DMD Fast Forward. Published on September 25, 2013 as doi:10.1124/dmd.113.053991

Cytochrome P4503A does not mediate the interaction between methadone and ritonavir/lopinavir

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DMD Fast Forward. Published on September 25, 2013 as DOI: 10.1124/dmd.113.053991
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Running Title Page

Running title: Methadone interaction with ritonavir/lopinavir

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Word count: Abstract: 248

Introduction: 412

Discussion: 1568

Number of references: 60

Abbreviations: AUC, area under the plasma concentration-time curve; C_{max}, peak plasma concentration; CL_H, hepatic clearance; CL_{IV}, systemic clearance; CL/F, apparent oral clearance; EDDP, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine; E_G, intestinal extraction; E_H, hepatic extraction; F_{oral}, bioavailability; P-gp, P-glycoprotein; Vss, steady-state volume of distribution; Vz/F, apparent volume of distribution.

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Abstract

Plasma concentrations of orally administered methadone are reduced by the HIV protease inhibitor combination ritonavir/lopinavir, but the mechanism is unknown. Methadone metabolism, clearance and drug interactions have been attributed to CYP3A4, but this remains controversial. This investigation assessed effects of acute (2d) and steady-state (2 weeks) ritonavir/lopinavir on intravenous and oral methadone metabolism and clearance, hepatic and intestinal CYP3A4/5 activity (using the probe substrate intravenous and oral alfentanil), and intestinal transporter activity (using oral fexofenadine), in healthy volunteers. Plasma and urine concentrations of methadone and metabolite enantiomers, and other analytes, were determined by mass spectrometry. Acute and chronic ritonavir/lopinavir reduced plasma methadone enantiomer concentrations in half, with an average 2.6- and 1.5-fold induction of systemic and apparent oral methadone clearances. Induction was attributable to stereoselectively increased hepatic methadone N-demethylation, hepatic extraction, and hepatic clearance, and there was a strong correlation between methadone N-demethylation and clearance. Methadone renal clearance was unchanged. Alfentanil systemic clearance and hepatic extraction, apparent oral clearance, and intestinal extraction were reduced to 25%, 16% and 35% of control, indicating strong inhibition of hepatic and intestinal CYP3A activities. Ritonavir/lopinavir (acute>chronic) increased fexofenadine exposure, suggesting intestinal P-glycoprotein inhibition. There was no correlation between methadone clearance and CYP3A activity. Acute and steady-state ritonavir/lopinavir stereoselectively induced methadone N-demethylation and clearance, despite significant inhibition of hepatic and intestinal CYP3A activity. Ritonavir/lopinavir inhibited intestinal transporters activity, but had no effect on methadone bioavailability. These results do not support a significant role for CYP3A or ritonavir/lopinavir-inhibitable intestinal transporters in single-dose methadone disposition.

Introduction

Methadone is a clinically efficacious and cost-effective analgesic, used to treat acute and chronic pain in a variety of settings (Nicholson, 2007; Chou et al., 2009; Kharasch, 2011). Methadone is also used to treat opiate addiction, and effectively prevents opiate withdrawal, diminishes illicit drug use, and reduces HIV/AIDS and other infectious diseases (Lobmaier et al., 2010). Methadone is used in various countries as a racemate, or as the single R-enantiomer, which has approximately 50 times greater mu opioid receptor activity than its antipode. Methadone pharmacokinetics and pharmacodynamics, including metabolism and clearance, are considered to have considerable and unpredictable inter- and intra-individual variability (Ferrari et al., 2004; McCance-Katz et al., 2010). Methadone drug interactions are a specific concern (McCance-Katz et al., 2010; Saber-Tehrani et al., 2011). Due to increased use, primarily for pain treatment, methadone prescribing has increased significantly in the past decades, but was also accompanied by a commensurate increase in toxicity and adverse events (Webster, 2005; Sims et al., 2007).

Highly active antiretroviral drugs used for the therapy of HIV and AIDS are notorious perpetrators of drug interactions. Influence on the activity of hepatic and extrahepatic enzymes such as cytochromes P450 (CYP) and glucuronosyltransferases, as well as on influx and efflux transporters in several organs, have been well-described, along with clinically significant consequences for pharmacokinetics, pharmacodynamics, and clinical therapeutics (Dickinson et al., 2010; Pal et al., 2011; Griffin et al., 2012).

Ritonavir-boosted lopinavir is one of several fixed-dose protease inhibitors combinations, in which ritonavir is used primarily to inhibit CYP3A-mediated elimination of the coadministered protease inhibitor, and thus enhance bioavailability and exposure. Ritonavir/lopinavir is known to inhibit the activity of CYP3A isoforms, and clinically may inhibit CYP3A activity but also induce CYP3A expression, potentially resulting in net induction or net inhibition of CYP3A activity (Dickinson et al., 2010; Pal et al., 2011; Griffin et al., 2012).

Ritonavir/lopinavir has been shown to decrease significantly methadone exposure (area under the

curve) and increase methadone clearance (Clarke et al., 2002; McCance-Katz et al., 2003), and to variably cause symptoms of opioid withdrawal in methadone-maintained patients (Clarke et al., 2002; Stevens et al., 2003). Cessation of ritonavir/lopinavir was associated with an arrhythmia, attributed to an acute increase in methadone exposure (Lüthi et al., 2007). Induction of methadone clearance was attributed to either or both CYP3A4 and CYP2D6 (McCance-Katz et al., 2003). Nevertheless, the actual mechanism by which ritonavir/lopinavir alters methadone disposition and clinical effects is unknown.

The purpose of this investigation was to determine the mechanism by which ritonavir/lopinavir alters methadone disposition and clinical effect, assessing CYP3A and/or P-glycoprotein-mediated methadone intestinal absorption, first-pass elimination, systemic clearance, intestinal and/or hepatic metabolism, and bioavailability, and to compare the effects of single-dose and steady-state ritonavir/lopinavir. A comprehensive crossover investigation in healthy volunteers evaluated the metabolism and clearance of both intravenous and oral methadone. Clearances of intravenous and oral alfentanil, a non-selective CYP3A4/5 (henceforth referred to as CYP3A) substrate, phenotyped hepatic and first-pass CYP3A activities, and oral fexofenadine probed P-gp (Kharasch et al., 2004b; Kharasch et al., 2005; Kharasch et al., 2007; Kharasch et al., 2008a; Kharasch et al., 2008b; Kharasch et al., 2009a; Kharasch et al., 2009b; Kharasch et al., 2011a; Kharasch et al., 2011b; Kharasch et al., 2012a; Kharasch et al., 2012b).

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Materials and Methods

Clinical Protocol

The investigation was a 3-part sequential crossover (control first, for logistical reasons), approved by the Washington University Institutional Review Board. All subjects provided written informed consent. Each subject was their own control. Subjects were normal healthy volunteers (smokers or nonsmokers) 18-40 yr, within 25% of ideal body weight (body mass index <30). Exclusion criteria were 1) major medical problems, 2) history of hepatic or renal disease, 3) use of drugs or herbals known to alter CYP3A activity, including females hormonal contraceptives, 4) known history of drug or alcohol addiction, or routine handling of addicting drugs in the regular course of employment, 5) family history of type 2 diabetes, 6) fasting blood glucose >110 mg/dl (because protease inhibitors can cause glucose intolerance), 7) HIV seropositivity (because monotherapy can cause HIV resistance). The final study cohort was 4 males and 8 females 29 ± 7 yr, range 20-40; 772 ± 13 kg, range 57-995). Subjects were instructed to consume no grapefruit, apples or oranges for 7d before and on any study day, no alcohol or caffeine for 1d before and on a study day, and no food or water after midnight before each study session.

First-pass CYP3A activity and intestinal P-gp (and other transporters) activity were evaluated on day 1 using oral alfentanil and fexofenadine as *in vivo* probes (Kharasch et al., 2004b; Kharasch et al., 2005; Kharasch et al., 2007; Kharasch et al., 2008b; Kharasch et al., 2009a; Kharasch et al., 2009b; Kharasch et al., 2011a; Kharasch et al., 2011b; Kharasch et al., 2012a; Kharasch et al., 2012b). Subjects received ondansetron (4 mg IV) for antinausea prophylaxis followed 30 min later by oral alfentanil (45 and 5 μg/kg with 100 ml water at control and ritonavir/lopinavir sessions, respectively), followed 1 hr later by fexofenadine (60 mg, 100 ml water). Venous blood was sampled for 48 hr after alfentanil dosing and plasma stored at -20°C for later analysis. Hepatic CYP3A activity was evaluated on day 2 using IV alfentanil as an *in vivo* probe (Kharasch et al., 2004b; Kharasch et al., 2007; Kharasch et al., 2008b; Kharasch et al., 2009a; Kharasch et al., 2009b; Kharasch et al., 2011a; Kharasch et al., 2011b; Kharasch et al., 2012a; Kharasch et al., 2012b). Subjects received ondansetron followed 30 min later by alfentanil bolus (15 and 5 μg/kg at control and ritonavir/lopinavir sessions, respectively). Venous blood was

sampled for 24 hr after alfentanil dosing. Subjects received a standard breakfast and lunch 3 and 6 hr, respectively, after alfentanil in both sessions.

Methadone metabolism and clearance were assessed on day 3 by simultaneously administering IV and oral methadone (Kharasch et al., 2004a; Kharasch et al., 2008a; Kharasch et al., 2009a; Kharasch et al., 2009b; Kharasch et al., 2012a; Kharasch et al., 2012b). Subjects received IV ondansetron followed 30 min later by IV racemic unlabelled (d0)-methadone HCl (6.0 mg, equivalent to 5.4 mg free base) and oral deuterated racemic (d5)-methadone HCl (11.0 mg, equivalent to 9.86 mg free base, with 100 ml water, synthesized and used under Investigational New Drug approval (Kharasch et al., 2004a).) Subjects received a standard breakfast and lunch 2 and 4 hr after methadone, respectively, and free access to food and water thereafter. Venous blood was sampled for 96 hr after methadone. Continuous urine samples were collected at 24, 48, 72, and 96 hr. Nausea and/or vomiting were treated with ondansetron (4 mg IV or 8 mg orally) as needed.

Hepatic and first-pass CYP3A, intestinal transporters activity and methadone disposition were assessed at baseline. After 1-2 months, subjects began taking ritonavir/lopinavir 100/400 mg (Kaletra, Abbott Labs) twice daily. Dosing was adjusted on study days (morning dose at breakfast) to preclude an acute effect from the morning dose. First-pass CYP3A and transporter activities were determined on day 2 of ritonavir/lopinavir, and methadone disposition on day 3. At steady-state ritonavir/lopinavir, first-pass CYP3A and intestinal transporters activity were assessed on day 15, hepatic CYP3A on day 17, and methadone disposition on day 17. Because the duration of ritonavir administration may affect the degree of CYP3A alteration (Hsu et al., 1998; Greenblatt et al., 1999; Kharasch et al., 2008a; Kharasch et al., 2008b), both acute and steady-state ritonavir/lopinavir were evaluated.

Sample size was determined using a simplified analysis (paired t-test) for the outcome variable methadone systemic clearance. A previous study found 22 and 33% interday/intrasubject variability in IV and oral methadone clearances, respectively (Kharasch et al., 2004a). To detect a 30% change in clearance, using a paired t-test, with 33% variability, 1-ß=0.8, α=0.05, would require 12 subjects.

Analytical Methods

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Plasma alfentanil and fexofenadine concentrations were quantified simultaneously by solid-phase extraction and electrospray liquid chromatography-mass spectrometry as described previously (Kharasch et al., 2005). Plasma and urine methadone and EDDP enantiomer concentrations were quantified by chiral liquid chromatography-tandem electrospray mass spectrometry as described (Kharasch and Stubbert, 2013).

Data Analysis

Pharmacokinetic data were analyzed using noncompartmental methods (Phoenix, Pharsight Corp, Mountain View, CA), assuming complete absorption, as described previously (Kharasch et al., 2004a; Kharasch et al., 2004b; Kharasch et al., 2007; Kharasch et al., 2008a; Kharasch et al., 2008b; Kharasch et al., 2009a; Kharasch et al., 2009b; Kharasch et al., 2012a; Kharasch et al., 2012b). Area under the plasma concentration-time curve (AUC) was determined using the trapezoidal rule. Systemic clearance (CL_{IV}) was $dose_{IV}/AUC_{IV,}$ apparent oral clearance (CL/F) was $dose_{oral}/AUC_{oral}$, bioavailability (F_{oral}) was ($AUC_{oral}/dose_{oral}$) x ($dose_{IV}/AUC_{IV}$), steady-state volume of distribution (V_{ss}) was mean residence time x CL, volume of distribution based on the terminal phase (Vz) was dose/(AUC x λ) where λ is the terminal elimination rate constant. Hepatic extraction ratio (E_H) was determined as (CL_{IV}/Q_p), where hepatic plasma flow (Q_p) was estimated as the product of hepatic blood flow (25.3 and 25.5 ml/kg in males and females) and hematocrit (estimated as 40 and 36 in males and females) and negligible extrahepatic metabolism was assumed. Gastrointestinal extraction ratio (E_G) was $1-F_{oral}/(F_H \times F_{abs})$ where the oral dose was assumed to be entirely absorbed and thus F_{abs} was considered to be unity, and $F_H=1-E_H$. Renal clearance (Cl_r and CL_r/F) was determined as: amount excreted in urine/$AUC_{0-\infty}$. EDDP formation clearance was determined from urine data as: Cl_f = fraction of dose recovered in urine x CL_{IV} and Cl_f/F = fraction of dose recovered in urine x CL/F_l, for IV and oral dosing, respectively. Hepatic clearance (CL_H) was $CL_{iv}-CL_r$.

Statistical Analysis

Differences between treatment groups for pharmacokinetic and effect parameters were analyzed using one-way repeated measures analysis of variance followed by the Student-Newman-Keuls test for

multiple comparisons, or paired t-tests, as appropriate (Sigmaplot 12.5, Systat Software, Inc, San Jose, CA). Non-normal data were log transformed for analysis, but reported as the non-transformed results. Statistical significance was assigned at $p < 0.05$. Results are reported as the arithmetic mean ± standard deviation (SD). Plasma AUC and urine data were also assessed as ratios (treated/control) and the geometric mean and 90% confidence interval of the geometric mean. Confidence intervals excluding 1.0 were considered statistically significant. Relationships between methadone metabolism and clearance were evaluated by linear regression analysis.

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Results

Both acute and steady-state ritonavir/lopinavir diminished plasma R- and S-methadone concentrations and exposure (Figure 1, Table 1). Plasma $AUC_{0-\infty}$ ratios (ritonavir/lopinavir/control) were significantly reduced to 0.5-0.7, with a slightly greater effect on S- than R-methadone, and a slightly greater reduction by acute vs steady-state ritonavir/lopinavir. IV R- and S-methadone systemic clearance, hepatic clearance, and hepatic extraction were significantly increased by both acute vs steady-state ritonavir/lopinavir. Methadone renal clearance was unaffected by ritonavir/lopinavir. Methadone N-demethylation was also increased by ritonavir/lopinavir, with the plasma R- and S-EDDP/methadone AUC_{∞} ratio (ritonavir/lopinavir/control) increased, with a greater effect on the S- enantiomer. S-EDDP formation clearance was significantly increased by acute but not steady-state ritonavir/lopinavir. When control and both ritonavir/lopinavir sessions were analyzed together, there was a significant positive correlation ($r=0.90$, $p<0.001$) between methadone N-demethylation (plasma EDDP/methadone AUC ratio) and systemic clearance after intravenous methadone administration (Figure 2A). IV methadone disposition was stereoselective, with greater exposure to S-methadone, and a time-dependent increase in the plasma methadone R/S concentration ratio, which was further amplified by acute ritonavir/lopinavir (Figure 2B).

Acute and steady-state ritonavir/lopinavir also diminished oral methadone plasma concentrations, with a greater effect of acute vs steady-state ritonavir/lopinavir (Figure 3, Table 2). Plasma $AUC_{0-\infty}$ ratios (ritonavir/lopinavir/control) were significantly reduced to 0.4-0.8, with a greater reduction by acute vs steady-state ritonavir/lopinavir, and slightly greater effect on S- than R-methadone. The apparent oral clearance of both methadone enantiomers was significantly increased by acute and steady-state ritonavir/lopinavir. Methadone bioavailability was unaffected, as was renal clearance. Oral methadone N-demethylation was apparently induced, with the plasma EDDP/methadone AUC_{∞} ratio (ritonavir/lopinavir/control) increased by acute and steady-state ritonavir/lopinavir, with a greater effect on S- than R-methadone N-demethylation. Oral methadone disposition was stereoselective, and the time-

dependent increase in the plasma methadone R/S concentration ratio augmented by acute, and less so, steady-state ritonavir/lopinavir (Figure 2C).

Both acute and steady-state ritonavir/lopinavir markedly inhibited hepatic and first-pass CYP3A activity. Alfentanil plasma concentrations are shown in Figure 4, and pharmacokinetic parameters provided in Table 3. The $AUC_{0-\infty}$/dose ratio (ritonavir/lopinavir/control) for oral alfentanil was increased 11- and 7-fold, respectively, by acute and steady-state ritonavir/lopinavir, and that of IV alfentanil was increased almost 5-fold by steady-state ritonavir/lopinavir. These results indicate 75% inhibition of hepatic alfentanil clearance and CYP3A activity, and 84-90% inhibition of first-pass ALF clearance and CYP3A activity. Both hepatic and intestinal alfentanil extraction were substantially inhibited.

Disposition of oral fexofenadine was used to probe the activity of intestinal P-gp, and potentially other intestinal transporters. Both acute and steady-state ritonavir/lopinavir, the former more than the latter, increased fexofenadine peak plasma concentrations and AUC, with no effects on apparent elimination half-life (Figure 5, Table 3).

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Discussion

This investigation determined the role of CYP3A and P-glycoprotein in the mechanism by which ritonavir/lopinavir alters methadone disposition, and compared effects of single-dose and steady-state ritonavir/lopinavir. Steady-state (14d) ritonavir/lopinavir (100/400mg twice daily) had previously been found to reduce steady-state oral methadone AUC_{0-24} (36%) and C_{max} (44%) (Clarke et al., 2002). These results were considered surprising, given protease inhibitors effects on CYP3A and the consideration at that time of methadone as a CYP3A substrate (Clarke et al., 2002). However, neither methadone pharmacokinetic parameters nor metabolism were assessed, and mechanism(s) of altered methadone concentrations were considered unclear. Another study found that 7d ritonavir/lopinavir twice daily caused a 26-28% reduction in AUC_{0-24} and C_{max}, and a 42% increase in racemic methadone clearance, and some patients suffered opiate-withdrawal (McCance-Katz et al., 2003). Reduced methadone exposure was attributed to net induction of metabolic clearance, involving CYP3A and/or CYP2D6, and possibly also intestinal P-gp. Nevertheless, it subsequently became clear that protease inhibitors do not cause net CYP3A induction (Dickinson et al., 2010), and CYP2D6 does not contribute materially to methadone clearance (Shiran et al., 2009), hence driving the need to understand the mechanism of the methadone interaction (Khalsa and Elkashef, 2010). This is the first investigation to evaluate ritonavir/lopinavir effects on IV methadone disposition, oral and IV methadone concurrently, methadone metabolism, renal excretion, the stereoselectivity of these effects, and the mechanisms by which they occur.

Steady-state ritonavir/lopinavir reduced total (R- and S-methadone) plasma concentrations by half after a single methadone dose, consistent with previous reports with steady-state methadone dosing (Clarke et al., 2002; McCance-Katz et al., 2003). Overall, there was an average 1.5-fold induction of systemic and apparent oral methadone clearances. Effects of acute (2d) ritonavir/lopinavir on clearances were greater, averaging 2.6-fold. Clearance induction was attributable to increased hepatic methadone N-demethylation, hepatic extraction, and hepatic clearance, and there was a strong correlation between methadone N-demethylation and clearance. Induction of IV and oral methadone N-demethylation was evidenced by increases in plasma EDDP/methadone ratios and EDDP formation clearances. Neither

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acute nor steady-state ritonavir/lopinavir altered methadone renal clearance. Ritonavir/lopinavir effects on methadone plasma concentrations, metabolism, and clearance were stereoselective. There was a time-dependent increase in plasma R/S-methadone concentration ratios, which were augmented by ritonavir/lopinavir. S-methadone systemic and oral clearances, hepatic clearance, hepatic extraction ratios, and EDDP/methadone ratios were induced approximately 2-fold, while those of R-methadone were induced less. Together, the major finding of this investigation suggests that ritonavir/lopinavir decreases methadone plasma concentrations via a stereoselective metabolic drug interaction, resulting from preferential induction of hepatic S-methadone clearance, in turn attributable to stereoselective (S->R-methadone) induction of hepatic methadone N-demethylation and clearance.

The second major result of this investigation was that ritonavir/lopinavir increased fexofenadine AUC and C_{max}. Effects after short-term exposure (3.7 and 2.5-fold increases in AUC and C_{max}) were greater than at steady-state (2.0- and 1.8-fold, respectively), and were not due to reduced elimination. These results are similar to the 4- and 2.9-fold increases in fexofenadine AUC caused by single-dose and steady-state ritonavir/lopinavir reported previously (van Heeswijk et al., 2006). When this investigation was designed, fexofenadine was considered selective for P-gp, and used to probe intestinal P-gp-mediated interactions based on existing recommendations. However fexofenadine is now known as a substrate for multiple transporters, with absorption influenced by intestinal P-gp-mediated efflux and organic anion transporting polypeptide (OATP) 1A2-mediated uptake, and hepatic OATP-mediated hepatic (Bailey et al., 2007; Matsushima et al., 2008). Changes in fexofenadine AUC caused by ritonavir/lopinavir are consistent with decreased fexofenadine intestinal efflux, and acute inhibition of P-gp, with partial P-gp induction (yet continued net P-gp inhibition) upon continuing exposure. Similar conclusions were articulated previously (van Heeswijk et al., 2006), and also reached using oral digoxin, considered to quantify primarily intestinal P-gp activity (Wyen et al., 2008). Ritonavir/lopinavir increased digoxin AUC and C_{max} 1.8- and 1.6-fold, respectively, interpreted as P-gp inhibition (Wyen et al., 2008). Ritonavir alone at steady state (300 or 400mg twice daily) increased digoxin AUC 1.4- to 1.9-fold (Ding et al., 2004; Kirby et al., 2012). In vitro, acute lopinavir exposure inhibited P-gp, while extended

exposure induced P-gp (Vishnuvardhan et al., 2003). Together these findings suggest that ritonavir/lopinavir inhibits intestinal P-gp.

Another potential explanation for ritonavir/lopinavir reduction of oral methadone concentrations, in additional to increased clearance, was diminished bioavailability. Methadone has been considered a P-gp substrate, and rodent studies suggested a role for intestinal P-gp in methadone absorption (Ortega et al., 2007). Nevertheless, despite apparent P-gp inhibition by ritonavir/lopinavir, there was no change in methadone bioavailability. Previous clinical studies also showed methadone bioavailability apparently unrelated to P-gp (or fexofenadine transporter) activity. Specifically, ritonavir, indinavir, ritonavir/indinavir, nelfinavir, and efavirenz, respectively, inhibited, inhibited, inhibited, induced, and induced fexofenadine efflux (suggesting altered P-gp activity), while methadone bioavailability was unchanged, unchanged, unchanged, decreased, and unchanged (Kharasch et al., 2008a; Kharasch et al., 2009a; Kharasch et al., 2009b; Kharasch et al., 2012a; Kharasch et al., 2012b). Together these results do not suggest that intestinal P-gp significantly mediates methadone absorption and first pass extraction, and thus transporter mechanisms of methadone bioavailability and drug interactions remain unclear.

The third major finding of this investigation was that acute and steady-state ritonavir/lopinavir strongly inhibited both hepatic and intestinal CYP3A. Steady-state ritonavir/lopinavir inhibited combined hepatic and intestinal CYP3A, and hepatic CYP3A activities, by 85% and 78%, respectively, as assessed by apparent oral and intravenous alfentanil clearances. Hepatic and intestinal alfentanil extractions were inhibited 78% and 65%, respectively. Effects of shorter-duration (2d) ritonavir/lopinavir on CYP3A were even greater, causing 91% inhibition of oral alfentanil clearance. Previously, combined hepatic and intestinal CYP3A, and hepatic CYP3A activities, were inhibited by ritonavir/lopinavir 92% and 77%, respectively (Yeh et al., 2006), and 82% and 76%, respectively (Wyen et al., 2008), as assessed by apparent oral and intravenous midazolam clearances. The present results recapitulate the ability of ritonavir to cause both acute CYP3A inhibition/inactivation, and mild induction of new CYP3A protein synthesis with continuing exposure, albeit with strong net CYP3A inhibition always predominant (Kirby et al., 2011b).

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The ability of ritonavir/lopinavir to inhibit CYP3A while simultaneously inducing metabolism mediated by other CYP isoforms is consistent with previous observations, whether ritonavir is used either alone at therapeutic doses or in combination with other protease inhibitors at boosting doses (Foisy et al., 2008). For example, steady-state ritonavir alone (400 mg twice daily) caused 90% inhibition of hepatic and first-pass CYP3A activity, while inducing CYP1A2, CYP2B6, and CYP2C9 3-, 2-, and 2-fold, respectively (Kharasch et al., 2008b; Kharasch et al., 2008c; Kirby et al., 2011a; Kirby et al., 2011b). Steady-state ritonavir/lopinavir decreased hepatic CYP3A activity 77%, while hepatic CYP1A2, CYP2C9, and CYP2C19 activities increased 43%, 29%, and 100% (Yeh et al., 2006). Ritonavir/lopinavir inhibited CYP3A-dependent elimination of lumefantrine, while concurrently inducing metabolism of artemether, attributed to induction of CPY2B6, CYP2C9, and/or CYP2C19 (Byakika-Kibwika et al., 2012). More specifically, and of particular pertinence to this investigation, is that ritonavir/lopinavir inhibits CYP3A while significantly inducing hepatic CYP2B6 activity (Piscitelli et al., 2000; Hogeland et al., 2007).

Effects of acute ritonavir/lopinavir to inhibit CYP3A and apparent P-gp activity, and to induce methadone clearances and apparent CYP2B6 activity, were greater after short-term compared with steady-state exposure. This is consistent with acute inhibition of P-gp and CYP3A, with partial P-gp and CYP3A induction (yet continued net inhibition). This may relate to ritonavir and/or lopinavir effects on nuclear receptors, and their regulation of CYPs 2B6 and 3A, and P-gp (Svärd et al., 2011). Relationships of these clinical observations to mechanism-based inactivation of CYPs by ritonavir are presently unknown (Lin et al., 2013).

This investigation provides mechanistic insights regarding methadone metabolism and clearance, and drug interactions. Although methadone plasma concentrations were known to be reduced by ritonavir/lopinavir, the mechanism was never explained (Clarke et al., 2002; McCance-Katz et al., 2003). For many years, methadone clearance was attributed to CYP3A4. Nevertheless, the present results provide strong evidence against CYP3A mediating ritonavir/lopinavir induction of methadone metabolism and clearance. Specifically, ritonavir/lopinavir caused 1.3- to 3-fold increases in intravenous

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and oral methadone enantiomers N-demethylation and clearance, despite 78% and 85% inhibition of hepatic, and combined intestinal and hepatic, CYP3A activity. Moreover, there was no correlation between apparent oral methadone clearance and combined intestinal and hepatic CYP3A activity (Figure 6), nor between IV methadone clearance and hepatic CYP3A activity. CYP3A appears not to mediate ritonavir/lopinavir effects on methadone disposition, and, more generally, these results do not support a predominant role for CYP3A in clinical N-demethylation and clearance of single-dose methadone.

The preponderance of other clinical evidence also vitiates the long-held notions that CYP3A4 activity determines clinical methadone N-metabolism and clearance, CYP3A4 interindividual variability is a major factor in variable methadone bioavailability, and CYP3A4 mediates methadone interactions with antiretrovirals and other drugs (Ferrari et al., 2004; McCance-Katz et al., 2010), which were extrapolated from *in vitro* methadone N-demethylation by CYP3A4 (Iribarne et al., 1996; Foster et al., 1999; Gerber et al., 2004; Chang et al., 2011). Strong hepatic and first-pass CYP3A inhibition by troleandomycin, and by ritonavir/indinavir, had no significant effects on methadone plasma concentrations, systemic or apparent oral clearance, hepatic clearance, or bioavailability (Kharasch et al., 2004a; Kharasch et al., 2009a). Ritonavir alone, and nelfinavir, induced methadone N-demethylation and clearance, despite strong hepatic and first-pass CYP3A inhibition (Kharasch et al., 2008a; Kharasch et al., 2008b; Kharasch et al., 2009b). Neither these, nor the present investigation, showed a significant correlation between CYP3A activity and methadone clearance or N-demethylation. Taken together, these investigations do not support a significant role for CYP3A in clinical single-dose methadone N-demethylation and clearance.

In contrast, accumulating evidence suggests CYP2B6 as a major determinant of clinical methadone N-demethylation and clearance. CYP2B6 catalyzes methadone N-demethylation *in vitro* as avidly as CYP3A4, and CYP2B6 but not CYP3A4 is stereoselective (Gerber et al., 2004; Kharasch et al., 2004a; Totah et al., 2007; Totah et al., 2008; Chang et al., 2011). Methadone clearance was doubled by efavirenz and rifampin, which induce CYP2B6 as well as CYP3A4, and by ritonavir, which induces CYP2B6 while inhibiting CYP3A (Kharasch et al., 2004a; Kharasch et al., 2008a; Kharasch et al.,

2008c). Increasing the activity of CYP2B6, which metabolizes methadone stereoselectively (Gerber et al., 2004; Kharasch et al., 2004a; Totah et al., 2007; Totah et al., 2008; Chang et al., 2011), increased plasma R/S methadone ratios (Kharasch et al., 2004a; Kharasch et al., 2008a; Kharasch et al., 2008c; Totah et al., 2008; Kharasch et al., 2012b), while inhibiting CYP3A, which metabolizes methadone non-stereoselectively, did not (Kharasch et al., 2009a; Kharasch et al., 2009b; Kharasch et al., 2012a). CYP2B6 inhibition by ticlopidine increased methadone enantiomers AUC, and reduced methadone N-demethylation and clearance (Kharasch and Stubbert, 2013). Pharmacogenetic studies also evidence CYP2B6 involvement in methadone disposition, with *CYP2B6*6/*6* homozygotes having higher dose-adjusted S-methadone plasma concentrations and/or lower dose requirements than heterozygotes or noncarriers (Crettol et al., 2006; Wang et al., 2011; Levran et al., 2013).

In summary, acute and steady-state ritonavir/lopinavir induced the clinical N-demethylation and clearance of single-dose methadone. This occurred despite significant inhibition of hepatic and intestinal CYP3A activity. Steady-state, and more so acute ritonavir/lopinavir, inhibited intestinal P-gp activity, but had no effect on methadone bioavailability. These observations together with previous results, do not support a significant role for CYP3A or ritonavir/lopinavir-inhibitable intestinal transporters in single-dose methadone disposition. In general, the results demonstrate the potential for CYP2B6- as well as CYP3A-mediated ritonavir/lopinavir drug interactions.

Acknowledgements

The technical assistance of Darain Mitchell and Jing Berry is gratefully appreciated

Authorship Contributions

Participated in research design: Kharasch

Conducted experiments: Kharasch,. Stubbert

Contributed new reagents or analytic tools: Stubbert

Performed data analysis: Kharasch, Stubbert

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Footnotes

This work was supported by National Institutes of Health grants [R01-GM63674], [R01-DA14211], [K24-DA0041] and [UL1-TR000448].

No author has any conflict of interest.

Figure legends

Figure 1. Effect of acute and steady-state ritonavir/lopinavir (RTV/LOP) on intravenous methadone disposition and metabolism. Shown are plasma R- and S-methadone and R- and S-EDDP concentrations. Subjects received 6.0 mg intravenous methadone HCl (5.4 mg free base). Each data point is the mean ± SD (n=12). Some SD values are omitted for clarity.

Figure 2. Effect of ritonavir/lopinavir (RTV/LOP) on methadone disposition. (A) Relationship between methadone N-demethylation (plasma EDDP AUC/methadone AUC ratio) and systemic methadone clearance after intravenous methadone administration. Data for each methadone enantiomer include control, acute and steady-state ritonavir/lopinavir pretreatments. Each data point is the result from an individual subject. There was a significant correlation between methadone metabolism and clearance (Pearson product moment correlation, $r=0.90$, $p<0.001$). (B and C) Plasma R/S-methadone concentration ratios in controls and after acute and steady-state ritonavir/lopinavir, for (B) intravenous and (C) oral methadone. Each data point is the mean ± SD (n=12). Some SD values are omitted for clarity.

Figure 3. Effect of acute and steady-state ritonavir/lopinavir (RTV/LOP) on oral methadone disposition and metabolism. Shown are plasma R- and S-methadone and R- and S-EDDP concentrations. Subjects received 11.0 mg oral methadone HCl (9.9 mg free base). Each data point is the mean ± SD (n=12). Some SD values are omitted for clarity.

Figure 4. Effect of acute and steady-state ritonavir/lopinavir (RTV/LOP) first-pass and hepatic CYP3A activity, assessed with alfentanil as a CYP3A probe. Shown are dose-adjusted plasma alfentanil concentrations after (A) oral and (B) intravenous administration. Subjects received 45 and 53 μg/kg oral alfentanil at the control and ritonavir/lopinavir sessions, respectively, and 15 and 5 μg/kg intravenous alfentanil at the control and ritonavir/lopinavir sessions, respectively. Each data point is the mean ± SD (n=12). Some SD values are omitted for clarity.

Figure 5. Effect of acute and steady-state ritonavir/lopinavir (RTV/LOP) on intestinal transporter

activity, assessed using fexofenadine as a transporter probe. Each subject received 60 mg oral fexofenadine on all occasions. Each data point is the mean ± SD (n=12).

Figure 6. Relationship between methadone apparent oral clearance and first-pass CYP3A activity. The latter was measured as the apparent oral clearance of alfentanil. Data for each methadone enantiomer include control, acute and steady-state ritonavir/lopinavir pretreatments. Each data point is the result from an individual subject. There was no significant correlation between the clearance of either methadone enantiomer and CYP3A activity.

Table 1 Intravenous methadone pharmacokinetic parameters

	Control	Acute ritonavir/lopinavir	Steady-state ritonavir/lopinavir	Control	Acute ritonavir/lopinavir	Steady-state ritonavir/lopinavir
	R-methadone			S-methadone		
C_{max} (ng/ml)	56 ± 28	36 ± 13*	36 ± 17*	78 ± 35	44 ± 17*	45 ± 22*
AUC_{0-96} (ng •hr •ml-1)	304 ± 81	213 ± 102*	234 ± 111*	441 ± 142	244 ± 161*	312 ± 201*
$AUC_{0-\infty}$ (ng •hr •ml^{-1})	427 ± 152	304± 160*	354 ± 213	547 ± 215	313 ± 238*	410 ± 284*
$AUC_{0-\infty}$ ratio (ritonavir-lopinavir/control)		0.65 (0.54, 0.79)	0.74 (0.59,0.93)		0.47 (0.34, 0.63)	0.64 (0.50, 0.83)
CL_{IV} (ml•kg^{-1}•min^{-1})	1.66 ± 0.57	2.80 ± 1.71*	2.49 ± 1.41*	1.37 ± 0.65	3.62 ± 3.17*	2.49 ± 1.99*
CL_H (ml•kg^{-1}•min^{-1})	1.54 ± 0.52	2.63 ± 1.61*	2.33 ± 1.31*	1.29 ± 0.63	3.47 ± 3.06*	2.38 ± 1.92*
Renal clearance (ml•kg^{-1}•min^{-1})	0.13 ± 0.09	0.16 ± 0.13	0.14 ± 0.13	0.08 ± 0.05	0.13 ± 0.10	0.10 ± 0.08
Elimination $t_{1/2}$ (hr)	48 ± 10	52 ± 21	54 ± 24	35 ± 10	37 ± 18	41 ± 15
Vss (L/kg)	6.2 ± 1.6	10.4 ± 4.9*	9.3 ± 2.8*	3.6 ± 1.0	8.3 ± 4.7*	6.8 ± 3.3
E_H	0.10 ± 0.04	0.17 ± 0.11*	0.15 ± 0.09*	0.08 ± 0.04	0.22 ± 0.20*	0.15 ± 0.13*
	R-EDDP			S-EDDP		
C_{max} (ng/ml)	0.39 ± 0.08	0.34 ± 0.12	0.43 ± 0.34	0.74 ± 0.22	0.68 ± 0.23	0.74 ± 0.36
AUC_{0-96} (ng •hr •ml^{-1})	20 ± 5	17 ± 5*	17 ± 5*	37 ± 10	32 ± 9	32 ± 8
AUC_{∞} (ng •hr •ml^{-1})	28 ± 7	26 ± 11	23 ± 8	45 ± 12	41 ± 12	40 ± 12
Elimination $t_{1/2}$ (hr)	48 ± 14	59 ± 35	44 ± 13	37 ± 12	40 ± 15	36 ± 10
AUC_{0-96} (EDDP/methadone)	0.08 ± 0.03	0.10 ± 0.05	0.08 ± 0.03	0.10 ± 0.04	0.21 ± 0.17*	0.15 ± 0.10
AUC_{∞} (EDDP/methadone)	0.09 ± 0.05	0.11 ± 0.06	0.08 ± 0.03	0.11 ± 0.04	0.22 ± 0.18*	0.15 ± 0.11
AUC_{0-96} (EDDP/methadone) ratio (ritonavir-lopinavir/control)		1.15 (0.98,1.36)	1.08 (0.99,1.17)		1.73 (1.35,2.22)	1.32 (1.13,1.55)
EDDP formation clearance (ml•kg^{-1}•min^{-1})	0.15 ± 0.08	0.17± 0.09	0.16 ± 0.08	0.21 ± 0.09	0.41 ± 0.26*	0.27 ± 0.13

Subjects received 6.0 mg IV methadone HCl at all sessions. Results are the arithmetic mean ± SD, except AUC ratios (ritonavir/lopinavir/control) which are the geometric mean (90% confidence interval). N=12, except renal clearances and EDDP formation clearances (N=10, due to incomplete urine collections in 2 subjects).

*Significantly different from control ($p<0.05$)

Table 2 Oral methadone pharmacokinetic parameters

	Control	Acute ritonavir/lopinavir	Steady-state ritonavir/lopinavir	Control	Acute ritonavir/lopinavir	Steady-state ritonavir/lopinavir
	R-methadone			S-methadone		
C_{max} (ng/ml)	14 ± 6	8 ± 4*	10 ± 4*	23 ± 10	10 ± 7*	15 ± 8*
t_{max} (hr)	5 ± 3	6 ± 6	5 ± 2	5 ± 3	6 ± 5	5 ± 3
AUC_{0-96} (ng •hr •ml^{-1})	462 ± 154	288 ± 201*	399 ± 190	690 ± 289	324 ± 285*	525 ± 332*
$AUC_{0-\infty}$ (ng •hr •ml^{-1})	672 ± 253	424 ± 292*	583± 301	862 ± 404	415 ± 397*	688 ± 435
$AUC_{0-\infty}$ ratio (ritonavir-lopinavir/control)		0.52 (0.38,0.70)	0.79 (0.61, 1.02)		0.35 (0.24, 0.50)	0.70 (0.50, 0.97)
CL/F (ml•kg^{-1}•min^{-1})	1.98 ± 0.74	4.94 ± 4.70*	2.81 ± 1.78*	1.70 ± 0.90	6.48 ± 5.83*	3.04 ± 3.16
Renal clearance (ml•kg^{-1}•min^{-1})	0.15 ± 0.11	0.15 ± 0.15	0.16 ± 0.15	0.09 ± 0.06	0.11 ± 0.12	0.11 ± 0.09
Elimination $t_{1/2}$ (hr)	50 ± 9	54 ± 29	49 ± 24	35 ± 8	36 ± 14	40 ± 16
Vz/F (L/kg)	8.4 ± 3.6	19.5 ± 15.4*	10.0 ± 4.2	4.9 ± 2.6	17.5 ± 14.3*	8.2 ± 5.4
F_{oral}	0.88 ± 0.20	0.79 ± 0.35	0.92 ± 0.24	0.87 ± 0.20	0.72 ± 0.31	0.92± 0.21
	R-EDDP			S-EDDP		
C_{max} (ng/ml)	1.4 ± 0.9	1.0 ± 0.5	1.4 ± 1.1	2.0 ± 1.5	1.8 ± 1.2	1.9 ± 1.1
AUC_{0-96} (ng •hr •ml^{-1})	46 ± 22	30 ± 14	39 ± 13	70 ± 36	54± 27 [a]	63± 17 [a]
AUC_{∞} (ng •hr •ml^{-1})	64 ± 40	40± 22	48± 18	96 ± 61	63± 30 [a]	74 ± 21
Elimination $t_{1/2}$ (hr)	43 ± 12	36 ± 14	36 ± 9	40 ± 17	35 ± 25	31 ± 6
AUC_{0-96} (EDDP/methadone)	0.10 ± 0.03	0.12 ± 0.07	0.11 ± 0.05	0.11 ± 0.05	0.20 ± 0.14	0.19 ± 0.17
AUC_{∞} (EDDP/methadone)	0.10 ± 0.04	0.12 ± 0.07	0.10 ± 0.05	0.12 ± 0.05	0.21 ± 0.15*	0.18 ± 0.17*
AUC_{0-96} (EDDP/methadone) ratio (ritonavir-lopinavir/control)		1.16 (0.92,1.45)	1.11 (0.95,1.29)		1.74 (1.32, 2.31)	1.46 (1.19,1.78)
EDDP formation clearance (ml•kg^{-1}•min^{-1})	0.16 ± 0.10	0.17 ± 0.13	0.18 ± 0.10	0.20 ± 0.12	0.36 ± 0.30	0.28 ± 0.13

Subjects received 11.0 mg oral methadone HCl at all sessions. Results are the arithmetic mean ± SD, except AUC ratios (ritonavir/lopinavir/control) which are the geometric mean (90% confidence interval). N=12, except renal clearances and EDDP formation clearances (N=10, due to incomplete urine collections in 2 subjects).

*Significantly different from control ($p<0.05$)

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Table 3. Pharmacokinetic parameters for intravenous and oral alfentanil and oral fexofenadine

	Control	Acute ritonavir/lopinavir	Steady-state ritonavir/lopinavir
IV alfentanil			
C_{max}/dose (ng/ml/mg)	100 ± 37		109± 42
$AUC_{0-\infty}$/dose (ng•hr•ml^{-1}/mg)	68 ± 26		383 ± 287*
$AUC_{0-\infty}$/dose ratio (ritonavir-lopinavir/control)			4.6 (3.2, 6.8)
CL_{IV} (ml•kg^{-1}•min^{-1})	3.89 ± 1.33		0.97 ± 0.61*
Elimination $t_{1/2}$ (hr)	1.5 ± 0.4		8.2 ± 3.1*
E_H	0.28 ± 0.08		0.06 ± 0.04*
Oral alfentanil			
C_{max}/dose (ng/ml/mg)	12 ± 4	30 ± 9*	28 ± 11*
$AUC_{0-\infty}$/dose (ng•hr•ml^{-1}/mg)	32 ± 17	367 ± 193*	246 ± 165*
$AUC_{0-\infty}$/dose ratio (ritonavir-lopinavir/control)		11.4 (7.4, 17.7)	6.9 (4.3 11.2)
CL/F (ml•kg^{-1}•min^{-1})	9.2 ± 4.6	0.8 ± 0.3*	1.5 ± 1.2*
Elimination $t_{1/2}$ (hr)	1.4 ± 0.3	8.8 ± 3.0*	7.7 ± 3.0*
F_{oral}	0.45 ± 0.09		0.84 ± 0.15*
E_G	0.40 ± 0.10		0.14 ± 0.13*
Oral fexofenadine			
C_{max} (ng/ml)	172 ± 90	440 ± 192*	303 ± 175*
$AUC_{0-\infty}$ (ng •hr •ml^{-1})	1180 ± 620	4420 ± 2180*	2470 ± 1360*
$AUC_{0-\infty}$ ratio (ritonavir-lopinavir/control)		3.7 (2.8,4.9)	2.0 (1.6,2.6)
CL/F (ml•kg^{-1}•min^{-1})	14.4 ± 6.1	4.0 ± 1.9*	7.4 ± 3.8*
Elimination $t_{1/2}$ (hr)	9.6 ± 4.8	7.8 ± 2.7	10.1 ± 3.0

Subjects received 15 and 5 µg/kg intravenous (IV) alfentanil and 45 and 5 µg/kg oral alfentanil, at control and ritonavir/lopinavir sessions, respectively. All oral fexofenadine doses were 60 mg. Peak alfentanil plasma concentration (C_{max}) and area under the concentration-time curve (AUC) are shown normalized to

dose. No other variables are dose adjusted. Results are the arithmetic mean ± SD (n=12), except the $AUC_{0-\infty}$/dose ratio (ritonavir-indinavir/lopinavir), which is the geometric mean (90% confidence interval).

*Significantly different from control ($p<0.05$)

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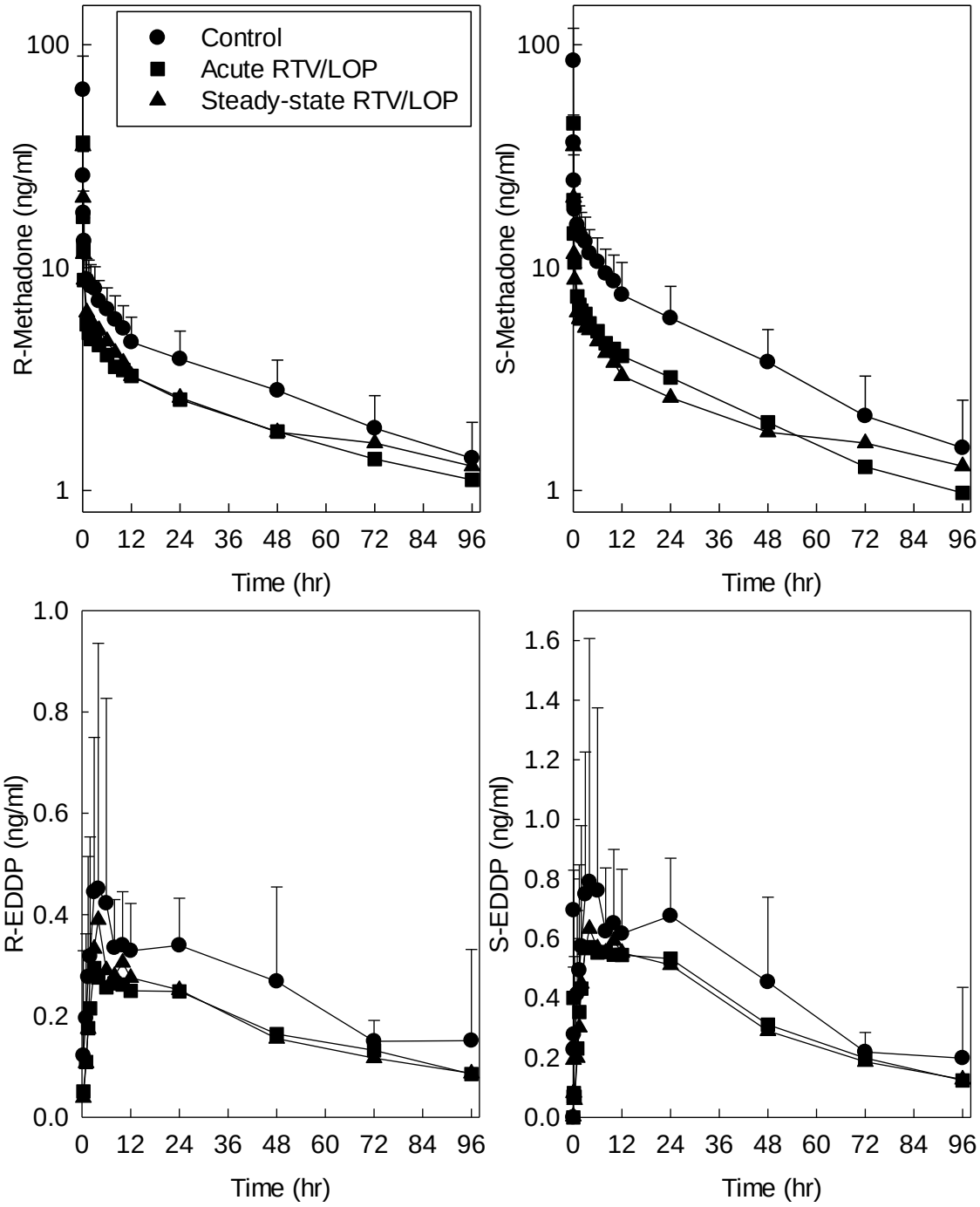

Figure 1

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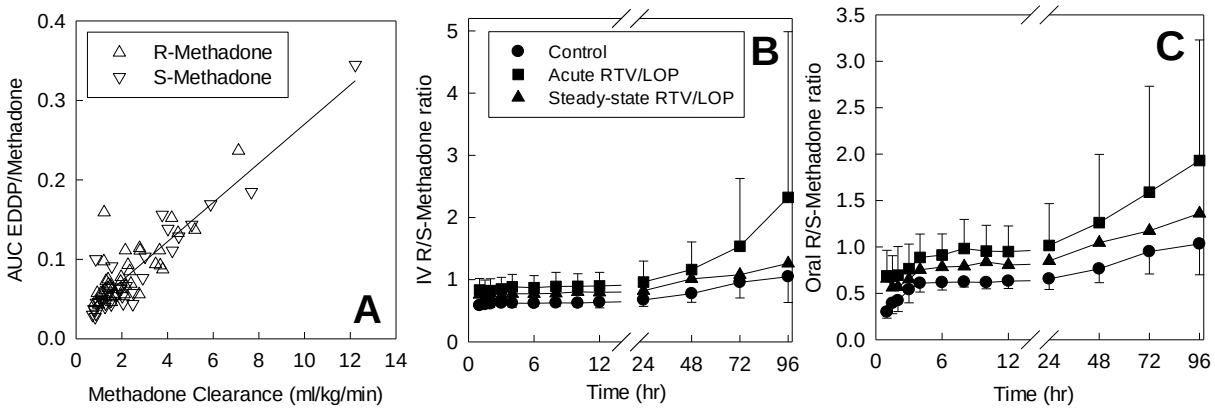

Figure 2

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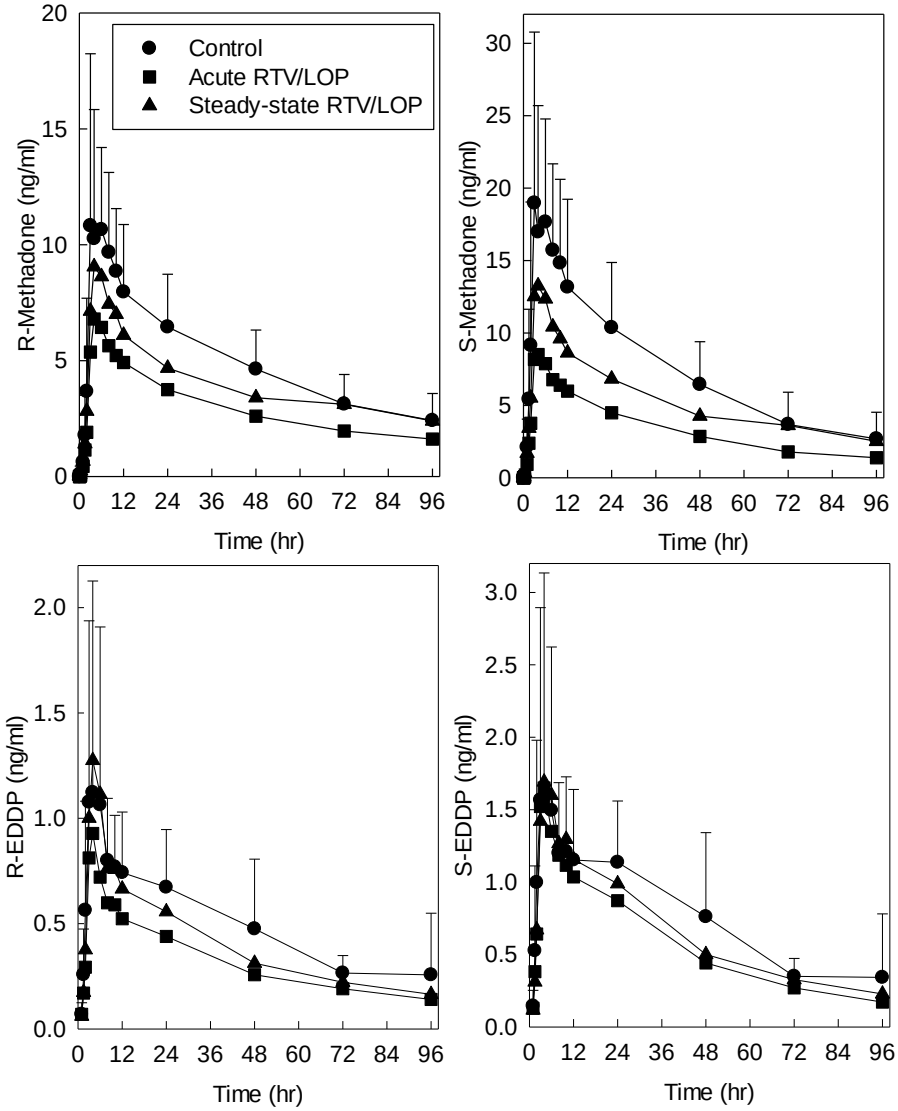

Figure 3

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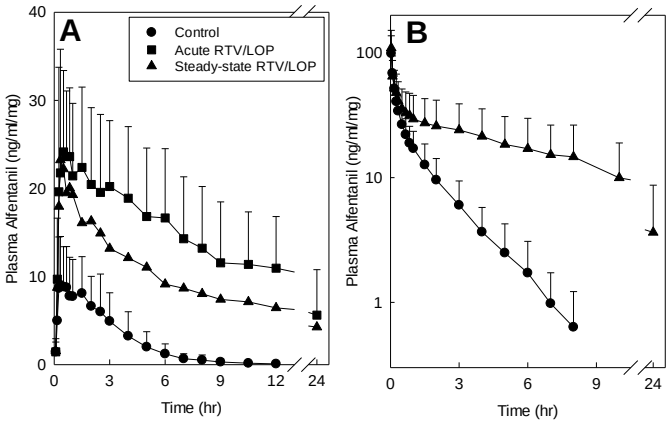

Figure 4

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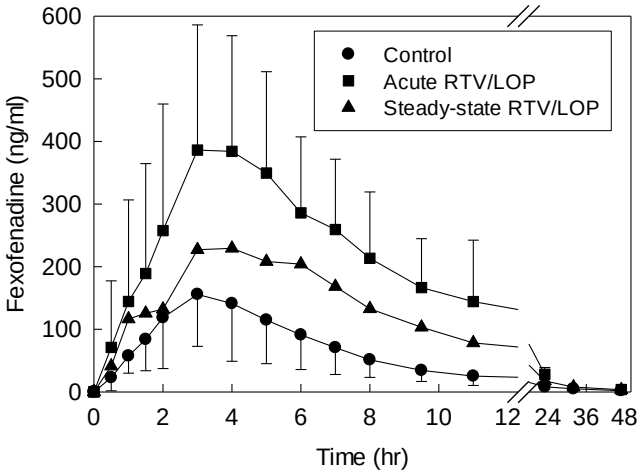

Figure 5

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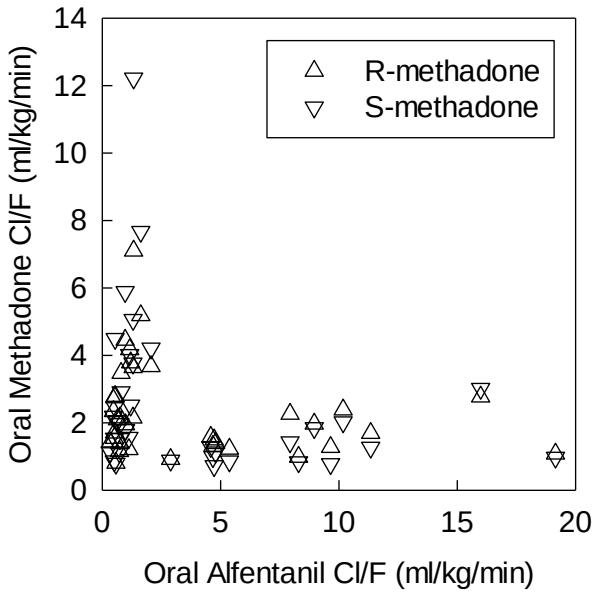

Figure 6