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The Mechanism of Autoinduction of Methadone N-demethylation in Human Hepatocytes

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Abstract

Background—There is considerable inter- and intraindividual variability in methadone metabolism and clearance. Methadone dosing is particularly challenging during initiation of therapy, due to time-dependent increases in hepatic clearance (autoinduction). Although methadone N-demethylation is catalyzed *in vitro* by cytochrome P4502B6 (CYP2B6) and CYP3A4, and clearance *in vivo* depends on CYP2B6, mechanism(s) of autoinduction are incompletely understood. In this investigation we determined mechanism(s) of methadone autoinduction using human hepatocytes.

Methods—Fresh human hepatocytes were exposed to 0.1–10 μ M methadone for 72 hr. Cells were washed and methadone N-demethylation assessed. CYP2B6, CYP3A4, and CYP3A5 mRNA, protein expression (by gel-free high performance liquid chromatography-mass spectrometry) and catalytic activity (bupropion hydroxylation and alfentanil dealkylation for

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CYP2B6 and CYP3A4/5, respectively) were measured. Mechanisms of CYP induction were characterized using pregnane X receptor and constitutive androstane receptor reporter gene assays.

Results—Methadone (10 μ M) increased methadone N-demethylation 2-fold, CYP2B6 and CYP3A4 mRNA 3-fold, and protein expression 2-fold. CYP3A5 mRNA was unchanged. CYP2B6 and CYP3A4/5 activities increased 2-fold. Induction by methadone enantiomers (R- vs S-methadone) did not differ. Induction was relatively weak compared with maximum induction by phenobarbital and rifampin. Lower methadone concentrations had smaller effects. Methadone was an agonist for the pregnane X receptor but not the constitutive androstane receptor.

Conclusions—Methadone caused concentration-dependent autoinduction of methadone N-demethylation in human hepatocytes, related to induction of CYP2B6 and CYP3A4 mRNA expression, protein expression, and catalytic activity. Induction was related to pregnane X receptor but not constitutive androstane receptor activation. These *in vitro* findings provide mechanistic insights into clinical autoinduction of methadone metabolism and clearance.

Introduction

Methadone maintenance therapy is used to treat opiate addiction, and effectively prevents opiate withdrawal, diminishes illicit drug use, and reduces human immunodeficiency virus/acquired immune deficiency syndrome and other infectious diseases.¹ Methadone is also used to treat acute, chronic, and cancer pain.²⁻⁴ Nevertheless, the clinical use of methadone is challenging. There is considerable and unpredictable inter- and intraindividual variability in methadone pharmacokinetics and pharmacodynamics, including metabolism and clearance, as well as susceptibility to drug interactions.^{5,6} This variability confers risks of drug accumulation and toxicity, or opiate withdrawal or inadequate analgesia, which can confound methadone use. This is particularly acute during the first few weeks of use. While there was a 13-fold increase in methadone prescriptions between 1997-2006,⁷ there was also an exponential increase in methadone toxicity, including a nearly 1800% increase in adverse events and a 390% increase in fatalities,^{8,9} which persist today.¹⁰

It is well recognized that the first 1-2 weeks of methadone treatment for pain therapy constitute the highest risk period for adverse events, most often relative overdose.¹¹⁻¹³ Mortality rates in the first 1-2 weeks are 10- to 100-fold greater than in the period thereafter.¹¹⁻¹³ This constitutes a major challenge, whether initiating pain therapy with methadone, using it as a second-line drug, or in an opioid rotation scheme.¹⁴ Contributing to the problem of interindividual variability in methadone disposition are the changes in methadone clearance during the initiation of therapy. Elimination of both IV and oral methadone undergoes time-dependent autoinduction (methadone-induced increases in methadone elimination) with repeated dosing.¹⁵⁻¹⁷ After at least one week, plasma concentrations decreased 25%-40%,¹⁸ and elimination half-life was reduced by half.¹⁷ Autoinduction is highly variable. Reductions in plasma methadone concentrations after 8d averaged 25%-40%, but were as much as 60% in some patients.^{17,18} Methadone autoinduction also occurs in rodents, along with induction of hepatic microsomal enzymes and methadone N-demethylation.¹⁹⁻²¹

Methadone in humans is cleared primarily by hepatic cytochrome P450 (CYP)-catalyzed metabolism, with some urinary excretion of unchanged drug. The most abundant metabolite is 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), which is pharmacologically inactive. Methadone clearance and N-demethylation are stereoselective. After considerable research, a consensus has emerged that methadone N-demethylation *in vitro*, both by human liver microsomes and expressed CYPs, is catalyzed most efficiently by CYP2B6 and CYP3A4 (while CYP3A5 is comparatively inactive), and only CYP2B6 N-demethylates methadone stereoselectively.²²⁻²⁶ In contrast, the relative importance of CYPs 2B6 and 3A4

in clinical methadone clearance remains unclear, and the identity of the CYP isoform responsible for methadone clearance remains controversial. Based on extrapolation of early *in vitro* studies of CYP3A4, methadone metabolism and clearance *in vivo* were attributed to CYP3A4.^{6,27-30} Conversely, several subsequent clinical investigations showed minimal or no role for CYP3A in single-dose methadone clearance.^{23,31-33} Moreover, several studies are consonant with CYP2B6, rather than CYP3A4, mediating clinical methadone metabolism and clearance.^{23,25,28,29,31,34-36}

Autoinduction of methadone elimination in humans has been ascribed to increased clearance and metabolism,¹⁶ and speculatively to hepatic CYP3A4 induction.^{37,38} Nevertheless, it appears from the above that CYP3A4 induction may not be the mechanism. Methadone autoinduction remains poorly understood, and yet clinically significant. A previous investigation in human hepatocytes showed that methadone induced CYP2B6 and CYP3A4 mRNA and protein expression, attributed to pregnane X receptor (PXR) and constitutive androstane receptor (CAR) activation.³⁹ That investigation did not measure CYP enzymatic activity, did not evaluate methadone enantiomer effects on enzyme induction, and used Western blot analysis to measure CYP expression. Western blot analysis, however, particularly of CYP2B6, has been reported to be only semiquantitative due to issues such as antibody affinity and specificity,⁴⁰ and Western blot analysis of hepatocyte CYP2B6 protein was reported to underestimate the degree of induction, compared with that of mRNA and enzyme activity.⁴¹ Recently, tandem mass spectrometry-based CYP protein quantification methods have been developed, which also allow the simultaneous absolute quantification of multiple cytochrome P450 isoforms.⁴² Therefore, the purpose of this investigation was to use primary human hepatocytes to test the hypothesis that autoinduction of human methadone clearance can result from increased methadone N-demethylation, in turn caused by induction of CYP2B6 and/or CYP3A4 mRNA, protein, and catalytic activity.

Methods

Chemicals and reagents

Alfentanil and methadone were obtained from the National Institutes of Drug Abuse through Research Triangle Institute (Research Triangle, NC). Williams E media was obtained from Lonza Walkersville (Walkersville, MD). Hepatocyte Supplement was obtained from Life Technologies (Durham, NC). Dimethyl sulfoxide (DMSO), rifampin, phenobarbital and bupropion hydrochloride were obtained from Sigma (St Louis, MO). Formic acid was obtained from Fisher Scientific (Pittsburg, PA) and acetonitrile was from Sigma and both were high-performance liquid chromatography (HPLC) grade. Noralfentanil, EDDP, and hydroxybupropion were obtained from Cerilliant (Round Rock, TX).

Hepatocyte Induction

Fresh plated human hepatocytes from 4 donor livers were generously provided by Life Technologies (Durham, NC). Donor descriptions are provided in Table 1. Hepatocytes were isolated from 4 donor livers, plated, and overlaid with Matrigel before shipping. Cells were plated at a density of 0.5×10^6 cells/well and microscopically examined for proper morphology and confluency before shipment. The shipping media (Hypothermasol FRS, Bioline Solutions, Bothell, WA) was removed and changed to supplemented William's E Media upon arrival and the cells allowed to equilibrate for 24 hours at 37°C/5% CO₂/95% humidity. A 1000x DMSO stock methadone was made for each concentration tested (0.1 μM, 1 μM and 10 μM) and indicated concentrations were achieved by diluting the DMSO stock into supplemented William's E media. Phenobarbital test solution was made by solubilizing compound directly into media and adding sufficient DMSO to equal 0.1%. Cells were incubated in drug-containing media (or vehicle control, 0.1% DMSO) for 72 hours,

with media/drug changed each day, using recommended assay conditions.⁴³ Cell morphology and confluency were examined microscopically each day and the plate discarded if any changes were observed.

CYP Activity

After the 72-hour induction period, hepatocytes were incubated with drug-free media for 1 hr at 37°C in a shaker incubator (approximately 60 rpm). Media was then changed to media that contained 500 µM bupropion and incubated for 40 min at 37°C. Media was sampled for analysis, and replaced with drug-free media for 1 hr. The wash media was then exchanged and the sequence of 40-min incubation, followed by a 1-hr washout period was performed for both 500 µM methadone and then 200 µM alfentanil. Racemic methadone N-demethylation was determined from the formation of racemic EDDP.^{23,44} CYP2B6 activity was determined by the standard probe racemic bupropion hydroxylation, assessed by the formation of racemic hydroxybupropion, and CYP3A4/5 activity was determined by alfentanil N-dealkylation, measured by the formation of noralfentanil.⁴⁵ At the end of each experiment, hepatocytes were frozen for later quantification of mRNA and CYP protein. Fold induction was calculated for each substrate by comparing the metabolic activity after methadone or phenobarbital treatment to that after the treatment with 0.1% DMSO.

Metabolite Analysis

Metabolite analysis was performed on an API 3200 triple-quadrupole mass spectrometer (EDDP and noralfentanil) and API 4000 QTRAP mass spectrometer (hydroxybupropion), each equipped with a Turbo Ion Spray ionization source (Applied Biosystems/MDS Sciex, Foster City, CA). Structurally comparable deuterated analogues were used as internal standards for each analyte. The HPLC system for the API 3200 mass spectrometer was a Shimadzu LC-20AC HPLC system (Shimadzu, Columbia, MD) while an Agilent 1100 series HPLC system was used the API 4000 mass spectrometer (Agilent, Wilmington, DE). The chromatographic separation was performed on a T3 column (50 × 2.1 mm, 3.5 µm) (Waters Corp, Milford, MA). The injection volume was 20 µl and the oven temperature was 25°C. HPLC mobile phase was (A) 0.1% formic acid and (B) 0.1% formic acid in methanol using a flow rate of 0.3 ml/min. The gradient program for EDDP was 35% B for 0 min, linear gradient to 60% B between 0 and 1.0 min, held at 60% until 2 min, linear gradient to 100% until 3 min, held at 100% B until 4 min, then re-equilibrated to initial conditions between 4.01 and 5.0 min; for noralfentanil it was 35% B for 0 min, linear gradient to 40% B between 0 and 0.5 min, held at 40% until 2.5 min, linear gradient to 100% until 3 min, held at 100% B until 4 min, then re-equilibrated to initial conditions between 4.0 and 5.5 min, and for hydroxybupropion it was 10% B for 0 min, linear gradient to 30% B between 0 and 1.0 min, held at 30% until 1.5 min, linear gradient to 100% until 2 min, held at 100% B until 2.5 min, then re-equilibrated to initial conditions between 2.5 and 6.0 min. Under these conditions, retention times for EDDP, noralfentanil and hydroxybupropion were 2.7, 2.9 and 1.5 min, respectively. Both Q1 and Q3 quadrupoles were optimized to unit mass resolution, and the mass spectrometer conditions were optimized for each analyte. The instrument was operated in positive-ion mode with an ion spray voltage of 5500 V. The curtain gas was set at 20, ion source gas 1 at 30, ion source gas 2 at 30 and the collision gas was set at 5. Analytes were monitored using Multiple Reaction Monitoring. Transitions for each analyte and internal standard were m/z 278.2→234.2 and m/z 281.2→234.2 for EDDP and EDDP d3; m/z 277.0→128.0 and m/z 282.0→128.0 for noralfentanil and noralfentanil d5; and m/z 256.1→238.1 and m/z 262.2→167.2 for hydroxybupropion and hydroxybupropion d6. Metabolites were quantified using area ratios and standard curves prepared using calibration standards in blank media.

Quantification of CYP proteins by HPLC-MS/MS

CYP protein quantification by HPLC-MS/MS was performed as described previously,⁴² with slight modification. Each sample (100 μ l) was precipitated with 600 μ l of acetone (-80° C) and centrifuged at 14,000 rpm for 15 min. The supernatant was discarded and the protein precipitate dried under vacuum for 30 min. 20 μ l of 100mM Tris (pH 8) was added to each sample along with 2 μ l of 10% ocytl β -D-glucopyranoside. Two μ l of 100 mM Tris(2-carboxyethyl)phosphine was then added and incubated at 60° C for 1h. Five μ l of 0.1 M methyl methanethiosulfonate was added and incubated at room temperature for 10 min. The mixture was then tryptically digested by first adding another 20 μ l of the TRIS solution followed by 25 μ g of trypsin and the resultant solution was digested 4 hours at 37° C. Finally, 2 pmol of each isotopically enriched synthetic peptide was added to the digest and analyzed by HPLC-MS/MS. HPLC-MS/MS was performed using a Shimadzu 30AD HPLC system coupled to an Acquity UPLC BEH C18 (2.1 $\times$ 100 mm) column (Waters). The peptides were separated with a linear gradient of 5-30% B over 8 min. Mobile phase A consisted of 2% ACN, 0.1% formic acid and mobile phase B consisted of 90% ACN, 10% water, 0.1% formic acid. The HPLC flow rate was 700 μ l/min. MS detection was performed on a 5500 QTRAP system (AB SCIEX) using a Turbo V source and Analyst software 1.5.1. The Scheduled MRM algorithm was used to maximize dwell time on each transition.

Quantitative polymerase chain reaction (PCR)

After the metabolic activity assays were performed, mRNA was isolated using an RNeasy kit (QIAGEN, Valencia, CA) following the manufacturer's protocol. mRNA was then converted to cDNA using Superscript VILO (Invitrogen, Carlsbad, CA) and quantified using a Synergy Mx plate reader (BioTEK, Winooski, VT). cDNA samples were then analyzed using Taqman Gene Expression Assay kits (Applied Biosystems, Carlsbad, CA) labeled with either FAM (CYP2B6 Hs03044635_g1, CYP3A4 Hs00604506_m1, or CYP3A5 Hs00241417_m1) or VIC (GAPDH, Hs02758991_g1) and were used according to the manufacture's protocol. Briefly, 75 ng of total cDNA was added to the premixed Taqman kit for each analyte and amplified using a 7500 FAST PCR instrument (Applied Biosystems, Carlsbad, CA) for 55 cycles. Each multiplexed reaction contained probes for both a cytochrome P450 and GAPDH and the amplification of each monitored by separate channels of the instrument. Data from each qPCR run were analyzed with SDS software (v. 1.3.1, Applied Biosystems, Foster City, CA) and the cDNA copy number for each sample was calculated using the formula below, where CT is the crossing threshold as determined by the instrument software. Fold induction was then calculated comparing the drug-treated sample to the vehicle control.

$$\text{copy number} = \text{average} \frac{1}{2}^{-\Delta\Delta CT}$$

Pregnane X receptor (PXR) Reporter Gene Assay

The PXR reporter gene assay was kindly performed by Puracyp, Inc (Carlsbad, CA) using their proprietary method. Briefly, HepG2 cells transfected with both a PXR response element and the luciferase gene were exposed to either, 0.1% DMSO, 10 μ M rifampin or methadone. After 24-hour incubation, both cell viability and luciferase activity were examined and fold induction calculated. The relative light unit values for each well was corrected for number of viable cells in that well and normalized assuming the amount of luminescence detected in response to treatment with 0.1% DMSO to be uninduced.

Constitutive androstane receptor (CAR) Reporter Gene Assay

The ability of methadone to act as an agonist for human CAR isoform 3 (CAR) was assessed using the CAR Reporter Assay System from Indigo Biosystems, Inc (State College, PA) and using the manufacturer's protocol. Briefly, Chinese Hamster Ovary cells transfected with both the CAR response element and a luciferase gene, were thawed, plated and incubated overnight (37°/5% CO₂/95% humidity) with either positive control (6-(4-Chlorophenyl)imidazole[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime, CITCO) or methadone. Eight concentrations of both were analyzed, and a vehicle control was included as a 0 nM treatment. After an overnight incubation the incubation media was removed and the provided Luciferase Detection Reagent was added to each well. The plate was then incubated at room temperature for 20 min and luminescence intensity quantified using a BioTEK, Synergy Mx plate reader (Winooski, VT) and reported in terms of relative light units. Percent activity was calculated such that the highest and lowest relative light unit values represent 0% and 100%, respectively. Curve fitting and ED₅₀ calculations were performed using SigmaPlot 12.3 (Systat Software, San Jose, CA) using the embedded functions. Each point represents the mean and standard deviation of three independent determinations.

Statistical Analysis

Hepatocyte induction incubations were performed in triplicate from each liver, using cells from 4 liver donors. Triplicate observations from each liver were averaged to obtain the result for that liver. For most experiments, results for three livers (n=3) were then reported as the arithmetic mean $\pm$ standard deviation. Data were analyzed according to standard approaches.^{43,46} The primary criterion for determination of induction was an increase in enzyme activity of at least 2-fold. A positive result in at least one of the donor hepatocytes was considered an indication of induction.

Results

Induction was measured in hepatocytes from four human livers after 72-hr incubation with 0.1-10 μ M methadone, with daily change of media and replenishment of methadone. Cells were washed to remove residual methadone before assessing catalytic activities. Formation of racemic EDDP from racemic methadone was increased 2-fold after incubation with 10 μ M methadone, while lower concentrations had lesser or no inductive effect (Figure 1). Phenobarbital (1 mM) and rifampin (10 μ M) were used as standard positive controls for hepatocyte induction. Induction of methadone N-demethylation was about half that observed for phenobarbital and rifampin (approximately 4-fold for both). An enantioselective assay for EDDP was also used to analyze the stereoselectivity of induction of R- and S-EDDP formation from racemic methadone.²⁴ No differences in R-versus S-EDDP formation rates from methadone were observed after induction by 10 μ M methadone, or by phenobarbital or rifampin, compared with controls (data not shown).

In vitro activities of CYP2B6 and CYP3A4/5 were measured by metabolism of bupropion to hydroxybupropion, and N-dealkylation of alfentanil to noralfentanil, respectively. Both CYP2B6 and CYP3A4/5 activities were induced approximately 2-fold by 10 μ M methadone compared with vehicle control (Figure 1). Lower methadone concentrations (0.1 and 1 μ M) did not increase CYP2B6 or CYP3A activities. Both phenobarbital and rifampin increased CYP3A4/5 activity 12-fold, and increased CYP2B6 activity 10-fold and 4-fold, respectively. The extent of both CYP2B6 and CYP3A4/5 induction by 10 μ M methadone was approximately 20% of that by phenobarbital.

Stereoselectivity of methadone enantiomer induction of hepatocyte CYP2B6 and CYP3A4/5 activities, and of methadone N-demethylation was also evaluated. There were no differences in the effects of R- versus S-methadone, at any concentration, on methadone metabolism or CYP2B6 and CYP3A4/5 activities (Figure 2).

To explore if the changes in hepatocyte CYP catalytic activity were due to increased synthesis of new protein, CYP2B6, CYP3A4 and CYP3A5 contents were quantified by HPLC-tandem mass spectrometry. The multiplex assay also quantified CYP1A2. CYP2B6 and CYP3A4 protein contents were both increased approximately 2-fold after incubation with 10 μ M methadone, compared to DMSO control (Figure 3). The extent of induction by 10 μ M methadone was approximately 20% of that by phenobarbital. Neither CYP1A2 nor CYP3A5 protein levels increased more than the 2-fold cut-off generally used as the criterion for induction. When compared to the 0.1% DMSO control, lesser content of each CYP isoform was detected after incubation with 1 μ M methadone.

After incubation with methadone, phenobarbital, rifampin or 0.1% DMSO, mRNA transcripts for CYP2B6, CYP3A4 and CYP3A5 were quantified (Figure 4). As with metabolic activity and protein contents, 10 μ M methadone but not lower concentrations increased mRNA levels more than 2-fold. Both the CYP2B6 and CYP3A4 transcripts increased approximately 3-fold compared to control. Assuming that 1 mM phenobarbital represents maximal induction,⁴³ 10 μ M methadone increased CYP2B6 transcripts to 36% of that for phenobarbital, and to 59% of that for rifampin. CYP3A4 transcript levels were approximately 19% of those for both phenobarbital and rifampin. Lower methadone concentrations had no effect on CYP2B6 or CYP3A4 mRNA expression, and CYP3A5 mRNA transcript levels were unchanged at all methadone concentrations.

To assess the ability of methadone to function as either a PXR or CAR agonist, reporter gene assays were conducted for both nuclear receptors (Figures 5 and 6). Three methadone concentrations (1 μ M-100 μ M) and a positive control (rifampin) were incubated with HepG2 cells transfected with both a PXR response element and the luciferase gene and the resulting luciferase activity used as an indication of the ability of the test compound to induce the transcription of genes under the PXR response element (Figure 5). At clinically relevant methadone concentrations (1 or 10 μ M) induction was approximately 2-fold, and at 100 μ M methadone induction was 13-fold. The positive control rifampin induced PXR-related luminescence more than 16-fold. This result demonstrated that methadone is a PXR agonist.

For the CAR assay (Figure 6) the relative light unit values for each sample were normalized such that the lowest and highest values were defined as 0% and 100%, respectively. For the methadone-treated samples no increase in luminescence was detected over the entire concentration range tested (10.2 nM-250 μ M), while the positive control (CITCO) gave a very robust signal (approximately 200-fold higher than that observed for methadone) with an EC₅₀ (224 nM) comparable to that provided by the manufacturer (220 nM). This result demonstrated the methadone is not a CAR agonist.

Discussion

Mortality rates related to accidental methadone overdose in the first 1-2 weeks of dosing are 10- to 100-fold greater than in the period thereafter.¹¹⁻¹³ Previous *in vivo* and *in vitro* studies have shown that with repeated dosing over approximately 2 weeks, methadone clearance increased 2- to 3-fold.¹⁹ The half-life of this autoinduction was estimated to be 94 hr.^{37,38} The autoinduction of methadone clearance has been attributed to induction of methadone N-demethylation, since urinary EDDP/methadone concentration ratios were increased 3-fold at steady-state.¹⁶ Initial methadone dosing paradigms which are incorrectly based on steady-

state clearance values, rather than on initial-dose clearance values, will result in plasma concentrations which are 2- to 3-fold greater than anticipated, and may result in unintended toxicity or even overdose. Thus, while autoinduction actually increases methadone clearance over time, it may be paradoxically contributing indirectly to untoward clinical outcomes, and accidental overdose, at initial dosing. Although the clinical phenomenon of autoinduction of methadone clearance has been well-described,^{15-17,37,47} the mechanism of methadone autoinduction is incompletely understood.

Fresh human hepatocytes cultured between a sandwich of rat tail collagen are considered the “gold standard” for evaluating *in vitro* induction of cytochrome P450 enzymes, because the cells retain “liver-like” morphology and expression of liver-specific proteins.⁴³ Therefore, this investigation used primary human hepatocytes to evaluate the mechanism of methadone autoinduction, testing the hypothesis that methadone N-demethylation undergoes upregulation.

The primary result was that methadone induced methadone N-demethylation in human hepatocytes, and at clinically relevant concentrations. This is the first report of such an effect. EDDP formation was upregulated 2-fold by 10 μ M racemic methadone, but not by 1 μ M methadone. Thus methadone clearly causes hepatic autoinduction. Actual clinical hepatic methadone concentrations are unknown, but maximum hepatic concentrations can be predicted using standard approaches,⁴⁸ which predict 3-21 μ M RS-methadone after 10-100 mg oral methadone. Therefore methadone N-demethylation was induced by hepatic (portal) methadone concentrations which would be achieved after oral dosing. In contrast, the hepatic methadone concentrations occurring at systemic steady-state (<2-3 μ M) methadone concentrations might not be expected to cause autoinduction. There was no difference in the effects of R- versus S-methadone on induction of CYP activity or methadone N-demethylation. The clinical implication is that autoinduction is unlikely to differ between the two marketed forms of methadone, racemic and single (R-) enantiomer. The 2-fold induction of hepatocyte methadone N-demethylation is similar to the 2-3-fold increase in methadone clearance and N-demethylation found clinically after several weeks of oral methadone.^{16,17} It is interesting to note that in rats, autoinduction was observed with oral, but not subcutaneous or intraperitoneal methadone, further suggesting route and concentration-dependence of autoinduction.^{19,21}

Autoinduction of methadone N-demethylation was associated with upregulation of both CYP2B6 and CYP3A4 catalytic activities, an observation also not previously reported. Catalytic activity of both CYPs was induced approximately 2-fold, as were protein and mRNA expression. CYP3A5 protein expression was also somewhat increased, but this could not contribute to autoinduction of methadone N-demethylation because CYP3A5 is comparatively inactive towards methadone.^{23,26} Both CYP2B6 and CYP3A4 are the major isoforms catalyzing methadone metabolism *in vitro*;²²⁻²⁶ however, there is a lack of agreement as to their relative contributions to metabolism and clearance clinically,^{6,22,23,28,31-33,35,36,49} and there is no information on their relative contribution to clinical methadone autoinduction. The relative contribution of CYP2B6 and CYP3A4 to autoinduction of methadone N-demethylation in hepatocytes is similarly unknown, and the present data do not inform any relative attributions. CYP2B6-mediated methadone N-demethylation is stereoselective, while that by CYP3A4 is not.²²⁻²⁶ Previous studies predicted,^{24,25} and later validated,²⁵ that clinical CYP3A4 induction would minimally affect the plasma R/S-methadone concentration ratio, whereas CYP2B6 induction would increase the ratio. That hepatocyte methadone autoinduction and induction by rifampin and phenobarbital in the present investigation did not alter the R/S-EDDP ratio, suggests that the relative contribution of CYPs 2B6 and 3A4 to methadone N-demethylation remained unchanged, regardless of the inducer. The finding that protein expression of all four CYP

isoforms decreased with 1 μ M methadone appears to be an assay artifact, and not supported by either a decrease in methadone metabolism or decreased mRNA levels.

Reporter gene assays were used to identify the role of the human xenobiotic receptors PXR and CAR in the methadone-mediated transcriptional up-regulation of CYP2B6 and CYP3A4. Human PXR and CAR are both members of the nuclear receptor 1I (NR1I) subfamily that include mediators of vitamin D signaling, and xenobiotic sensors.⁵⁰ While CAR is the closest mammalian relative of PXR, and is activated by some of the same ligands, it is less promiscuous than PXR and displays fundamental differences from PXR with regard to cellular regulation and ligand interaction.⁵⁰ The PXR reporter gene assay showed a strong response to methadone. However, the hCAR reporter gene assay did not show any activation, up to 250 μ M methadone. This suggests that methadone upregulation of CYP2B6 and CYP3A5 is mediated by PXR but not CAR. The observation that CYP2B6 and CYP3A4 activity, protein and mRNA were similarly induced by methadone, and that methadone activated PXR but not CAR, is similar to the previous finding of upregulation of both CYP2B6 and CYP3A4 by the PXR ligand rifampicin, but preferential upregulation of CYP2B6 over CYP3A4 by a CAR-specific ligand.⁵¹

Results of this investigation show some similarities and differences compared with a previous report.³⁹ Methadone was reported to induce the expression of CYP2B6 and CYP3A4 mRNA and expression, although effects on CYP activity and on methadone N-demethylation, as well as the enantioselectivity of these effects, were not evaluated.³⁹ Thus, both studies showed that methadone upregulated CYP2B6 and CYP3A4 mRNA. Transcript levels were higher in the previous study; however, differences may be due to the shorter incubation time (24 hr) than the current investigation (72 hr). Zhang *et al.* showed that CYP2B6 mRNA peaked after 6-24 hr of phenobarbital or rifampin exposure and decreased to 90% of peak expression by 72 hr. CYP3A4 mRNA peaked after 48 hr and decreased 25% by 72 hr.⁴¹ CYP protein expression and activity, however, were greatest after 72 hr.⁴¹ In the present investigation, a 72-hr time point was selected based on enzymatic activity as the primary endpoint, with protein and mRNA quantification as confirmatory, and consistency with Food and Drug Administration guidance for in vitro induction studies.^{43, a} After 72 hr, there was approximately 2-fold induction of CYP2B6 and CYP3A4 protein expression by 10 μ M methadone and smaller increases in CYP1A2 and CYP3A5 protein expression, using HPLC-MS/MS. In contrast, the previous study, using Western blot analysis, found that CYP2B6 protein level did not increase when incubated with up to 50 μ M methadone, and CYP3A4 protein was either unchanged or increased in two different hepatocyte preparations.³⁹ In preliminary experiments using Western blot, we found that CYP3A4 protein was increased by methadone, phenobarbital, and rifampin, but CYP2B6 protein expression could not be reliably quantified, despite using several different antibodies as well as a novel Taqman chemistry-based protein expression assay (results not shown). Others also found that analysis of hepatocyte CYP2B6 expression by Western blot was not sensitive or quantitative, and underestimated induction compared with mRNA and enzyme activity.⁴¹ The use of mass spectrometry for CYP quantification eliminates many of the selectivity and specificity issues often associated with Western blot analysis. Other factors, including hepatocyte donors and the longer incubation times used herein, may explain differences between the present and previous investigations.³⁹ The last difference was the apparent mechanism of CYP2B6 and CYP3A4 induction. The present investigation found evidence for methadone activation of human PXR but not CAR. In contrast, Tolson *et al.* reported methadone activation of both PXR and CAR.³⁹ Nevertheless, CAR activation occurred only at concentrations exceeding 25 μ M methadone, and the response was weak compared to a

^a<http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm>

standard positive control. Differences between the present and previous CAR assays include the cell line and vector used, and, given also the complexity of CAR-mediated gene regulation, the primary conclusion of both investigations is that methadone autoinduction is primarily PXR-mediated.

In addition to autoinduction of methadone metabolism and clearance, other factors may also contribute to inter- and/or intraindividual variability in methadone disposition. These may include genetic variability in CYP expression and activity (particularly CYP2B6)⁵² and drug interactions. These require further elucidation.

In conclusion, these *in vitro* studies demonstrate that methadone causes autoinduction of methadone N-demethylation in human hepatocytes, in turn related to induction of CYP2B6 and CYP3A4 catalytic activity, protein expression, and mRNA expression, in turn mediated by PXR activation. These *in vitro* findings parallel previous clinical evidence of autoinduction of methadone metabolism and clearance during the first two weeks of therapy, and provide insights into the mechanism by which this occurs.

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Figure 1. Methadone induction of methadone metabolism and CYP2B6 and CYP 3A4/5 activities in human hepatocytes

Primary hepatocytes (donors 1284, 1290 1331) were cultured for 3 d with the indicated concentration of methadone or 0.1% dimethyl sulfoxide (DMSO), and media was replaced daily. Metabolism of racemic methadone (RS-EDDP formation), CYP2B6 activity (bupropion hydroxylation), and CYP3A4/5 activity (alfentanil metabolism to noralfentanil) was then determined as described in the Methods. Fold induction results represent normalization the 0.1% DMSO vehicle control. Phenobarbital (1 mM) and rifampin (10 μM) were also evaluated as positive controls. Each data point is the mean $\pm$ SD of three livers (n=3), each determined in triplicate

Figure 2. Stereoselectivity of methadone enantiomers induction of methadone metabolism and CYP2B6 and CYP 3A4/5 activities in human hepatocytes

Media containing R-, S-, or RS-methadone or 0.1% dimethyl sulfoxide (DMSO) was added to primary hepatocytes daily for 3 d. Stereoselective N-demethylation of RS-methadone (R- or S-EDDP formation), CYP2B6 activity (bupropion hydroxylation), and CYP3A4/5 activity (alfentanil metabolism to noralfentanil) was determined as described in the Methods. Fold induction is normalized to the 0.1% DMSO vehicle control. Each data point is the mean $\pm$ SD of triplicate determinations from a single liver (HL 1336).

Figure 3. Methadone effects on CYP protein expression in human hepatocytes

Media containing the indicated concentration of methadone, phenobarbital, rifampin or 0.1% dimethyl sulfoxide (DMSO) was added to primary hepatocytes (donors 1284, 1290 1331) daily for 3 d. Subsequent to the determination of catalytic activity, cells were then lysed. Protein content of CYPs 1A2, 2B6, 3A4, and 3A5 was determined in a single analysis by LC-MS/MS. Fold induction results represent normalization to the 0.1% DMSO vehicle control. Phenobarbital (1 mM) and rifampin (10 μM) were evaluated as positive controls. Each data point is the mean $\pm$ SD of three livers (n=3), each determined in triplicate.

Figure 4. Methadone effects on CYP mRNA expression in human hepatocytes

Media containing the indicated concentration of methadone, phenobarbital, rifampin or 0.1% dimethyl sulfoxide (DMSO) was added to primary hepatocytes (donors 1284, 1290 1331) daily for 3 d. mRNA content was determined by quantitative polymerase chain reaction (PCR) as described in the Methods. GAPDH PCR was performed in parallel as an internal control and the calculated fold induction corrected for differences in GAPDH copy number. Fold induction results represent normalization the 0.1% DMSO vehicle control. Phenobarbital (1 mM) and rifampin (10 μ M) were positive controls. Each data point is the mean $\pm$ SD of three livers (n=3), each determined in triplicate.

Figure 5. Methadone effects on PXR activation

Pregnane X receptor (PXR) activation was assessed using a reporter gene assay, using a proprietary method and cell line (Puracyp, Inc). HepG2 cells were stably transfected with the PXR response element and a luciferase gene. Cells were plated and incubated in the presence of the indicated concentration of methadone, 10 μ M rifampin or 0.1% dimethyl sulfoxide (DMSO) for 48 hours, and luciferase activity and cell viability determined as described in the Methods. Results (mean $\pm$ SD, n=3) are expressed as the increase in luminescence for each incubation condition compared to that for the 0.1% DMSO control and are corrected for cell viability.

Figure 6. Methadone effects on CAR activation

Methadone effects were assessed using a human constitutive androstane receptor (CAR) reporter gene assay. Proprietary cells, transfected with a CAR responsive luciferase reporter gene, were incubated with varying concentrations of methadone (▼) or CITCO (●), the positive control. After an overnight incubation the luciferase detection reagent was added to the cells and luminescence quantified. Relative light units were normalized such that the lowest response and highest level of response were defined as 0% and 100%, respectively. The ED_{50} for CITCO (224 nM) was determined by nonlinear regression analysis. Each data point is the mean $\pm$ SD (n=3).

Table 1

Liver Donor Demographics.

Donor ID	Sex	Race	Age	Smoker	Alcohol Use
Hu 1284	Male	Caucasian	51	No	No
Hu 1290	Female	Caucasian	52	No	Yes, Rarely
Hu1331	Male	Caucasian	75	Yes	Yes
Hu1336	Female	African-American	47	Unknown	Unknown