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

Received January 31, 2010; accepted April 21, 2010

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-diphenylpyrrolidine (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_m (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 $40.6 \pm 2.8 \mu\text{M}$ 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. 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-

This work was supported in part by the National Institutes of Health National Center for Research Resources [Grant K24-RR020815]; and the National Institutes of Health National Institute for General Medical Sciences [Grants T32-GM008425, U01-GM061373].

Parts of this work were previously presented at the following conference: Lu WJ, Desta Z, and Flockhart DA (2010) Methadone is a potent and mechanism-based inhibitor of metabolism CYP19 (aromatase). *Cancer Genomics, Epigenomics, and the Development of Novel Therapeutics*; 2009 Feb 5–9; Waikoloa, HI. American Association for Cancer Research, Philadelphia, PA.

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

doi:10.1124/dmd.110.032474.

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

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.

tions and are therefore vulnerable to drug-drug interactions (Wechsules 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 use of the drug over the past decade (Kharasch et al., 2009). In addition, the effect of methadone itself on the metabolism or pharmacokinetics of other medications is understudied.

Although 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 (Nanovskaya et al., 2004; Hieronymus et al., 2006). 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, for patients with metastatic breast cancer who are in pain, methadone may often be coprescribed with a potent aromatase inhibitor. Last, the variable pharmacokinetics of methadone via autoinhibition or autoinduction may be explained in part by its effects on aromatase.

For these reasons and as a first step toward 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.

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 iodide (EDDP iodide) and 2-ethyl-5-methyl-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, 17 β -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 (electrophoresis purity reagent) was obtained from Bio-Rad Laboratories (Hercules, CA). All solutions of methadone, testosterone, 17 β -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 insect 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 incubations 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 MgCl_2 , 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 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 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 tested 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 MgCl_2 , 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 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 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 $\times$ 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 $\times$ 150 mm, 3.5- μm particle size; Phenomenex, Torrance, CA), a Nova-Pak C₁₈ Guard column (4 μm ; Waters, Dublin, Ireland). An isocratic 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 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 interday coefficients of variation of 2.4 and 5.3%, respectively. The limit of quantification of EDDP was 5 pmol on column, with intra- and interday 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, San Jose, 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, noncompetitive, and uncompetitive). The equations for kinetic and inhibition analyses of the data were constructed with NONMEM V (version 1.1; ICON, Hanover, MD, and University of California San Francisco, San Francisco, CA), using 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_{inact} and K_I . K_I and k_{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 picomoles per minute per picomole of CYP19, and the percentage of remaining activity was calculated as shown in eq. 1:

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

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 0 (100%). The extent of inhibition was expressed as $1 - \text{percentage of remaining enzyme activity}$.

The pseudo first-order rate constant for enzyme inactivation, λ , was estimated from the aggregation of all the data using eq. 2:

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

The relationship between λ and inhibitor concentration was fit to eq. 3:

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

where k_{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 that there was a negligible change in $[I]$ during the incubation period and that loss of enzyme was due to inactivation. As indicated above, we fit all of the data for each inhibitor concentration to obtain a single estimate for k_{inact} and a single estimate for K_I using eqs. 1 to 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 of 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 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.

Metabolism of Methadone by CYP19. 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 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. Because 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-dependent manner. When CYP19 was preincubated in the presence of 1 mM methadone and then incubated with a V_{max} 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

FIG. 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.

FIG. 3. Recombinant human CYP19 was able to catalyze methadone metabolism to EDDP. Racemic methadone at a range of 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.

conditions without methadone, no decrease in enzyme activity was observed, and the V_{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_{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_{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 (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

FIG. 4. Kinetic analysis of mechanism-based inhibition of CYP19 by racemic methadone. A, time- and concentration-dependent inactivation. Methadone (MTD) (10, 50, 100, 200, and 500 μM) was preincubated 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 (gray circles) were plotted as the percentage 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.

FIG. 5. Mechanism-based inhibition of CYP19 by racemic methadone, EDDP, and EMDP. Methadone (MTD), EDDP, and EMDP (100 μ M) were preincubated with recombinant human CYP19 (0.1 μ M) for 0, 5, 10, 15, or 20 min, and then activity of CYP19 (0.01 μ M) was assessed in incubations carried out in duplicate ($n = 2$). Representative data were plotted as log percent CYP19 activity observed at zero minutes of preincubation.

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_m of 4 μ M for the conversion of testosterone to estradiol when 0.01 μ M CYP19 was used, whereas the K_m reported for this reaction in placental microsomes was 0.2 μ M (Zharikova et al., 2006). Likewise, the inactivation constant K_I that we observed was 40.6 μ M, but these data were obtained with a CYP19 concentration of 0.1 μ 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 μ 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 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 diltiazem (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 (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 (Kalgutkar 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.

Acknowledgments. *R*-Methadone and *S*-methadone were generously provided by the National Institute on Drug Abuse. We are grateful to David Jones, Ph.D., for valuable advice on experimental design and review of the manuscript and to David Thacker, Pharm.D., and Evan Ogburn, M.Sc., for assistance during the development of the chromatographic assays used.

References

- Bäckström T (1995) Symptoms related to the menopause and sex steroid treatments. *Ciba Found Symp* **191**:171–180.
- Deshmukh SV, Nanovskaya TN, and Ahmed MS (2003) Aromatase is the major enzyme metabolizing buprenorphine in human placenta. *J Pharmacol Exp Ther* **306**:1099–1105.
- Eap CB, Bourquin M, Martin J, Spagnoli J, Livoti S, Powell K, Baumann P, and Déglon J (2000) Plasma concentrations of the enantiomers of methadone and therapeutic response in methadone maintenance treatment. *Drug Alcohol Depend* **61**:47–54.
- Eap CB, Buclin T, and Baumann P (2002) Interindividual variability of the clinical pharmacokinetics of methadone: implications for the treatment of opioid dependence. *Clin Pharmacokinet* **41**:1153–1193.
- Foster DJ, Somogyi AA, and Bochner F (1999) Methadone *N*-demethylation in human liver microsomes: lack of stereoselectivity and involvement of CYP3A4. *Br J Clin Pharmacol* **47**:403–412.
- Fredheim OM, Moksnes K, Borchgrevink PC, Kaasa S, and Dale O (2008) Clinical pharmacology of methadone for pain. *Acta Anaesthesiol Scand* **52**:879–889.
- Hallinan R, Byrne A, Agho K, McMahon CG, Tynan P, and Attia J (2009) Hypogonadism in men receiving methadone and buprenorphine maintenance treatment. *Int J Androl* **32**:131–139.
- Hieronimus TL, Nanovskaya TN, Deshmukh SV, Vargas R, Hankins GD, and Ahmed MS (2006) Methadone metabolism by early gestational age placentas. *Am J Perinatol* **23**:287–294.
- Hollenberg PF, Kent UM, and Bumpus NN (2008) Mechanism-based inactivation of human cytochromes p450s: experimental characterization, reactive intermediates, and clinical implications. *Chem Res Toxicol* **21**:189–205.
- Iribarne C, Dreano Y, Bardou LG, Menez JF, and Berthou F (1997) Interaction of methadone with substrates of human hepatic cytochrome P450 3A4. *Toxicology* **117**:13–23.
- Jones DR, Gorski JC, Hamman MA, Mayhew BS, Rider S, and Hall SD (1999) Diltiazem inhibition of cytochrome P-450 3A activity is due to metabolite intermediate complex formation. *J Pharmacol Exp Ther* **290**:1116–1125.
- Kalgutkar AS, Obach RS, and Maurer TS (2007) Mechanism-based inactivation of cytochrome P450 enzymes: chemical mechanisms, structure-activity relationships and relationship to clinical drug-drug interactions and idiosyncratic adverse drug reactions. *Curr Drug Metab* **8**:407–447.
- Kharasch ED, Bedynek PS, Park S, Whittington D, Walker A, and Hoffer C (2008) Mechanism of ritonavir changes in methadone pharmacokinetics and pharmacodynamics: I. Evidence against CYP3A mediation of methadone clearance. *Clin Pharmacol Ther* **84**:497–505.
- Kharasch ED, Hoffer C, Whittington D, and Sheffels P (2004) Role of hepatic and intestinal cytochrome P450 3A and 2B6 in the metabolism, disposition, and mitotic effects of methadone. *Clin Pharmacol Ther* **76**:250–269.
- Kharasch ED, Hoffer C, Whittington D, Walker A, and Bedynek PS (2009) Methadone pharmacokinetics are independent of cytochrome P4503A (CYP3A) activity and gastrointestinal drug transport: insights from methadone interactions with ritonavir/indinavir. *Anesthesiology* **110**:660–672.
- Kim TW, Alford DP, Malabanan A, Holick MF, and Samet JH (2006) Low bone density in patients receiving methadone maintenance treatment. *Drug Alcohol Depend* **85**:258–262.
- Kleber HD (2008) Methadone maintenance 4 decades later: thousands of lives saved but still controversial. *JAMA* **300**:2303–2305.
- Krantz MJ, Kutinsky IB, Robertson AD, and Mehler PS (2003) Dose-related effects of methadone on QT prolongation in a series of patients with torsade de pointes. *Pharmacotherapy* **23**:802–805.
- Levine B, Wu SC, Dixon A, and Smialek JE (1995) Site dependence of postmortem blood methadone concentrations. *Am J Forensic Med Pathol* **16**:97–100.
- Morton EB (2007) *Methadone Pharmacology*. Ph.D. thesis, University of Adelaide, Adelaide, South Australia.
- Nanovskaya TN, Deshmukh SV, Nekhayeva IA, Zharikova OL, Hankins GD, and Ahmed MS (2004) Methadone metabolism by human placenta. *Biochem Pharmacol* **68**:583–591.
- Ortiz de Montellano PR, Mico BA, Mathews JM, Kunze KL, Miwa GT, and Lu AY (1981) Selective inactivation of cytochrome P-450 isoenzymes by suicide substrates. *Arch Biochem Biophys* **210**:717–728.
- Osawa Y, Higashiyama T, Toma Y, and Yarbrough C (1997) Diverse function of aromatase and the N-terminal sequence deleted form. *J Steroid Biochem Mol Biol* **61**:117–126.
- Pohlman A, Boaz HE, and Sullivan HR (1971) Synthesis and identification of metabolites resulting from the biotransformation of DL-methadone in man and in the rat. *J Med Chem* **14**:194–197.
- Pond SM, Kreek MJ, Tong TG, Raghunath J, and Benowitz NL (1985) Altered methadone pharmacokinetics in methadone-maintained pregnant women. *J Pharmacol Exp Ther* **233**:1–6.
- Senay EC (1985) Methadone maintenance treatment. *Int J Addict* **20**:803–821.
- Serane VT and Kurian O (2008) Neonatal abstinence syndrome. *Indian J Pediatr* **75**:911–914.
- Shiran MR, Lennard MS, Iqbal MZ, Lagundoye O, Seiwright N, Tucker GT, and Rostami-Hodjegan A (2009) Contribution of the activities of CYP3A, CYP2D6, CYP1A2 and other potential covariates to the disposition of methadone in patients undergoing methadone maintenance treatment. *Br J Clin Pharmacol* **67**:29–37.
- Silverman R (1988) *Mechanism Based Inactivation: Chemistry and Enzymology*. CRC Press, Boca Raton, FL.
- Sims SA, Snow LA, and Porucznik CA (2007) Surveillance of methadone-related adverse drug events using multiple public health data sources. *J Biomed Inform* **40**:382–389.
- Szeto HH, Clapp JF 3rd, Larrow RW, Hewitt J, Inturrisi CE, and Mann LI (1981) Disposition of methadone in the ovine maternal-fetal unit. *Life Sci* **28**:2111–2117.
- Totah RA, Sheffels P, Roberts T, Whittington D, Thummel K, and Kharasch ED (2008) Role of CYP2B6 in stereoselective human methadone metabolism. *Anesthesiology* **108**:363–374.
- Wang JS and DeVane CL (2003) Involvement of CYP3A4, CYP2C8, and CYP2D6 in the metabolism of (*R*)- and (*S*)-methadone in vitro. *Drug Metab Dispos* **31**:742–747.
- Weschules DJ, Bain KT, and Richeimer S (2008) Actual and potential drug interactions associated with methadone. *Pain Med* **9**:315–344.
- Wilkinson GR (2005) Drug metabolism and variability among patients in drug response. *N Engl J Med* **352**:2211–2221.
- Zharikova OL, Deshmukh SV, Nanovskaya TN, Hankins GD, and Ahmed MS (2006) The effect of methadone and buprenorphine on human placental aromatase. *Biochem Pharmacol* **71**:1255–1264.

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