250μl. The final CYP19 concentration used was 0.01μM when testosterone was the substrate. When methadone was the substrate, the CYP concentration used was 0.05μM. When testosterone was the substrate, the reaction was prewarmed for 5 min at 37°C, initiated by addition of the NADPH-generating system, and incubated at 37°C for 10 min. When methadone was the substrate the final incubation was for 30 min. All reactions were terminated by the addition of 20μL of 60% (w/v) perchloric acid, followed by an immediate vortex and then by placing the tubes on ice.

**Reversible Inhibition of Recombinant CYP19**

Solutions of testosterone and tested inhibitor were mixed, and methanol was removed by drying in speed vacuum before the incubation. Incubations were then carried out in the same way as described in general incubation conditions except that the final testosterone and CYP19 concentrations were 4 μM and 0.01μM, respectively. The inhibition of CYP19 by methadone or letrozole was determined by measuring the conversion rate of testosterone to estradiol.

**Mechanism-based Inhibition of Recombinant CYP19**

Recombinant CYP19 was preincubated with various concentrations of inhibitors. All preincubations contained tested inhibitor, 0.1 μM CYP19 and 100mM sodium phosphate buffer (pH = 7.4), NADPH-generating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate, 3.3 mM MgCl2, and 0.4 U/ml glucose 6-phosphate dehydrogenase) in a final volume of 250μl. Preincubations were initiated and carried out in the same way as described for general incubation conditions. After a period of time, each 250μl preincubation mixture was diluted 10 times with a solution containing testosterone, sodium phosphate buffer and NADPH-generating
system to reach a final incubation mixture of 50 µM testosterone and 0.01 µM CYP19. The dilution was to eliminate competitive inhibition as a possible cause of the inhibitory effect. This incubation mix was then incubated at 37°C for 10 min. The reaction was terminated the same way as described in general incubation conditions. The extent of inactivation of CYP19, expressed as the percent activity remaining after preincubation, was determined by measuring the conversion rate of testosterone to estradiol.

**Extraction**

All samples were extracted immediately after the incubation. First, 25 µL of 500µM norgestrol was added to each sample as an internal standard. The incubation mixture was centrifuged at 14,000rpm for 5min at room temperature. The supernatant layer was made alkaline by adding 500µL of 1M glycine-NaOH buffer (pH 11.3) and extracted by adding 6mL of ethyl acetate. This mixture was vortex-mixed for 10 seconds and then centrifuged at 26,000rpm for 15min. The organic layer was transferred to 13×100-mm glass culture tubes and evaporated to dryness. The resulting residue was reconstituted with mobile phase (50% 10mM monobasic potassium phosphate, 40% acetonitrile and 10% methanol) and analyzed as described below immediately.

**Quantifications of Estradiol and EDDP Formation**

High performance liquid chromatography (HPLC) assays with ultraviolet (UV) detection were developed for the quantification of testosterone conversion to estradiol and of methadone conversion to EDDP. The HPLC-UV system was comprised of a Waters (Milford, MA) model 510 HPLC pump and a Waters model 717plus autosampler, coupled with a Waters 486 tunable absorbance detector. The separation system consisted of a Zorbax SB-C<sub>18</sub> column (4.6 x 150mm, 3.5µm particle size, Phenomenex, Torrance, CA), a Nova-Pak C<sub>18</sub> Guard column (4 µm; Waters, Inc., Ireland). An isocratic elution was used to separate the compounds. The mobile phase consisted of 50% 10mM monobasic potassium phosphate, 40% acetonitrile and
10% methanol without PH adjustment. The eluate was introduced, at 0.8 ml/min to the UV detector with a run time of 25 min. Under these conditions, the retention times of estradiol, EDDP, testosterone, methadone, norgestrol and EMDP were approximately 8, 10, 12, 15, 20 and 35 min, respectively.

Peak areas for each peak were obtained from an integrator, and peak area ratios with internal standard were calculated. Standard curves were estimated by linear regression of peak area ratios. Quantification of samples was carried out by applying the linear regression equation of the standard curve to the peak area ratio. The microsomal activity data represent individual data points or the mean of duplicate assays. Duplicates did not vary by more than 10%. The limit of quantification for estradiol was 2.5 pmol on column, with intra- and inter-day coefficients of variation of 2.4% and 5.3% respectively. The limit of quantification of EDDP was 5 pmol on column, with intra- and inter-day coefficients of variation of 5.5% and 7.3% respectively.

**Kinetic Analyses**

Formation rates of metabolite at different substrate concentrations were fit to appropriate enzyme kinetic models using SigmaPlot (Version 10.0, Systat software, Port Richmond, CA). Data were best fit to a one site Michaelis-Menten equation. The rates of metabolite formation from substrate probes in the presence of the test inhibitors were compared with those for control in which the inhibitor was replaced with vehicle. The inhibition data were fit to different models of enzyme inhibition (competitive, non-competitive, and uncompetitive). The equations for kinetic and inhibition analyses of the data were constructed using NONMEM V v1.1 ® (ICON, Hanover MD and UCSF), utilizing the nonlinear regression functionality. The appropriateness of the fit was determined by visual inspection and by using the objective function, residual patterns, residual sums of squares, and precision of the parameter estimates. The correlation coefficient and its corresponding statistical significance were determined by conventional methods.
Determination of $k_{\text{inact}}$ and $K_i$

$K_i$ and $k_{\text{inact}}$ values were estimated using an approach that involves a simultaneous fit of all the data. Data from experiments documenting the time course of inactivation were used for the calculation of inhibition kinetic parameters. Estradiol formation was expressed as pmol/min/pmol CYP19 and the percentage of remaining activity was calculated as shown in Equation 1

$$\% \text{ of remaining enzyme activity} = \frac{E_t}{E_0} \times 100 \quad (1)$$

where $E_t$ is the enzyme activity expressed as estradiol formation at time $t$, and $E_0$ is the average estradiol formation at preincubation time zero (100%). The extent of inhibition was expressed as $(1 - \%)$ of remaining enzyme activity.

The pseudo first-order rate constant for enzyme inactivation, $\lambda$, was estimated from the aggregation of all the data using Equation 2.

$$E(t) = E_0 \cdot e^{-\lambda \cdot t} \quad (2)$$

The relationship between $\lambda$ and inhibitor concentration was fit to the following equation:

$$\lambda = k_{\text{inact}} \cdot [I]/(K_i + [I]) \quad (3)$$

where $k_{\text{inact}}$ is the rate constant for inactivation, $[I]$ is inhibitor or inactivator concentration, and $K_i$ is the inactivator concentration that produced half the maximal rate of inactivation, analogous to a $K_m$. Equation 3 assumes there was negligible change of $[I]$ during the incubation period and that loss of enzyme was due to inactivation. As indicated above, we fit all the data for each inhibitor concentration to obtain a single estimate for $k_{\text{inact}}$ and a single estimate for $K_i$ using equations 1-3.
Results

Testosterone Metabolism by Recombinant CYP19

To measure the activity of enzyme, the rate of aromatization of testosterone to 17-β-estradiol was used as a marker of CYP19 activity. Under the conditions used, CYP19 was able to catalyze the generation of estradiol from testosterone with a $V_{max}$ of 9.1 pmol/min/pmol P450 and $K_m$ of 4.0 µM. (data not shown). A concentration of testosterone at the $K_m$ was chosen to test for reversible, competitive inhibition of CYP19 by methadone. At 50 µM testosterone, the rate of estradiol formation was at $V_{max}$. This concentration was therefore chosen for experiments to test the mechanism-based inhibition of CYP19 by methadone. The rate of estradiol formation was used as a measure of CYP activity.

Reversible Inhibition of CYP19 by Methadone

Figure 2 shows the rate of estradiol formation from 4 µM testosterone in the presence of a range of methadone concentrations from 0 to 2mM. No effect of methadone on the rate of metabolism of this probe for CYP19 was observed under these conditions, even at the highest concentration of methadone used. The selective CYP19 inhibitor letrozole (1µM) used as positive control, was able was to completely block the formation of estradiol.

Metabolism of Methadone by CYP19

Since no competitive inhibition of CYP19 was observed under the conditions used, the possibility that methadone itself could be metabolized by CYP19 under these conditions was tested. Metabolism of methadone to EDDP by recombinant human CYP19 was observed. Experiments to characterize this metabolism were then carried out under conditions where secondary metabolism from EDDP to EMDP was not detected. Figure 3 depicts the rate of EDDP generation from methadone across a range of methadone concentrations (0 – 2000 µM).
These data indicate metabolism of methadone to EDDP with a $K_m$ of 314 µM and a $V_{max}$ of 7.6 pmol/min/pmol of P450. The enzyme converting methadone to EDDP was confirmed to be CYP19 by demonstrating that the conversion of methadone (1000 µM) to EDDP was completely inhibited by 1 µM letrozole (data not shown).

**Mechanism Based Inhibition of CYP19 by Methadone**

Since methadone metabolism to EDDP could be catalyzed by CYP19, pilot experiments were conducted to test the ability of methadone to inactivate CYP19 activity in a time dependant manner. When CYP19 was pre-incubated in the presence of 1mM methadone and then incubated with a $V_{max}$ concentration of testosterone (50 µM), this resulted in a log linear time-dependant loss of CYP19 activity (data not shown). If enzyme was pre-incubated under the same conditions without methadone, no decrease in enzyme activity was observed and the $V_{max}$ activity of estradiol formation was maintained. This inactivation was also dependant on methadone concentration. When 0 – 500 µM racemic methadone was pre-incubated for 25 min, inhibition of 50% of activity was seen at approximately 50 µM methadone, while 30% inhibition was brought about by the presence of 10 µM methadone (data not shown). When the individual enantiomers (0 – 500 µM, over 5 – 20 min) were separately tested under the same conditions, no stereoselectivity was observed (data not shown).

To estimate the kinetics of the interaction between methadone and CYP19 during preincubation, a range of methadone concentrations and preincubation times were tested. Figure 4A depicts the preincubation time and concentration dependence of the effect of methadone on CYP19 activity. Control incubations were carried out in the presence of NADPH but without methadone. The rates of metabolism at 0 preincubation time for each concentration were consistent with the $V_{max}$ activity of CYP19 under these conditions. It was therefore possible to normalize the data at each concentration to the activity seen at 0 min of preincubation. Kinetic analyses of the rates of
enzyme inactivation in the presence of methadone were carried out using NONMEM®, and predicted time curves for inactivation at each methadone concentration were obtained (Figure 4A). The $K_I$, and $k_{inact}$ values were calculated to be $40.6 \pm 2.8\mu\text{M}$, and $0.061 \pm 0.001\text{min}^{-1}$ respectively. A close correlation between observed and predicted rates of CYP19 was observed (Figure 4B), indicating that the data are reliably represented by the model.

Since methadone is metabolized by CYP19, we tested the possibility that inhibition of CYP19 by methadone might be due to the metabolites formed. We directly tested the metabolites (EDDP and EMDP) in both reversible and mechanism based experimental designs in the same way as described for methadone itself. No competitive inhibition was observed when EDDP or EMDP were tested at concentrations up to $100 \ \mu\text{M}$ (Data not shown). When mechanism based inhibition was tested, inactivation of CYP19 was observed by EDDP or EMDP at concentrations as low as $10 \ \mu\text{M}$ (Data not shown). Figure 5 shows that the rate of inactivation by EMDP (100 $\mu\text{M}$) was slower than that by either EDDP (100 $\mu\text{M}$) or racemic methadone (100 $\mu\text{M}$), which appeared equipotent.
Discussion

The effective use of methadone in the treatment of pain and heroin addiction is compromised by poor understanding of the metabolic routes involved in its disposition. One direct consequence is inability to anticipate drug interactions in patient populations who are commonly co-prescribed multiple medications. In this study we demonstrated that CYP19 is able to metabolize methadone and that methadone is a mechanism-based inhibitor of the enzyme.

Our data showed no clear competitive inhibition of CYP19 by methadone using recombinant human CYP19. Ahmed et al. (Zharikova, et al., 2006) reported competitive inhibition with a $K_i$ of 393 µM in human placental microsomes but, consistent with the present data, they did not demonstrate competitive inhibition in recombinant CYP19. Although we have not yet tested placental microsomes, it may be anticipated that a lower $K_i$ would be obtained if methadone acts as a mechanism-based inhibitor in the placenta.

We observed that human recombinant CYP19 was able to metabolize methadone to the dominant metabolites that are observed in vivo and that this can be completely blocked by the specific aromatase inhibitor letrozole. These data are consistent with those reported by Ahmed et al., who reported methadone metabolism in human placental microsomes, and by recombinant CYP19 (Nanovskaya, et al., 2004). This finding may be important. First, it is possible that CYP19 contributes to the clearance of methadone in vivo, as suggested by a number of clinical observations. Methadone clearance has been documented to increase during pregnancy, when placental CYP19 has high activity (Pond, et al., 1985), (Nanovskaya, et al., 2004). It is possible that fetal CYP19 activity could be responsible in part for the methadone withdrawal observed in neonates of methadone patients (Serane and Kurian, 2008), (Szeto, et al., 1981). That said, the extent to which CYP19 metabolizes methadone in general is unclear,
and withdrawal from methadone is often unanticipated, idiosyncratic and is poorly understood. Second, since the biochemical aromatization of testosterone by CYP19 is initiated by the removal of a methyl group, and since CYP19 is also able to remove methyl groups from methadone, cocaine (Osawa, et al., 1997), and buprenorphine (Deshmukh, et al., 2003), it is possible that CYP19 is involved in the demethylation of other drugs.

We have shown that methadone is able to bring about time- and concentration-dependant inhibition of CYP19, consistent with the action of an irreversible mechanism-based or “suicide” inhibitor. The data meet established criteria (Silverman, 1988) for the involvement of methadone as a mechanism-based inhibitor of CYP19. Such inhibitory activity is dependent on the catalytic mechanism and activity of the enzyme itself, which must first bind the drug and then catalytically activate it (Jones, et al., 1999). The activated moiety irreversibly alters the enzyme and removes it permanently from the pool of active enzyme. It is important to note that the concentration of CYP19 used in our experiments was relatively high. We observed a $K_m$ of 4 µM for the conversion of testosterone to estradiol when 0.01 µM CYP19 was used, while the $K_m$ reported for this reaction in placental microsomes was 0.2 µM (Zharikova, et al., 2006). Similarly, the inactivation constant $K_i$ that we observed was 40.6 µM, but these data were obtained with a CYP19 concentration, (0.1 µM), ten times higher. Lower values may be obtained at the lower enzyme concentrations likely to be present in cells in vivo. In addition, while R-methadone therapeutic concentrations observed in plasma at steady state are in the 0.5 – 1 µM range (Eap, et al., 2000), the concentration of this highly hydrophobic drug in tissues and at the CYP19 active site may be substantially higher (Levine, et al., 1995).

Since methadone appears able to inhibit CYP19, it follows that methadone may alter the metabolism and disposition of endogenous testosterone and androstenedione. Lower concentrations of estradiol and of FSH have been documented in men taking methadone
(Hallinan, et al., 2009), and low bone mineral density that may be due to low estrogen concentrations has been documented in 83% of patients in a methadone maintenance treatment program (Kim, et al., 2006). Conceivably, other side effects of methadone that include flushing, muscle pain and symptoms reminiscent of estrogen withdrawal (Senay, 1985), (Backstrom, 1995) may be explained in part by the drug’s action on CYP19.

Unanticipated accumulation of methadone in patients vulnerable to auto-inhibition may contribute to unexpectedly high methadone concentrations with resultant potentially lethal consequences. Methadone accumulation does not occur universally, and multiple factors may underlie the auto-inhibition observed in some patients, in contrast to the auto-induction observed in others (Morton EB, 2007). A poor understanding of the effects of methadone on its own clearance over time also impedes our ability to dose accurately, to anticipate and manage withdrawal in multiple clinical settings. These include the withdrawal that neonates of methadone-treated mothers’ experience (Serane and Kurian, 2008), the withdrawal symptoms that often occur during pregnancy (Pond, et al., 1985), and that occur routine use of methadone to treat pain or heroin addiction.

Our data indicate that EDDP appeared to be as potent a mechanism-based inhibitor of CYP19 as methadone itself, while EMDP appeared weaker. This suggests that these metabolites may contribute to the inhibitory effect on CYP19, although they are present at lower concentrations than methadone in vivo (Kharasch, et al., 2008). Mechanism based inhibition can involve the formation of a metabolite intermediate complex (MIC) that inactivates the enzyme and takes it out of the active pool (Jones, et al., 1999). Such inhibition brought about by metabolite-intermediate complex formation has been demonstrated for a number of N-demethylated drugs including diltiazem (Jones, et al., 1999) and erythromycin (Ortiz de Montellano, et al., 1981). Compared to reversible inhibition, irreversible inhibition more frequently results in unfavorable
drug-drug interactions as the inactivated P450 enzyme has to be replaced by newly synthesized protein (Kalgutkar, et al., 2007), and severe clinical consequences may result (Wilkinson, 2005).

Mechanism-based inhibition can result from bioactivation to a reactive intermediate that interacts with the heme prosthetic group of a cytochrome P450, or that covalently modifies the apoprotein (Hollenberg, et al., 2008), (Kalgutkar, et al., 2007). While it is possible in situations where one species is responsible for MIC formation to measure the kinetics of spectral change caused by interaction with heme, methadone and both its metabolites all appear to be active mechanism based CYP19 inhibitors in this study. As a result, we were not able to determine the kinetics of MIC formation by the individual species, and the precise molecular mechanism responsible for this mechanism-based inactivation requires further study.

We have shown that CYP19 is able to demethylate methadone in vitro, and that this drug is a mechanism-based inhibitor of CYP19. While the clinical relevance of this interaction is not clear, it is possible that this contributes to the variability in methadone clearance, to drug-drug interactions caused by methadone via CYP19 inhibition, and to the side effects observed in individual patients.
Acknowledgements

R- and S-methadone were generously provided by the National Institute on Drug Abuse. The authors are grateful to David Jones PhD for valuable advice on experimental design and review of the manuscript, and to David Thacker PharmD and Evan Ogburn MSc for assistance during the development of the chromatographic assays used.
Reference List


Footnotes

This work was supported in part by the National Institutes of Health National Center for Research Resources [Grant K24RR020815] and the National Institute for General Medical Sciences, [Grants T32GM008425, U01GM061373].

This work was presented in part at the AACR meeting on Cancer Genomics and Epigenomics in Hawaii on February 9th, 2009.

Reprint Requests:

Wenjie Jessie Lu
Division of Clinical Pharmacology
Room 7123 Myers,
1001 West 10th Street,
Indianapolis, IN 46250
317-630-8795
317-630-8185 (Fax)
lu20@iupui.edu
Legends

Figure 1. Dominant Route of Methadone Metabolism. Methadone is N-demethylated to the primary metabolite, EDDP. EDDP is further N-demethylated to the secondary metabolite, EMDP.

Figure 2. Methadone is Not a Reversible Inhibitor of Recombinant Human CYP19. Testosterone (4 µM) was incubated with a range of racemic methadone concentrations and recombinant human CYP19 for 10 min, and CYP19 activity was determined by measuring the generation of estradiol from testosterone in three experiments. Bars represent the average rate of estradiol formation in duplicate samples at increasing concentrations of methadone or in the presence of 1 µM letrozole (positive control). The dotted line indicates the average rate obtained in the absence of methadone.

Figure 3. Recombinant Human CYP19 was Able to Catalyze Methadone Metabolism to EDDP. A range of racemic methadone concentrations was incubated with recombinant CYP19 (0.05 µM) for 30 min and the amounts of EDDP generated were determined by HPLC as described in three experiments. Individual points represent the mean of duplicate incubations.

Figure 4. Kinetic Analysis of Mechanism Based Inhibition of CYP19 by Racemic Methadone. A) Time- and concentration-dependent inactivation. Methadone (MTD 10, 50, 100, 200, 500 µM) was pre-incubated with human recombinant CYP19 (0.1 µM) for 0, 5, 10, 15, or 20 min, and then activity of CYP19 (0.01 µM) was assessed in incubations carried out in duplicate (n=2). Individual data points (●) were plotted as the percent of CYP19 activity observed at 0 min of preincubation. The curve of each plot represents the line of best fit for the inactivation reaction at the concentration indicated using NONMEM®. B) Correlation Between
Observed and NONMEM® Predicted Values of Percent CYP19 Activity Remaining. The solid line represents the line of equality. A correlation coefficient ($R^2$) of 0.9906 was obtained.

**Figure 5. Mechanism Based Inhibition of CYP19 by Racemic Methadone, EDDP and EMDP.** Methadone (MTD), EDDP and EMDP (100 µM) were pre-incubated with recombinant human CYP19 (0.1 µM) for 0, 5, 10, 15, or 20 min, and then activity of CYP19 (0.01 µM) was assessed in incubations carried out in duplicate ($n=2$). Representative data were plotted as the log percent of CYP19 activity observed at zero min of preincubation.
Figure 1

- Methadone
- EDDP
- EMDP
Figure 2

Rate of Estradiol Generation (pmol/min/pmol P450)

Methadone (μM)

0 50 100 500 1000 1500 2000 Letrozole 1μM
Figure 3

![Graph showing enzymatic activity of Methadone with concentration](image)

- $V_{max} = 7.64$ pmol/min/pmol CYP19
- $K_m = 314 \mu M$
Figure 5

Log (% CYP19 Activity) vs. Preincubation Time (min)

- MTD 100 μM
- EDDP 100 μM
- EMDP 100 μM