DMD Fast Forward. Published on September 22, 2009 as doi:10.1124/dmd.109.028605

DMD 28605

The Effect of Rifampin and Nelfinavir on the Metabolism of Methadone and Buprenorphine in Primary Cultures of Human Hepatocytes

David E. Moody, Wenfang B. Fang, Shen-Nan Lin, Denise M. Weyant, Stephen C. Strom and Curtis J. Omiecinski

Center for Human Toxicology, Department of Pharmacology and Toxicology, University of Utah, Salt Lake City, Utah (D.E.M., W.B.F., S.N.L.); Center for Molecular Toxicology and Carcinogenesis, Department of Veterinary and Biomedical Sciences, Pennsylvania State University, University Park, Pennsylvania (C.J.O, D.M.W.); and Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania (S.C.S.)

Running Title: Buprenorphine and Methadone Metabolism in Human Hepatocytes

Author for Correspondence: David E. Moody, Ph.D.
Center for Human Toxicology
University of Utah
417 Wakara Way, Suite 2111
Salt Lake City, UT 84108
801-581-5117 (phone) / 801-581-5034 (fax)
david.moody@utah.edu

Number of text pages: 13
Number of tables: 3
Number of figures: 3
Number of references: 38
Number of words in the abstract: 249
Number of words in the introduction: 917
Number of words in the discussion: 1294

Abbreviations used: AUIC, area under the incubation time-concentration curve; EDDP, 2-ethylidene-1,5-dimethyl-3,3-dipehylpyrrolidine; EMDP, 2-ethyl-5-methyl-3,3-diphenylpyrroline; DMSO, dimethylsulfoxide; CYP, cytochrome P450; UGT, UDP-glucuronosyl transferase; UDPGA, uridine 5'-diphosphoglucuronic acid; HLM, human liver microsomes; HSIM, human small intestine microsomes; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry;

DMD Fast Forward. Published on September 22, 2009 as DOI: 10.1124/dmd.109.028605
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

We tested the hypothesis that primary cultures of human hepatocytes could predict potential drug interactions with methadone and buprenorphine. Hepatocytes (5 donors) were preincubated with DMSO (vehicle), rifampin or nelfinavir prior to incubation with methadone or buprenorphine. Culture media (0-60 minute) was analyzed by liquid chromatography-tandem mass spectrometry for R- and S-methadone and R- and S-2-ethylidene-1,5-dimethyl-3,3-dipehylpyrrolidine (EDDP) or for buprenorphine, norbuprenorphine, and their glucuronides (B-3-G and N-3-G). R- and S-EDDP were detected in 3/5, 4/5 and 5/5 media from cells pretreated with DMSO, nelfinavir and rifampin. R-EDDP increased 3.1- and 26.5-fold, and S-EDDP 2.5- and 21.3-fold after nelfinavir and rifampin. The rifampin effect was significant. B-3-G production was detected in media of all cells incubated with buprenorphine, and accounted for most buprenorphine loss from culture media; it was not significantly affected by either pretreatment. Norbuprenorphine and N-3-G together were detected in 3/5, 4/5 and 5/5 donors pretreated with DMSO, nelfinavir and rifampin; norbuprenorphine in 1/5, 1/5 and 2/5. While there was a trend for norbuprenorphine (2.8-, 4.9-fold) and N-3-G (1.7-, 1.9-fold) to increase after nelfinavir and rifampin, none of the changes were significant. To investigate low norbuprenorphine production, buprenorphine was incubated with human liver and small intestine microsomes fortified to support both N-dealkylation and glucurondation; N-dealkylation predominated in small intestine, glucuronidation in liver microsomes. These studies support the hypothesis that methadone metabolism and its potential for drug interactions can be predicted with cultured human hepatocytes, but for buprenorphine, combined effects of hepatic and small intestinal metabolism are likely involved.

Introduction

Methadone (Eap, et al. 2002) and buprenorphine (Cowan, et al. 1977) are full and partial mu-opioid receptor agonists, respectively, that are used as analgesics and as replacement therapy in the treatment of opioid dependence (Eap, et al. 2002; Johnson, et al. 2000). In both types of usage, co-administration of other medications is common. Many of the patients being treated for opioid dependence are, or were, intravenous drug abusers. HIV infection and acquired immunodeficiency syndrome is therefore a common co-morbidity (Cohn 2002), and co-treatment with antiretroviral drugs, as well as, drugs for accompanying co-morbidities, such as tuberculosis, are common.

Many of these co-medications are recognized to cause drug interactions at sites of drug metabolism. Indeed, a number of antiretroviral agents have been found to alter the pharmacokinetics of methadone. Most, including the nonnucleoside reverse transcriptase inhibitors efavirenz, nevirapine, and etravirine, and the protease inhibitors lopinavir/ritonavir combination, and nelfinavir (McCance-Katz 2005), induce methadone metabolism. The more potent inducers have been associated with signs of treatment failure (i.e., withdrawal syndrome). Methadone metabolism is also induced in vivo by treatment with the antitubercular agent rifampin (Kharasch, et al. 2004; Kreek, et al. 1976); this induction was also associated with withdrawal syndromes (Kreek, et al. 1976). More recently, studies on the interaction of buprenorphine with antiretrovirals have been conducted. Elfavirenz and delavirdine caused significant pharmacokinetic changes but had no apparent effect on efficacy (McCance-Katz, et al. 2006a); ritonavir moderately increased plasma area under the time-concentration curves (AUC) of buprenorphine and norbuprenorphine, while nelfinavir and lopinavir/ritonavir combination had minimal effect on buprenorphine pharmacokinetics; none affected therapeutic efficacy (McCance-Katz, et al. 2006b). Atazanavir alone or in combination with ritonavir significantly increased plasma AUCs of buprenorphine and norbuprenorphine. In this case there were signs of increased sedation (McCance-Katz, et al. 2007). Studies to date suggest that methadone is more susceptible than buprenorphine to potential drug interactions with antiretroviral agents. Differences in the metabolism of methadone and buprenorphine may well define differences in their susceptibility to drug interactions.

Methadone is used as a racemic mixture of R- and S-methadone, with the R-enantiomer having greater potency (Eap, et al. 2002). It is primarily metabolized by N-demethylation, which is followed by spontaneous cyclization to 2-ethylidene-1,5-dimethyl-3,3-dipehylpyrrolidine (EDDP), which may be further N-demethylated to 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP). These metabolites retain the R- and S-enantiomeric relationship. While other sites of metabolism have been noted, including reduction to methadol and p-hydroxylation of the phenyl rings, the resultant metabolites are not readily detected and making determination of their contribution to clearance of the parent drug hard to determine. The N-demethylation of methadone has been attributed to cytochrome P450 (CYP) 3A4, 2B6, 2D6 and 2C19 (Iribarne, et al. 1996; Kharasch, et al. 2004; Moody, et al. 1997). CYP2B6 preferably forms S-EDDP (Crettol, et al. 2005; Totah, et al. 2008).

Buprenorphine is primarily N-dealkylated to norbuprenorphine; both are extensively conjugated by UDP-glucuronosyltransferases (UGT) (Cone, et al. 1984). The formation of norbuprenorphine was initially found to be performed by CYP3A4 (Iribarne, et al. 1997), subsequent studies also demonstrated the involvement of CYP2C8 (Moody, et al. 2002; Picard, et al. 2005). Recently a number of sites of side-chain and phenyl ring hydroxylations have been identified (Chang, et al. 2006; Picard, et al. 2005). The side-chain hydroxylation products of buprenorphine (M1) and norbuprenorphine (M3) are present at sufficient amounts to be identified in the urine (but not plasma) of subjects taking therapeutic doses of sublingual buprenorphine (Chang, et al. 2006), but their quantitative contribution to the clearance of buprenrophine has not been determined due to lack of reference material. M1 is formed by both CYP3A4 and 2C8; M3 is formed primarily by 3A4 (Chang, et al. 2006); they too are modulated by anti-retroviral medications (Moody, et al. 2009). The glucuronidation of buprenorphine is primarily carried out by UGT1A1 and 2B7, and that of norbuprenorphine by UGT1A1 and 1A3 (Chang and Moody 2009). Glucuronidated products of both M1, and to a lesser extent M3, are detected in urine; however, the UGT(s) involved have not been studied.

Advances in the development of primary hepatocyte culturing methodology have established this in vitro system as a robust and differentiated model of in vivo liver induction responses and in vivo drug metabolism (Olsavsky, et al. 2007). Therefore, in this study, we hypothesized that primary cultures of human hepatoctyes could be used to predict and to better define the in vivo

interactions reported between methadone and buprenorphine with antiretroviral and antitubucular agents. In our investigation, we used the protease inhibitor, nelfinavir, as a model antiretroviral agent, as it has been studied previously in vivo with both methadone (McCance-Katz 2005) and buprenorphine (McCance-Katz, et al. 2006b), along with the prototypical inducer and antitubercular agent, rifampin, which has been studied in vivo with respect to methadone disposition (Kharasch, et al. 2004; Kreek, et al. 1976).

The response of methadone metabolism to nelfinavir and rifampin qualitatively resembled the response seen in vivo. Buprenorphine glucuronidation was unresponsive to either treatment, which was consistent with in vivo studies on nelfinavir, but not expected with rifampin. In addition, hepatocytes incubated with buprenorphine only occasionally produced norbuprenorphine. To further explore this ambiguity between the ability of hepatocytes to glucuronidate but not N-dealkylate buprenorphine, human liver microsomes (HLM) and human small intestine microsomes (HSIM) were incubated with buprenorphine under conditions that favored both the glucuronidation and N-dealkylation pathways. N-dealkylation was a more prominent component of buprenorphine metabolism in the HSIM. These findings lead us to propose the hypothesis that the small intestine plays an important role in the N-dealkylation of buprenorphine in humans.

Methods

Materials. Buprenorphine, d_4-buprenorphine, norbuprenorphine, d_3-norbuprenorphine, buprenorphine-3-ß-D-glucuronde, norbuprenorphine-3-ß-D-glucuronde, racemic methadone, racemic d_3-methadone, racemic EDDP perchlorate, and racemic d_3-EDDP perchlorate were purchased from Cerilliant (Round Rock, TX). Uridine 5'-diphosphoglucuronic acid (UDPGA), alamethicin, D-glucose-6-phosphate monosodium salt, glucose-6-phosphate dehydrogenase, ß-NADP sodium salt, EDTA disodium salt, magnesium chloride, and ammonium carbonate were obtained from Sigma-Aldrich (St. Louis, MO). Liver samples (unused donor tissue) were obtained from Tissue Transformation Technologies (Edison, NJ). HSIM (enterocyte) were obtained from BD-Gentest (Woburn, MA). Solid-phase extraction columns (endcapped C8 500 mg/6 ml) were purchased from United Chemical Technologies, Inc. (Bristol, PA). Organic solvents used for extractions were HPLC grade or higher. Aqueous reagents were prepared in purified water (specific resistance > 18.2 mΩ/cm) obtained by a Milli-Q Plus water purification system (Millipore, Billerica, MA).

Incubation with Primary Cultures of Human Hepatocytes. Primary human hepatocytes from 5 different donors were obtained from the University of Pittsburgh, through the Liver Tissue Cell Distribution System, NIH Contract #N01-DK-7-0004 / HHSN267200700004C. Culture details have been reported previously (Olsavsky, et al. 2007). Briefly, hepatocytes were plated on collagen-coated 6-well dishes, and within 48 h after cell attachment, a dilute overlay of Matrigel (BD Biosciences, San Jose, CA) was added to the cultures dropwise (90 µL of ice-cold 10 mg/ml Matrigel; 225 µg/ml per well) with swirling in 2.5 ml/well of William's Media E supplemented with 1% penicillin/streptomycin, 10 mM HEPES, 20 µM glutamine, 25 nM dexamethasone, 10 mM insulin, 30 mM linoleic acid, 1 mg/ml BSA, 5 ng/ml selenious acid, and 5 µg/ml transferrin. Forty eight hours following Matrigel additions, cells were exposed to either rifampicin (8.93 µg/ml), nelfinavir (2.0 µg/ml) or DMSO (vehicle control) for 96 h, then treated with buprenorphine (2.5 ng/ml, 5 ng/ml or 10 ng/ml), methadone (100 ng/ml, 300 ng/ml or 400 ng/ml) or DMSO. The first time point (time 0) was collected immediately after chemical addition, through removal of 300 µL of media. Subsequently, additional 300 µL media aliquots were removed at 15, 30, 45 and 60 min intervals for LC-ESI-MS/MS analysis. The assays were

conducted in duplicate, i.e., extracts from 2 wells/treatment arm were analyzed in parallel. During the hepatocyte culture period, media was replenished every 48 h.

In vitro Incubations of Buprenorphine with HLM and HSIM. HLM were prepared from human liver by differential centrifugation as described by Chang et al. (Chang, et al. 2006). The HLM used in these experiments were a pool from livers of five different donors. The HSIM purchased from BD-Gentest were described as from mature enterocytes prepared from both duodenum and jejunum sections. The incubation mixture (final volume 500 µl) contained incubation buffer (0.1 M phosphate buffer, pH 7.4 with 1.0 mM EDTA and 5.0 mM $MgCl_2$) and was fortified to support both N-dealkylation and glucuronidation by addition of a NADPH-generating system composed of 10 mM glucose-6-phosphate, 1.2 mM NADP, and 1.2 units of glucose-6-phosphate dehydrogenase, as well as 2 mM UDPGA, and 50 µg/mg protein alamethicin. HLM or HSIM were added to achieve a final concentration of 0.5 mg prot/ml. Buprenorphine was at 5 ng/ml. The reaction was initiated by addition of the NADPH generating system and UDPGA, samples were incubated for the times indicated at 37˚C, the reaction was terminated by addition of 200 µl methanol and samples were quickly moved to –75˚C storage until analysis.

Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS/MS) Enantiomer-Selective Determination of R- and S-Methadone and R- and S-EDDP. LC-ESI-MS/MS was performed essentially as described by Moody et al. (Moody, et al. 2008). One hundred microliter aliquots of hepatocyte culture media were fortified with d_3-methadone and d_3-EDDP as internal standard, subjected to liquid-liquid extraction using methyl t-butyl ether, dried and reconstituted in 100 µl of 10 mM ammonium acetate/methanol (9:1, v/v). Chromatographic separation was achieved with a Hewlett-Packard (Agilent Technologies, Palo Alto, CA) 1100 series HPLC system equipped with a Chiral-AGP column (50 x 2.0 mm, 5 µm, Analytical, Pompton Plains, NJ). A Finnigan/Thermo (San Jose, CA) Quest TSQ triple-stage quadrupole MS was used for analysis. All analytical batches included two sets of calibrators ranging from 2.5 to 500 ng enantiomer/ml, and duplicate quality control samples at 5.0, 100 and 425 ng enantiomer/ml. Calibrators and QCs were prepared in hepatocyte culture media from separately prepared stocks.

LC-ESI-MS/MS Determination of Buprenorphine, Norbuprenorphine, Buprenorphine-3-Glucuronide and Norbuprenorphine-3-Glucuronide. LC-ESI-MS/MS was performed essentially as described by Huang et al. (Huang, et al. 2006). For hepatocyte culture media, 0.25 ml aliquots were used; for microsomal incubates, the entire mixture along with the methanol used to terminate the reaction was used. d_4-Buprenorphine and d_3-norbuprenorphine were added as internal standards for buprenorphine and buprenorphine-3-glucuronide, and norbuprenorphine and norbuprenorphine-3-glucuronide, respectively. Samples were subjected to solid-phase extraction with C18 columns, the eluates dried and reconstituted in 0.1% formic acid in water:acetonitrile (98:2, v/v). Chromatographic separation was achieved using a Surveyor LC system (Thermo-Finnigan, San Jose, CA) equipped with an YMC ODS-AQ 3 µ column (50 x 2 mm, Waters, Milford, MA). A TSQ-Quantum (Thermo-Finnigan, San Jose, CA) triple-stage quadrupole MS was used for analysis. All analytical batches included two sets of calibrators ranging from 0.1 to 50 ng/ml, and duplicate quality control samples at 0.25, 25 and 40 ng/ml. For hepatocyte culture media analyses, calibrators and QCs were prepared in hepatocyte culture media; for microsomal analyses, calibrators and QCs were prepared in drug-free plasma. Separately prepared stocks were used for calibrators and QCs, respectively.

Data Analysis and Statistics. All incubations were performed in duplicate. Substrate utilization and product formation were plotted versus time of incubation. From these curves, the $AUIC_{0\text{-}60min}$ were determined using the trapezoidal rule. For hepatocyte incubations the results from the 5 donors across the three treatment groups (DMSO controls, rifampin, and nelfinavir) were first compared using repeated measures ANOVA. If a significant difference was found at two-tailed $p < 0.05$, the Tukey posthoc test was used to determine differences between groups (two-tailed $p < 0.05$). GraphPad InStat version 3.0 (GraphPad software, San Diego, CA) was used for these determinations. Comparison between R- and S-methadone utilization and R- and S-EDDP formation were made using Students paired t-test using Excel:mac 2001 (Microsoft, Seattle, WA).

Results

Incubations of methadone and buprenorphine with primary cultures of human hepatocytes were performed using hepatocytes prepared from five different donors. The demographics of the donors are shown in Table 1.

The mean results for R- and S-methadone utilization and R- and S-EDDP formation in hepatocytes pretreated with the vehicle (DMSO), rifampin and nelfinavir are shown in Figure 1. The mean and individual derived $AUIC_{0\text{-}60min}$ for the time-course of R- and S-methadone utilization and R- and S-EDDP formation are shown in Table 2. R- and S-methadone loss from hepatocyte culture media was continuous for the 60-minute time period. It did not differ between treatment groups or between R- and S-methadone within any treatment group. Time-dependent R- and S-EDDP production in the hepatocyte culture media was identified in 3 of 5, 4 of 5 and 5 of 5 donors receiving respective pretreatment with DMSO, nelfinavir and rifampin, and incubated with 300 ng/ml racemic methadone. R- and S-EDDP production increased with increasing methadone concentration in all treatment groups (data not shown). The amount produced by rifampin pretreatment was significantly greater (26.5- and 20.1-fold increases compared to vehicle controls in $AUIC_{0\text{-}60min}$ for R- and S-EDDP, respectively) than with either other treatment group. The $AUIC_{0\text{-}60min}$ R- and S-EDDP production after nelfinavir were 3.1- and 2.5-fold compared to the DMSO vehicle control, but these did not reach significance. The ratio of S- to R-EDDP production was 1.76; this decreased to 1.42 and 1.41 after treatment with rifampin and nelfinavir, respectively.

The mean results for buprenorphine utilization and norbuprenorphine, buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide formation in hepatocytes pretreated with the vehicle (DMSO), rifampin and nelfinavir are shown in Figure 2. The mean and individual derived $AUIC_{0\text{-}60min}$s for the time-course of buprenorphine utilization and norbuprenorphine, buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide formation are shown in Table 3. These results were derived from hepatocytes incubated with 5 ng/ml buprenorphine. Buprenorphine loss from hepatocyte culture media was continuous over time. At the latter time-points there was a tendency for greater loss in hepatocytes pretreated with rifampin and nelfinavir, but the changes in $AUIC_{0\text{-}60min}$ (1.43- and 1.13-fold relative to vehicle control for

rifampin and nelfinavir, respectively) did not reach significance. Buprenorphine-3-glucuronide was detected in hepatocyte culture media in a time-dependent manner from all donors, and accounted for the majority of buprenorphine loss. There were no significant differences in buprenorphine-3-glucuronide formation between treatment groups. Only small amounts of norbuprenorphine or its glucuronide were detected in hepatocyte culture media; norbuprenorphine in 1 of 5, 2 of 5 and 1 of 5 donors pretreated with DMSO, rifampin and nelfinavir; norbuprenorphine-3-glucuronide in 3 of 5, 4 of 5 and 4 of 5 donors pretreated with DMSO, rifampin and nelfinavir. While norbuprenorphine production tended to increase relative to vehicle control with pretreatment with rifampin (4.9-fold increase in $AUIC_{0\text{-}60min}$) and nelfinavir (2.6-fold increase in $AUIC_{0\text{-}60min}$) these were not significant and were based on positive findings in 2 and 1 donor. For norbuprenorphine-3-glucuronide $AUIC_{0\text{-}60min}$ there was no significant change between treatment groups. If the combined formation of norbuprenrophine and norbuprenorphine-3-glucuronide is considered the tendency for increased $AUIC_{0\text{-}60min}$ relative to vehicle control continued (1.35-fold with rifampin and 1.19-fold with nelfinavir), but these were also not significant (data not shown). In general, the production of all three metabolites increased with increases in buprenorphine concentration, but there were some cases where no norbuprenrophine or norbuprenorphine-3-glucuronide was detected at either of the concentrations of buprenorphine used (data not shown).

The primary cultures of human hepatocytes did not replicate the formation of norbuprenorphine seen in humans or HLM incubated with buprenorphine supplemented with a source of NADPH (Chang and Moody 2005; Huang, et al. 2006; Moody, et al. 2002). The potential for the intestines to form norbuprenorphine was determined by comparing production of buprenorphine metabolites in HLM and HSIM. To allow comparative production of glucuronide and N-dealkylation products buprenorphine was incubated with microsomes along with both a source of NADPH and UDPGA along with the facilitator of glucuronidation, alamethicin (Figure 3). Under these conditions, HLM produced all 3 metabolites with the following $AUIC_{0\text{-}60min}$, norbuprenorphine (0.34), buprenorphine-3-glucuronide (0.81) and norbuprenorphine-3-glucuronide (0.27). The formation of the latter coincided with decreases in norbuprenorphine over time; their combined $AUIC_{0\text{-}60min}$ (0.61) was still less than that of buprenorphine-3-glucuronide. HSIM were also active in metabolizing buprenorphine, the $AUIC_{0\text{-}60min}$ for buprenorphine utilization was 29% of that in HLM. HSIM produced both

norbuprenorphine and buprenorphine-3-glucuronide with respective AUC of 0.40 and 0.41. No norbuprenorphine-3-glucuronide formation was detected. If alamethicin was omitted from the incubation, glucuronidation was reduced in both systems, but the ratio of $AUIC_{0\text{-}60min}$s of N-dealkylation to glucuronidation remained higher in the HSIM (3.85) than HLM (2.46).

Discussion

The primary purpose of this study was to test the hypothesis that primary cultures of human hepatocytes could predict potential drug interactions with methadone and buprenorphine.. This was done using a highly defined, serum-free two-dimensional sandwich system, that configures hepatocytes with collagen I as the substratum together with a dilute extracellular matrix overlay and a defined serum-free medium containing nanomolar concentrations of the synthetic glucocoricoid, dexamethasone. This sandwich culture model preserves many liver characteristics, including: tight junctions, gap junctions and bile canaliculi morphology (Hoffmaster, et al. 2004; Olsavsky, et al. 2007; Page, et al. 2007), expression of basolateral and canalicular domains of the plasma membrane with maintenance of polarized hepatic transport (Annaert and Brouwer 2005; Hoffmaster, et al. 2004), preservation of hepatocyte differentiation markers, hepatic nuclear receptors, drug metabolizing activities (Kern, et al. 1997; Olsavsky, et al. 2007; Page, et al. 2007; Sidhu and Omiecinski 2004), and maintenance of cellular induction responses to phenobarbital, a sensitive indicator of a well-differentiated hepatocyte (Olsavsky, et al. 2007; Sidhu and Omiecinski 2004). These studies represent the first reported results of incubation of methadone and buprenorphine with cultured hepatocytes. They confirm the concept that human primary hepatocytes are viable models for certain of the drug interaction responses with these agents, but also raise questions about the mode of disposition of both drugs.

A major difference between *in vitro* incubation of methadone with HLM and cultured hepatocytes is the much greater disappearance of methadone from the culture medium than the formation of EDDP. In microsomal systems we have observed smaller excesses of methadone use (e.g. 125-150%) over EDDP production (Moody and Chang, unpublished observations), but nothing near the 10 to 30-fold observed here with hepatocytes. One can speculate that this could arise from a large contribution of non-microsomal metabolism and/or a time-dependent uptake of methadone into the hepatocytes. While other metabolites of methadone have been observed, the formation of the methadols and ring hydrozylation products are consistent with CYP-mediated reactions. Few reports on the involvement of drug transporters on methadone disposition are available beyond findings that it is not closely linked to p-glycoprotein genetics (Crettol, et al. 2006), nor is its disposition impacted by inhibition of intestinal p-glycoprotein activity

(Kharasch, et al. 2009). Based on our current results, studies on the effects of other drug transporters on methadone disposition appear warranted.

The drug interaction results obtained in the current study with methadone are generally consistent with the clinical interaction studies that have been conducted previously. Our data demonstrated that rifampin significantly increased N-demethylation of methadone in the cultured hepatocytes. Similar effects were obtained in clinical studies (Kharasch, et al. 2004; Kreek, et al. 1976). Of note, we detected a decrease in the ratio of S- to R-EDDP production, suggesting that CYP3A was induced to preferentially over CYP2B6 following rifampin exposures in hepatocytes (Totah, et al. 2008). Although the trend was evident, we did not find a statistically significant increase in methadone metabolism after nelfinavir treatment, although these effects were reported clinically (McCance-Katz, et al. 2004). One potential explanation for our results is interindividual variability in responsiveness. Although 3 donors were quite responsive to the inductive effects of nelfinavir, the other 2 donors were not. In addition, nelfinavir has been reported as a mixed inhibitor and inducer of CYP3A4 (Dixit, et al. 2007), effects that may be differentially manifested among individuals.

Buprenorphine disappearance from culture media was matched almost one-to-one by metabolite formation. This does not exclude drug transporter involvement, but if present there was an egress-ingress equilibrium. The response of buprenorphine metabolism to nelfinavir was similar to the clinical interaction study where no significant changes occurred in the plasma AUCs of buprenorphine, norbuprenorphine, buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide (McCance-Katz, et al. 2006b). No clinical study on the effect of rifampin on buprenorphine pharmacokinetics has yet been published, but the known induction of CYP3A4 by this agent (Kharasch, et al. 2004) would be expected to have an impact on the metabolism of buprenorphine, if N-dealkylation was occurring in the hepatocytes. There was, however, little indication of active N-dealkylation of buprenorphine occurring in the hepatocytes we studied. This is difficult to reconcile with respect to the known *in vivo* pharmacokinetics of buprenorphine and its *in vitro* metabolism as studied here and earlier.

Plasma AUCs of norbuprenorphine and its glucuronide are significant. Expressed relative to the buprenorphine AUC, the respective ratios are 2.73 and 9.84 (Huang, et al. 2006). It is

possible that the low relative rate of formation of norbuprenorphine compared to buprenorphine-3-glucuronide in hepatocytes could be because glucuronide expression is better maintained in culture than CYPs, and so cultured hepatocytes are just not a good model for in vivo metabolism of buprenorphine. However, we consider this possibility unlikely in that the 2-D sandwich model used for the culture of the primary hepatocytes in these experiments has undergone relatively extensive characterization in these respects. For example, in the report by Olsavsky et al. (Olsavsky, et al. 2007) whole genome microarray expression analyses were performed in a series of hepatocyte cultures obtained from 10 different donors. Functional assessment by gene ontology categorization revealed that over 80% of the 221 and 196 genes assigned to the 'transferase' and 'oxidoreductase' categories, respectively, were unchanged from that of pooled human liver samples, with the majority of the rest exhibiting slight increases relative to liver tissue. Analysis at the individual gene level for a series of CYPs, hydrolases, transferases, ABC transporters and nuclear receptor expression levels also were largely consistent with levels measured in liver. Induction capacities of the cultured hepatocytes, in particular for phenobarbital, were also robustly maintained. This points to other factors that may explain the paucity of norbuprenorphine formation detected in cultured hepatocytes.

Metabolites released from cultured hepatocytes not only reflect potential excretion into sinusoids and eventually into the systemic circulation, but also excretion into the biliary cannuliculi. Cone et al. (Cone, et al. 1984) examined buprenorphine and norbuprenorphine in urine and feces; in feces they found predominantly buprenorphine and buprenorphine-3-glucuronide. Buprenorphine-3-glucuronide excreted in the bile could be deconjugated by bacterial glucuronidases, accounting for the high concentration of parent drug in the gut, that in turn provides substrate for small intestinal metabolism. These findings are consistent with a hypothesis that buprenorphine is primarily glucuronidated in the liver, while it is primarily N-dealkylated in the small intestine. Our current *in vitro* studies in HLM and HSIM, however, are consistent with the idea that both organs participate in buprenorphine N-dealkylation.

Recent studies have examined *in vitro* clearance of buprenorphine in systems that promote both CYP-mediated and glucuronidative metabolism (Cubitt, et al. 2009; Kilford, et al. 2009; Mohutsky, et al. 2006). These have been limited to just monitoring of buprenorphine use and have employed supra-therapeutic concentrations of buprenorphine. We have now used such a

combined system that employed therapeutic concentrations of buprenorphine and monitored specific metabolite formation, as well as buprenorphine use. While both routes of metabolism were present in both sources of microsomes, the ratio of N-dealkylation to glucuronidation was greater in the HSIM. Glucuronidation was limited to buprenorphine and not detected for norbuprenorphine. Recently, Cubitt et al. (Cubitt, et al. 2009) have also demonstrated that HSIM can metabolize buprenorphine. Their study, however, used supra-therapeutic concentrations (1 μM, ≈ 460 ng/ml) and only monitored buprenorphine use under conditions supplemented with either an NADPH-generating system or glucuronidation. We have now demonstrated specific buprenorphine N-dealkylation and glucuronidation in human small intestine tissue at therapeutic concentrations. This is consistent with the growing knowledge of drug metabolism capabilities of the small intestine. In terms of the present study it appears that we can currently only hypothesize that intestinal metabolism is a contributor to buprenorphine N-dealkylation. Further studies will be required to elucidate the relevance of minimal buprenorphine N-dealkylation in cultured hepatoctyes to the *in vivo* and microsomal metabolism of buprenoprhine.

References

Annaert PP and Brouwer KL (2005) Assessment of drug interactions in hepatobilliary transport using rhodamine 123 in sandwich-cultured rat hepatocytes. *Drug Metab Dispos* **33:** 388-394.

Chang Y and Moody DE (2005) Effect of benzodiazepines on the metabolism of buprenorphine in human liver microsomes. *Eur J Clin Pharmacol* **60:** 875-881.

Chang Y and Moody DE (2009) Glucuronidation of buprenorphine and norbuprenorphine by human liver microsomes and UDP-glucuronosyltransferases. *Drug Metab Lett* **3:** 101-107.

Chang Y, Moody DE and McCance-Katz EF (2006) Novel metabolites of buprenorphine detected in human liver microsomes and human urine. *Drug Metab Dispos* **34:** 440-448.

Cohn JA (2002) HIV-1 infection in injection drug users. *Infect Dis Clin North Am* **16:** 745-770.

Cone E, Gorodetzky C, Yousefnejad D, Buchwald W and Johnson R (1984) The metabolism and excretion of buprenorphine in humans. *Drug Metab Dispos* **12:** 577-581.

Cowan A, Lewis JW and MacFarlane IR (1977) Agonist and antagonist properties of buprenorphine, a new antinociceptive agent. *Br J Pharmacol* **60:** 537-545.

Crettol S, Deglon JJ, Besson J, Croquette-Krokar M, Hammig R, Gothuey I, Monnat M and Eap CB (2006) ABCB1 and cytochrome P450 genotypes and phenotypes: Influence on methadone plasma levels and response to treatment. *Clin Pharmacol Ther* **80:** 668-681.

Crettol S, Deglon JJ, Besson J, Croquette-Krokkar M, Gothuey I, Hammig R, Monnat M, Huttemann H, Baumann P and Eap CB (2005) Methadone enantiomer plasma levels, CYP2B6, CYP2C19, and CYP2C9 genotypes, and response to treatment. *Clin Pharmacol Ther* **78:** 593-604.

Cubitt HE, Houston JB and Galetin A (2009) Relative importance of intestinal and hepatic glucuronidation - impact on the prediction of drug clearance. *Pharm Res* **26:** 1073-1083.

Dixit V, Hariparsad N, Li F, Desai P, Thummel KE and Unadkat JD (2007) Cytochrome P450 enzymes and transporters induced by anti-human immunodeficiency virus protease inhibitors in human hepatocytes: Implications for predicting clinical drug interactions. *Drug Metab Dispos* **35:** 1853-1859.

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.

Hoffmaster KA, Turncliff RZ, LeCluyse EL, Kim RB, Meier PJ and Brouwer KL (2004) P-glycoprotein expression, localization, and function in sandwich-cultured primary rat and human hepatocytes; relevance to the hepatobilliary disposition of a model opioid peptide. *Pharm Res* **21:** 1294-1302.

Huang W, Moody DE and McCance-Katz EF (2006) The in vivo glucuronidation of buprenorphine and norbuprenorphine determined by liquid chromatography-electrospray ionization-tandem mass spectrometry. *Ther Drug Monit* **28:** 245-251.

Iribarne C, Berthou F, Baird S, Dreano Y, Picart D, Bail JP, Beaune P and Menez JF (1996) Involvement of cytochrome P450 3A4 enzyme in the N-demethylation of methadone in human liver microsomes. *Chem Res Toxicol* **9:** 365-373.

Iribarne C, Picart D, Dreano Y, Bail J-P and Berthou F (1997) Involvement of cytochrome P450 3A4 in *N*-dealylation of buprenorphine in human liver micrsomes. *Life Sci* **60:** 1953-1964.

Johnson RE, Chutuape MA, Strain EC, Walsh SL, Stitzer ML and Bigelow GE (2000) A comparison of levomethadol acetate, buprenorphine and methadone for opioid dependence. *N Engl J Med* **343:** 1290-1297.

Kern A, Bader A, Pichlmayr R and Sewing K-F (1997) Drug metabolism in hepatocyte sandwich cultures of rats and humans. *Biochem Pharmacol* **54:** 761-772.

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 miotic 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. *Anesthesiology* **110:** 660-672.

Kilford PJ, Stringer R, Sohal B, Houston JB and Galetin A (2009) Prediction of drug clearance by glucuronidation from in vitro data: use of combined cytochrome P450 and UGP-glucuronosyl transferase cofactors in alamethicin-activated human liver microsomes. *Drug Metab Dispos* **37:** 82-89.

Kreek MJ, Garfield JW, Gutjahr CL and Giusti LM (1976) Rifampin-induced methadone withdrawal. *N Engl J Med* **294:** 1104-1106.

McCance-Katz EF (2005) Treatment of opioid dependence and coinfection with HIV and hepatitis C virus in opioid-dependent patients: The importance of drug interactions between opioids and antiretroviral agents. *Clin Infect Dis* **41:** (Suppl 1) S89-S95.

McCance-Katz EF, Moody DE, Morse GD, Friedland G, Pade P, Baker J, Alvanzo A, Smith P, Ogundele A, Jatlow P and Rainey PM (2006a) Interactions between buprenorphine and antiretrovirals. I. The nonnucleoside reverse-transcriptase inhibitors efavirenz and delavirdine. *Clin Infect Dis* **43:** (Suppl 4) S224-S234.

McCance-Katz EF, Moody DE, Morse GD, Ma Q, DiFrancesco R, Friedland G, Pade P and Rainey PM (2007) Interaction between buprenorphine and atazanavir or atazanavir/ritonavir. *Drug Alc Depend* **91:** 269-278.

McCance-Katz EF, Moody DE, Smith PF, Morse GD, Friedland G, Pade P, Baker J, Alvanzo A, Jatlow P and Rainey PM (2006b) Interactions between buprenorphine and antiretrovirals. II. The protease inhibitors nelfinavir, lopinavir/ritonavir, and ritonavir. *Clin Infect Dis* **43:** (Suppl 4) S235-S246.

McCance-Katz EF, Rainey PM, Smith P, Morse GD, Friedland G, Gourevitch M and Jatlow P (2004) Drug interactions between opioids and antiretroviral medications: Interaction between methadone, LAAM, and nelfinavir. *Am J Addict* **13:** 163-180.

Mohutsky MA, Chien JY, Ring BJ and Wrighton SA (2006) Predictions of the in vivo clearance of drugs from rate of loss using human liver microsomes for phase I and phase II biotransformations. *Pharmaceut Res* **23:** 654-662.

Moody DE, Alburges ME, Parker RJ, Collins JM and Strong JM (1997) The involvement of cytochrome P450 3A in the N-demethylation of *l*-α-acetylmethadol (LAAM), norLAAM and methadone. *Drug Metab Dispos* **25:** 1347-1353.

Moody DE, Chang Y, Huang W and McCance-Katz EF (2009) The in vivo response of novel buprenorphine metabolites, M1 and M3, to antiretroviral inducers and inhibitors of buprenorphine metabolism. *Basic Clin Pharmacol Toxicol* **105**: 211-215.

Moody DE, Lin SN, Chang Y, Lamm L, Greenwald MK and Ahmed MS (2008) An enantiomer selective liquid chromatography-tandem mass spectrometry method for methadone and EDDP validated for use in human plasma, urine and liver microsomes. *J Anal Toxicol* **32:** 208-219.

Moody DE, Slawson MH, Strain EC, Laycock JD, Spanbauer AC and Foltz RL (2002) A liquid chromatographic-electrospray ionization-tandem mass spectrometric method for determination of buprenorphine, its metabolite, norbuprenorphine, and a co-formulant, naloxone, that is suitable for in vivo and in vitro metabolism studies. *Anal Biochem* **306:** 31-39.

Olsavsky KM, Page JL, Johnson MC, Zarbl H, Strom SC and Omiecinski CJ (2007) Gene expression profiling and differentiation assessment in primary human hepatocyte cultures, established hepatoma cell lines, and human liver tissue. *Toxicol Appl Pharmacol* **222:** 42-56.

Page JL, Johnson MC, Olsavasky KM, Strom SC, Zarbl H and Omiecinski CJ (2007) Gene expression profiling of extracellular matrix as an effector of human hepatocyte phenotype in primary cell culture. *Toxicol Sci* **97:** 384-397.

Picard N, Cresteil T, Djebli N and Marquet P (2005) In vitro metabolism study of buprenorphine: evidence for new metabolic pathways. *Drug Metab Dispos* **33:** 689-695.

Sidhu JS and Omiecinski CJ (2004) Phenobarbital responsiveness as a uniquely sensitive indicator of hepatocyte differentiation status: requirement of dexamethasone and extracellular matrix in establishing the functional integrity of cultured primary rat hepatocytes. *Exp Cell Res* **292:** 252-264.

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.

Footnotes

Unnumbered footnote to the title:
This work was supported by the National Institutes of Health [Grants R01 DA10100 (D.E.M.) and GM066411 (C.J.O.)]. Normal human hepatocytes were obtained through the Liver Tissue Cell Distribution System, Pittsburgh, PA, which was funded by the National Institutes of Health [Contract N01-DK-7-004 / HHSN267200700004C].

Unnumbered footnote to the title:
For projects unrelated to the current study, Dr. Moody has received research funding and consultant fees from Reckitt Benckiser, the manufacturer of buprenorphine.

Legends for Figures

Figure 1. Time course of A) R-methadone and B) S-methadone utilization and C) R-EDDP and D) S-EDDP formation in media from cultured human hepatocytes that had been preincubated with DMSO, rifampin or nelfinavir and incubated with 300 ng/ml racemic methadone. Results are the mean of results for 5 donors, each incubated in duplicate.

Figure 2. Time course of A) buprenorphine utilization and B) norbuprenorphine, C) buprenorphine-3-glucuronide (Bup-3-Gluc) and D) norbuprenorphine-3-glucuronide (Norbup-3-Gluc) formation in media from cultured human hepatocytes that had been preincubated with DMSO, rifampin or nelfinavir and incubated with 5 ng/ml buprenorphine. Results are the mean of results for 5 donors, each incubated in duplicate.

Figure 3. Time course of buprenorphine (Bup) utilization and norbuprenorphine (Norbup), buprenorphine-3-glucuronide (B3G) and norbuprenorphine-3-glucuronide (N3G) formation in A) human liver microsomes (HLM) and B) human small intestine microsomes (HSIM) incubated with 5 ng/ml buprenorphine. Results are the mean of duplicate incubations.

DMD Fast Forward. Published on September 22, 2009 as DOI: 10.1124/dmd.109.028605
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Donor demographics

DONOR	AGE	GENDER	RACIAL	CLINICAL INFORMATION
A	53	F	Caucasian	Resection, non-malignant growth in liver
B	44	M	Caucasian	Resection, colon cancer with metastasis to liver
C	60	F	Caucasian	Resection, cholangiocarcinoma
D	69	F	Caucasian	Resection, hepatocellular carcinoma
E	49	M	Caucasian	Resection, metastatic colon cancer, chemotherapy

DMD Fast Forward. Published on September 22, 2009 as DOI: 10.1124/dmd.109.028605
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. Area under the incubation time curve for R-methadone (R-Meth) and S-methadone (S-Meth) utilization and R-EDDP and S-EDDP formation in media from cultured hepatocytes treated with DMSO, rifampin or nelfinavir and incubated with 300 ng/ml racemic methadone.

		Utilization		Production	
Treatment	Donor	R-Meth	S-Meth	R-EDDP	S-EDDP
		$(\mu mol)(ml)^{-1}(min)$		$(nmol)(ml)^{-1}(min)$	
DMSO	A	5.27	4.84	22.1	32.8
	B	5.11	5.24	0.0	0.0
	C	6.00	5.78	0.0	0.0
	D	6.06	6.07	15.9	21.3
	E	4.36	4.45	19.2	46.3
	Mean ± SD	5.36 ± 0.70	5.28 ± 0.66	11.4 ± 10.7 [a]	20.1 ± 20.4 [a]
Rifampin	A	4.03	3.79	359	586
	B	5.58	5.75	480	506
	C	6.01	5.81	133	213
	D	6.36	6.64	181	286
	E	4.95	4.97	355	548
	Mean ± SD	5.39 ± 0.70	5.39 ± 1.07	302 ± 142 [b]	428 ± 167 [b,*]
Nelfinavir	A	1.73	2.22	22.7	32.5
	B	5.11	4.98	0.0	0.0
	C	5.24	5.17	34.7	43.3
	D	7.22	7.01	58.7	102
	E	5.99	5.49	62.5	74.9
	Mean ± SD	5.06 ± 2.04	4.97 ± 1.73	35.7 ± 25.9 [a]	50.5 ± 39.2 [a]
	Repeated Measures ANOVA p	0.861	0.678	0.0013	0.0003

a,b – Groups sharing same letter are not significantly different ($p < 0.05$) based on Tukey posthoc test when repeated measures ANOVA showed significance ($p < 0.05$) between groups.

* - Significant difference ($p < 0.05$) between R- and S- enantiomers based on Students paired t-test.

DMD Fast Forward. Published on September 22, 2009 as DOI: 10.1124/dmd.109.028605
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3. Area under the incubation time curve for buprenorphine (Bup) utilization and norbuprenorphine (Nor), buprenorphine-3-glucuronide (B-3-G), and nobuprenorphine-3-glucuronide (N-3-G) formation in media from cultured hepatocytes treated with DMSO, rifampin or nelfinavir and incubated with 5 ng/ml buprenorphine.

		Utilization	Production		
Treatment	Donor	Bup	Nor	B-3-G	N-3-G
			(nmol)(ml)$^{-1}$(min)		
DMSO	A	95.7	0.0	52.2	14.7
	B	113	0.0	49.4	10.4
	C	63.1	0.0	36.8	0.0
	D	70.3	0.0	60.0	0.0
	E	109	5.8	92.7	4.1
	Mean ± SD	90.2 ± 22.5	1.2 ± 2.6	58.2 ± 21.0	5.4 ± 6.5
Rifampin	A	124	0.0	34.8	13.4
	B	179	0.0	83.5	17.1
	C	68.5	12.7	39.5	3.8
	D	76.4	0.0	56.0	3.1
	E	196	17.0	43.7	0.0
	Mean ± SD	129 ± 58	5.9 ± 8.3	51.5 ± 19.5	7.5 ± 7.4
Nelfinavir	A	78.3	0.0	61.0	10.4
	B	106	0.0	95.3	13.4
	C	118	0.0	56.4	0.0
	D	79.1	0.0	82.2	3.6
	E	130	17.0	80.9	14.6
	Mean ± SEM	102 ± 23	3.4 ± 7.6	75.2 ± 16.1	8.4 ± 6.4
	Repeated Measures ANOVA p	0.150	0.241	0.102	0.647

Figure 1

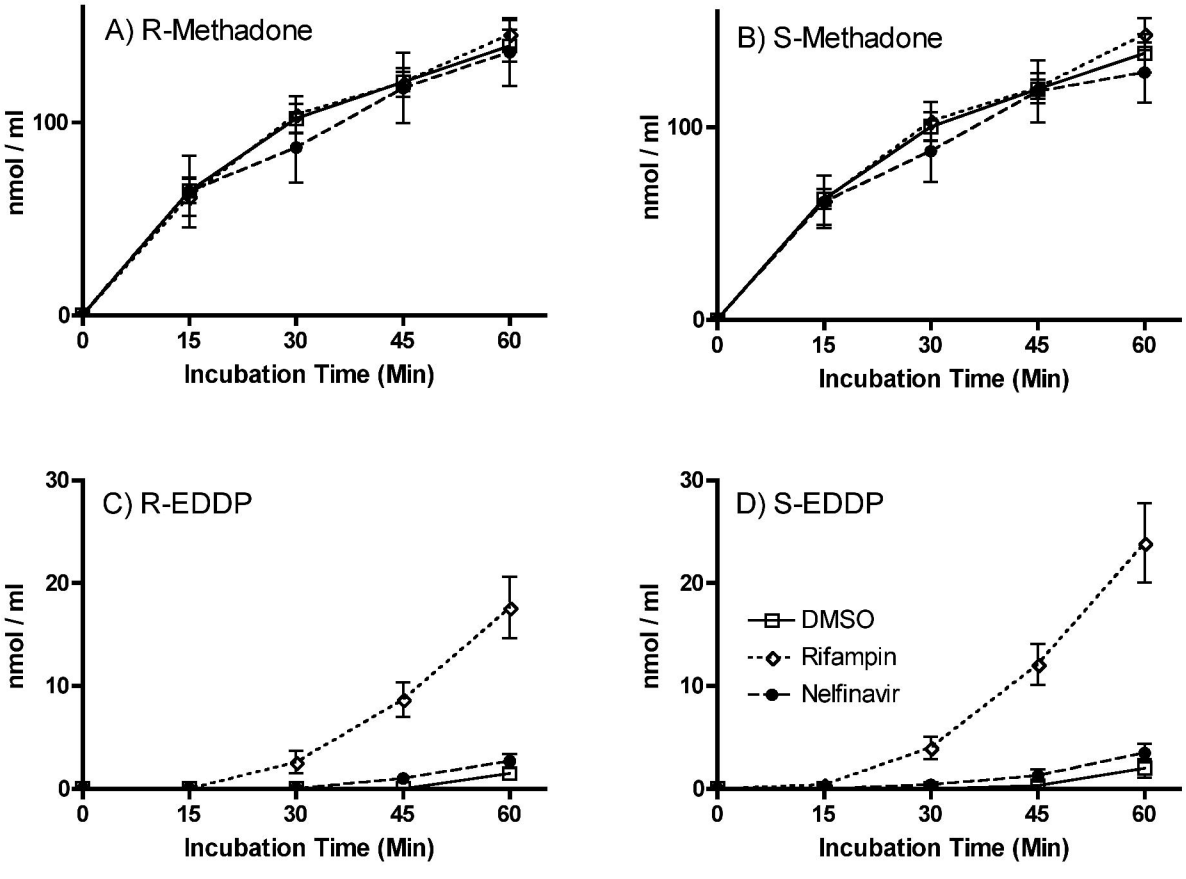

A) R-Methadone
B) S-Methadone
C) R-EDDP
D) S-EDDP
nmol / ml
Incubation Time (Min)
0
15
30
45
60
100
20
10
DMSO
Rifampin
Nelfinavir

Figure 2

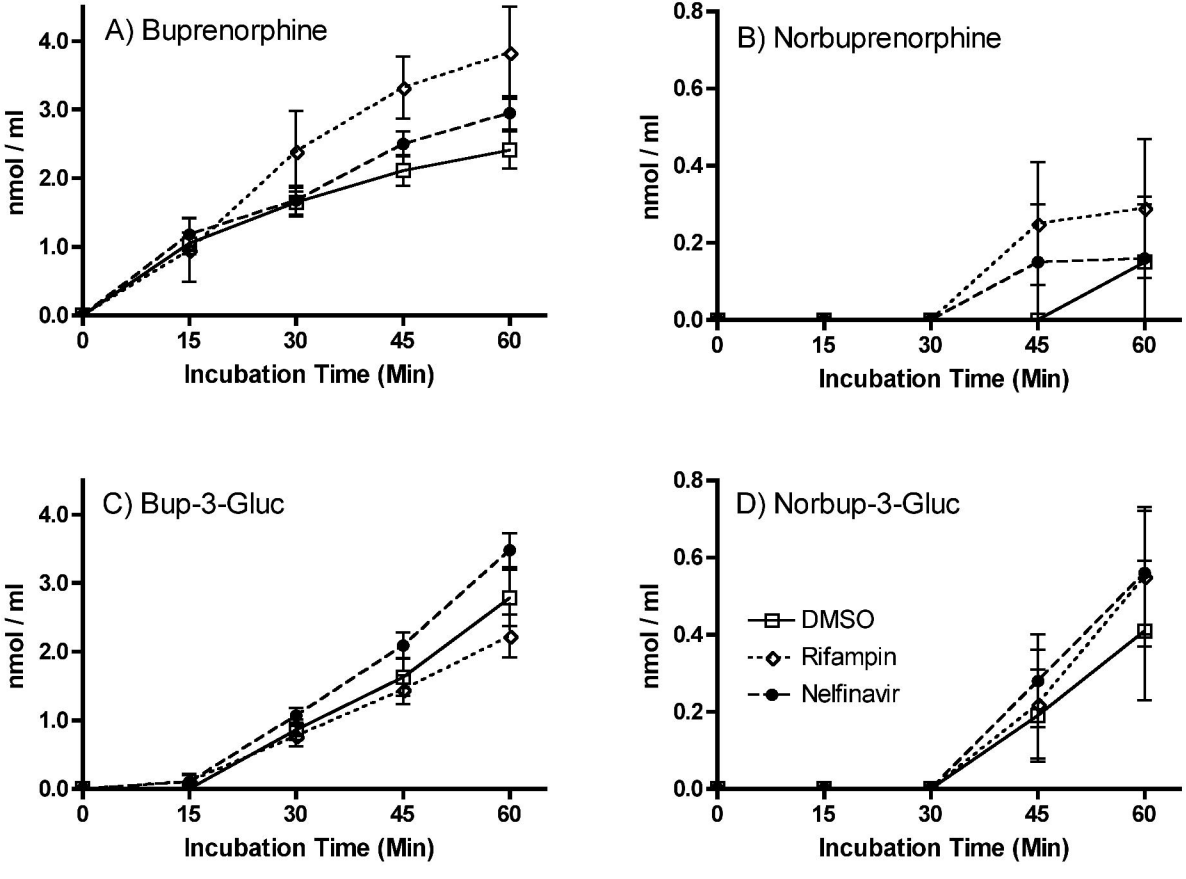
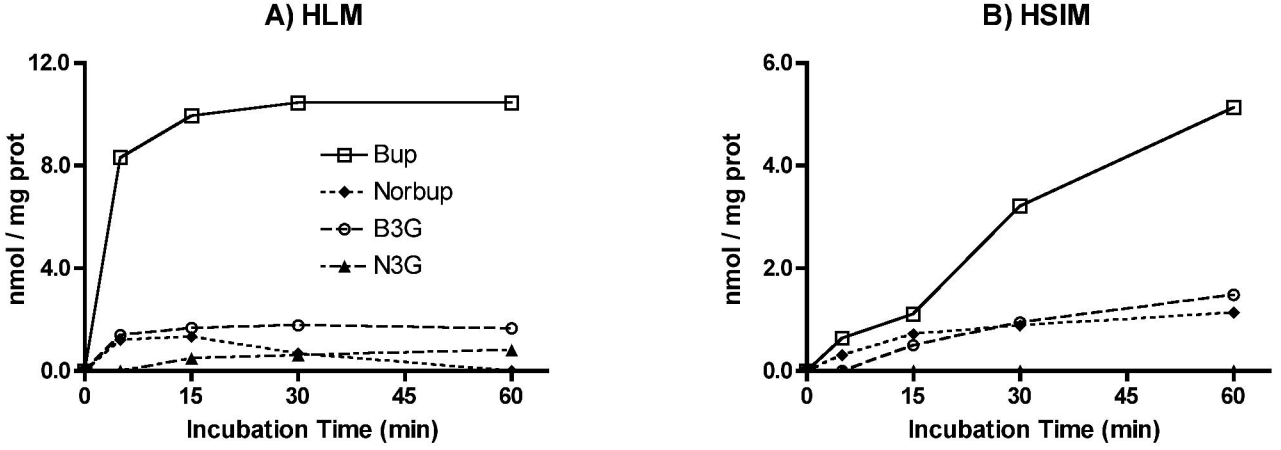

A) Buprenorphine
nmol / ml
4.0
3.0
2.0
1.0
0.0
0
15
30
45
60
Incubation Time (Min)
B) Norbuprenorphine
nmol / ml
0.8
0.6
0.4
0.2
0.0
0
15
30
45
60
Incubation Time (Min)
C) Bup-3-Gluc
nmol / ml
4.0
3.0
2.0
1.0
0.0
0
15
30
45
60
Incubation Time (Min)
D) Norbup-3-Gluc
nmol / ml
0.8
0.6
0.4
0.2
0.0
DMSO
Rifampin
Nelfinavir
0
15
30
45
60
Incubation Time (Min)

Figure 3


A) HLM
12.0
8.0
4.0
0.0
nmol / mg prot
Bup
Norbup
B3G
N3G
0
15
30
45
60
Incubation Time (min)
B) HSIM
6.0
4.0
2.0
0.0
nmol / mg prot
0
15
30
45
60
Incubation Time (min)