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

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

Division of Clinical Pharmacology, Departments of Medicine and Pharmacology, Indiana University School of Medicine, Indianapolis, Indiana

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ABSTRACT:
The peripheral conversion of testosterone to estradiol by aromatase is the primary source of endogenous estrogen in postmenopausal women. Studies indicating that placental aromatase is able to metabolize methadone to its primary metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidin (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 or its metabolite EDDP or 2-ethyl-5-methyl-3,3-diphenylpyrrolidine (EMDP) was incubated for 30 min with testosterone at the $K_i$ (4 μM). To test for mechanism-based inhibition, microsomal preincubations were performed for up to 30 min using racemic methadone (1–1000 μM), R- or S-methadone (0.5–500 μM), or EDDP or EMDP (10 and 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-dependent 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 ± 2.8 μM and 0.061 ± 0.001 min$^{-1}$, respectively. No stereoselectivity was observed. Methadone is metabolized by CYP19 and may act as a potent inhibitor of CYP19 in vivo. These findings may contribute to variability in methadone clearance, to drug-drug interactions, and to side effects observed in individual patients.

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 increasing, prescriptions having grown in the United States by 1300% between 1997 and 2006 (Kharasch et al., 2009), primarily as the result of its 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 interindividual 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. Although methadone is generally administered as a racemic mixture, consisting of equal amounts of two enantiomers; the R-, levo-, $l$-, or (+)- and the S-, dextro-, $d$-, or (−)-methadone, these enantiomers differ in pharmacokinetic and pharmacodynamic properties. The unpredictability of the effects of methadone due to these multiple characteristics results in a high incidence of inappropriate over- and underdosing. 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 (Krantz et al., 2003; Kalgutkar et al., 2007; Sims et al., 2007; Kharasch et al., 2009).

The dominant metabolism of methadone to its primary demethylated metabolite, EDDP, and then to its secondary metabolite, EMDP, is well documented (Fig. 1) (Pohland et al., 1971), but the enzymatic route by which these reactions occur in the body is controversial (Shiran et al., 2009). Because 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 often have coprescriptions for many other medica-

ABBREVIATIONS: EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EMDP, 2-ethyl-5-methyl-3,3-diphenylpyrroline; HPLC, high-performance liquid chromatography; P450, cytochrome P450; MIC, metabolite intermediate complex.
Methadone inhibition of CYP19

Methadone is N-demethylated to the primary metabolite, EDDP. EDDP is further N-demethylated to the secondary metabolite, EMDP.

![Diagram of Methadone Metabolism](https://example.com/diagram.png)

Fig. 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.

Reversible inhibition of recombinant CYP19.

Solutions of testosterone and testosteron 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 and 0.01 μM, 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 testosteron inhibitor, 0.1 μM CYP19 and 100 mM 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 250 μl. Preincubations were initiated and carried out in the same way as described for general incubation conditions. After a period of time, each 250-μl preincubation mixture was diluted 10 times with a solution containing testosteron, sodium phosphate buffer, and NADPH-generating system to reach a final incubation mixture of 50 mM 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 percentage of activity remaining after preincubation, was determined by measuring the conversion rate of testosterone to estradiol.

Materials and Methods

**Chemicals and Reagents.** R(-)-Methadone and S(+)-methadone were generously provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine hydrochloride (EMDP hydrochloride) were obtained from Alltech Associates (Deerfield, IL). Norgestrel was obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Racemic methadone hydrochloride, 17β-estradiol, testosterone, β-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, high-performance liquid chromatography (HPLC)-grade acetonitrile, and methanol were purchased from Thermo Fisher Scientific (Waltham, MA). Glycine (electroerosis purity reagent) was obtained from Bio-Rad Laboratories (Hercules, CA). All solutions of methadone, testosterone, 17β-estradiol, norgestrel, and letrozole were prepared by dissolving each compound in methanol and were stored at -20°C.

For recombinant human CYP19 microsomal preparations, baculovirus insecct cell-expressed human CYP19 (with oxidoreductase) was purchased from BD Biosciences (San Jose, CA). All microsomal preparations were stored at -80°C until used.

**Testosterone and Methadone Metabolism In Vitro by Recombinant CYP19.** General incubation conditions were as follows. All incubations were performed 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 a speed vacuum before the incubation. All incubations contained recombinant human CYP19 in 100 mM sodium phosphate buffer (pH 7.4), with an 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 250 μl. The final CYP19 concentration used was 0.01 μM when testosterone was the substrate. When methadone was the substrate, the P450 concentration used was 0.05 μM. 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 20 μl 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 testosteron 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 and 0.01 μM, 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 testosteron inhibitor, 0.1 μM CYP19 and 100 mM 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 250 μl. Preincubations were initiated and carried out in the same way as described for general incubation conditions. After a period of time, each 250-μl preincubation mixture was diluted 10 times with a solution containing testosteron, sodium phosphate buffer, and NADPH-generating system to reach a final incubation mixture of 50 mM 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 percentage of 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 norgestrel (500 μM) was added to each sample as an internal standard. The incubation mixture was centrifuged at 14,000 rpm for 5 min at room temperature. The supernatant layer was made alkaline by adding 500 μl of glycine-NaOH (1 M) buffer (pH 11.3) and extracted by adding 6 ml of ethyl acetate. This mixture was vortex-mixed for 10 s and then centrifuged at 26,000 rpm for 15 min. 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% 10 mM monobasic potassium phosphate, 40% acetonitrile, and 10% methanol) and analyzed as described below.

**Quantifications of Estradiol and EDDP Formation.** HPLC assays with UV detection were developed for the quantification of testosterone conversion to estradiol and of methadone conversion to EDDP. The HPLC-UV system comprising 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-C18 column (4.6 × 150 mm, 3.5-μm particle size; Phenomenex, Torrance, CA), a Nova-Pak C18 Guard column (4 μm, Waters, Dublin, Ireland). An isotropic elution was used to separate the compounds. The mobile phase consisted of 50% 10 mM 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, norgestrel, 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. Samples were quantified by applying the linear regression equation of the standard curve to the peak area ratio. The interassay variation of 5.5 and 7.3%, respectively.

Mechanism-Based Inhibition of CYP19 by Methadone. Because 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 performed under conditions in which secondary metabolism from EDDP to EMMP 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<sub>m</sub> of 314 μM and a V<sub>max</sub> 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).

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Mechanism-Based Inhibition of CYP19 by Methadone. Because methadone metabolite to EDDP could be catalyzed by CYP19, pilot experiments were conducted to test the ability of methadone to inactivate CYP19 activity in a time-dependent manner. When CYP19 was preincubated in the presence of 1 mM methadone and then incubated with a V<sub>max</sub> concentration of testosterone (50 μM), this resulted in a log-linear time-dependent loss of CYP19 activity (data not shown). If enzyme was preincubated under the same

Results

Testosterone Metabolism by Recombinant CYP19. To measure the activity of enzyme, the rate of aromatization of testosterone to 17β-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<sub>max</sub> of 9.1 pmol/min/pmol of P450 and K<sub>m</sub> of 4.0 μM (data not shown). A concentration of testosterone at the K<sub>m</sub> was chosen to test for reversible, competitive inhibition of CYP19 by methadone. At 50 μM testosterone, the rate of estradiol formation was at V<sub>max</sub>. 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 P450 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 2 mM. 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 (1 μM), used as positive control, was able to completely block the formation of estradiol.

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conditions without methadone, no decrease in enzyme activity was observed, and the $V_{\text{max}}$ activity of estradiol formation was maintained. This inactivation was also dependent on methadone concentration. When 0 to 500 μM racemic methadone was preincubated for 25 min, inhibition of 50% of activity was seen at approximately 50 μM methadone, whereas 30% inhibition was produced 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 performed in the presence of NADPH but without methadone. The rates of metabolism at 0 preincubation time for each concentration were consistent with the $V_{\text{max}}$ 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 performed with NONMEM, and predicted time curves for inactivation at each methadone concentration were obtained (Fig. 4A). The $K_i$ and $k_{\text{inact}}$ values were calculated to be 40.6 ± 2.8 μM, and 0.061 ± 0.001 min$^{-1}$, respectively. A close correlation between observed and predicted rates of CYP19 was observed (Fig. 4B), indicating that the data are reliably represented by the model.

Because 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 was tested at concentrations up to 100 μM (data not shown). When mechanism-based inhibition was tested, inactivation of CYP19 was observed by EDDP or EMDP at concentrations as low as 10 μM (data not shown). Figure 5 shows that the rate of inactivation by EMDP (100 μM) was slower than that by either EDDP (100 μM) or racemic methadone (100 μM), which appeared to be 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, for whom multiple medications are commonly coprescribed. 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 and colleagues (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 seen in vivo, and that this metabolism can be completely blocked by the specific aromatase inhibitor letrozole. These data are consistent with those noted by Ahmed and colleagues (Nanovskaya et al., 2004), who reported methadone metabolism in human placental microsomes and by recombinant CYP19. 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 (Szeto et al., 1981; Serane and Kurian, 2008). That said, the extent to which CYP19 metabolizes methadone in general is unclear, and withdrawal from methadone is often unanticipated, is idiosyncratic, and is poorly understood. Second, because the biochemical aromatization of testosterone by CYP19 is initiated by the removal of a methyl group and because 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-dependent 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 $K_{i}$ of 4 $\mu$M for the conversion of testosterone to estradiol when 0.01 $\mu$M CYP19 was used, whereas the $K_{i}$ reported for this reaction in placental microsomes was 0.2 $\mu$M (Zharikova et al., 2006). Likewise, the inactivation constant $K_{i}$ that we observed was 40.6 $\mu$M, but these data were obtained with a CYP19 concentration of 0.1 $\mu$M, 10 times higher. Lower values may be obtained at the lower enzyme concentrations likely to be present in cells in vivo. In addition, although $R$-methadone therapeutic concentrations observed in plasma at steady state are in the 0.5 to 1 $\mu$M 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).

Because methadone seems to be 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). It is conceivable that other side effects of methadone, which include flushing, muscle pain, and symptoms reminiscent of estrogen withdrawal (Senay, 1985; Bäckström, 1995), may be explained in part by the drug’s action on CYP19.

Unanticipated accumulation of methadone in patients vulnerable to autoinhibition may contribute to unexpectedly high methadone concentrations with resultant potentially lethal consequences. Methadone accumulation does not occur universally, and multiple factors may underlie the autoinhibition observed in some patients, in contrast to the autoinduction observed in others (Morton, 2007). The poor understanding of the effects of methadone on its own clearance over time also impedes our ability to dose accurately, to anticipate withdrawal, and to 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 the withdrawal symptoms that occur with routine use of methadone to treat pain or heroin addiction.

Our data indicate that EDDP seemed to be as potent a mechanism-based inhibitor of CYP19 as methadone itself, whereas EMDP seemed to be weaker. This finding suggests that these metabolites may contribute to the inhibitory effect on CYP19, although they are present at lower concentrations than that of 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 dilazepem (Jones et al., 1999) and erythromycin (Ortiz de Montellano et al., 1981). Compared with reversible inhibition, irreversible inhibition more frequently results in unfavorable drug-drug interactions because the inactivated P450 enzyme has to be replaced by newly synthesized protein (Kalugtukar 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 (Kalugtukar et al., 2007; Hollenberg et al., 2008). Although it is possible in situations in which 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 seemed 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. Although the clinical relevance of this interaction is not clear, it is possible that it 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.
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References

Address correspondence to: Wenjie Jessie Lu, Division of Clinical Pharmacology, Room 7123 Myers, 1001 W. 10th St., Indianapolis, IN 46202. E-mail: lu20@iupui.edu